

Journal of Coastal Life Medicine

journal homepage: www.jclmm.com



Document heading

doi:10.12980/JCLM.1.2013D317

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A study on the effect of using mangrove leaf extracts as a feed additive in the progress of bacterial infections in marine ornamental fish

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PEER REVIEW

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Comments

It is good to concentrate on all aspect of fish infection by isolation disease causing bacterial pathogens, control of infection by mangrove extract and induction of immune response in fish by feed. Moreover, they checked its immune response in different days.

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ABSTRACT

Objective: To ascertain the feasibility of using sustainable natural resources in maintaining disease free fish in such establishments.

Methods: The infected marine ornamental fishes were collected from the hatchery condition and causative bacteria were identified by morphology and biochemical techniques. The antibacterial activity and disease resistant capability of mangrove plant leaf extract were investigated against fish pathogens.

Results: Based on the *in vitro* assay, methanol extract of *Avicennia marina* was exhibited good inhibition activity at the concentration of 220, 200, 175 and 150 µg/mL against *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Vibrio parahaemolyticus*, and *Vibrio anguillarum* respectively. The experimental trial reveals feeding marine ornamental fish with feed incorporated with a methanol leaf extract of *Avicennia marina*, increases their survival and reduces their susceptibility to infections from the isolated bacteria.

Conclusions: The mangrove leaves have potential to control the infections caused by *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Vibrio parahaemolyticus* and *Vibrio anguillarum*.

KEYWORDS

Infected fish, Challenge experiment, Leukocytes, Disease resistance, Mangrove herbal, Bacterial infections

1. Introduction

Ornamental fish keeping is growing rapidly as a hobby on a global scale. Fish keeping is considered as the second largest hobby next to photography. Keeping colourful tropical fish as pets or for decorative purposes is a wide spread hobby all over the world and in 2008 the "Global Live Trade" in this industry was valued at US\$ 392 million. The growth in this sector has picked up during the last decade as an important production sector that can augment the household income, especially in the rural sector of developing countries[1]. Recent developments in the marine aquarium technology, development of synthetic sea slat mix and the vast range of water quality monitoring kits and filter equipment have

enabled the marine fish keeping hobby to expand even in to non-coastal areas around the world. The number of people that keep marine aquarium fish in homes has been estimated to be around 1.5–2 billion around the world[2,3].

Disease and uncontrolled mortality in fish tanks have been the main reason for most people to withdraw from this hobby. Majority of diseases affecting fish are infectious, caused by opportunistic viruses and bacteria or parasitic invasions. Out of these, diseases of bacterial origin have become a chronic problem in ornamental fish health management. They can either be the primary or secondary cause of the disease condition taking advantage of a breach in the fish integument or a compromise in its immune system[4].

Common symptoms typical of a bacterial infection in fish

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Foundation Project: Supported by University Grants Commission (UGC), New Delhi for providing the financial assistance. Reference No. U.G.C. No. 33-384 / 2007 (SR).

Article history:

Received 29 Jul 2013

Received in revised form 8 Aug, 2nd revised form 15 Aug, 3rd revised form 19 Aug 2013

Accepted 20 Sep 2013

Available online 28 Oct 2013

are inflamed areas on the body or fins, raised scales, skin ulcers, pop eye or exophthalmia, dropsy or swollen abdomen, fin and gill rot. Dropsy is caused by a bacterial infection in the peritoneal cavity including the kidneys; causing fluid accumulation in the body cavity. Gills are a primary target in most infections. On infection, the gill tissues may become necrotic causing ensuing death of the fish. Bacteria invade the body of the fish causing damage to internal organs or whole systems.

Antibiotics are useful additions to any fish–health manager’s toolbox, but should be treated only as tools and not ‘magic bullets’. The ability of antibiotics to help eliminate the causative bacteria in a fish disease situation depends on a number of factors. They merely control the growth of bacteria in a fish long enough for the immune system of the fish to take over and eliminate the disease causing bacteria. However, specific antibiotics fail as a result of the development of resistance to the given antibiotic by the microorganism[5]. Due to these reasons much attention has been paid to using herbal and traditional medicines in the recent years[6].

Mangroves and halophytes are being used in a wide range of applications including in the control of bacterial, fungal and viral diseases. They have been used in traditional medicine to treat various diseases for centuries[7]. Some mangrove plants have been screened for their antiviral, antibacterial, antiulcer and anti–inflammatory activities[7–9].

The present study has been conducted with the aim of developing a protocol for using extracts of *Avicennia marina* (*A. marina*) and *Rhizophora apiculata* (*R. apiculata*) leaves in controlling common bacterial infections, thus facilitating a ‘disease–free marine ornamental aquaculture’ with the use of appropriate sustainable herbal preparations.

2. Material and methods

2.1. Sample collection and observation of symptoms

Samples of infected marine ornamental fish were collected from the hatchery of our Centre at Parangipettai. Different sets of clinical symptoms and behavioral changes have been observed in these infected fish and these symptoms have been categorized.

2.2. Signs of disease

Scrapings from the body surface and fins of infected fish were observed under a microscope to identify and record the presence of moulds and other parasites.

2.3. Isolation of pathogens

The liver, kidney, spleen and blood from the fish that were collected and transferred from the hatchery to the laboratory were removed for isolation of bacteria. These samples were homogenized, serially diluted and inoculated on Zobell

marine agar plates 2216 (ZMA) (Hi Media™, Mumbai) along with 50% seawater. After being cultured for 24 h at 30 °C, the uniform colonies were sub cultured for further processing[10].

2.4. Characterization and identification of pathogens

The isolated bacteria were identified in concurrence with Bergey’s Manual of Systematic Bacteriology. They were characterized based on colony morphology, gram nature, shape, motility and their behavior in selective and differential media. Further, the following biochemical techniques were performed on the isolates to identify the bacteria based on their reactions: Indole, methyl red, voges proskeur, citrate utilization, nitrate reduction, production of hydrogen sulphide, urease, catalase, oxidase and fermentation of glucose, galactose, fructose, lactose, glycerol, maltose, mannitol, raffinose, sucrose, xylose, glycerol, utilization of blood, starch, casein, phosphate, decarboxylate of lysine and ornithine. The results of biochemical characterization were compared with those appearing in previous reports[11] and subsequently with standard data of Bergey’s Manual of Systemic Bacteriology and were identified as *Pseudomonas fluorescens* (*P. fluorescens*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Vibrio anguillarum* (*V. anguillarum*) and *Vibrio parahaemolyticus* (*V. parahaemolyticus*).

2.5. Collection and extraction of mangrove leaves

Leaves of mangroves *A. marina* and *R. apiculata* were collected from the man–made mangrove stretches in the Vellar estuary of Parangipettai (11°29’24” N and 79°45’36” E). Spoilt parts of the leaves were removed. The remaining parts were washed with sterile distilled water and dried in the shade. They were then powdered with the help of a mechanical grinder. A known quantity of mangrove leaf powder was mixed in different types of solvents such as acetone, chloroform and methanol. After 48 h of incubation in the shaker, the supernatant was collected, dried in vacuum desiccators and stored in sterile containers[12].

2.6. Preparation of discs

The mangrove leaf extracts (500 µg) was dissolved in dimethyl sulfoxide and was filtered using a syringe filter with a pore size of 0.22 µm. Sterile discs of 6 mm diameter (Hi–Media™) were loaded with different concentrations of extracts and dried later. The dried discs were stored in sterile containers till further use. Solvent loaded discs were prepared and used as the negative control. A commercial antibiotic, oxytetracycline, was used as the positive control.

2.7. Antimicrobial activity

Antibiotic sensitivity of the isolated organisms was tested by disc diffusion method using Kirby–Bauer technique. Surface of the Mueller–Hinton agar was uniformly inoculated with the help of a cotton swab. Prior to inoculation, the swab

stick was dipped into bacterial suspension having a visually equivalent turbidity to 0.5 McFarland standards. By making use of the template drawn, extract-loaded discs were placed on the solidified Mueller Hinton agar containing the test organisms. Commercial oxytetracycline was used as a positive control and solvent loaded discs were used as a negative control. Inoculated plates were incubated at 37 °C for 24 h. Tests were performed in triplicate. On the next day, plates were read by measuring the diameter of the inhibition zone.

2.8. Minimum inhibitory concentration (MIC)

Broth dilution method was used to find out the minimal inhibitory concentration [13]. Stock concentration of mangrove leaf extract was prepared using DMSO. Mueller Hinton broth was prepared, sterilized and mixed with known concentrations of different extracts. This mixture was swirled carefully until a complete mixing of extract and broth was achieved. It was then inoculated with test organisms (0.5 McFarland). The tubes were incubated at 37 °C for 24 h and the MIC was recorded based on the growth of the organisms assessed through the spectrophotometer at 620 nm.

2.9. Fish collection and acclimatization

A total of 200 healthy, sub-adult clownfish, each weighing around 20–25 g, were obtained from the marine ornamental fish hatchery of the Experimental Centre and transferred to a cement tank with a water capacity of 2500 liters. This tank was filled with UV treated water. The fish were maintained in the same tank for 10 d with continuous aeration and were fed with boiled oyster meat twice a day. During the period of the experiment the water was maintained at a temperature of (28.2 ± 1.4) °C, pH at (8.2 ± 0.3), salinity of (28.0 ± 2.2) PSU and dissolved oxygen level of (5.8 ± 0.6) mg/L. Ammonia and nitrite levels in the water were below detectable levels.

2.10. Re-infection and experimental infection

After the acclimatization period, 80 specimens of active fish were shifted to the experimental tanks. The fiber glass tanks had a volume of 200 liters and were filled with 150 liters of UV-sterilized water. The bacterial culture was centrifuged at 1000 g for 10 min at 4 °C. The supernatant was discarded and the isolated bacterial pellets were washed thrice and re-suspended in phosphate-buffered saline (PBS) at a pH of 7.4. The optical density of the solution was adjusted to 0.5 at 456 nm which corresponded to 1×10^6 , 1×10^7 , 1×10^8 CFU/mL. Same amounts of bacterial pellets were dissolved in experimental groups of 10 fish each in duplicate along with PBS. The control group was exposed only to PBS. Intra-peritoneal injection method was not used for challenging since the fish were small in size. The fish were carefully observed for any behavioral changes or mortality once they had been challenged. It was noted that the clinical symptoms observed in the challenged fish were the same as those in aquarium tanks prior to the onset of the experiment.

2.11. Re-isolation

Blood, spleen, liver and kidney of the challenged fish were aseptically removed and streaked on NA and ZMA 2216 agar. After incubation at 28 °C for 48 h, bacteria were identified with the same methodology as in the first isolation. The results were then compared.

2.12. Feed preparation

As the leaf extract of *A. marina* expressed considerable inhibitive activity in the antimicrobial assay, it was incorporated into three experimental diets at rates of 1%, 2% and 4%. Feed not containing any mangrove leaf extracts was used as the control. Diets were prepared as per the basal ratio suggested by Dhayanithi [14]. The basal diet contained proteins, carbohydrates, lipids, moisture, fiber, vitamins and minerals. To prepare the feed, the ingredients (acetes, wheat flour, groundnut oil cake, rice bran, cod-liver oil and vitamin complex) were finely powdered and mixed well with gelatin solution, adhering to the appropriate amounts of the active ingredients. The pH of the mixture was adjusted to 7.0 ± 0.1. The mixture was cold-extruded, made into pellets, air dried and stored at room temperature.

2.13. The experiment

Healthy fish, numbering 120 were divided into 4 groups. Each group contained 10 fish in 3 sub groups. One group was fed with the control feed; second, third and fourth groups of fish were fed with the experimental feed containing 1%, 2% and 4% of the prepared extract of *A. marina* respectively. The fish were fed at a rate of 5% of their body weight. As the average individual body weight of the selected fish was around 20 g, the daily feed amount was 1 g per fish. This corresponds to 0 (in the control), 10, 20 and 40 mg of the herbal extract in the different groups respectively. The feeding was carried out twice a day till the end of the experiment.

2.14. Sampling

Five randomly chosen fish from each group were taken for sampling at weekly intervals. Blood was collected from the caudal vein of the fish with the help of a plastic syringe, which was rinsed with an anticoagulant (EDTA). Part of the blood was added to an equal volume of 10% tri sodium citrate and stored at 40 °C until further analysis.

2.15. Estimation of white blood cells (WBC)

The blood samples containing diluting fluid were incubated for 5 min until complete haemolysis of red blood cells occurred. Subsequently the blood mixture was placed on a haemocytometer and the number of white blood cells was counted. The result was calculated with the following formula:

$$\text{Number of WBC/mm}^3 = \frac{\text{No. of WBC} \times \text{Dilution}}{\text{Area counted} \times \text{Depth of the fluid}}$$

(Dilution=25, Area counted=4, Depth of the fluid=0.1 mm)

2.16. Clinical trial and disease resistance experiment

After one month, fish in the experimental tanks were exposed to a 24 h culture of the same bacterial pathogens; *P. fluorescens*, *P. aeruginosa*, *V. anguillarum* and *V. parahaemolyticus*. This was done with a standardized concentration of bacterial isolates in pellet form under aseptic conditions. The appropriate doses of the virulent bacteria were standardized based on the results of the challenge study and used for clinical trial and the disease resistance experiment. The control group was exposed to the same amount of buffer without the pathogenic bacteria. Subsequently, the fish were observed for specific symptoms. All groups were maintained in triplicate.

3. Results

The major symptoms observed in the marine ornamental fish in our tanks were: sluggish behavior, twirling, erratic movement, exophthalmia, fin rot, hemorrhages at the base of fins, mouth, skin and muscles, tail rot and iris opaqueness (Figure 1).

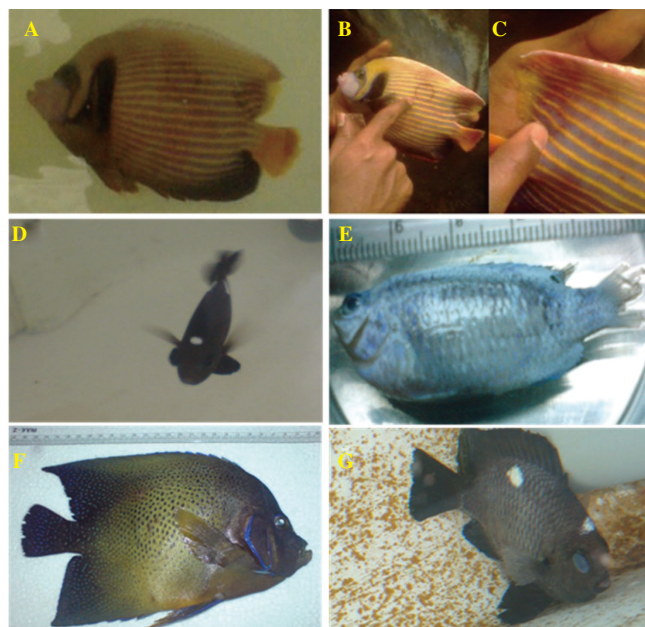


Figure 1. Major symptoms observed in the marine ornamental fish in the tanks. A: Hemorrhages at fins, B and C: Hemorrhages at body surface of infected emperor angelfish, *Pomacanthus imperator*, D: Infected three spot damsel fish, *Dascyllus trimaculatus* with exophthalmia, E: Fin rot and tail rot on Electric blue damsel, *Chrysiptera cyanea*, F: Iris cloudy symptoms in Koran angelfish, *Pomacanthus semicirculatus*, G: Iris cloudy symptoms in *Dascyllus trimaculatus*.

Based on the results of comparative colony morphology and biochemical characteristics, *P. aeruginosa* was identified as the causative agent of the bacterial infection of the emperor angel fish with symptoms such as hemorrhages at fin bases and body surface. Likewise, *V. anguillarum* was found to

cause opacity of the eye in Koran angel fish (*Pomacanthus semicirculatus*) and the three-spot damsel fish (*Dascyllus trimaculatus*). *P. fluorescens* was isolated from the electric blue damsel fish (*Crysiptera cyanea*) that was showing symptoms such as fin and tail rot. *V. parahaemolyticus* was identified as the agent causing exophthalmia in the three-spot damsel fish, *Dascyllus trimaculatus*. The results of the bio-chemical characterization of isolated bacteria from the infected fishes are shown in Table 1.

Table 1

Results of bio-chemical characterization of isolated bacteria from the infected marine ornamental fish, expressed various symptoms.

	MOFP1	MOFP2	MOFP3	MOFP4
Gram staining	–	–	–	–
Shape	Rod	Rod	Sickle	Sickle
Motility	+	+	+	+
Indole test	–	–	+	+
Methyl red	–	–	–	+
Voges Proskauer	–	–	+	–
Citrate utilization	+	+	+	–
Urease	+	+	–	+
H ₂ S	–	–	–	+
Nitrate reduction	–	+	+	+
Catalase	–	–	+	+
Oxidase	+	+	+	+
Glucose	+	+	+	+
Galactose	+	+	+	+
Fructose	+	+	+	+
Glycerol	–	–	–	–
Lactose	–	–	–	+
Maltose	+	–	+	–
Mannitol	+	–	+	+
Raffinose	–	+	+	–
Sucrose	–	–	+	–
Xylose	+	–	+	–
Blood haemolysis	–	–	+	+
Starch	–	–	–	+
Casein	+	+	–	+
Phosphatase	–	+	–	–
Lysine decarboxylase	–	–	–	+
Ornithine decarboxylase	–	–	–	+
Fluorescent pigment	+	+	–	–
Production of pyocyanin	–	+	–	–

MOF1: *P. fluorescens*, MOFP2: *P. aeruginosa*, MOFP3: *V. anguillarum*, MOFP4: *V. parahaemolyticus*

Acetone, chloroform, and methanol extracts of *A. marina* and *R. apiculata* were tested for their antimicrobial activity and it could be seen that the extracts were active at 500 µg/disc concentrations (Table 2). Methanol extract of *A. marina* exhibited the minimum inhibitory concentration at 220, 200, 175 and 150 µg/mL against *P. fluorescens*, *P. aeruginosa*, *V. parahaemolyticus* and *V. anguillarum* respectively.

Table 2

Antimicrobial activity of mangrove leaves against bacterial isolates from infected marine ornamental fishes.

Bacteria	<i>A. marina</i>			<i>R. apiculata</i>		
	Acetone	Chloroform	Methanol	Acetone	Chloroform	Methanol
<i>P. fluorescens</i>	17±0.4	20±0.5	19±0.2	14±0.7	12±0.4	12±0.4
<i>P. aeruginosa</i>	19±1.4	22±0.4	21±0.4	13±0.7	11±0.4	14±0.6
<i>V. anguillarum</i>	17±1.3	00±0.0	22±0.4	16±1.2	14±0.1	17±0.6
<i>V. parahaemolyticus</i>	19±0.1	11±0.5	21±0.2	18±0.7	08±0.6	12±0.4

The symptoms such as sluggish behavior, twirling, erratic movement, exophthalmia, fin rot, hemorrhaging at the base of fins, tail rot and eye opaqueness, were reproduced in the healthy fish after they were challenged with the bacterial isolates obtained from the effected fish. It was noticed that the same type of bacteria caused different sets of symptoms in different specimens. The fish were found to be free of external parasites during the period of the experiment.

Appropriate colonies of bacteria were isolated from the internal organs of the challenged fish. The cause of death could be established by isolating the same organisms from the blood, liver, spleen and kidneys of the affected fish. These isolates were identified through microscopic and macroscopic techniques and conformational biochemical techniques using selective and differential media.

The temperature, salinity, pH, nitrate, nitrite and ammonia levels were monitored in different tanks at regular intervals. Subsequently they were standardized and maintained at a temperature of (28.2±1.4) °C, pH at (8.2±0.3), salinity at (28 ±2.2) PSU and the dissolved oxygen level at (5.8±0.6) mg/L. The ammonia and nitrite levels in the water were below detectable levels.

Isolates of *P. fluorescens*, *P. aeruginosa*, *V. anguillarum* and *V. parahaemolyticus* were used for challenging the group of healthy fish at concentrations of 1×10⁶, 1×10⁷ and 1×10⁸ colony forming units (CFU)/mL (Table 3).

Table 3

Percentage of mortality caused by bacterial isolates.

Name of the bacteria	Percentage of mortality (%)		
	1×10 ⁶	1×10 ⁷	1×10 ⁸
<i>P. fluorescens</i>	60	75	80
<i>P. aeruginosa</i>	40	50	75
<i>V. anguillarum</i>	75	80	80
<i>V. parahaemolyticus</i>	90	90	90

Based on the results of the challenge experiment, it was concluded that the 4 bacterial isolates that were obtained possessed pathogenic qualities and that they were the causes of mortality in the tested groups of fish. Estimation of leukocyte levels was carried out for each experimental group of fish during the experimental period. It could be seen clearly that the fish in the groups fed with the extract supplemented feed reacted with a gradual increase of leukocyte levels in the blood showing an active immune response as against in the control group (Figure 2).

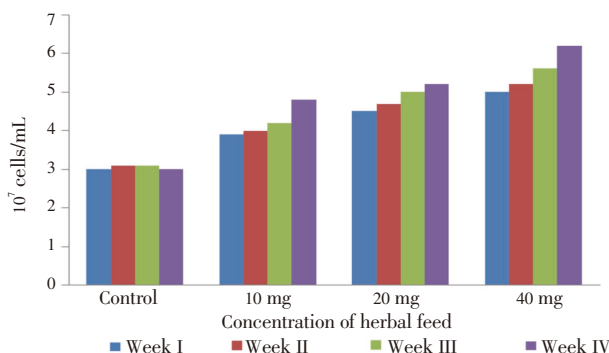


Figure 2. Estimation of leukocyte count from the experimental group fed with mangrove herbal diet and without herbal diet (control).

After the elapse of a period of one month of feeding with the extract incorporated fish, they were challenged with the isolates of the pathogenic bacteria as shown in Figure 3. The resultant mortality as against the control group is shown in the same figure. It can be seen from this data that the feed supplement mixed with mangrove extract could enhance the digestion process and stimulate an active metabolism.

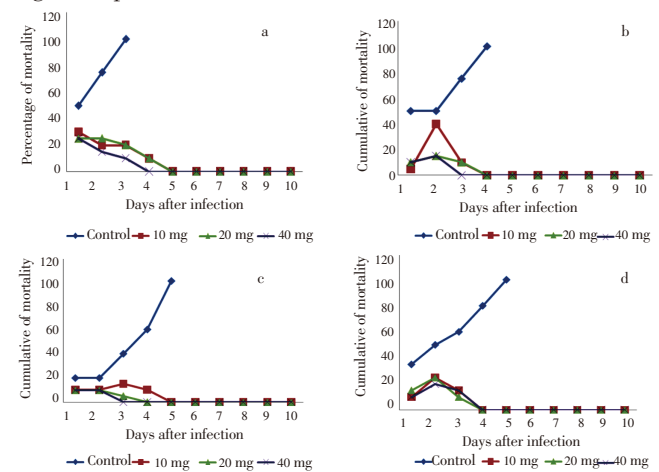


Figure 3. Disease resistant experiment of *A. marina* extract against the four types of bacterial isolates. a: *Pseudomonas fluorescens*, b: *P. aeruginosa*, c: *V. anguillarum*, d: *V. parahaemolyticus*

4. Discussion

Maintaining tanks with live marine fish and invertebrates as pets and a hobby has become a popular activity in the recent past and is growing at a global scale. Bacterial infections have become a chronic problem in hatchery operations as well as export and import establishments of marine ornamental fish, causing hundreds of thousands of dollars in monetary loss and unnecessary loss of animal life. Yet the symptoms and treatment protocols for these diseases have not been standardized.

The present study looks in to developing components of sustainable technology in the production of marine ornamental fish. Four types of bacteria were isolated from infected marine ornamental fish and were identified to be *P. fluorescens*, *P. aeruginosa*, *V. anguillarum* and *V. parahaemolyticus*. These results conform to earlier reports that a majority of bacterial infections in fish are caused by Gram-negative organisms and includes the following pathogenic genera: *Aeromonas*, *Citrobacter*, *Edwardsiella*, *Flavobacterium* (flexibacter), *Pseudomonas*, and *Vibrio*. A study of literature on previous research work shows that *Vibrio alginoliticus*, *Pseudomonas* sp., *Aeromonas hydrophila* are the predominant bacterial pathogens causing diseases in ornamental fish[12]. The present study suggests that the underlying reason for the occurrence of mass mortalities in the marine ornamental fish hatchery and aquariums is mostly bacterial infections. Further, it was confirmed that these fish were affected with infections of *P. fluorescens*, *P. aeruginosa*, *V. anguillarum* and *V. parahaemolyticus*.

The infected fish showed various symptoms including haemorrhagic lesions on the skin and at the base of fins. Similar symptoms were previously reported in Tench fry,

Silver carp and bighead as caused by *P. fluorescens*[15]. Among the *Pseudomonads*, *P. aeruginosa* and *P. fluorescens* have been considered as opportunistic pathogenic species in aquaculture[16]. However, other species of the genus may also induce serious infection like *Pseudomonas putida* infection in Rainbow trout[17], *Pseudomonas anguilliseptica* in Eel, *Anguilla japonica*[18], *Pseudomonas chlororaphis* in Amago trout, *Oncorhynchus rhodurus*[19].

The re-infection study (Koch's postulate experiment) was carried out to assess the virulence and pathogenicity of the bacteria under study. In this experiment, fish were challenged with bacterial isolates previously isolated from various infected marine fish. The challenged fish showed symptoms characteristic to the same pathogens used in the experiment. The bacteria were isolated from these fish. Based on this fact, the bacterial isolates were classified as strict pathogens and opportunistic pathogens. Externally affected fish had hemorrhages at the base of fins, mouth and skin muscles with fading pigments. The internal symptoms included an enlarged spleen. The bacterium, *V. anguillarum*, was isolated from infected fish showing fading of skin coloration, erythema (bloody blotches) at the base of the fins and around the mouth[20].

The occurrence of bacterial strains associated with fish diseases that show resistance to commonly used antibiotics is a worldwide problem in aquaculture. This phenomenon has received considerable attention in the recent years and its importance continues to increase due to the absence of a methodology for more effective and safer use of antibiotics[21].

Use of herbal medicinal preparations as antimicrobial agents to combat this situation can become a sustainable solution for this problem. These traditional systems of health care and longevity[22] are still being rejected by many due to a lack of standardization of the procedures and methods of their use[23]. To our knowledge, no studies have focused on the methodology of preparation, optimizing dosages, duration of effective treatment and on the whole, effectiveness of mangrove leaf extracts in the management of marine ornamental fish production. Therefore, the present work has attempted to deal with this need of information on the effect of mangrove extracts on the innate immune and disease resistance responses of clownfish. Based on previous reports[24], levels of 10, 20 and 40 mg/kg of mangrove extract was incorporated in to feed for measuring the its effect on the immune response and disease resistance of clownfish.

Molecules derived from natural products have had an excellent record of providing novel chemical structures for the development of new therapeutic agents. Many of the world's most valuable and successful medicines have been derived from sources in nature. An antimicrobial agent originating from marine halophytes is an immediate necessity in developing novel marine pharmaceuticals. Literature on antibacterial studies of natural product on fish pathogens is comparatively rare[25].

Five different extracts of two mangrove herbs were screened for their inhibiting activity against four different bacterial pathogens in the present study. Among them, methanol

extract of *A. marina* effectively controlled the growth of all four bacterial isolates used in the current experiment. This complies with the results obtained by earlier report[26], who used extracts of the plants *Murraya koenigii*, *Psoralea corylifolia* and *Quercus infectoria* were screened against the bacterial pathogens such as *P. aeruginosa*, *Staphylococcus aureus* and *Vibrio harveyi* isolated from infected Indian white shrimp *Fenneropenaeus indicus*. They found them to have a strong antimicrobial activity against these isolates. Kathiresan[7] have showed that methanolic extracts of *Eucalyptus globules*, *Punica granatum*, *Artemisia mozicana*, and *Bovvonia arborea* possessed strong *in vitro* antibacterial activity against *Staphylococcus aureus*, *E. coli*, *Pseudomonas* sp., and *Candida* sp.

Dhayanithi et al[10] have recently reported that methanol and ethanol extracts of neem, *Azadirachta indica* express good *in vitro* activity against *Aeromonas hydrophila*, *Enterobacter* sp., *E. coli*, *P. aeruginosa*, *Proteus* sp., *Streptococcus* sp., *Vibrio cholerae*, *V. alginolyticus*, *V. parahaemolyticus* and *Yersinia enterocolitica*.

Developing challenge models is one of the first steps in vaccine or pharmaceutical development for animal diseases. Fish can be infected by bath with a chosen bacterial concentration or by introduction of diseased fish. It should also be noted that in some studies no difference in mortality rates was observed at the end of the experiments between control and experiment groups through intracoelomic challenge methods[27]. Since, the marine ornamental fish are small in size, exposure by prolonged bath has been standardized as the challenge route. The present experiment determined a challenge model for *P. fluorescens*, *P. aeruginosa*, *V. anguillarum* and *V. parahaemolyticus* against Clownfish. The effective dose was standardized as 1×10^6 , 1×10^7 and 1×10^8 colony forming units (CFU)/mL necessary to cause 60% to 90% mortality in the challenged group of fish. It is concluded that the 4 bacterial isolates possess pathogenic effects and cause mortality in the fish challenged with them. In most of the studies, a lethal dose of bacteria/CFUs that should kill 90% of the fish population is chosen as target bacteria/CFU dose; however these numbers do vary among different research groups[27,28].

Immunostimulants and adjuvants used in fish vaccines are of interest, as they offer an alternative to drugs, chemicals and antibiotics currently used in fish culture to control diseases. Medicinal herbs as immunostimulants in aquaculture have received increased attention in the last two decades, not only for their immune stimulating functions, but also for their growth promoting effects with hardly any side effects. It has been shown that immunostimulants of herbal origin are capable of enhancing immune responses and reduce mortality due to viral, bacterial and/or parasitic infections in Carp[29,30].

The results of the present study showed that experimental fish fed with 10, 20 and 40 mg/kg dose of the methanol extracts of *A. marina* significantly enhanced leucocytes amount on the second week. Leukocyte cells are the most important cellular components of the innate immune system of fish[31].

Their phagocytal activity is a primitive defense mechanism and an important characteristic of the nonspecific immune system^[32]. Herbal medicine extracts can also enhance phagocytosis in various fish species^[33].

The mortality in tested groups was significantly reduced to 80%, 75% and 85% against *P. fluorescens*, 60%, 85%, 85% against *P. aeruginosa*, 85%, 90%, 90% against *V. anguillarum* and 75%, 75%, 80% against *V. parahaemolyticus* where, the fish were fed with 10, 20 and 40 mg/kg respectively. Mortality in the untreated (control) group was 90%. Hence, it is proposed that mortality in the herbal feed supplement group had significantly reduced in comparison with the control group. Previous studies in this line also show that dietary supplementation and intraperitoneal injection of herbal leaves extracts were enhanced the resistance against pathogenic bacteria, *Aeromonas hydrophila*^[33]. The present finding is in agreement with the results of Harikrishnan et al.^[34] in *Paralichthys olivaceus* fed with a diet containing extract of *Punica granatum* leaf powder and with Harikrishnan^[35] on tiger shrimp with the fraction of *Solanum nigrum*. They also demonstrated that the administration of this fraction within 6 and 3 d prior to an intraperitoneal challenge with *Eclipta tarda* significantly increased the survival rate in the tested fish. The resistance against *Aeromonas hydrophila* was enhanced in *Labeo rohita*, fed with 0.5% of achyranthes^[30]. It is important to estimate the increased protection in the ethanol and methanol triherbal extract treated fish to determine the efficacy of an immunostimulant.

In this study the challenged groups showed reduced mortality when compared with the control group. These results indicate that methanol extract of *A. marina* was capable of activating the immune system of clownfish. This has a promising role in ornamental aquaculture to prevent diseases and outbreaks. Further investigation of the use of mangrove extract preparations for disease prevention in aquaculture is necessary.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

Authors are thankful to the authorities of the Annamalai University for providing facilities. The authors are thankful to the University Grants Commission (UGC), New Delhi for providing the financial assistance. Reference No. U.G.C. No. 33–384/2007 (SR).

Comments

Background

Antibacterial activity of mangrove (*A. marina* and *R. apiculata*) leaf extract against fish pathogens *P. fluorescens*,

P. aeruginosa, *V. parahaemolyticus*, and *V. anguillarum*. Feed trial experiments with methanol leaf extract of *A. marina* in marine ornamental fish to increases fish survival and reduce their susceptibility to infections.

Research frontiers

Bacterial infections in marine fishes and mostly ornamental fishes are serious issue. And in aquaculture, chemical antibiotics are completely banned. So here they tried mangrove extracts to inhibit bacterial pathogens and they made feed experimental trials.

Related reports

Dhayanithi et al (2012), already studied that *Vibrio alginoliticus*, *Pseudomonas* sp., *Aeromonas hydrophila* are the predominant bacterial pathogens causing diseases in ornamental fish. Velmurugan and Citarasu (2010), extracted the plants *Murraya koenigi*, *Psoralea corylifolia* and *Quercus infectoria* and were screened against the bacterial pathogens such as *P. aeruginosa*, *Staphylococcus aureus* and *Vibrio harveyi*. Citarasu (2010) stated that immunostimulants of herbal origin are capable of enhancing immune responses and reduce mortality due to viral, bacterial and/or parasitic infections in carp.

Innovations and breakthroughs

They mixed that mangrove extract *A. marina* in fish feed and studied their immune response and disease resistant are innovative. And they stated that this extract was capable of activating the immune system of clownfish.

Applications

In this research they are experimenting mangrove extract for inducing fish immune response including with antibacterial activity.

Peer review

It is good to concentrate on all aspect of fish infection by isolation disease causing bacterial pathogens, control of infection by mangrove extract and induction of immune response in fish by feed. Moreover, they checked its immune response in different days.

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