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

Nagare Sujit K.1, Deshpande Mangirish N.2* and Dighe Pearl K.3

1Department of Pharmacognosy, Indira Institute of Pharmacy, Sadavali (Devrukh), Ratnagiri 415804, Maharashtra, India
2Department of Pharmacology, PES’s Rajaram and Tarabai Bandekar College of Pharmacy, Farmagudi, Ponda Goa 403401, India
3Department of Pharmaceutics, PES’s Rajaram and Tarabai Bandekar College of Pharmacy, Farmagudi, Ponda Goa 403401, India

*Corresponding Author

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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 10^6 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 cholerae*; Multi drug resistant *Streptococcus pyogenes*, *Acinetobacter sp.*, *Salmonella typhi*, *Staphylococcus aureus*, *Salmonella typhi*, *Staphylococcus aureus*. Fungal Strains *Aspergillus fumigatus*, *Rhodotorula sp.*, *Cryptococcus neoformans*, *Candida albicans*, *Aspergillus 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 FePO₄ 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).

**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 neoformans, 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, Cryptococcus neoformans,* 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 (LJ 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

<table>
<thead>
<tr>
<th>Strain code</th>
<th>Bacterial taxons</th>
<th>Multi drug resistant taxons</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIOHAM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.3</td>
<td>- -</td>
<td>- -</td>
</tr>
<tr>
<td>4.1</td>
<td>+ +</td>
<td>- -</td>
</tr>
<tr>
<td>4.2</td>
<td>+ +</td>
<td>- -</td>
</tr>
</tbody>
</table>

EC- Escherichia coli, PA- Pseudomonas aeruginosa, SA- Staphylococcus aureus, ST- Salmonella typhi, SF- Shigella flexineri, KS- Klebsiella sp., VC- Vibrio 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

<table>
<thead>
<tr>
<th>Strain code</th>
<th>Fungal taxons</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NIOHAM</td>
<td>AF</td>
<td>RS</td>
<td>CN</td>
<td>CA</td>
<td>AN</td>
</tr>
<tr>
<td>11.3</td>
<td>+ +</td>
<td>- -</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4.1</td>
<td>+ + +</td>
<td>+ +</td>
<td>+</td>
<td>-</td>
<td>+ +</td>
</tr>
<tr>
<td>4.2</td>
<td>+ + +</td>
<td>+ +</td>
<td>+</td>
<td>-</td>
<td>+ +</td>
</tr>
</tbody>
</table>

AF- Aspargillus fumigatus, RS- Rhodotorula.sp., CN-Cryptococcus neoformans, 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 NIOHAM 11.3 were studied against Vibrio cholerae, MDR Acinetobacter and Aspargillus fumigatus. The MIC of NIOHAM 11.3 for Vibrio cholerae, MDR Acinetobacter 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 NIOHAM 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. aeruginosa, 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 NIOHAM 11.3 displayed a MIC at 50-100 mcg/ml. On the other hand NIOHAM 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

<table>
<thead>
<tr>
<th>Test microorganisms (Bacteria)</th>
<th>MIC of isolated compound in g/ml</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Bacterial clinical isolates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. <em>S.typhi</em> (B4)</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>2. <em>Klebsiella</em> sp (B6)</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>3. <em>Vibrio</em> cholera (B7)</td>
<td>125</td>
<td></td>
</tr>
<tr>
<td>B. Fungal Clinical isolates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. <em>Aspergillus fumigatus</em> (F1)</td>
<td>62.5</td>
<td></td>
</tr>
<tr>
<td>2. <em>Cryptococcus neoformis</em> (F4)</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>3. <em>Aspergillus niger</em> (F5)</td>
<td>T</td>
<td></td>
</tr>
<tr>
<td>C. MDR Clinical isolates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. MDR <em>Salmonella typhi</em> (D2)</td>
<td>125</td>
<td></td>
</tr>
</tbody>
</table>

NT: Not tested

Table 4: Antitubercular Activity

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Compounds</th>
<th>50 mcg/ml</th>
<th>100 mcg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>NIOMAM 11.3</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>3.</td>
<td>NIOMAM 4.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>STD.</td>
<td>Streptomycin</td>
<td>+ +</td>
<td>+ +</td>
</tr>
</tbody>
</table>

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

According to Hill (1996), the interactions between marine invertebrates and microorganisms 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 *Ircinia* 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
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 grown 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 *Haliclona* 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.

**References**


