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Assessment of Antimicrobial Activity of Bioactives Isolated from Marine Sponge Associated Microorganisms

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Abstract: Bacteria isolated from *Ircinia* and *Haliclona* marine sponges (Porifera) were tested for antimicrobial and antifungal activity against a panel of human and animal pathogens. The LJ (Lowenstein Jensen) media technique was also used to test the antitubercular properties of these isolated extracts. When three marine species were extracted in methanol or ethyl acetate, they showed antimicrobial activity. The most effective strains were NIO MAM 11.3, NIO MAM 4.2, and NIOMAM 4.1, which killed all of the microorganisms tested. Two strains were cultivated in large numbers using Zobells Marine Broth medium. Sponge bacteria from the genus *Ircinia* showed activity against *Vibrio cholera*, multidrug-resistant *Acinetobacter*, and *Aspergillus fumigatus*. In contrast, bacteria from the genus *Haliclona* showed significant antibacterial activity against *Staphylococcus aureus, Staphylococcus typhi, Shigella, Klebsiella* sp. and *Vibrio cholerae.*

Keywords: Marine sponges, *Ircinia, Haliclona,* Zobells, Antimicrobial, Antibacterial, Antifungal, *Staphylococcus aureus*, *Vibrio cholerae*

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Introduction

Substantial amounts of structurally unique and physiologically active metabolites may be found in marine species. Bacteria, cyanobacteria, and fungus are only few of the microorganisms that most marine invertebrates host in the extracellular and intracellular spaces of their tissue (Arajo *et al*., 2017). In 1966, researchers discovered and defined the first antibiotic

produced by marine bacteria. Halicondrin B (cytotoxic substance) was isolated from *Halichondria okadai* sponge off the coast of Novel Zealand. It is one of several chemically distinct compounds of marine origin with varied biological properties that are now being studied and/or turned into new medications (Murray *et al*., 1999). Marine sponges, one of the earliest animal phyla (Wang, 2006), are the primary source of the natural chemicals discussed here. About 15,000 species have been described, however, this likely understates the real diversity (Hentschel *et al*., 2006). Sponge filter feeding removes microorganisms from seawater (15 x 106 bacteria ml^{1}) at a high rate (up to 24 m^{3} kg^{1} sponge^{1} day^{1}) (Thomas *et al*., 2010).

Bacteria in sponge primorphs extract has demonstrated powerful angiogenesis inhibitor, while marine epiphytic bacteria associated with nutrient-rich algal surface invertebrates have been found to create antibacterial secondary metabolites that limit settling of potent competitor (Thakur *et al*., 2005). To find substances with potential medicinal applications, it is routine practice to screen organic extracts from marine sponges and other marine creatures (Deshpande *et al*., 2010).

Recent research has shown that bacteria living in commensal or symbiotic relationships with marine invertebrates are responsible for producing bioactive natural products that were first identified from marine sponges. Known challenges in developing pharmaceuticals from sponges might be mitigated via the isolation and growth of related microorganisms that create bioactive chemicals (de Rosa *et al*., 2003). The microorganisms found in association with marine invertebrates offer a potentially untapped and superior BioSource for new pharmaceutical research because, according to screening results, they contain a higher percentage of antibiotics and cytotoxic activities than those found in sediments and sea water (de Rosa *et al*., 2000).

In light of these findings, we looked for therapeutic actions derived from marine sources that were new, intriguing, and possibly beneficial. Antibacterial, antifungal, and antitubercular properties of methanolic and ethyl acetate extracts of 12 related bacterial strains were tested *in vitro* against hospital-isolated bacteria (MDR) and fungal strains. This research is a part of a larger initiative to screen marine creatures for various biological functions, with the hope of finding new compounds with promising medicinal applications. Because of the wealth of biological knowledge they may provide and the commercial opportunities they present, more thorough studies of marine sponge symbionts are warranted.

Materials and Methods

Sample collection:

SCUBA diving was used to collect samples from subtidal habitats at depths of 10 to 15 m at several places 2 nautical miles off the southeast Indian coastline (Tamil Nadu, India). They were frozen immediately and sent to the lab to isolate epibionts and endosymbionts. Dona-Paula, Goa's National Institute of Oceanography (NIO) has the samples.

Isolation of Sponge Associated Bacteria:

Sponge samples of both the *Ircinia* sp. and *Haliclona* sp. species were washed in salt water to kill any bacteria. The sponge was cut into pieces 2 cm x 2 cm in size, and then the pieces were washed three times in sterile seawater (0.22 m filtered) and vortexed to remove loosely attached bacteria. The pieces were then placed on ZMA plates with Nystatin for bacterial isolation, and on CA agar plates with streptomycin for fungal isolation (Kyung *et al*., 2001). Sponge samples for Endosymbiont isolation were placed in sterile sea water, given a 20-second vortex, and then decanted; 70% ethanol was added to wash and remove any symbionts that were not tightly attached; finally, the sponge pieces were crushed and dilutions were made from 10^{-1} to 10^{-5}, after

which they were spread out on plates.

Growth and Maintenance of Clinical Isolates:

All the sixteen clinical isolates (pathogens) were grown on Zobells marine agar collected from government medical college Goa. The following test strains were used- bacterial strains *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella typhi*, *Shigella flexineri*, *Klebsiella* sp., *Vibrio cholera*; Multi drug resistant *Streptococcus pyogenes*, *Acinetobacter* sp., *Salmonella typhi*, *Staphylococcus aureus*. Fungal Strains Aspargillus fumigatus, Rhodotorula sp., Cryptococcus neofemans, Candida albicans, Aspargillus niger. All the test strains were isolated from hospitalized patient from Government Medical College and maintained on ZMA at 37 °C and sub cultured every two weeks.*Screening for bioactive metabolite production by marine bacteria*:

All the marine bacteria isolated as above were cultured in 100 ml Zobells marine broth (peptone 0.5 g, yeast extract 0.1 g, and $FePO_4$ 0.01 g dissolved in 75 ml of sterile sea water and 25 ml of sterile water, pH-7.2-7.4) for the production of secondary metabolites in 250 ml flasks, the flasks were incubated at 28 °C/180 rpm for 96 h.

Extract preparation:

Isolated strains from marine sponge were purified and subjected for primary screening with 100 ml of ZMB media, after 96 h incubation the broth were centrifuged at 8000 rpm for 10 min and cell pellets were separated and sonicated with 100 ml methanol thrice. The filtered extracts were concentrated on rotary vacuum evaporator and the centrifuged broth extracted with ethyl acetate thrice and concentrated (Ely *et al*., 2004). These extracts of all isolated strains were screened for bioassay where NIOMAM 11.3, NIOMAM 4.1, NIOMAM 4.2 shown good results and taken for further study.

Mass Culture:

Seed culture of NIOMAM 11.3, NIOMAM 4.2 strains that have shown good activity were mass cultured. For mass culture inoculation 100 ml Zobells Marine Broth media were prepared in 250 ml Erlenmeyer flask for 28 °C/180 rpm for 24 h. This seed culture (100 ml each) inoculated in 5 L Erlenmeyer flask containing 2L Zobells Marine Broth and cultured with shaking (28 °C/180 rpm). After 96 h incubation culture was centrifuged at 8000 rpm for 10 min, cell pellets were sonicated thrice with methanol filtered and broth were extracted with ethyl acetate and both organic fractions are concentrated under vacuum on a rotary evaporator at low temperature to get crude methanolic and ethyl acetate extract.

Purification of crude extracts by column chromatography:

Niomam 11.3: 476 mg loaded on sephadex LH-20 column with the solvent used for elution was (1:1) methanol: chloroform 47 fractions were collected. The TLC of the fractions was developed in 5% methanol: chloroform and sprayed with 5% methanol: sulphuric acids, according to separation pattern fractions are combined and then bioactive fraction-I (1-15) was submitted for spectroscopic study.

Niomam 4.2: 475mg initially extracts subjected for purification by sephadex LH- 20, ODS (octa desyl silane) but there was no separation. So using flash chromatography did the purification.

Antimicrobial screening:

Antibacterial activity of crude extract:

Antibacterial activity was investigated against seven bacterial strains, four fungal strains, and five multidrug-resistant bacterial strains using Kirby-Bauer disc diffusion (Becerro *et al*., 1994). A 6 mm Whatman No. 1 filter paper disc was autoclaved for 15 min at 121 °C. Extracts were impregnated on sterile discs (crude extract with unknown concentration). Surface inoculating agar plates with a broth culture of the examined microorganisms was homogenous.

Every sample included 1.2 x 108 CFU/ml. The impregnated discs were spaced apart on the medium and incubated at 37 °C for 24 h. The

positive control was a disc with 10 mg of streptomycin. Methanolic and ethyl acetate marine organism extracts caused growth inhibitory halos that were measured in mm.

Antifungal activity of crude extract:

As in the antibacterial test, the Kirby-Bauer disc diffusion technique was used to measure antifungal activity against *Aspergillus fumigatus*, *Cryptococcus neofemans*, *Aspergillus niger*, *Rhodotorula*, and *Candida albicans*. Different extracts impregnated sterile discs (crude extract with unknown concentration). 1.2 x 108 CFU/ml were inoculums. Nystatin was a 100-unit/disc positive control. The plates were incubated for 18 h at 24 °C. Methanolic marine organism extract growth inhibition halos were measured in mm. Assays were duplicated.

Antibacterial and Antifungal Activity of Purified Compound (*MIC*):

The following test organisms were used to determine liquid medium MICs against serial micro dilution (Antal *et al*., 2005). MDR *Salmonella typhi*, *Klebsiella* sp., *Vibrio cholera*, *Aspergillus fumigatus*, *Cryptoccocus neofemans*, and *Aspergillus niger*. Inoculum standardisation was done using 24 h cultures of all test organisms diluted by 10-fold serial dilution with sterile water from 10^{-1} to 10^{-8} and plated on sterile Muller Hilton agar plates. The colonies on petridish was counted. Dissolving 10 mg of the isolated bioactive component in 1 ml of solvent produced a 10 mg/ml stock solution. Diluted clinical pathogenic isolates yielded 106 CFU/ml. 0.2 ml of 10-mg/ml stock solution (using suitable solvent) of the test material was added to 1.8 ml of seeded broth to create 1000 µg/ml (1st tube). To make 500 µg/ml test solution, 1 ml from the first tube was added to 1 ml of seeded broth. The test compounds and reference drugs were serially diluted two-fold to achieve 1000 to 7.81 µg/ml, then incubated at 37 °C for 24 h and 27 °C for 48 h for bacteria and fungi, respectively. Visual comparison determined MIC (presence of turbidity due to growth). The transparent tube's MIC is the lowest antibiotic concentration without growth.

Antitubercular activity:

The Lowenstein–Jensen egg medium (L J Medium) was used for the antitubercular screening, and the H37Rv strain was used as the test subject (Friedman *et al*., 2003). *Mycobacterium tuberculosis* H37Rv Strain was added to LJ Medium that included both standard medication and control LJ Medium. This medium was also inoculated with LJ Medium. The inoculation medium was kept in an incubator at 37 °C for a period of six weeks.

Results and Discussion

In the present study twelve associated strains were isolated from *Ircinia* sp., and *Haliclona* sp. sponges. All the isolates showed a positive response in inhibition zone assay against bacteria, MDR and fungal strains. Out of twelve strains, three (25%) were found to be bioactive metabolite producer. The bioactivity differed from strain to strain and both broad spectrum and species-specific activities were found. Three strains (NIOMAM 11.3, NIOMAM 4.1 and NIOMAM 4.2) exhibited broad-spectrum activity. In the present investigation methanolic extract inhibited *Vibrio cholera,* MDR *Acinetobacter* and *Aspergillus fumigatus. Ircinia* sp. associated organism exhibited mild antimicrobial activity against *Vibrio cholera, MDR Acinetobacter* and *Aspergillus fumigatus* but rest of the clinical isolates tested were insensitive to it (Tables 1, 2). Among the ten marine strains isolated from the sponge *Haliclona* sp., two of them showed broad spectrum of activity against all clinical isolates. Both the strains NIOMAM 4.1 and NIOMAM 4.2 have shown inhibitory action against *S. typhi, Klebsiella* sp., *Vibrio cholerae,* and *Cryptococcus neoformans*. The strain showed static activity against *Shigella flexineri, S. pyogenes, Acinetobacter* sp., *S. typhi, S. aureus, Aspergillus fumigatus, Rhodotorula* sp., *Aspergillus niger*.

MIC was evaluated for the isolated bioactive metabolite against several test organisms as per serial micro-dilution method (Rifai *et al*., 2005).

Table 1: Antibacterial Activity Against Pathogenic Bacterial & MDR Isolates

Strain code	Bacterial taxons							Multi drug resistant taxons			
NIOMAM	EC	PA	SA	ST	SF	KS	VC	SP	AP	ST	SA
11.3	- -	- -	- -	- -	- -	- -	+ +	- -	+ +	- -	- -
4.1	+ +	- -	+ +	+ +	+ +	+ + +	+ + +	+ +	+	+ + +	+ +
4.2	+ +	- -	+ +	+ +	+ + +	+ + +	+ + +	+ + +	+	+ + +	+ +

EC-*Escherichia coli*, PA- *Pseudomonas aeruginosa*, SA-*Staphylococcus aureus*, ST- *Salmonella typhi*, SF-*Shigella flexineri*, KS-*Klebsiella sp.*, VC-*Vibio cholerae*, MDR SA- Multi drug resistant *Staph pyogenes*, AP- *Acinetobacter sp*, ST- *S.typhi*, SA-*S.aureus*. (- -) No activity, (+) weak activity (7 – 10 mm), (+ +) moderate activity (10 – 15 mm), (+ + +) significant activity (15 – 20 mm)

Table 2: Antifungal Activity Against Pathogenic Fungal Isolates

Strain code	Fungal taxons				
NIOMAM	AF	RS	CN	CA	AN
11.3	+ +	- -	- -	- -	- -
4.1	+ + +	+ + +	+ + +	- -	+ + +
4.2	+ + +	+ + +	+ + +	- -	+ + +

AF- *Aspargillus fumigatus*, RS- *Rhodotorula.*sp., CN-*Cryptococcus neofemans*, CA-*Candida albicans*, AN- *Aspargillus niger*, (- -) No activity, (+) weak activity (7 – 10 mm), (+ +) moderate activity (10 – 15 mm), (+ + +) significant activity (15 – 20 mm)

The MIC of purified extract of NIOMAM 11.3 were studied against *Vibrio cholerae, MDR Acenetobacter and Aspargillus fumigatus.* The MIC of NIOMAM 11.3 for *Vibrio cholerae,* MDR *Acenetobacter* and *A. fumigatus* was 125 µg/ml, 62.5 µg/ml, 62.5 µg/ml; whereas standard showed MIC 31.25 µg/ml, 62.5 µg/ml, 31.25 µg/ml, respectively. The MIC of NIOMAM 4.2 for *S. typhi, Klebsiella* sp., *Vibrio cholerae, A. niger, A. fumigatus* was 62.5 µg/ml and for *Cryptococcus neoformans* 31.25 µg/ml. Whereas the standard was shown in range of 31.25-62.5 µg/ml respectively (Table 3). The secondary metabolites of host sponge *Dendrila nigra* associated *Streptomyces* sp. extracellular protein showed promising antibacterial activity against *P.aeurigunosa, M.luteus, B.cereus, E.coli, S.typhi, S.aureus, V. fisheri, Bacillus subtilis* (Carballeira *et al*., 2004). Literature indicates that sponge and associated symbionts contain potent antimicrobial, Antitubercular secondary metabolites *et al*., 2004).

Based from preliminary survey, we then focused our attention on Antitubercular activity of isolated bioactive compound and determined its MIC against *M. tuberculosis* H37Rv (Hill, 1996). The results of this were more precise (Table 4). In this assay NIOMAM 11.3 displayed a MIC at 50-100 mcg/ml. On the other hand NIOMAM 4.2 showed very weak activity at higher concentration. From these results we can conclude that *M. tuberculosis* H37Rv is susceptible to the different isolated extracts tested. In the past two decades, however, there has been a decline in the discovery of new lead compound from common soil derived microorganisms. For this reason the cultivation of marine microorganisms has become a major focus in the search for the next generation of pharmaceutical

Table 3: Determination of MIC for an Isolated Bioactive Compound against Bacteria, Fungi and Multi Drug Resistant Strains

Test microorganisms (Bacteria)	MIC of isolated compound in ∟g/ml		
	NIOMAM 11.3	NIOMAM 4.2	Standard
A. Bacterial clinical Isolates			Streptomycin
1. *S.typhi(B4)*	NT	125	NT
2.. *Klebsiella.sp (B6)*	NT	125	31.25
3. *Vibrio.cholera (B7)*	125	125	31.25
B. Fungal Clinical Isolates			Nystatin
1. *Aspargillus fumigatus (F1)*	62.5	125	62.5
2. *Cryptoccocus neoformis (F4)*	N	62.5	31.25
3. *Aspargillus niger (F5)*	T	125	31.25
	N		
	T		
C. MDR Clinical Isolates			Ciprofloxacin
1. MDR *Salmonella typhi (D2)*	125	NT	62.5

NT:- Not tested

Table 4: Antitubercular Activity

S. No.	Compounds	50 mcg/ml	100 mcg/ml
1.	NIOMAM 11.3	- +	+ +
3.	NIOMAM 4.2	- -	- -
STD.	Streptomycin	+ +	+ +

+ +: Denotes No Growth; + -: Denotes Growth with Less Than 20 Colonies; - -: Denotes the Growth

agents. There is wide scope for further improvement of the activity through various lead molecule development strategies. Butanolic extract of cyclic peptides from *Ircinia* sp*.* sponge symbionts showed promising cytotoxic activity carried out on mouse lymphoma cells(Barry *et al*., 1977). Antifouling potential and antibacterial properties of *Ircinia ramosa* sponge associated bacteria during different collection period were already proven (Thakur and Anil, 2000). Similar observation of antimycobactericidal action has been reported in literature for 2- methoxylated fatty acid extracted from marine sponge and symbionts against *Mycobacrerium* sp. (Crews and Harrison, 2000). In the present study the extract obtained from *Haliclona* sponge associated bacteria showed promising broad spectrum of activity; *Ircinia* sponge associated bacteria showed good activity against *Vibrio cholerae*. Sponge extract from the genus *Haliclona* also showed the antibacterial and antifungal activity against various clinical isolates (Selvin and Lipton 2004).

According to Hill (1996), the interactions between marine invertebrates and micro-organisms that may serve as food or which reside either permanently or temporarily within marine microorganisms are very complicated and have not yet been fully explored. As a result, it is possible that these symbionts play a substantial role in the defence of the host sponge against various infectious agents and predators. The harmful compounds (secondary metabolites) that were assumed to be accumulating in sponge tissue were secreted by symbionts (Selvin *et al*., 2004). The pharmaceutical sector has an interest in discovering the primary origin of sponge's secondary metabolites. This work sheds light on the significance of symbionts associated with I*rcinia* sp. and *Haliclona* sp. as a useful resource for the identification of new bioactive molecules and drugs. The phylogenetic study of all of the strains as well as the process of isolating a bioactive chemical from NIOMAM 4.1 are both

currently under progress.

Characterization of bioactive metabolite producer strains:

Morphological characterizations of the antagonistic isolates NIOMAM 11.3, 4.1, 4.2 were determined using standard methods (Jing *et al*., 2020). Strain NIOMAM 11.3 grew as cream off white translucent circular bacterium on ZMA and was further characterized as gram-negative small rod, it is also catalase and oxidase positive. Strain NIOMAM 4.1 grew as white bacterium on ZMA; it was gram-negative cocci characterized as catalase positive and delayed oxidase positive. Strain NIOMAM 4.2 was grew white opaque irregular bacterium on ZMA; it was identified as gram-negative cocci and further characterized as catalase positive and delayed oxidase positive.

Conclusion

Marine sponge-associated organisms from Southeast Indian *Ircinia* and *Heliclona* sponges exhibit many biological functions. We studied the most intriguing species, NIOMAM 11.3, 4.1, and 4.2. NIOMAM 11.3 and 4.2 species methanolic extract showed the potential. The three active strains, which showed antibacterial and antifungal activity, are promising. Strain NIOMAM 4.1 is currently being studied. This active molecule is being structurally elucidated. The extracted bioactive component was efficacious against Multi Drug Resistant strains, demonstrating the value of bacteria from sponges *Ircinia* and *Haliclona* as a source of new bioactive molecules.

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