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Antimicrobial activity of marine resources against the fish pathogens isolated from *Mugil cephalus*

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INTRODUCTION

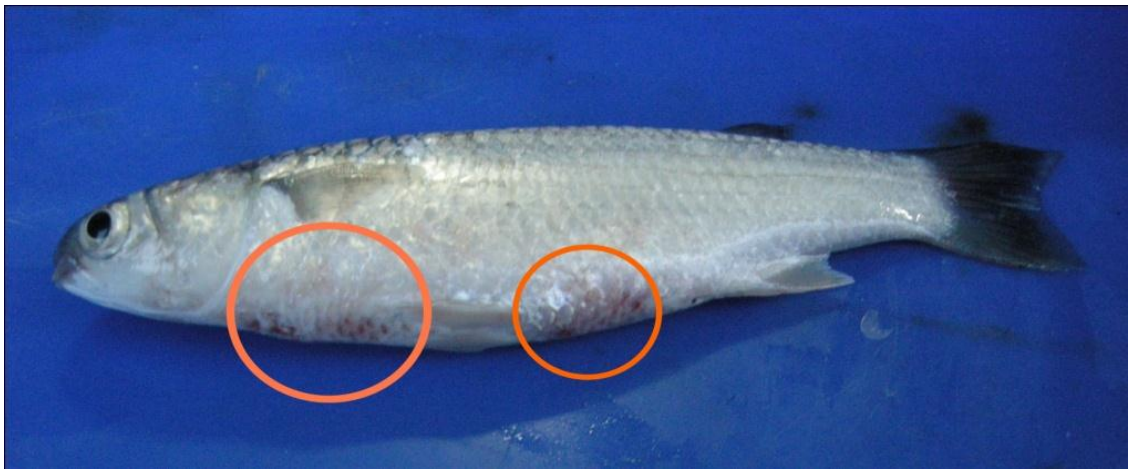
Fish and Fishery products have much economic activity all over the world. Generally, fishes are one of the main food components for humans for its high nutritional value (Eze *et al.,* 2011). However, the bacterial fish diseases constitute major challenges for the sustainable aquaculture production. Particularly, the fin rot diseases, tail rot diseases, skin ulcer, hemorrhagic septicemia and gall bladder, enteric septicemia, furunculosis, ulcerative disease etc., have been caused by bacteria (Eze *et al.,* 2011). The non indigenous pathogens include, *Clostridium botulinum, Listeria, monocytogenes, Staphylococcus aureus, Salmonella* sp, *Shigalla s*p, *Escherichia coli*, etc. which contaminate the fishes and its habitats. The indigenous bacterial pathogens *viz., Vibrio* sp, and *Aeromonas* sp. etc are naturally living in the fish habitat (Eze *et al.,* 2011). Nevertheless, the exploration of antimicrobial therapeutics from marine resources for disease free fish culture *Mugil cephalus* are not attempted so far. In this connection, the present study has made an attempt to findout the potential leads for the development of antimicrobial therapeutics for disease free fish culture.

MATERIALS AND METHODS

Isolation and identification of bacterial pathogens from infected fish

Mugil cephalus L. is one of the most important estuarine fish in the world by virtue of its wide distribution, commercial importance and consumer preference. It is widely distributed in coastal waters and estuarine of the tropical and sub tropical zone of all seas (Thomson, 1963). Studies on the various aspects of biology and fishery of this species revealed the importance as a food fish and its role in the economy of various regions. Mullet are dark above (blue to green with some brown) and silvery on the sides. Conspicuous stripes on sides are formed from dark spots at the base of scales. Dorsal and anal fins are unscaled. The second dorsal fin begins directly above the point where the anal fin begins. The anal fin has 3 spines and 8 soft rays; juveniles have 2 spines and 9 soft rays (Robins *et al.,* 1986).

The infected fish *Mugil cephalus* were collected from ---------- coast, Tamil Nadu, India (Lat. 9° 44’ 12’’ N and Long. 79° 10’ 14’’ E). The infected parts of the collected fish were aseptically removed by using sterile forceps and scissors.



The collected sample was homogenized in sterile mortar and pestle using phosphate buffer as solvent. The homogenized samples were serially diluted up to 10^6. One millilitre of diluted sample from each dilution was poured into sterile petri dishes followed by sterilized molten agar medium (HiMedia Laboratories Private Limited, Mumbai) *viz.,* Tergitol 7 agar, Barid parker agar, *Salmonella Shigella* agar, TCBS agar, *Aeromonas* isolation medium, *Bacillus cereus* agar base, Caprylate Thallous agar (CT agar). After solidification, all the plates were inverted and incubated in a thermostat incubator to allow the growth of bacteria. Triplicates were maintained for each dilution and for each medium. After incubation, the colonies appeared on the respective agar plates which showed morphologically identical to the colonies

appeared in HiMedia Laboratory Manual, Mumbai were restreaked thrice in the respective agar medium for pure culture. Finally, the cultures were stored in respective agar slants for further use and identification.

Re-Infection Studies

Grey mullet (*M. cephalus* L.) of 20g average weight were obtained from ---------- ---------- coastal region and it certified as diseases free. The fish were maintained in aquaria with aerated seawater at 28°C for 2 weeks and fed with artificial formulated feed pellet in order to adapt to laboratory conditions. All fish were anaesthetized with MS-222 (10% w/v) and then injected subcutaneously with 0.1 ml of a bacterial suspension (10^6 cells) diluted with phosphate buffered saline (PBS). In each treatment, twenty fishes were injected with five isolated pathogenic strains and twenty other fish were injected with 0.1 ml of sterile PBS as controls. The fish were then replaced into the aquaria in seven days and held under the same conditions before injection. The morbidity and death of the fish were monitored daily for 7 days and the virulence effect of isolated fish pathogens were calculated by the Kozinska *et al.* (2002).

Collection of marine resources:

Different mangrove plant species *viz*., *Avicennia marina, Acanthus ilicifolius, Bruguiera cylindrica, Ceriops decandra, Excoecaria agallocha, Lumnitzera racemosa, Rhizophora apiculata* and *Rhizophora mucronata* with different parts (Leaf, collar, hypocotyl, bark, stilt root, stem and flower) and ten different whole seaweeds extracts *viz., Enteromorpha intestinalis, Ulva lactuca, Acanthopora spicifera, Caulerpa racemosa, Sargassum microystum, Dictyota dichotoma, C. scalpelliformis, Gracilaria corticata, Turbinaria decurrens* and *C. toxifolia* and four seagrass species (*Syringodium isoetifolium, Cymodocea serrulata, Halophila ovalis* and *Enhalus sp.*) with two different parts (Leaf and Root) were washed thrice in sterile distilled water to remove adhering soil particles and salts and were tested for the antibacterial activity.

Identification of Marine Halophytes

The taxonomic identifies of the mangrove plants, seaweeds and seagrass taxonomic identities were confirmed by standard monograph. The plant species and parts used for the present study are mentioned in Tables 1-3.

Table 1. Name of the Mangrove plants and their plant parts chosen for antibacterial activity

Plant species	**Parts used**
Bruguiera cylindrica	Hypocotyl
Ceriops decandra	Leaf, collar and hypocotyl
Lumintzera recemosa	Leaf
Rhizophora apiculata	Bark, collar, hypocotyl
Rhizophora muronata	Bark, collar, hypocotyl and flower
Avicennia marina	bark and flower
Acanthus ilicifolius	Leaf
Excoecaria agallocha	Leaf

Table 2. Name of the seaweeds chosen for the antibacterial activity

Plant species	**Parts used**
Enteromorpha intestinalis	Whole
Ulva lactuca	Whole
Acanthopora spicifera	Whole
Caulerpa racemosa	Whole
Sargassum microystum	Whole
Dictyota dichotoma	Whole
C.scalpelliformis	Whole
Gracilaria corticata	Whole
Turbinaria decurrens	Whole
C.toxifolia	Whole

Table 3. Name of the seagrasses chosen for the antibacteial activity

Plant species	**Parts used**
Syringodium isoetifolium	Leaf and Root
Cymodocea serrulata	Leaf and Root
Halophila ovalis	Leaf
Enhalus sps.	Leaf

Extraction of bioactive compounds:

About 500 g of marine halophytic samples were subjected for size reduction to coarse powder. The powder was defatted with petroleum ether (50-60°C) and then extracted with 1 L of ethanol and water mixture (3:1) by percolation method. The ethanolic extract was concentrated by using rotary flash evaporator (Superfit , India) to get the fine residues and further lyophilised (BENCHTOP 2K) to remove the excess organic residues. The residual extract was further used for the screening of antibacterial activity.

RESULTS AND DISCUSSION

The present study made an attempt to isolate the bacterial fish pathogens from infected fish species *Mugil cephalus*. Five morphologically different strains were identified (Table 4) and the isolated bacterial fish pathogens were marked as 1-5.

Table 4: Morphological characteristics of isolated fish bacterial pathogens

S. No.	Form	Elevation	Margin	Colour of the Colony
1.	Circular	Flat	Undulate	Creamy white
2.	Circular	Flat	Undulate	Creamy
3.	Irregular	Flat	Undulate	Creamy
4.	Irregular	Flat	Undulate	Creamy
5.	Circular	Flat	Undulate	Creamy

The present study also made an attempt for re-infection studies in healthy fishes to prove the Koch's postulates and the results revealed that, the maximum (70%) level of strong virulence was identified with 01 infected pathogens, further the minimum percentage of week virulence was identified with 03 (30%) and none of the pathogens were showed avirulence in fishes. There was no death or sign of disease in control group (Table 5).

Table 5: Virulence for *Mugil cephalus* infected with fish pathogens (n=20)

Level	% of strains				
	01	**02**	**03**	**04**	**05**
Strongly virulent	70% (14)	-	-	-	-
Virulent	30% (6)	55% (11)	60% (12)	60% (12)	55% (11)
Weakly virulent	-	45% (10)	30% (6)	40% (8)	45% (9)
Avirulent	-	-	-	-	-

Values in parenthesis showed number of animals

The isolated fish pathogens were identified as *E.coli, Citrobacter freundii, Vibrio harveyi, Staphyllococcus aureus* and *Shigella flexneri.* Previously, Sujatha *et al.,* (2011) isolated *E. coli, Salmonella typhi, Pseudomonas aeruginosa, Vibrio cholerae and Shigella Dysenteriae* in *Megalaspis cordyla* and *Priacanthus hamrur* from habitat. Moreover, El-Hady and El-khatib isolated *F.columnare, Ps.fluorescens, V.harvey*i, and *S.aureus* from the *Oreochromis niloticus*. Several authors reported that, most of the edible fishes such as *Mugil cephalus* and *Oreochromis* sp., were mostly infected with the bacterial pathogens *viz., Streptococcus, Mycobacterium, Vibrio* sp., *Bacillus subtilis* (Hubbert, 1989; Nigrelli and Vogel, 1963; Anon, 1992; Al-Sunaiher *et al.* 2010; Nagvenkar, 2006; Ampofo and Clerk, 2010). Generally, the fish diseases are caused by the water contamination and toxins (Chen *et al.,* 2010). In addition that, this bacterial pathogenic diversity was frequently encountered with aquaculture (Levin and Bull, 2004).

Bacteria are important pathogens for both cultivated and wild fish and are responsible for serious economic losses. Some bacteria cause only surface diseases as skin or gill infections, especially *flexibacteria*, but some inflict systemic disease (Inglis *et al.,* 2001). The prevalent fish diseases in fish farms are usually initiated by bacteria. There are basically two types of bacteria producing disease obligate pathogens and facultative pathogens. Facultative pathogens can survive indefinitely in water and, when environmental conditions are conducive, infectious fish diseases may spread. Many potentially pathogenic bacteria of fish normally exist in a commensal association with the host or live free in the environment. Both these types of bacteria become pathogenic when the fish is immuno compromised by some form of stressor

(Kirjusina *et al.,* 2007). Fish bacterial infections can occur as a bacteremia, which implies the presence of bacterial organisms in the bloodstream without clinical signs.

Disease control and management in fish culture systems has become one of the major problems as the shrimp bacterial pathogens are becoming more and more resistant to the conventional therapeutic drugs used in the industry and thus the shrimp farmers suffer from heavy financial losses. So, there is a need for the search of novel bio-active compounds with therapeutic potential which can be used to control the bacterial diseases in an eco-friendly manner (Patil *et al.*, 2001).

Plants that have been adapted to thrive greater than 0.5% NaCl are called halophytes *eg*. mangroves and seaweeds. Mangroves are woody plants that grow at the interface between land and sea in tropical and sub-tropical latitudes, which are often classified either as excretives and succumbers or excluders and includers. Excretives / excluders have glandular cells capable of secreting excess salts from plant organs *eg*. *Avicennia germinas.* Succumbers / includers used to accumulate water within large vacuoles to minimize salt toxicity *eg*. *Salicornia, Suaeda, Sesuvium portulacastrum, Allenrolfea, Arthrocremum, Halimione.* Seaweeds are floating submerged plants of shallow marine meadow, having salt tolerance, because the osmolarity of cytoplasm is adjusted to match the osmolarity of the seawater, so that desiccation of the plant does not occur. Marine halophytes have been traditionally known for treating variety of biological activity. But, few studies were done on infectious diseases. In view of this, the present study is aimed to initiate to screen the marine halophytes against bacterial fish pathogens. Based on this, all the identified bacterial fish pathogens were subjected for the antibacterial activity with the crude extracts obtained from mangrove plants, seaweeds and seagrasses by disc diffusion assay. The values were interpreted as zone of inhibition in millimeter in diameter. The antibacterial activity reveals that, among the mangrove plant species, the crude extracts from the bark of *Rhizophora mucronata, R. apiculata,* the collar from *R. mucronata* and *Ceriops decandra* showed maximum antibacterial activity (9.0mm dia.) against the bacterial fish pathogens *E.coli* and the bark from *R. apiculata* showed maximum antibacterial activity (9.0mm dia) against the bacterial fish pathogens *Shigella flexnerii* (Table 6).

Table 6. Antibacterial activity of mangrove extracts against isolated fish pathogens

Name of the Species	**Parts**	***E. coli***	***Citrobacter freundii***	***Vibrio harvey*i**	***Staphyllococcus aureus***	***Shigella flexneri***
		Zone of inhibition in millimeter diameter				
Rhizophora mucronata	Bark	9.0	8.0	7.0	8.0	8.0
Rhizophora mucronata	Hypocotyl	8.0	-	-	6.0	-
Rhizophora mucronata	Still Root	7.0	6.0	-	6.0	-
Rhizophora mucronata	Collar	9.0	7.0	7.0	8.0	8.0
Ceriops decandra	leaf	6.0	-	-	6.0	-
Ceriops decandra	Collar	9.0	6.0	8.0	6.0	6.0
Ceriops decandra	Hypocotyl	7.0	6.0	6.0	6.0	6.0
Bruguiera cylindrica	Hypocotyl	6.0	-	-	7.0	6.0
Lumnitzera racemosa	Leaf	8.0	7.0	6.0	6.0	8.0
Rhizophora apiculata	Bark	9.0	8.0	7.0	8.0	9.0
Rhizophora apiculata	Hypocotyl	6.0	-	7.0	-	-
Rhizophora apiculata	Collar	6.0	-	-	6.0	-
Avicennia marina	Bark	6.0	-	-	6.0	-
Avicennia marina	Flower	7.0	6.0	7.0	6.0	6.0
Acanthus ilicifolius	Leaf	7.0	6.0	-	6.0	6.0
Excoecaria agallocha	Leaf	6.0	-	-	-	6.0

Values between plant species and plant parts area found Significant at $p<0.05$ level

Likewise, the crude extract obtained from the seaweed *Ulva lactuca* showed maximum antibacterial activity (9.0mm dia.) against *E.coli*, the *Acanthopora spicifera* showed maximum antibactetial activity (8.0mm dia.) against *Vibrio harveyi,* the *Caulerpa racemosa* showed maximum antibacterial activity against *E.coli* and *Shigella flexnerii* (9mm dia.), *Citrobacter freundi* and *Staphyllococcus aureus* (8.0mm dia), the *Caulerpa toxifolia* showed maximum antibacterial activity against *E.coli* (9mm dia.), *Staphyllococcus aureus* (8.0mm dia) and *Shigella flexneri* (8.0mm dia.) (Table 7). Besides that, the crude extract from the seagrass *Halophila ovalis* showed maximum antibactetial activity (7.0mm dia.) against *Vibrio harveyi* (Table 8).

Table 7. Antibacterial activity of seaweed extracts against isolated fish pathogens

Name of the Species	**Parts**	***E. coli***	***Citrobacter freundii***	***Vibrio harveyi***	***Staphyllococcus aureus***	***Shigella flexneri***
		Zone of inhibition in millimeter diameter				
Enteromorpha intestinalis	Whole	7.0	6.0	6.0	7.0	6.0
Ulva lactuca	Whole	9.0	-	6.0	6.0	-
Acanthopora spicifera	Whole	7.0	6.0	8.0	7.0	6.0
Caulerpa racemosa	Whole	9.0	8.0	7.0	8.0	9.0
Sargassum microystum	Whole	6.0	-	7.0	-	-
Dictyota dichotoma	Whole	6.0	-	-	6.0	-
C. scalpelliformis	Whole	6.0	-	-	6.0	-
Gracilaria corticata	Whole	7.0	6.0	7.0	6.0	6.0
Turbinaria decurrens	Whole	7.0	6.0	6.0	6.0	6.0
C. toxifolia	Whole	9.0	6.0	7.0	8.0	8.0

Values between plant species and plant parts area found Significant at $p<0.05$ level

Table 8. Antibacterial activity of seagrasses extracts against isolated fish pathogens

Name of the Species	Parts	*E. coli*	*Citrobacter freundii*	*Vibrio harvey*i	*Staphyllococcus aureus*	*Shigella flexneri*
		Zone of inhibition in millimeter diameter				
Syringodium isoetifolium	Leaf	6.0	-	-	6.0	-
Syringodium isoetifolium	Root	-	-	-	-	-
Cymodocea serrulata	Leaf	6.0	-	6.0	-	-
Cymodocea serrulata	Root	-	-	-	-	-
Halophila ovalis	Leaf	6.0	-	7.0	6.0	-
Enhalus sp.,	Leaf	6.0	-	-	6.0	-

Values between plant species and plant parts area found Significant at $p<0.05$ level

It reveals that, all the selected marine halophytes showed various ranges of biological activities and the maximum percentage of biological activity was recorded with the bark of *R. mucronata*. It might be due to the presence of secondary metabolites such as tannins, sterols, anthocyanins (Giffoni *et al.*, 2009), poly phenol (Rey *et al.,* 2000), triterpenoides, betulinic acid (Kamaraj *et al*., 2008), diterpeniod (Rahuman *et al*, 2008), saponin (Perich *et al.*, 1995), monoterpene and lectins (Ayo, 2010) terpeniod (Domenech *et al,* 2009), coumarin, isoflavonoidis (Joseph *et al.*, 2009), alkaloids (Lee, 2001) and polysaccharides (Qiu *et al*, 2007) agents. Similar reports are also reported with the non halophytic plant extracts *viz, Toddalia asiatica* (Bandara *et al*., 1990), *Aalypha indica* (Govindarajan*,et al.,* 2008), *Ocimum canum, O.sanctum* (Kamaraj *et al*.2008), *Cryptomeria japonica* (Cheng *et al*.2008), *Spilanthes calva* (Pandey *et al* 2007), *Ajuga remota* (Sharma *et al*, 2005), *Leucas aspera* (Bagavan,*et al*, 2008), and *Abutilon indicum, Jatropha gossypifolia, Euphorbia thymifolia* (Rahuman *et al*, 2008). Further attempt has been initiated to findout the efficacy of the most promising extracts for the treatment of fish diseases in fish under field conditions.

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