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BIOACTIVE POTENTIAL OF SPONGE ASSOCIATED BACTERIA -A PRELIMINARY STUDY

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ABSTRACT

The rich diversity of marine bacteria associated with marine invertebrates, including sponges has received major attention in pharmaceutical industries due to high scope for novel bioactive metabolites. In this study, bacteria associated with the sponges were isolated and characterized. The bioactive metabolites produced by these bacteria were extracted with ethyl acetate, hexane and chloroform. The bioactivity of crude metabolites was screened by using well and disk diffusion method against bacterial pathogens. A total of 70 bacteria were isolated from six different types of sponges. All the isolates were characterized and identified up to genus level. The results indicated that out of 70 bacterial isolates, only five bacterial species exhibited inhibitory activity against the tested pathogens such as Bacillus sp., E. Coli, Salmonella sp., Pseudomonas aeruginosa, Candida albicans and Aspergillus flavus and the strains were named as SgM1, SgM2, SgM3, SgM4 and SgM5. Among these, the SgM3 strain was suspected as Micrococcus sp., strain based on biochemical analysis, and it showed high inhibitory activity against bacterial pathogens, such as Bacillus sp., (19 mm), Salmonella sp., (20 mm) and Pseudomonas aeruginosa (16 mm) and also against fungus Candida albicans (7mm) both in well and disk diffusion assay. The crude ethyl acetate extract of SgM3 showed Minimum Inhibitory Concentration (MIC) of 20 µg/ml against Bacillus sp., and Salmonella sp., whereas the MIC value of crude extract of SgM1 was 20 µg/ml against Bacillus sp., and 40 µg/ml against Aspergillus flavus fungal pathogen. The Minimum Bactericidal Concentration (MBC) was 1.56 ml against both bacterial and fungal pathogens. Further, the active compound was characterized by thin layer chromatography (TLC) technique and the R_f value was 0.65 which revealed its bioactive potential. The present study reports that the sponge associated bacteria had the ability to produce a novel bioactive compound that may be a promising candidate for the therapeutic and biomedical application.

KEY WORDS

Marine bacteria, Antimicrobial activity, Sponge, Crude metabolites.

INTRODUCTION:

The marine ecosystem comprises a richest source of biologically active compounds from marine organism such as marine animals including invertebrates, marine plants and marine microbes. The marine sponges are the filter feeder, sessile organism and they harbor a rich source of microbes with their body system [1] and 30% biomass of the sponge tissue have 10^{10} microbial load. Based on the microbial abundance they are categorized into: low microbial abundance (LMA 10^5-10^6) and the high microbial abundance (HMA 10^8-10^{10}) [2]. They play a major role in protecting the sponges from predation, potential pathogen, competitors and fouling organism. Some kind of toxic metabolic products are also produced by symbiotic microbes; these toxic substances were potential and effective for pharmaceutical and biotechnological applications [3]. They have the property of anti-cancer, anti-viral and anti-inflammatory activities [4]. Sponge-associated marine

microorganisms have received a renewed attention with respect to their production of secondary metabolites; also, several studies have been reported on their symbiotic association with microbes and their bio-active compounds [5]. Among the bacterial isolates from marine sources, *Bacillus* is the most significant producers of compounds with antimicrobial activity [6-10]. Currently the marine genus *Pseudomonas* sp., have also been found to produce the potential biologically active novel metabolites and have the significant activity against pathogens [11]. Similarly, the genus of *Micrococcus* associated with *Spirastella* sponge produced a urethanase compound that was used in the beverage industry to remove cancer causing compounds [12]. During the past 2 to 3 decades, several novel compounds have been isolated from marine organisms and many of them have been reported to have biological activities [13]**.** The attention of natural products by chemist and pharmaceutical companies are mainly focused on sponge derived compounds and as of now more than 10,000 compounds were recovered from marine sources largely from sponges; several compounds are in clinical trials [12]. Thus, this study primarily focuses on the isolation and identification of bacteria from marine sponge, and subsequent screening of these bacteria for antimicrobial activities against the clinically relevant bacterial pathogens such as *E. coli, Bacillus* sp*., Pseudomonas aeruginosa* and *Salmonella* sp., and fungal pathogens *Candida albicans,* and *Aspergillus flavus*

MATERIAL AND METHODS:

Collection of Samples:

Six different types of sponges were collected from Mandabam and Rameshwaram in Tamil Nadu, India. All the samples were collected by hand picking method and placed in new polythene bags and transported to the laboratory in an ice chest (4° C) and the samples were processed immediately for the isolation of bacteria in an aseptic condition.

Isolation and Identification of bacterial strains:

The associated form of bacteria was isolated by standard method; sponges were rinsed with tap water to remove the debris present in the upper portion then again washed with sterile distilled water. Samples were cut into small pieces using sterile scissors, ground with mortar and pestle. Approximately, 1g of the sample was taken and transferred into 250 ml Erlenmeyer flasks, containing 99 ml of sterile phosphate buffered saline (PBS) (or) sponge dissociation medium (SDM), and shaken continuously for 30 minutes to separate the associated form of bacteria. Further tenfold dilution was made with PBS up to desired level and 0.1 ml was taken from the diluted sample and plated onto the Zo Bell Marine Agar (ZMA) and Nutrient agar (NA) medium. Plates in duplicate were incubated at room temperature (25-28°C) for 3-5 days. At the end of the incubation period the different morphological colonies were selected at random and purified for the screening of bioactivity.

Primary screening:

All the pure isolates obtained from sponges with different morphological features were primarily screened for their bioactivity by cross streaking method against various bacterial pathogens such as *Bacillus* sp., *Salmonella* sp., *Pseudomonas aeruginosa* and *E. coli.* The test strains were streaked perpendicularly across pathogens in Muller Hinton Agar (MHA) plate and the plates were incubated at 37° C for 24 h. The highly active isolates were selected for further screening by well and disk diffusion method and identified through biochemical and microscopic characterization.

Identification of symbiotic bacteria:

All the positive potent strains were identified through phenotypic characteristics such as micromorphology (Simple staining, Gram staining, Endospore staining and Motility), and biochemical characteristics (Indole production, Methyl red and Voges – Proskauer test, Citrate utilization, Catalase, Oxidase, Lipid hydrolysis, Gelatin hydrolysis and Carbohydrate fermentation tests by adopting standard procedure (Bergeys Manual of systematic Bacteriology, 2nd Edition, 2001-2012).

Metabolite production and extraction:

The bacterial metabolite production was carried out using modified nutrient broth (MNB) (Calcium chloride 0.015 g, Magnesium sulfate 0.02 g, Potassium chloride 0.01g, Disodium hydrogen phosphate 0.05g, Sodium chloride 0.01g, and Glucose 1%, in 1000 ml, with pH 7.4) and sterilized at 121° C for 15 minutes. A single colony from the nutrient agar plate was inoculated into 10 ml of sterile MNB media incubated at room temperature for 18 h. After incubation, the bacterial culture was aseptically transferred into 150 ml conical flask containing 50 ml of sterile MNB and incubated at room temperature for 36 h. Then, the culture in the flask was centrifuged at 10,000 Rpm for 20 minutes at 4° C and

the cell free supernatant was collected. Finally, extraction was made with hexane, ethyl acetate and chloroform. The concentrated crude organic phase was then collected and dried at room temperature. The dried crude metabolites were dissolved in respective solvent and the bioactivity was determined.

Secondary screening:

The bioactivity of crude metabolites was analyzed through well diffusion and disk diffusion method with MHA plates. The 18 h old test pathogens were plated as lawn culture, then made a well by using gel puncher and left it for 30 minutes. After 30 minutes different concentration of crude metabolites ranged between 10 and 50 µg/ml placed into the well and incubated at 37° C for 48 h. Similarly, in disk diffusion assay the same concentration was used and the concentrated disk was placed onto the MHA plates and incubated at 37° C for 48 h to test the inhibitory activity of crude metabolites against bacterial and fungal pathogens.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC):

The crude active metabolites were screened for their MIC and MBC. The MIC was performed by using 96-well micro-titer plate. The crude metabolite was taken in the concentration ranged between 10 and 100 µg/ml, the bacterial and fungal pathogens were grown in the nutrient broth and sabouraud dextrose broth respectively. The cells were seeded in micro-titer plate; the nutrient broth and sabouraud dextrose broth were maintained as a control. The above-mentioned concentration of crude metabolites were taken and seeded into the well and incubated at 37° C for 24 h, later the plates were analyzed in a spectrophotometer at 600 nm. The MBC was analysed with sterile Muller Hinton Broth (MHB) by the tube dilution method; 1 ml of broth was dispensed into the sterile tubes and labeled from 1 to 5. A stock of MHB containing 25 mg/ml crude extract was prepared and two-fold dilutions were made in sterile test tubes aseptically. Then, the tube was inoculated with equal volume of overnight culture, the tube 6 was used as control containing sterile medium and tube 7 was used as viable organism. The final concentration of extraction in each tube numbered after dilution 25, 12.5, 6.25, 3.125, 1.56 mg/ml and incubated at 37° C for 24 h and examined the growth.

Characterization by Thin Layer Chromatography:

The active crude metabolites were further characterized by thin layer chromatography. The crude metabolite was dissolved in respective solvent. Fractions were collected and characterized by thin layer chromatography followed with the polar and non-polar solvent system such as ethyl acetate: methanol: water (20:2:7, v/v/v) and chloroform: methanol (9:1v/v). The crude metabolites were spotted by using capillary tubes on to the silica coated plate allowed to dry after that the plates was developed with the solvent mobile phase in the above-mentioned solvent system for 30 minutes. The developed plates were observed through UV-transilluminator and the retention factor were calculated by the formula:

Rf value = Distance travelled by the solute / Distance travelled by the solvent

RESULTS:

Screening of antibacterial activity of the isolated marine bacteria:

A total of 70 bacterial strains were obtained from six different sponges. In this study, all the isolates were primarily screened against human bacterial pathogens, namely *Bacillus* sp.*, E. coli, Salmonella* sp., *Pseudomonas aeruginosa* and fungal pathogens *Candida albicans* and *Aspergillus flavus* in cross streak method. Among these, only five strains (SgM1, SgM2 SgM3, SgM4 and SgM5) inhibited more than one pathogen. Interestingly, the strain SgM1 and SgM3 was found to have prominent inhibitory activity against bacterial (Table 1) and fungal pathogens (Table 3).

Identification of Symbiotic Bacteria:

All the five promising strains namely SgM1, SgM2, SgM3, SgM4, SgM5 were subjected to various phenotypic characters. Among these, the high potent strains SgM1 and SgM3 was identified as *Micrococcus* sp., based on their biochemical characters. The strain SgM1 and SgM3 was Gran positive which produced oxidase and Catalase enzymes. Also, the strain was utilized citrate and fermented carbohydrates (Table 2).

Table-1: Primary screening of five potential isolates against bacterial pathogens-Cross streaking method

Cross Streak Method				
Bacterial Strains	***Bacillus* sp.,**	***E. coli***	***Salmonella* sp.,**	***Pseudomonas aeruginosa***
SgM1	+	+	++	++
SgM2	++	+	+++	++
SgM3	+++	+	+++	++
SgM4	+++	+	++	+
SgM5	+	+	++	+++

Table -2: Biochemical Characterizations of Potential Strains SgM1 and SgM3

Biochemical Tests	**Results**	
	SgM1	**SgM3**
Cultural characteristics	Small Creamy, Translucent	Small round convex colony
Isolated medium	Artificial Sea water in ZMA	Sponge dissociation medium in NA
Simple staining	Cocci	Cocci
Gram staining	+ve Cocci	+ve cocci
Catalase	+	+
Oxidase	+	+
TSI	Alkali	Alkali no H2s
Citrate utilization	+	+
Carbohydrate fermentation	+	+
Indole	+	+
MR-VP	-	-
Gelatin hydrolysis	+	-
Motility with H_2S	-	Motile no H_2S

Table-3: Antimicrobial activity of ethyl-acetate crude extracts obtained in secondary screening

S.No	**Crude extract**	**Zone of Inhibition (mm)**									
		***Bacillus* sp.,**		***E. coli***		***Salmonella* sp.,**		***Pseudomonas aeruginosa***		***Candida albicans***	
		W.D	**D.D**	**W.D**	**D.D**	**W.D**	**D.D**	**W.D**	**D.D**	**W.D**	**D.D**
1	**SgM1**	18	5	16	11	23	15	7	19	5	7
2	**SgM2**	-	-	18	13	-	11	-	16	-	-
3	**SgM3**	19	5	7	-	20	12	13	16	-	-
4	**SgM4**	10	10	-	14	-	10	18	19	-	-
5	**SgM5**	15	-	7	-	-	15	-	-	-	-

Secondary Screening of extracted crude metabolites:

The crude ethyl acetate extract of all the five strains with different concentration (10, 20, 30, 40 and 50 µg/ml) were tested against 4 bacterial strains and 2 fungal pathogens and the test results of 50 µg/ml concentration are given in Table 3. The results revealed that the Strain SgM3 inhibited well the growth of two bacterial pathogens *Bacillus* sp., (19 mm), *Salmonella* sp., (20 mm) in well diffusion method, whereas *Pseudomonas aeruginosa* inhibited well with 16 mm zone of inhibition in disk diffusion assay. However, SgM3 did not show any activity against fungal strains *Candida albicans* and *Aspergillus flavus*. Interestingly, the strain SgM1 showed inhibitory activity against all bacterial pathogens and on fungus *C. albicans* both in well and disk diffusion assay.

Determination of MIC and MBC:

The crude metabolites were further determined for their MIC and MBC activity. MIC was carried out in microtitre plate, whereas MBC was carried out by tube dilution assay. All the five crude metabolites were subjected for their ability to inhibit the growth of various bacterial and fungal pathogens like *Bacillus* sp., *E. coli, Salmonella* sp., *Pseudomonas aeruginosa*, *Candida albicans,* and *Aspergillus flavus* and the results are presented in Table 4 and 5 and Figure 1 and 2. Among these five isolates the MIC was observed in SgM3 strains with 20µg/ml as a lowest concentration against *Bacillus* sp., and *Salmonella* sp., On the other

hand, the MBC was fluctuated between 30 and 60 µg/ml concentrations for the remaining isolates (Table 4). Similarly, MBC was also done and the results revealed that the MBC was observed in SgM3 strains, inhibition was attained in 1.56 ml concentration against *Salmonella* sp., and *Pseudomonas aeruginosa*. However, the MBC was observed with 12.5 ml against all the remaining organism.

Figure-1: Determination of Minimum Inhibitory Concentration of crude extract of the potential isolates against bacterial pathogens

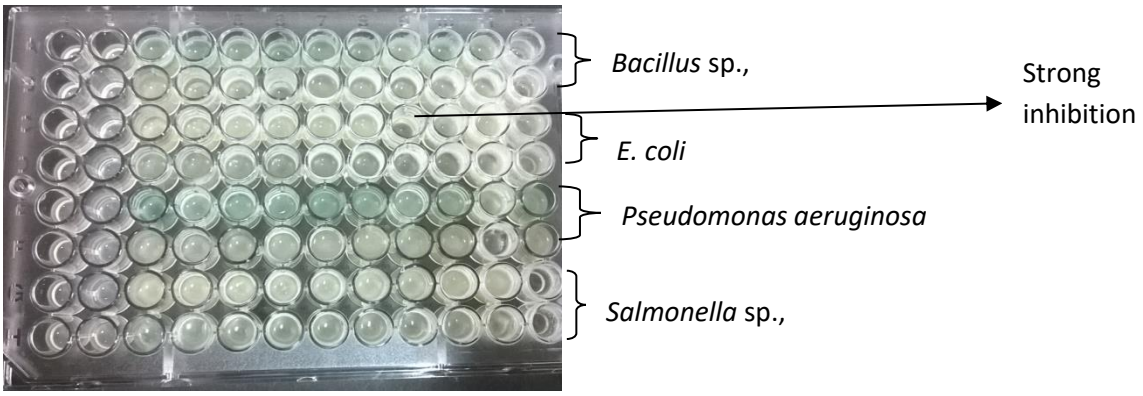


Fig. 2 Determination of Minimum Inhibitory Concentration of crude extract of the potential isolates against fungal pathogens.

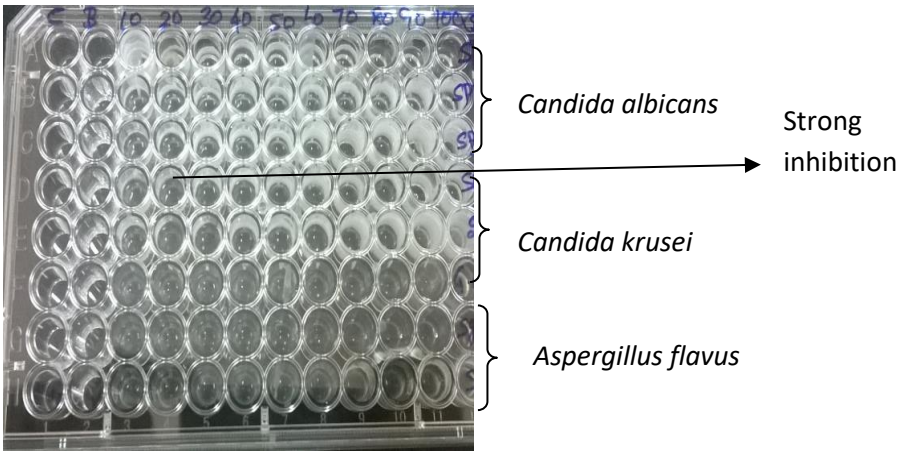


Table-4: Determination of Minimum Inhibitory Concentration (MIC) of the potential isolates (µg/ml).

Bacterial isolates	*Bacillus* sp.,	*E. coli*	*Salmonella* sp.,	*Pseudomonas aeruginosa*	*Candida albicans*	*Aspergillus flavus*
SgM1	20	60	50	50	60	40
SgM2	30	40	30	30	-	-
SgM3	20	60	20	30	-	-
SgM4	50	60	50	50	-	-
SgM5	30	10	80	40	-	-

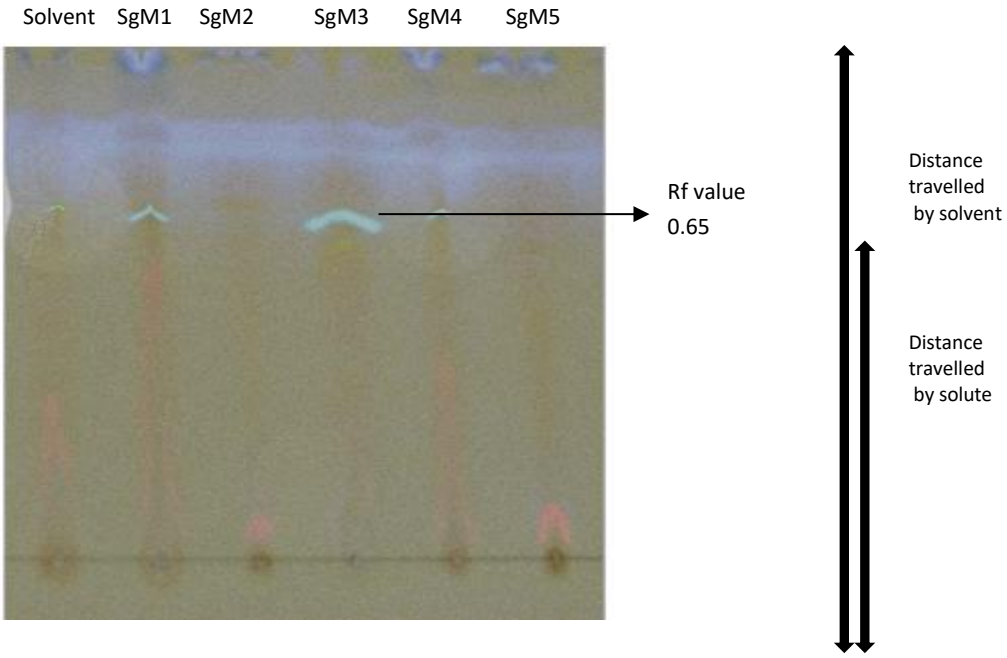
Table - 5: Determination of Minimum Bactericidal Concentration of the potential isolates (mg/ml)

Bacterial isolates	*Bacillus sp.,*	*E. coli*	*Salmonella sp.,*	*Pseudomonas aeruginosa*	*Candida albicans*	*Aspergillus flavus*
SgM1	25	6.25	12.5	6.25	12.5	12.5
SgM2	25	12.5	12.5	12.5	-	12.5
SgM3	25	25	1.56	1.56	-	-
SgM4	6.25	-	-	12.5	12.5	6.25
SgM5	6.25	25	6.25	25	-	-

Characterization of crude extract by Thin Layer Chromatography (TLC):

The crude metabolites were subjected to TLC analysis for the separation of bioactive compounds. The active fractions were collected and separated on the TLC plate, then the plate was developed in ethyl acetate: methanol: water (20:2:7, v/v/v) on silica gel plates. The plate was observed under UV transilluminator and the R_f value was 0.65 (Figure 3). The spot showed blue color on UV illumination. The active compound was further characterized for further analysis.

Figure- 3: The TLC plate showing the separation of active compounds



DISCUSSION:

The search of novel antimicrobials has gained the urgency because of increased development of resistant by pathogens against one or more antibiotics. Marine bacteria have been recognized as an important source for novel bioactive compounds. During the last decade, several bioactive compounds have been isolated from marine bacteria which are new sources for development of medically useful compounds [1]. Recent studies indicated that the symbiotic bacteria showed the specificity against pathogenic bacteria, they may also contain several bioactive metabolites and potentially able to inhibit the target organism [14]. In this study, we have mainly focused on the therapeutically applicable bioactive compound obtained from sponge associated microbes against different human pathogens.We have selected the pathogens based on the property of human pathogen in nature, biofilm producers, and drug resistant of both bacterial and fungal strains such as *Bacillus* sp., *Salmonella* sp., *Pseudomonas aeruginosa, Candida albicans* and *Aspergillus flavus.* A total of 70 strains was morphologically selected and all the

colonies were found to circular (60%) and translucent (20%). Based on this morphological physiological and biochemical properties, *Bacillus* sp., *Pseudomonas* sp., *Vibrio* sp., and *Micrococcus* sp., were identified based on biochemical properties [15]. Among these, the strains SgM1 and SgM3 showed promising activity against pathogens particularly the strain SgM1 exhibited moderate activity against *Candida albicans* and *Aspergillus flavus*. In general, the active compound obtained from any kind of natural samples might have inhibitory activity against either bacteria or fungi. However, in our study it was found that the strain SgM1 had inhibitory activity against both in bacterial as well as fungal pathogens. The present findings is completely endorsed by the works published elsewhere [17 and 18]. The strains SgM1 and SgM3 were isolated from marine sponges collected from Gulf of mannar region which is a considered as a pollution free environment. Marine environment is a complex environment and various physico-chemical parameters control the distribution and activity of free living microbes, symbionts and another macro life. Some group of microbes which has been associated in sessile marine sponges as a biofilm might have produced a toxic substance to prevent themselves from predators. Thus, the bacteria isolated and screened for bioactive potential has gained importance for therapeutical use and we consider these two strains as a novel microbe.

Also, in the present study the MIC and MBC of the crude extracts obtained from SgM1 and SgM3 were attempted. It was found that the MIC was 20 µg/ml and the MBC was 1.56 mg/ml against bacterial pathogens. Similar results was reported from marine compounds obtained from bryozoans, acidians, seaweed and sponges [17-20] and these organisms are the potential candidate to produce a novel bioactive compound have the biological acivities against pathogens. In another study, a potentially biologically active compound isolated from tunicate associated bacterium and named as Harman [18-19]. Interestingly, in support of the present work "sinimol" a sponge associated bacterium producing compound named as nor –harman had a good activity in MIC and MBC assay [19]. The crude metabolite was further characterized by thin layer chromatography, which is widely used method to fractionate the antimicrobial compounds isolated from natural materials. TLC is considered as a good resolution for evaluation of active compound, crude compound of metabolites produced under UV-transilluminator at 234nm and the Rf value was calculated as 0.65. This finding is in agreement with several findings of previous studies and reported that the ethyl acetate eluted compound in TLC plates Rf value was measured as 0.60,0.72,0.79 for the solvent system [16]. Further analysis of this crude extracts is on the way in our laboratory and results will be published.

CONCLUSION:

The discovery of novel antibiotics is necessary for resistant organism due to emergence of multi drug resistance. This study reports the novel finding of antimicrobial drug from marine resources which can be useful for multi drug resistant pathogenic organism. The isolated marine bacteria might exhibit a symbiotic relationship with the sponges and may produce novel bioactive compounds. Further study on marine bacteria associated with sponges will be of immense useful for the biomedical researches to screen for new compound for therapeutic applications.

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