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Investigation of bioactivity of extracts of Marine Sponge, *Spongosorites halichondrioides* (Dendy, 1905) from western coastal areas of India

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ABSTRACT

Objective: Sponges (phylum Porifera) are sessile marine invertebrates and are known to be the richest source of pharmacologically-active compounds. This work was taken to investigate the antibacterial, antifungal activity and cytotoxicity from marine sponge. **Method:** In this study the marine sponge *Spongosorites halichondrioides* crude extracts were investigated for three bioassays. The first is an antimicrobial test against *Proteus vulgaris*, *Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella typhi*, *Klebsiella pneumonia*, *Escherichia coli*, *P. aeruginosa* and the second is an antifungal test against three pathogenic fungi, *Aspergillus flavus*, *Aspergillus niger* and *Metarhizium anisopliae*. The third is a cytotoxicity test using larva of *Artemia salina*, for detection of cytotoxic activity in the extracts. **Result:** For all the three bioassays, extracts were found to be bioactive. This result suggests that this marine sponge is able to produce biologically active agents required for an overall defense against their predators. **Conclusions:** Further GC MS was done and the fragmentation pattern, showed the presence of sterol esters and terpenoids in the active extracts.

1. Introduction

Marine organisms have been widely known as potential sources of bioactive compounds. Sponges are a diverse group with about 5 000 species known around the world. They are believed to have developed secondary metabolites that are active against different strains of microorganisms as part of their defences and survival in the marine environment. More than 5 300 different products are known from sponges and their associated microorganisms, and more than 200 new metabolites from sponges are reported each year. In addition to the unusual nucleosides, bioactive terpenes, sterols, peptides, alkaloids, fatty acids, peroxides and frequently halogenated amino acid derivatives have been described from sponges[1]. A number of sponges possessing antimicrobial and antifungal activities have already been discovered. The secondary metabolites of marine organisms have been studied extensively over the past 30 years, since a small number of academic chemists began to isolate and elucidate novel compounds from marine sources in the 1970's. Drug discovery research from marine organisms has been accelerating and now involves interdisciplinary research including biochemistry, biology, ecology, organic chemistry, and pharmacology. Recently, much attention has been given to marine organisms due to their considerable biodiversity that has been found in the widespread oceans that cover over 70% of the world[2]. Among the first bioactive compounds from marine sources, spongouridine and spongothymidine from the Caribbean sponge (*Cryptotheca crypta*), were isolated serendipitously in the early 1950s. They were approved as an anticancer drug (cytosine arabinoside, Ara-C) and an antiviral drug (adenine arabinoside, Ara-A), respectively, 15 years later[3]. The evolution of antibiotic-resistant pathogenic bacteria has stimulated the search for alternative antimicrobial agents from alternative sources including sources from the ocean. In one of the studies, extracts from the sponge *Cinachyrella* sp., *Haliclona* sp., and *Petromica citrina* showed antibacterial activity against 61% of the CNS strains, including strains resistant to conventional antibiotics. Extracts from *P. citrina* showed the largest spectrum of inhibitory activity. The aqueous extract inhibited 51% of the CNS strains and presented a bactericidal effect over susceptible and multiresistant-bacteria at a minimal inhibitory concentration of 1.024 μg/ml. This study showed the potential of marine sponges as

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new sources of antibiotics and disinfectants for the control of CNS involved in bovine mastitis[4]. The marine sponges *Acanthella elongata, Axinella donnani, Callyspongia diffusa, Callyspongia subarmigera*, and *Echinodictyum gorgonoides* were studied for in vitro antibacterial properties against eight virulent marine fish pathogens and showed species specific antibacterial activity[5]. Another similar study from Indian south coastal areas of Tamil Nadu used the marine sponges *Callyspongia diffusa, Echinodictyum gorgonoides, Callyspongia reticutis, Gelliodes cellaria*, and *Thalysias vulpine* showed the antifungal activity against the various fungal strains such as the *Aspergillus niger*, *Pencillium notatum* and *Candida albicans* by using the agar well diffusion. The sponge crude extracts seems to have effective cytotoxic property that was detected by Brine shrimp assay method[6]. Marine sponge *Dendrilla nigra* has also shown prevalence of antiinflammatory, antioxidant, antibacterial anticancer activities. The GCMS inferred the presence of mixture of fatty acids which might be responsible for the activities reported[7]. In a similar attempt, *Spongosorites halichondrioides* (Phylum Porifera) which is a green colored sponge, turning yellow at the base attached to rocks and shells which is commonly found on sub tidal rocky beaches of Khardanda, Mumbai, India was collected for the study. They are multicellular organisms containing a system of chambers and passageways that allow water to circulate constantly through the body. The body size and shape is highly variable. The aim of this study was to evaluate the non-polar and polar extracts from S. halichondriodes for antimicrobial and antifungal activity. The brine shrimp toxicity test was used to evaluate the extracts for toxicity and potential cytotoxic activity.

2. Materials and Methods

Methanol, n- hexane, Ethyl acetate, n- Butanol was purchased from Qualigens Fine Chemicals, Mumbai, India., whereas Dimethyl sulfoxide (DMSO) was purchased from Sigma Aldrich® (Poole, Dorset, UK). Nutrient broth, Mueller Hinton Broth and Saboraud's broth was bought from HIMEDIA® (Himedia Laboratories Pvt. Ltd, Mumbai, INDIA). *Staphylococcus aureus* (ATCC 6538P), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 19429), *Salmonella typhi* (NCIM 2501), *Bacillus subtilis* (ATCC 6633), *Proteus vulgaris* (ATCC 9484), *Klebsilla pneumonia* (NCIM 2957) and antifungal strains *Aspergillus flavus* (ATCC 11492, 13450), *Aspergillus niger* (ATCC 16880), *Metarhizium anisopliae* (NCIM 1311) were obtained from the National Collection of Industrial Microorganisms, NCL, Pune, India. The Brine Shrimps eggs were purchased from Aquaworld (Chennai, India) and sea salt was prepared locally by evaporating water collected from the Arabian Sea, along the western Mumbai Coast.

2.1 Collection of Sponges

Sea Sponges, *Spongosorites halichondrioides* (Dendy, 1905) were collected by hand picking during low tide from fish landing center, Khardanda beach, Mumbai, (19°4′20″N 72°49′57″E) India. The sponge samples were carried in sterile ethyl polythene bags in sea water and transferred to the lab aseptically. The preparation of the spicules for the identification purpose was carried out by Acid Digestion method[6].The specimen was sent to Zoological Survey of India, Chennai for identification confirmation and is registered in MBRC/ZSI, Chennai.

2.2 Extraction and Fractionation Methodology

Sponge (*Spongosorites halichondrioides*) was collected from the Khardanda coast regions and cleaned from the associated marine organisms and extraneous matter. Samples were shade dried for three days until they were fully air dried and preserved thereafter at −4 ℃ until further use. Dried samples were immersed in methanol for three cycles depending on the weight of the material and allowing enough time to achieve color fading of the biomass and to get optimal extraction of the sample. Each cycle of extraction at room temperature was carried out overnight with stirring in automatic shaker. The methanolic extracts were collected and dried under vacuum to give a solid residue. This was attained using a Buchi rotary evaporator (Model RE 111) at < 40 ℃. Dissolved the residue in smallest possible volume of methanol in water (10% v/v) and fractionated against n-hexane, chloroform followed by ethyl acetate and *n*-BuOH. Each fraction was dried under vacuum using a rotavapor to give a solid residue. All fractions were then subjected to TLC and bioactivity assay as well[7].

2.3. Media Preparation

Mueller Hinton Agar and Sabourauds Agar (Himedia, India) were used for antibacterial and antifungal bioassay respectively. MHA was prepared by dissolving 19 g in 500 mL of distilled water and brought to boil to completely dissolve. Similarly Sabourauds agar 65 g was suspended 1 000 mL of distilled water. It was mixed well and dissolved with frequent agitation and dissolved upon boiling. Sterilization was achieved by autoclaving at 121 ℃ for 15 min. The media was dispensed (20 mL) onto the pre-sterilized Petri dishes yielding uniform depths. They were then covered and allowed to cool and solidify at room temperature. Whatman No. 1 Filter paper discs (5 mm diameter) were prepared and sterilized by autoclaving.

2.4. Antimicrobial Assay

Antimicrobial activity was performed using Disc Diffusion method [8.9]. The sterile disks were impregnated with hexane, Chloroform and Butanol extracts which were dissolved in DMSO. Agar plates were surface inoculated uniformly from the broth culture of the tested microorganisms. In all cases, the concentration was approximately 1.5 $\times 10^8$ CFU/ml (0.5 Mc Farland standard turbidity). The impregnated disks were placed on the medium suitably spaced apart and the plates were incubated at 37 ℃ for 24 h. Disk of Ampicillin for antibacterial and Amphotericin B (Himedia Mumbai, India) for antifungal, disk impregnated with DMSO was

used as controls. The bacterial plates were incubated at 37 ℃ overnight and fungal plates were incubated at 35 ℃ for 48 h. The diameter of inhibitory zones (mm) was measured. Extracts disk were used as negative control. The diameter (mm) of the growth inhibition zones caused by the hexane, Ethyl acetate, Butanol, Chloroform extracts of marine sponge was examined. All the assays were carried out in triplicate.

Presence of a clear circular zone around the sample impregnated disc was used as an indicator of activity. Antibacterial and Antifungal activity was expressed in terms of diameter of Zone of Inhibition. It was measured in mm using scale and recorded. For each extract, MIC was calculated by dilution method using disc diffusion method. The lowest concentration which showed no bacterial growth was considered as MIC.

2.5. Brine Shrimp/Artemia salina Bioassay

The larvae of *Artemia salina* were obtained after incubation of the eggs in a brine shrimp media at 28 ℃ for 24h[10]. The toxicity was determined after 24 h of exposure. The assay was performed for methanol, hexane, ethyl acetate and aqueous extract in 96 well polystyrene micro titer plates. Briefly, stock solutions (10 mg/mL) of all extracts were prepared by dissolving them in DMSO. Different levels of concentrations (1 000, 100, 10, 1 and 0.1 μg/mL) were prepared by drawing different volumes from the stock solutions and then added into vials, each containing 10 brine shrimps larvae. The volume was then adjusted to 5 mL with artificial sea water prepared by dissolving 3.8 g of sea salt in 1L of distilled water. Each level of concentration was tested in duplicate. The negative control contained brine shrimp, artificial sea water and DMSO (0.6%) only. The numbers of dead larvae were counted after 24 h of incubation under the microscope and the percentage mortality was calculated using the mean of three triplicates and the LC_{50} values of the samples were calculated.

3. Results

3.1 Antimicrobial Activity

Table 1 represents the antibacterial and antifungal activities of the extracts and shows the highest activity of hexane extract for both the studies in the form of maximum zone of inhibition. All the solvent extracts from the Sponge exhibited activity against gram positive, gram negative microorganism tested. Air dried S. halichondriodes non polar and polar extracts were found to be active against both bacteria and fungi. The hexane extract exhibited the highest activity for bacteria and fungi both. MIC Results showed prominent activity of n- hexane against bacterial strain *Bacillus subtilis* at 0.8 mg/ml (Table 2). GC- MS analysis was done for Methanol, hexane and Ethyl acetate extracts for understanding of active compounds present (Figures 1-3).

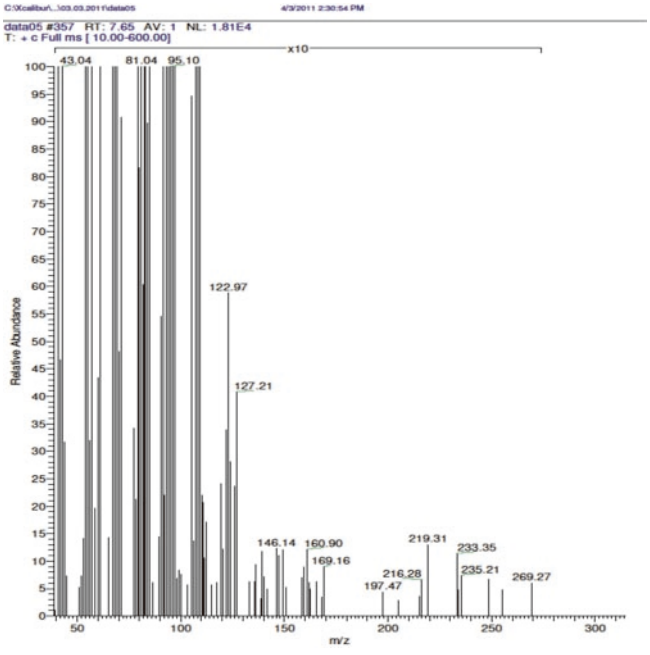

Figure 1. GC-MS of Methanol extract showing presence of compounds responsible for antimicrobial and brine shrimp toxicity.

Table 1
Antimicrobial activity of extracts from *S. halichondriodes*.

Crude extract	Zone of Inhibition (mm) ±Std. Deviation								
	B. subtilis	*S. aureus*	*P. vulgaris*	*S. typhi*	*K.pneumonia*	*E. coli*	*A.niger*	*A.flavus*	*M. anisoplaie*
n-Hexane	12±2.7	10.8±3	8±3	13±0.9	7±1	9.9±0.6	12.5±2.2	9.4±1.1	8.14±1.06
Chloroform	7±.05	8±0.7	7±0.3	7±1.4	7±0.45	8.6±2	9.6±1.5	10.3±2.0	10.3±3.2
Butanol	9.2±1.5	7.3±1	9.2±2.3	8.7±1.5	6+ 0.40	8±1.07	11±1	10.6±1.1	12.3±1.5
Std. Ampicillin (10 μg)	20±0.5	12±0.5	15±0.5	15±0.5	15±0.5	14±0.5	NA		

All the results are mean of triplicates.

Table 2
Minimum inhibitory concentration by serial tube dilution of different crude extract.

Crude Extract with stock conc.(20mg/mL)	Minimum Inhibitory Concentration in (mg/mL)					
	Bacillus subtilis	*Staphylococcus aureus*	*Proteus vulgaris*	*Salmonella typhi*	*Klebsiella pneumonia*	*Escherichia coli*
n-Hexane	0.8	1.75	2.5	0.8	2.5	1.75
Chloroform	2.5	1.75	2.5	2.5	2.5	1.75
Butanol	2.5	5	2.5	5	10	5

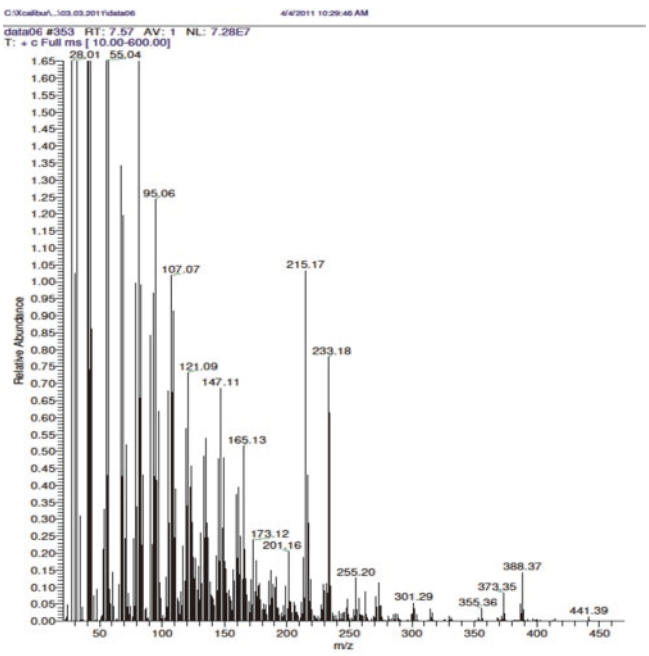

Figure 2. GC- MS of Hexane extract showing presence of compounds responsible for higher antimicrobial activity.

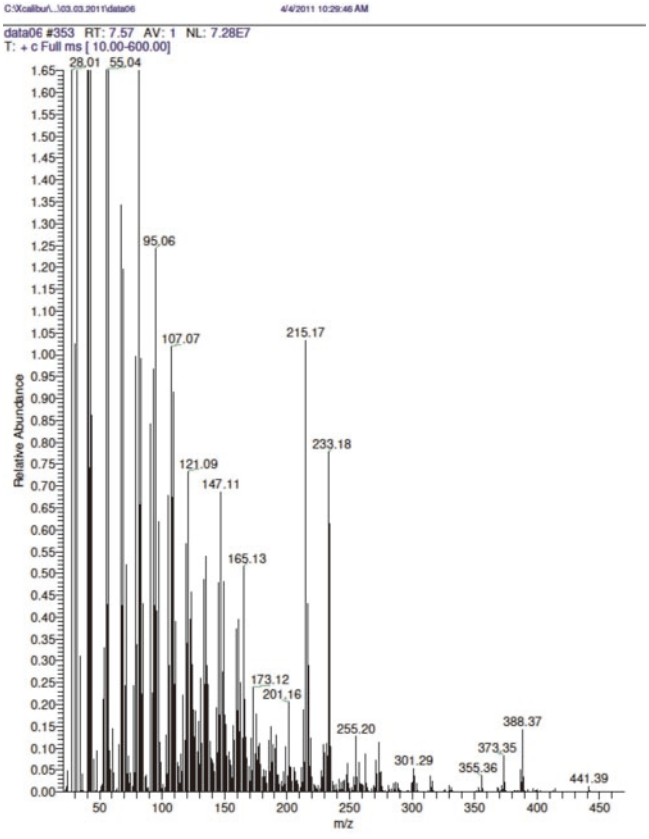

Figure 3. GC- MS of Ethyl Acetate extract showing presence of compounds responsible for antimicrobial and brine shrimp toxicity.

3.2. Brine Shrimp toxicity

Tables 3-6 show the results of brine shrimp lethality assay obtained from the four extracts. These results indicate that the extracts of *Spongosorites halichondriodes* were toxic to *A. salina* in dose - dependent manner. It was clearly observed that the total mortality (100% mortality) was recorded at the highest concentration (1 000 μg/ml) for Methanol extract.

The LC_{50} concentration of each extract was determined. The result revealed that the LC_{50} values of the Hexane and Butanol extract showed high toxicity and LC_{50} of both extract 5.62 μg/mL and 5.24 μg/mL. Methanol and Ethyl acetate extract showed LC_{50} of 1 000 and 11.48 μg/mL, respectively, and they are therefore less toxic than methanol and Butanol extracts. Methanol extract has the highest LC_{50} value of 1 000 μg/mL and is considered as the least toxic among the assayed solvent extracts cultures as confirmed by *Artemia salina* lethality assay. Cyclophosphamide was used as a standard with LC_{50} dose of 16.31 μg/mL. However, the results showed direct relationship to the fractional extraction performed. As the methanol was the first solvent used for total extraction and further solvents were used for fractionation so does the toxicity increases according the distribution of polar and non polar toxicants.

Table 3

Brine shrimp activity of methanol extract from *S. halichondriodes*.

Dose level (μg/mL)	Initial Nauplii	Average Number died after 24 h	% Mortality
1 000	10	5.33	53.33
100	10	3.33	33.33
10	10	2.66	26.66
1	10	1.33	13.33
0.1	10	0.33	3.33
0	10	0	0

All the results are mean of triplicates.

Table 4

Brine shrimp activity of hexane extract from *S. halichondriodes*.

Dose level (μg/mL)	Initial Nauplii	Average Number died after 24 h	% Mortality
1 000	10	10	100
100	10	10	100
10	10	5.66	56.66
1	10	2.33	23.33
0.1	10	0.33	3.33
0	10	0	0

All the results are mean of triplicates.

Table 5

Brine shrimp activity of ethyl acetate extract from *S. halichondriodes*.

Dose level (μg/mL)	Initial Nauplii	Average Number died after 24 h	% Mortality
1 000	10	10	100
100	10	6	60
10	10	4.33	43.36
1	10	3.33	33.33
0.1	10	0.66	6.66
0	10	0	0

All the results are mean of triplicates.

Table 6
Brine shrimp activity of butanol extract from *S. halichondriodes*.

Dose level (μg/mL)	Initial Nauplii	Average Number died after 24 h	% Mortality
1 000	10	9.66	96.66
100	10	8.33	83.33
10	10	5.33	53.33
1	10	3.33	33.33
0.1	10	1.33	13.33
0	10	0	0

All the results are mean of triplicates.

4. Discussion:

The most interesting species of marine sponges reported in terms of different activities are Ircinia felix, Topsentia ophiraphidites and Pandaros acanthifolium. Bioassay guided isolation and characterization of the active components from these species may yield useful candidates in the search for new pharmaceutical leads[11]. A new bis (indole) alkaloid (9) of the hamacanthin class along with topsentin class (1-4), and hamacanthin class (5-8) compounds topsentin class (1-4) from Spongosorites sp. have been studied for Sortase A inhibiting activity. The fibronectin- binding activity data highlighted the potential of these compounds for the treatment of *S. aureus* infections via inhibition of Sortase activity[12]. Spongosorites sp. have been reported to produce bis (indole) alkaloids, topsentin class (1-4), and hamacanthin class (5-8) compounds. In the evaluation of antimicrobial activity against various strains of bacteria and fungi, compounds of the hamacanthin class exhibited more potent antibacterial activity than those of the topsentin class. Deoxytopsentin (1) and hamacanthin A (5) also exhibited significant antibacterial activity against methicillin-resistant *Staphylococcus aureus*, with MIC values of less than 12.5 μg/mL. In the antifungal activity test, hamacanthins, especially hamacanthin A, showed potent inhibitory activity against medically important pathogenic fungi. Bis (indole) alkaloid compounds also exhibited moderate cytotoxicity against cancer cell lines[15]. In one study, a marine epiphytic bacterium *Pseudovibrio* sp. D323 produced tropodithietic acid (TDA) which was identified to be responsible for the antibacterial activity of the marine epiphytic bacterium *Pseudovibrio* sp. D323 and related strains. Phenol was also produced by these bacteria but was not directly related to the antibacterial activity[16]. Various metabolites from marine sponges and tunicates have been shown to possess antimicrobial, cytotoxic or antiparasitory properties, in agreement with the need for soft-bodied organisms to develop the art of chemicals against their predators.

Brine shrimp results obtained from air dried sponge samples showed strong toxicity with LC_{50} dose ranging from 5.24-1 000 μg/mL (Table 7). The fractionated extraction also separates the portion of toxins in polar extracts and the non polar extract. This suggests the speculation of the toxic characteristic of *S. halichondriodes* for potential to yield anticancer agents in future. Both non polar and polar extracts from *S. halichondriodes* were also evaluated for their antimicrobial and antifungal properties. The extracts showed promising antimicrobial activity against both Gram negative and Gram positive bacterias. *Spongosorites halichondriodes* has shown good antimicrobial, antifungal and brine shrimp toxicity activities in this investigation. GC-MS analysis of the methanol, Hexane and ethyl acetate crude extract was done for the understanding of active chemical compounds present. The MS spectra was searched for match in Mass bank and the fragmentation pattern of compounds has indicated the presence 5 α - Choletane- 3 α - ol, Dihydrocholesterol, Cholesterol, 3- β, 6- α Dihydroxy -5 α - Cholan-24- oic acid methyl ester in methanol extract. The same compounds were mostly present in Ethyl acetate extract. However, the hexane extract showed the fragment patterns for Terpenoids like Citronellyl acetate and butyrate along with Pregnanediol. All these compounds have sufficient information of possessing antibacterial and antiinflammatory activity. This study confirms the relationship between absence of predators and pharmacological activity of marine organisms.

These results suggest the further study of *Spongosorites halichondriodes* as a potential source for newer class of cytotoxic compounds. The high brine shrimp lethality corroborates with literature reports that the sponges are huge producers of toxins and are harmful and toxic to livestock and fishes.

Conflict of interest statement

The authors declare that they have no competing interest.

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