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The Antibacterial and Antibiotic Potentiating Activity of Marine Sponge *Ircinia fusca* Against Fish Pathogens

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Abstract

Aquaculture is a lucrative industry in several Asian countries and is a rapidly developing sector in the Indian subcontinent mainly along the coastal regions. The major constraint as well as limiting factor for sustainable fish production is the disease associated with cultured fishes. Hence, it is need of the hour to develop a cost-effective treatment for preventing or controlling disease outbreaks. The marine environment is a rich source that holds a boundless collection of unexplored bioactive compounds. Among the marine populations, sponges remain unique because of the presence of elite bioactive compounds in them. Therefore, in the current study, due emphasis was given to discovering the eco-friendly utilization of marine sponge *Ircinia fusca* for developing novel bioactive compounds against fish diseases. Antibacterial study of *Ircinia fusca* was carried out by well diffusion method, minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) against five fish pathogens (*Citrobacter freundii*, *Flavobacterium spp, Aeromonas hydrophila, Vibrio harveyi*, and *Vibrio parahaemolyticus*). Methanolic extract of *Ircinia fusca* showed potent antibacterial and antibiotic potentiating activity against fish pathogens. The present study reveals that *Ircinia fusca* is a potent producer of antimicrobial agents which can be developed into a new antibiotic drug. Further research should be done to develop these potential candidates for aquaculture and pharmaceutical sectors in the future.

Keywords: Antibacterial activity; Antibiotic potentiating activity; Marine sponge; *Ircinia fusca*; Fish pathogens

Introduction

Aquaculture, including the culture of both animals and plants in marine, brackish and freshwater environments is one of the booming industries globally. The global fish production in 2018 is estimated to be about 179 million tons [1] including the ornamental fish culture. India is one of the major contributors to world production, currently ranks second in aquaculture production in the world [2]. India is a rich resource of both freshwater and marine ornamental fishes. However, India's contribution to the international ornamental fish trade is negligible and Ranking 31st in the world [3].

The major challenge associated with fish production is the outbreak of diseases, which can cause a huge loss not only in edible fish production but also in the ornamental sector. Chemicals and chemotherapeutic agents such as antibiotics are the conventional methods of treating these diseases. However, they are effective agents for the treatment of bacterial diseases but can result in the

accumulation of drugs in aquatic habitat led to the formation of antibiotic-resistant bacteria, the buildup of antibiotics inside fishes, termination of beneficial microorganisms of the digestive tract and modifications in the microbiota of the aquatic environment [4, 5]. Methods to reduce antibiotics in the aquatic environment are still a topic to research. Approaches like natural biodegradation of antibiotics are performed for the eradication of these problems but, they are time-consuming and not an adequate option for their elimination [6].

To date, there is no effective system to control the diseases and their impacts on the environment. Hence, there is an urge to discover new strategies to control bacterial infection to sustain the aquaculture industry. Our oceans are unexplored treasures of many natural bioactive compounds, showing potential effects against many bacterial infections. In this study, we aim to develop new natural alternatives to control the pathogenic bacterial infection in fishes, which can contribute to the development of aquaculture and the ornamental industry.

Materials and Methods

Sampling and identification: Sponge sample was collected on April, 2019 from Kovalam beach situated at 8° 22' 0.01" North latitude and 76° 59' 48.01" East longitude in Thiruvananthapuram district, Kerala, India. The sponge surfaces, as well as pores, were cleaned with sterile water for it was contaminated with small marine organisms, sand and other debris. The sponge was dried, chopped and stored in a sterile plastic bag at -20℃ [7]. Taxonomic identification of the sample was done by standard identification procedure using spicules [8, 9]. The spicules were separated using nitric acid and using identification keys the sample was identified as *Ircinia fusca.*

Extraction: The dried sponge was extracted using solvents: hexane, chloroform, ethyl acetate and methanol for 48hr and filtered with Whatman's No. 1 filter paper. After the extraction process, the solvents can be gently and efficiently removed and recovered with the help of a rotary evaporator so that the extract obtained will be more concentrated and dried at room temperature. Dried samples were weighed and redissolved in dimethyl sulfoxide (DMSO) to attain a final concentration of 1mg/ml and stored at low temperature (-20°C) until further use.

Microorganisms: The bacterial strains were purchased from Microbial Type Culture Collection and Gene Bank (MTCC). *Aeromonas hydrophila subsp. hydrophila* (MTCC 1739), *Vibrio harveyi* (MTCC 3438), *Vibrio parahaemolyticus* (MTCC 451), *Citrobacter freundii* (MTCC 1658), and *Flavobacterium sp.* (MTCC 2495) are the fish pathogens used for the experiments. All the bacterial strains were maintained on agar slants and stored at 4°C until use. The bacterial strains were sub-cultured in Muller Hinton Agar (MHA) at 37°C for 24 hr prior to the antibacterial assays.

Chemicals: Medium: Muller Hinton Agar, Nutrient Agar, Nutrient Broth, Muller Hinton broth were procured from Hi-media India Pvt. Ltd., Vadhani Industrial Estate, Mumbai, India.

Antibacterial activity

Antibacterial assay (well diffusion method): The microbial growth inhibitory potential of different extracts of sponges were screened using the agar well diffusion method [10] and [11]. The pre-inoculated cultures were made to the turbidity of 0.5 McFarland standard turbidity (10^6 CFU/ml). These inoculums were uniformly spread on the MH agar plate using a sterile L rod. Using a sterile cork borer, wells were made into the media and 100 µl of the extract was transferred into the well. Tetracycline and 5% DMSO were used as positive drug control and solvent control respectively. The plates are incubated in an incubator at 37°C for 24 hours. After incubation, the zone of inhibitions was measured in mm. The experiments were performed in triplicates for each extract.

Minimum inhibitory concentration assay: Extracts that displayed significant activity (IZD > 8mm) were tested for MIC using 96 well

micro titer plates by microdilution technique. The MIC value has been defined as the lowest concentration required to inhibit the growth of bacteria [12]. MIC assay evaluates the ability of the extract to prevent microbial growth. 100µl of Muller-Hinton broth (MH broth) was added into all 8 wells (1-8 rows) in the microtiter plate and 100µl of the extract was added to the first well. The first well should be mixed thoroughly and serially diluted up to 8 wells and discard 100µl from the 8th well. Then add 5µl of bacterial culture 0.5 McFarland standard turbidity (10^6 CFU/ml) in all the wells. 10th and 11th wells are taken as media control and culture control. The plates were incubated overnight at 37ºC. The results were observed by physical observation and p-iodonitrotetrazolium chloride (0.2 mg/ml) was added to evaluate bacterial growth by forming purple coloration and the experiment was performed in triplicates.

Minimum bactericidal concentration: Extracts that displayed significant MIC values (below 100µg/ml) were further evaluated for their minimum bactericidal concentration (MBC). MBC is the lowest concentration required to eliminate 99.9% of the tested microorganisms. To evaluate the bactericidal concentration of the extracts, a loop full of culture medium with no visible growth is streaked on agar plates. The culture plates were kept overnight in an incubator at 37ºC. After incubation, the number of bacterial colonies were calculated. The extract can be considered bactericidal, when MIC = MBC or if MBC = 1, 2 or 3 dilutions above MIC [13].

Antibiotic potentiating assay: The synergistic effect of the extract with conventional antibiotics was evaluated by antibiotic potentiating assay [14]. A preliminary experiment using *Aeromonas hydrophila, Vibrio harveyi, and Vibrio parahaemolyticus* with tetracyclin was performed by microdilution assay to determine the sub-inhibitory MIC concentration to execute the experiment. Briefly, antibiotic potentiating activity was determined by testing the sponge extract at sub-inhibitory concentrations i.e., MIC/2 in association with standard antibiotics against the fish pathogens. All the experiments were performed in triplicates and the Fractional Inhibitory Concentration (FIC) was determined as follows:

$$\text{Fractional Inhibitory Concentration (FIC)} = MIC_{\text{Antibiotic + extract}} / MIC_{\text{Antibiotic alone}}$$

The experimental results were interpreted as follows: FIC ≤ 0.5 (synergistic), > 0.5 to 4 (indifferent) or > 4 (antagonistic)

Chemical characterisation: Methanolic extracts of *Ircinia fusca* were re-dissolved in DMSO and was subjected to chemical characterisation according to the standard procedures of Harborne [15]

Result and Discussion

Recent scientific developments in the field of aquaculture helped in the better understanding of the aquatic systems which led

Table 1: Diameter of the zone of inhibition of different extracts of *Ircinia fusca* against fish pathogens.

	Hexane	Ethyl Acetate	Methanol	Chloroform	Standard Tetracyclin
Citrobacter freundii	12.33± 0.58	15.00± 0.00	15.67±0.58	14.67±0.58	16.67±0.58
Flavobacterium spp	15.00± 1.00	14.67±0.58	16.67±1.15	14.00±0.00	14.33±0.58
Aeromonas hydrophila	16.33± 0.58	16.67± 0.58	18.00±1.00	15.00±1.00	13.00±0.00
Vibrio harveyi	16.33± 0.58	11.33 ±0.58	15.67±0.58	15.33±0.58	19.67±0.58
Vibrio parahaemolyticus	14.00± 0.00	17.00±1.00	17.00±0.00	16.33±0.58	12.00±0.00

All the results were expressed as Mean ± S.D., n=3.

to the efficient management of the aquaculture sustainably [16]. Aquaculture plays a significant and promising role in providing employment, nutrition and food security in developing countries [17]. Outbreaks of bacterial infections are one of the major challenges which affect aquaculture systems resulting in low productivity and high economic loss. Inadequate use of antibiotics led to the development of multiple drug-resistant pathogens which added momentum to explore new antibacterial agents from natural sources for aquaculture systems [18]. Several studies have proposed using natural extracts against bacterial infections [19, 20]. Screening of marine sponges and their metabolites for antibacterial agents is a recent trend worldwide [21, 22]. Here, we screened the antibacterial activity and antibiotic potentiating activity of marine sponge *Ircinia fusca* collected from Vizhinjam coast, Kerala. The present study is the first report on the antibacterial activity of *Ircinia fusca* against fish pathogens.

Agar well diffusion is a commonly accepted method for evaluating the antibacterial activity of samples [23]. A clear growth inhibition zone of pathogens can be visualized and is measured in millimeters. Screening of antibacterial activity of the four extracts against five fish pathogens was performed by well diffusion assay. Methanolic extract displayed significant antibacterial activity compared with other extracts against fish pathogens. Methanolic extract of sponge *Ircinia fusca* shows the largest IZD of 18mm against the fish pathogen *Vibrio parahaemolyticus* (Table 1). Methanolic extract displayed higher antibacterial activity against *Flavobacterium spp* with an IZD value of 16.67 ± 1.15mm compared to standard tetracyclin (IZD of 14.33 ± 0.58mm). The increasing orders of antimicrobial activity of different extracts are so on: methanol extract > chloroform extract > ethyl acetate extract > hexane extract. Significant antibacterial activity against fish pathogens was displayed by methanolic extract, while hexane extract showed weaker activity. Gram-negative bacteria are found to be more resistant compared to Gram-positive bacterial strains due to their typical cell wall structure [24]. Our studies revealed that sponge extracts of *Ircinia fusca* exhibited stronger antibacterial activity against Gram-negative fish pathogens which might be attributed to the synergistic effect of several antibacterial compounds present in the methanolic fraction of *Ircinia fusca*. Annie Selva Sonia et al., [25] reported the antibacterial activity of five marine sponges collected from Kanyakumari against eight fish pathogens. The phytochemical analysis disclosed the presence of glycosides and carbohydrates as the active components of methanolic extract. Several studies have reported the antibacterial potential of glycosides [26, 27].

The methanolic extract which shows better antibacterial activity (IZD > 10mm) was chosen to determine Minimum Inhibitory Concentration (MIC) by microdilution method to search the therapeutic concentration of the active extract. Methanolic extract of *Ircinia fusca* exhibited potent antibacterial activity displaying MIC value of 37.5µg/ml against *Citrobacter freundii, Aeromonas hydrophila, Vibrio harveyi* and *Vibrio parahaemolyticus* (Table 2). For extracts, the antibacterial activity can be considered significant if MIC values is < 100 µg/ml and moderate if 100< MIC <625 µg/ml [28]. Najiah et al. [29] reported that methanolic extract of *S. aromaticum, P.odorata* and *M. koenigii* displayed the lowest MIC values indicating the bacteriostatic activity of these extracts against fish pathogens.

Table 2: Minimum inhibitory concentration of the active extract of *Ircinia fusca* against fish pathogens

	MIC of methanolic extract of *Ircinia fusca*
Citrobacter freundii	37.5µg/ml
Flavobacterium spp	75µg/ml
Aeromonas hydrophila	37.5µg/ml

Vibrio harveyi	37.5µg/ml
Vibrio parahaemolyticus	37.5µg/ml

Table 3: Minimum Bactericidal Concentration of the active extract of *Ircinia fusca* against fish pathogens

	MBC of methanolic extract of *Ircinia fusca*
Citrobacter freundii	150 µg/ml
Flavobacterium spp	-
Aeromonas hydrophila	37.5µg/ml
Vibrio harveyi	-
Vibrio parahaemolyticus	37.5µg/ml

The MBC is considered as the lowest concentration required for killing the microorganisms. The MBC was performed by culturing the microorganisms in agar plates, which showed complete inhibition of the visible growth in MIC test. Results showed that methanolic extract of the sponge *Ircinia fusca* displays bactericidal activities against *Aeromonas hydrophila* (37.5µg/ml), *Vibrio parahaemolyticus* (37.5µg/ml) and *Citrobacter freundii* (150µg/ml) (Table 3). The present study indicated that methanolic extract of *Ircinia fusca* might contain pharmacologically active antibacterial compounds against *Aeromonas hydrophila* and *Vibrio parahaemolyticus.* The distinction between bactericidal and bacteriostatic antibacterial agents is an important concept in developing potential antibiotics. The antibacterial activity can be discriminated as bactericidal, i.e. which kills the bacteria or bacteriostatic that inhibit the bacterial growth. Our study confirmed that the antibacterial activity of methanolic extract of *Ircinia fusca* is bactericidal in action, hence it can be considered for further investigations to explore new antibacterial compounds from *Ircinia fusca*.

Based on the low MBC values, methanolic extract was chosen to evaluate its antibiotic potentiating activity against *Vibrio parahaemolyticus, Aeromonas hydrophila* and *Citrobacter freundii* by selecting MIC/2 as appropriate sub-inhibitory concentration. The synergistic effect was observed when a combination of methanolic extract of *Ircinia fusca* and tetracyclin was used against *Vibrio parahaemolyticus* with a FIC value of 0.25 (Table 4). Furthermore, the methanolic extract exhibited indifferent activity

Table 4: Antibiotic potentiating effect of methanolic extract of *Ircinia fusca* against fish pathogens

	Concentration of *Ircinia fusca* methanolic extract (µg/ml)	**MIC (µg/ml) of antibiotic tetracyclin in the absence and presence of extract**	**Fractional inhibitory concentration (FIC)**	**Antibiotic potentiating effect**
Citrobacter freundii	0	50	1	Indifferent
	MIC/2	50		
Aeromonas hydrophila	0	25	0.5	Indifferent
	MIC/2	12.5		
Vibrio parahaemolyticus	0	6.25	0.25	Synergy
	MIC/2	1.56		

when it was associated with tetracyclin against *Aeromonas hydrophila* and *Citrobacter freundii*. The emergence of new antibiotic-resistant strains is always a major concern in aquaculture [30]. Exploring the synergism of antibiotics with natural extract is a novel concept with great potential. Hence it is the need of the hour to expand the antibacterial spectrum to prevent antibiotic resistance by using natural extract with antibiotics [31]. The synergistic potential of marine extracts and standard antibacterial drugs has been frequently reported worldwide [32]. The antibiotic potentiating activity of sponge extract is selective to certain fish pathogenic strains and hence it can be used to suppress the production of antibiotic-resistant strains in aquaculture.

Conclusion

The undesirable consequences made by the use of antibiotics in fishes crave the path for the investigation of alternative methods to address the problem. Marine sponges have a natural defense system against pathogenic bacteria in the aquatic environment. Therefore, the antibacterial property of the marine sponge (*Ircinia fusca*) extracts can be exploited as an alternative method for treating bacterial diseases. The potential of extracts of sponge *Ircinia fusca* as an antibacterial agent in aquaculture is vast and needs more scientific research regarding their mode of action and formulations. Thus, more attention is needed towards the development of natural antibacterial agents from marine sponges which can contribute to producing novel therapeutics for treating bacterial diseases in fishes.

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