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Screening Twelve Species of Sponges for Biomedical Activity in Gulf of Mannar Tuticorin Coast

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Abstract To screen the antibacterial efficacy of various solvent extracts of 12 species marine sponges against some selected bacteria and fungi human pathogenic bacteria. Crude extracts were prepared from the selected marine sponge using different solvents namely, ethyl acetate and methanol and were tested for their antibacterial activity against human pathogenic bacteria using disc diffusion method. Minimum inhibitory concentration (MIC) was also performed for selected solvent extracts for all the bacterial species. A suitable positive control was also maintained. Among the marine sponge 12 species screened were found to be more active than 2 sponges. It was observed that the ethyl acetate extracts of the marine sponge showed higher inhibitory activity for the selected bacterial species than methanol solvent extracts. The results revealed that the crude ethyl acetate extracts seem to be a good source material in identifying the effective pure antibacterial compound in the sponges. The present study showed that the ethyl acetate extracts of marine sponges such as exhibited good antimicrobial activity. But the ethyl acetate of *Aurora globostellata* (Carter) and *Spirastrella inconstans* var. *moeandrina* Dendy sponge possessed highest antibacterial activity than methanol extracts and so it could be useful in seeking active principles against human pathogenic bacteria.

Keywords Antimicrobial activity; Phytochemical analysis; Human pathogens; Marine sponges

1 Introduction

Marine secondary metabolites are organic compounds produced by microbes, sponges, seaweed and other microorganism (Attaway and Zaborsky, 1993). The host organism biosynthesis these compounds as non-primary or secondary metabolites to protect themselves and to maintain homeostasis in their environment (Selvin, 2002). Some of these secondary metabolites offer avenues for developing potent drugs (Selvin and Lipton, 2004). Retrospective of research in this field indicated a number of diverse biologically active compounds have been isolated from marine sponges, the number of compounds taken-up for the field trial/clinical use is scanty. Hence screening many marine sponges for bioactive compound could bring many novel compounds in to light. In this context the present study was initiated to screen new sponges for biopotentials.

There are few reports on the antibacterial activity of bioactive compounds identified in sponges. Burkholder and Ruetzler (1969) revealed that 18 of 31 sponges tested showed antimicrobial effects, of which some are very strong against a range of gram-positive and gram-negative bacteria. Samples of 28 demosponges collected along fresh coast indicated a high antibacterial activity (Amade et al., 1987). Antibacterial activity of *Dendrilla nigra* had been reported by Ivanova et al (1993, 1994). Selvin and Lipton (2002, 2004) had reported the presence of antimicrobial bioactive compounds in the sponges *Axinella donnani* and *Clathria gorgonoides*. According to Selvin and Lipton (2004) most of the available reports on antibacterial property of sponges revealed their activity on gram positive bacteria. Venkateswarlu and Biabani (1995) had reported that the dichloromethane-methanol (1:1) extract of the sponge *Phycopsis* sp. collected from Tuticorin coast of India, exhibited antibacterial activity. The bromo-pyrrole alkaloids found in *Agelas dispar* showed moderate antibiotic activity against gram

positive bacteria such as *B. subtilis* and *S. aureus* (Caiferi et al., 1998). The *Latrunculia brevis* and *Prianos* sp. were contained potent antibacterial Discorhabdin D which was chemically characterized as pyrrolophenanthroline alkaloid (Ford and Capon, 2000). Extracts made from *Sigmoceptrella* sp. collected as by catch during traveling operation in the Great Australian Bay sent has inhibitory against many bacteria (Ovenden and Capon, 1999). The bioactive compound Arenosclerins A-C derived from the sponge *Arenosclero brasiliensis* was very effective against 12 antibiotic resistant bacteria isolated from a hospital (Torres et al., 2002). Sipkema et al (2005) reviewed the various bioactive potential present in the marine sponges.

The potency of sponge derived medicines lies in the fact that each of these thousands of metabolites and their derivatives has its own specific dose related inhibitory effect, efficacy and potential (diminished) side effect that determine its suitability for medicinal use. Leone et al (2008) has isolated Exiguaquinol from the sponge *Neopetrosia exigua* and this was found to inhibit the bacterial enzymes *Helicobacter pylori* (Glutamate racemase that inter converts L-glucose and D-glucose) needed for the construction of bacterial cell walls. A cytotoxic and antibacterial bromophenol was isolated from a sponge *Dysidea* sp. (Zhang et al., 2008). The sponge *Suberea mollis* was found to contain the moderately antibacterial Subereaphenol A (Shaala et al., 2008). Halicyclamine A isolated from the sponge *Haliclona* sp. inhibited the inosine−5′−dehydrogenase (IMPDH) and anti-*Mycobacterium tuberculosis* activity (Blunt et al., 2010).

In India, Gulf of Mannar region is a rich bed for valuable marine sponges. As marine sponges open new avenues to develop novel drugs, a preliminary screening was made on the available sponges, hitherto unstudied for their bio-medical potential. In this direction the sponges available from Gulf of Mannar coast were collected and from this collection four species of sponges with good antimicrobial response were further chosen to select the best species for an in-depth study.

2 Results and Discussion

In the preliminary screening, 12 species of sponges were tested for antimicrobial activities. Of the 12 species of sponge tested, the extracts of four species were chosen for further study. Both ethyl acetate and methanol extracts of the sponges were tested. Of the two solvents used, the extracts of ethyl acetate exhibited more antimicrobial activity than methanol (see Supplementary data, Table 1, 2). Hence ethyl acetate extracts were used further. Based on the antimicrobial activity, 4 species of sponges (TCN-8 and TCN-10) were selected for further study.

The results of the microbial assays for the crude extracts of 12 sponges are presented in Table 2 and Table 3.

As can be seen from the results, several of the extracts assayed showed a good antibacterial activity especially against *E. coli* (NCIM 2065), *P. aeruginosa* (NCIM 5031), *B. subtilis* (NCIM 2063) and *S. aureus* (NCIM 2079). When compared to methanol extract, the ethyl acetate extracts of all the sponges showed a high antibacterial activity. Both Gram positive and Gram negative bacteria were sensitive to the crude extracts. Fungi especially *S. cerevisiae* (NCIM 3054) and *A. niger* (NCIM 501) are more resistant than *C. albicans* (NCIM 3102) (Table 4).

Of the ethyl acetate and methanol extracts of twelve sponges tested, the methonolic extracts of the sponge *S. inconstans* var. *moeandrina*, and *A. globostellata* were highly sensitive to all the microbes tested (Figure 1). The three sponges belonged to the species *S. inconstans* were good antimicrobial agents among this other species. Although these species were collected from two different locations, they showed a good antibacterial activity. Hence *S. incontans* var. *moendrina* Dendy was selected for in depth study and compound isolation (Figure 2). To extract the antimicrobial bioactive principles from the sponges, ethyl acetate was found to be good solvent than methanol. Ethel acetate was found to be good solvent that methanol. Ethyl acetate extract of *S. inconstans* var. *moendrina* Dendy inhibited growth of the bacteria *S. abony* effectively (19.66±0.88 mm) when compared to the inhibitory role of all the other extracts.

Table 3 Screening of ethyl acetate extracts of four selected marine sponges for biochemical constituents

Sponges	Steroids	Triterpen-oids	Reducing suger	Alkaloids	Phenolic compound	Saponin	Xantho protein	Tannin	Flavan-oids	Aromatic acid
Aurora globostellata (TCN -8)	-	-	-	+	-	+	-	-	+	-
Spirastrella inconstans var. *moeandrina* Dendy (TCN -10)	+	-	+	+	-	+	-	+	+	-

Note: (+) Present; (-) Absent

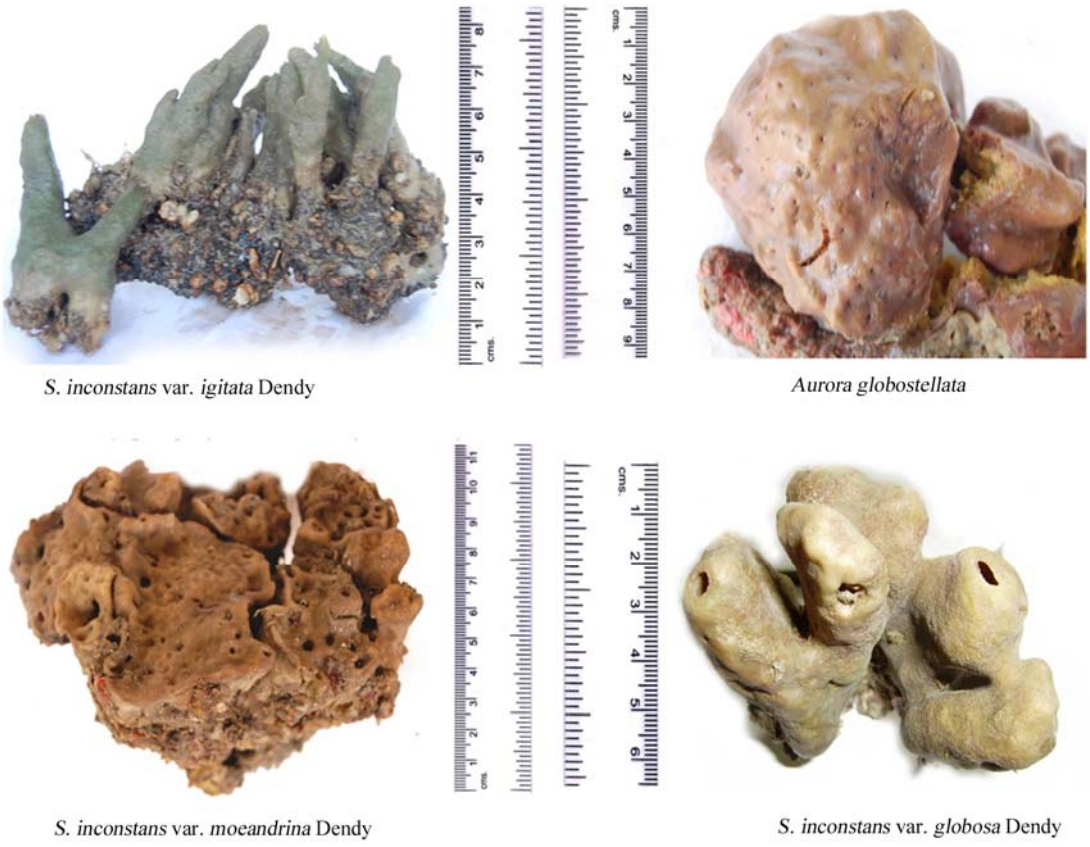

Figure 1 The four species selected for biomedical study

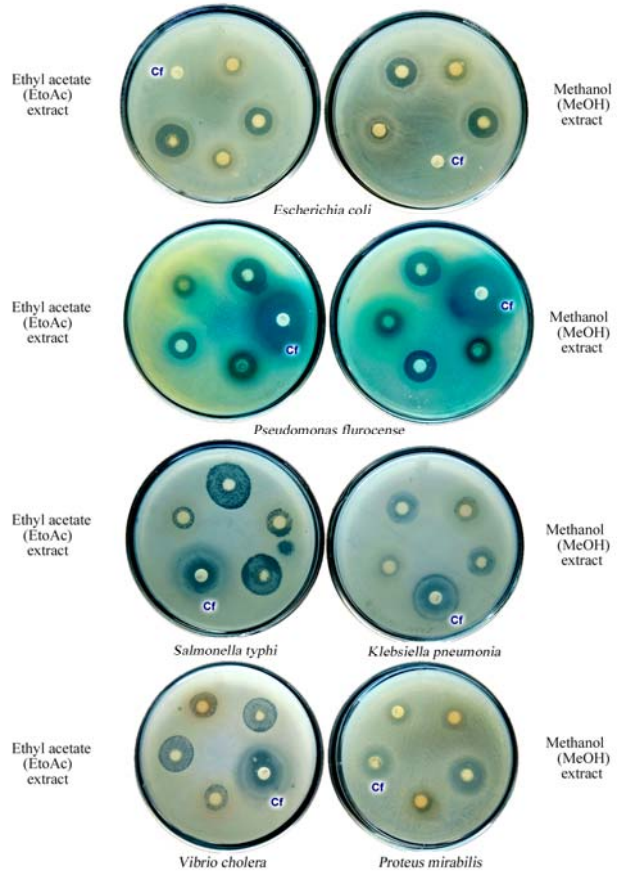

Figure 2 Antimicrobial screening and antibacterial activity of well assay method

Antifungal activity was also observed in the extracts of the sponges. *C. albicans* was sensitive to all the extracts tested and *S. cerevisiae* was resistant to both ethyl acetate and methanol extracts of sponges. The presence of chemical constituents like steroids, Tri terpenoids, Reducing sugar, Alkaloids, phenolic compound, Saponin, Xantho protein, Tannin, Flavanoids and Aromatic acid, were tested in the selected four species of sponges (Table 3).

Steroid was present in all the four species except in *A. globostellata* (TCN-8), triterpenoid was present only in reducing sugar was absent in *A. globostellata* (TCN-8) alkaloids, saponin and flavanoids were present in the species *A. globostellata* (TCN-8), phenolic compound, xantho protein, aromatic acid were absent in all the species was present in *S. inconstans* var. *moeandrina* (TCN-10) *S. inconstans* var. *globosa* (RSM-13).

This is not surprising as the sponge belonging to this genus and collected from different regions is reported to possess wide variety of compounds with different biological activities. Thus, *Haliclona* sp. from Indonesia yielded a triterpene ketide, Halicotriol B with weak antimicrobial activity against *S. aureus* and *Bacillus subtilis* (Crews and Harrison, 2000). The antifungal papuamine has been reported by Baker et al (1988) from a *Haliclona* sp. Fahy et al (1988) report a major antimicrobial alkaloid haliclonadiamine together with antifungal papuamine from *Haliclona* sp. of Palau. Antifungal aminoalcohols have been identified from a new species of *Haliclona* from Queensland (Clark et al., 2001). Charan et al (1996) report antimicrobial Haliclonacyclamines. It is therefore expected that the activity found by us in the extract of *H. cribricutis* could have, at least partially, been contributed by any one of the above compounds isolated from this genus. Organisms belonging to the same genus are bound to have common chemical constituents. Parameswarn et al (1992) report significant anti-viral and antibacterial activities in petroleum ether and ethyl acetate fractions of *H. cribricutis* and the activity observed against *K. pneumoniae* and *Vibrio parahaemolyticus* was attributed to o-demethyl renierones. *Ircinia* sp. exhibited mild antibacterial activity only against *S. aureus* but all the fungal strains tested were insensitive to it.

A number of cytotoxic compounds are reported from this genus. These include 73-deoxychondropsin-A from an Australian *Ircinia ramosa.* Chondropsin-C was found in a Philippine *Ircinia* species (Rashid et al., 2001). Moderately cytotoxic cumulated ketene irciniketene has been reported from *Ircinia selaginea* collected from Guangxi Province, China (Yan et al., 2001). Cytotoxic kohamaic acids A and B are known to be constituents of Ircinia species from Okinawa (Kokubo et al., 2001). Three tricarbocyclic sesterterpenoids of the cheilanthane class isolated from a Queensland Ircinia species were found to be inhibitors of MSK-1 and MAPKA-2 protein kinases (Buchanan et al., 2001). Though cytotoxic compounds are reported from this genus, there are no reports of any antimicrobial activity in the extracts.

Table 4 Screening of selected sponges for chemical constituents

Sample no.	Test	Reaction	Present(+)/absent (-)
1	Test for Steroids: Cold extract + minimum amount of $CHCl_3$ + 3 drops of acetic anhydride + 2 drops of Conc. sulphuric acid	Purple colour solution changing to blue or green	+
2	Test for Triterpenoids: Cold extract + piece of tin + 3 Drops of thionyl chloride	Violet or purple colour solution	+
3	Test for Reducing sugar:		
a	Cold extract + equal volume of Fehling A&B + heated over water bath	Red precipitate	
b	Cold extract + Tollens reagent and heated over bath	Silver mirror purple colour	+
c	Cold extract + Molish reagent	Purple colour	
4	Test for Aalkaloids:		
a.	Cold extract + 2N HCl (aqueous layer decanted) + 2 drops of Mayers reagent (KI + HgCl)	Pale yellow or white precipitate	+
b.	Cold extract + acetic acid (aqueous layer decanted) + few drops of Dragendroff's reagent	Orange or red orange precipitate	
5	Test for Phenolic compound: Cold extract + Neutral $FeCl_3$	An intense blue or violet coloration	+
6	Test for Saponin: Cold extract + water mixture shaken vigorously	Formation of foamy layer	+
7	Test for Xantho proteins: Cold extract + concentrated Nitric acid + excess Ammonia	Red orange precipitate	+
8	Test for Tannins: Cold extract + basic lead acetate	White precipitate	+
9	Test for Flavanoids: Cold extract + bit of Mg^{2+} drops of Con. HCl Heated and then cool	Red or orange red color	+
10	Test for Aromatic acids: Cold extract + Saturated Sodium bicarbonate	Brisk effervescence	+

3 Materials and Methods

3.1 Collection of sponges, sample preparation and extraction

The sponges 22 species were collected from the low inter tidal pools at Bay of Bengal from Gulf of Mannar Biosphere reserve of Tuticorin coast (8° 47′ N, 78° 8′ E) and Rameswaram coast (9° 28′ N, 79° 12′ E) at 4~5 m depth from Tamil Nadu, India. The sponges were collected by an eco-friendly bulk collection by catch in the fishing nets. From the all twelve sponges identified, tissues samples were incised out and (100 g) were washed with sea water, air dried and chopped into small size before being ground into fine paste. Using the paste the ethyl acetate (EtOAc), and methanol (MeOH) were carried out. The extraction was carried out in triplicates for 48 h. the extract was stored in dark container and left in deep freezer at −20℃. After 48 h the extract was

filtered through Whatmann filter paper (No: 2) and concentrated using vacuum rotary evaporator (Super fit, Bangalore). The concentrated extract was used for antimicrobial study. Among the twenty two sponges, four species were selected for further study based on the screening tests (Figure 1).

3.2 Antimicrobial activity

For the antimicrobial screening 5 species of bacterial isolates and three species of fungal isolates were selected. The bacterial and fungal strains were obtained from National Collection of Industrial Microorganisms (NCIM), Pune, India.

Escherichia coli (NCIM 2065), *Salmonella abony* (NCIM 2257), *Pseudomonas aeruginosa* (NCIM 5031), (Gram negative bacteria) *Bacillus subtilis* (NCIM 2063), *Staphylococcus aureus* (NCIM 2079), (Gram positive bacteria) strains were used. *Candida albicans* (NCIM 3102), *Saccharomyces cerevisiae* (NCIM 3054), unicellular fungi and *Aspergillus níger* (NCIM 501) mold fungi were used as fungal test microorganisms.

3.3 Antibacterial activity of well assay method

Assays were performed according to the standard guidelines of the National Committee for Clinical Laboratory Standards (NCCLS, 1999) using a modified Kirby–Bauer well assay method. A sterile stainless steel borer (6 mm) was used to make well in the medium. All the organisms were stored at −20℃ until use. Cells were grown at 3℃ in Mueller-Hinton broth to an OD_{420} = 1.9 (approximately 10^5 CFU/mL), and were transfer to Muller Hinton agar for bacteria, and Sabouraud dextrose agar for yeasts and fungi. Broth cultures were swabbed onto respective agar medium to achieve a lawn of confluent microbial growth separately for each strain. Four wells were bored in each plate. The sponge extract 100 μg/mL was loaded in to the well and to find out the inhibitory potential. The plates were incubated for bacteria at 37℃ 24 h and fungi were grown at 28℃ for 48 h. The growth of bacteria and fungi around each well was observed carefully and the diameter of the zone of inhibition around each well was measured using a Hi-media zone reader Triplicate plates were maintained for each test.

3.4 Preliminary screening of sponges for chemical constituents

Qualitative analysis of the chemicals present was carried out using methods described by Harborne (1998). The freshly prepared sponge extracts were analyzed for the presence of various constituents as described by Okawori et al (2008) (Table 4).

3.5 Thin Layer Chromatography (TLC)

The sponges with higher antibacterial activities were taken for TLC studies. *Aurora globostellata* (Carter) (TCN-8), *Spirastrella inconstans* var. *moeandrina* Dendy. (TCN-10). Thin layer chromatography plates were prepared by using with Si-gel F254 grade (Merck, Darmstadt, Germany) as stationary phase. Liquid mobile phases were either semipolar (CH_2Cl_2: MeOH; 9:1, v/v) or non polar (Hexane: EtOAc; 8: 2, v/v). Reversed phase (RP) was used for polar fractions. The mobile phase systems were MeOH: H_2O; 3:7, 8:2 and 1:1 (v/v). A one-dimensional ascending development technique was used to detect the constituents of an extract on TLC plate. Visual detection was done in daylight and under UV light at a wave length of 254 nm. The results were given in (Figure 3, 4)

3.6 Column Chromatography

In this study, two different sizes of columns were used. The columns was prepared by using Silica gel as a packing material which is used as stationary phase. (Normal phase Column Chromatography) Si-gel 60~120 mesh with a particle size of 0.004~0.063 mm (Merck) different combinations of organic solvents such as hexane, ethyl acetate and methanol were used by step gradient or isocratic elution (Figure 3, 4).

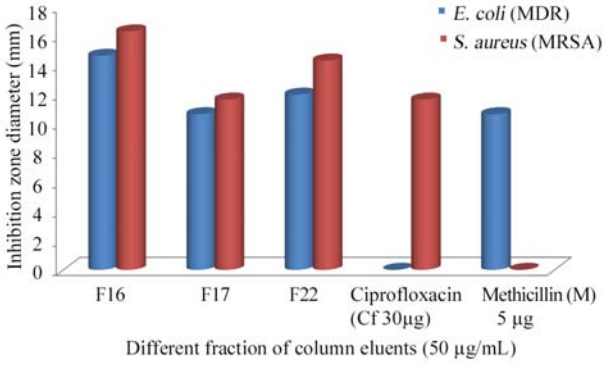

Figure 3 Antibacterial activity of purified compounds from marine sponge *Spirastrella inconstans* var. *moeandrina* Dendy

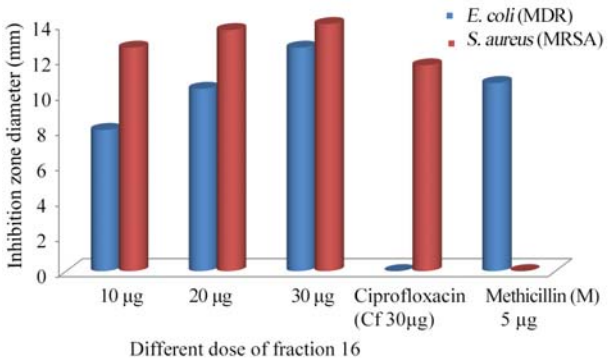

Figure 4 Antibacterial activity of purified compounds from marine sponge *Aurora globostellata*

3.7 Sponge extracts elution

Based on the antimicrobial assays, the extract that registered the maximum antimicrobial activity was selected for further study. EtoAc extract was very effective in antimicrobial properties so the ethyl acetate extract were chosen for further separation. The crude EtOAc extract (13.2 g) was applied over a flash chromatography column of silica gel 60 (150 g) and eluted with a solvent gradient system of EtOAc and MeOH, Fractions (50 mL) being collected as follow:

The various fractions were collected from followed solvent system: 1) Hexane 100%; 2) Hexane 99%: ethyl acetate 1%; 3) Hexane 98%: ethyl acetate 2%; 4) Hexane 96%: ethyl acetate 4%. Up to 22nd fraction were eluted. The fractions thus obtained were once again evaporated and concentrated. They were again assayed for antibacterial, antifungal activity and spectral studies.

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Table 1 Antimicrobial activity of ethyl actate (EtoAc) extract of 22 species of sponges collected from Gulf of Mannar region

Sponge	Specimen No.	Inhibition zone diameter (mm) for different microorganisms							
		Escherichia coli (NCIM 2065)	*Salmonella abony* (NCIM 2257)	*Pseudomonas aeruginosa* (NCIM 5031)	*Bacillus subtilis* (NCIM 2063)	*Staphylococcus aureus* (NCIM 2079)	*Candida albicans* (NCIM 3102)	*Saccharomyces cerevisiae* (NCIM 3054)	*Aspergillus níger* (NCIM 501)
Heteronema erecta	TCN-1	13.00±0.57	10.33±0.33	15.00±0.57	13.66±0.33	14.00±0.57	10.66±0.66	0.00±0.00	10.33±0.33
Callyspongia diffusa	TCN-2	15.00±0.57	14.66±0.33	12.00±0.00	13.00±0.57	14.66±0.88	8.66±0.66	8.66±0.66	10.33±0.33
Spirastrella inconstans var. *digitata*	TCN-3	19.66±0.33	21.00±0.57	19.66±0.33	18.00±0.57	20.00±0.57	13.66±0.33	12.33±0.33	14.33±0.33
Spirastrella vagabunda	TCN-4	15.00±0.57	12.66±0.66	16.33±0.33	12.00±0.57	17.00±0.57	13.00±0.57	0.00±0.00	9.00±0.57
Sigmadocia carnosa	TCN-5	10.66±0.66	14.66±0.66	13.33±0.33	12.66±0.66	12.33±0.33	10.00±0.00	9.66±0.88	0.00±0.00
Spongia officinalis	TCN-6	13.00±0.57	15.66±0.33	16.00±0.57	14.33±0.33	12.33±0.33	12.00±0.00	8.66±0.33	0.00±0.00
Hyattella cribriformis	TCN-7	9.33±0.66	12.33±0.33	10.33±0.33	12.00±0.57	11.33±0.33	10.66±0.66	0.00±0.00	8.33±0.33
Aurora globostellata	TCN-8	15.33±0.33	13.33±0.66	11.66±0.33	11.66±0.33	15.33±0.66	12.66±0.66	9.00±0.57	10.00±0.57
Callyspongia fibrosa	TCN-9	7.66±0.33	12.00±0.57	10.33±0.33	14.00±0.57	13.00±0.57	8.33±0.33	0.00±0.00	0.00±0.00
Spirastrella inconstans var. *moeandrina*	TCN-10	15.33±0.66	14.00±0.57	12.33±1.20	12.33±0.33	14.00±0.57	11.33±0.66	10.33±0.33	11.66±0.88
Spongia sp.	TCN-11	9.33±0.33	13.00±0.57	10.33±0.33	11.00±0.57	11.00±1.00	10.33±0.88	0.00±0.00	9.33±0.33
Spirastrella inconstans var. *globosa*	RSM-13	16.66±0.88	19.66±0.88	15.00±0.57	17.33±0.66	17.00±0.57	15.00±0.57	11.00±0.57	13.66±0.33

Table 2 Antimicrobial activity of methanol (MeOH) extract of 22 species of sponges collected from Gulf of Mannar region

Sponge	Specimen No	Inhibition zone diameter (mm) for different microorganisms							
		Escherichia coli (NCIM 2065)	*Salmonella abony* (NCIM 2257)	*Pseudomonas aeruginosa* (NCIM 5031)	*Bacillus subtilis* (NCIM 2063)	*Staphylococcus aureus* (NCIM 2079)	*Candida albicans* (NCIM 3102)	*Saccharomyces cerevisiae* (NCIM 3054)	*Aspergillus níger* (NCIM 501)
Heteronema erecta	TCN-1	10.66±0.66	13.00±0.57	13.00±0.57	14.33±0.33	12.33±0.33	12.33±0.33	0.00±0.00	11.00±0.57
Callyspongia diffusa	TCN-2	13.33±0.33	15.66±0.88	14.00±0.57	12.00±0.00	14.66±0.66	11.00±0.57	8.66±0.33	10.00±0.00
Spirastrella inconstans var. *digitata*	TCN-3	15.66±0.33	16.33±0.33	15.66±0.33	13.66±0.33	19.00±0.57	13.33±0.88	11.00±0.57	11.00±0.57
Spirastrella vagabunda	TCN-4	13.00±0.57	10.66±0.66	12.33±0.33	12.00±0.00	14.00±0.57	11.33±0.66	0.00±0.00	9.33±1.33
Sigmadocia carnosa	TCN-5	9.66±0.33	12.33±0.33	11.66±0.33	12.66±0.33	10.33±0.33	11.00±0.57	0.00±0.00	0.00±0.00
Spongia officinalis	TCN-6	11.66±0.33	13.00±0.57	13.00±0.57	11.00±0.57	10.33±0.33	10.33±0.33	0.00±0.00	0.00±0.00
Hyattella cribriformis	TCN-7	7.66±0.33	10.33±0.33	8.00±0.00	11.00±0.57	10.00±1.15	11.00±1.00	0.00±0.00	9.33±0.33
Aurora globostellata	TCN-8	13.00±0.57	12.00±0.57	13.33±0.66	10.66±0.66	12.66±0.66	10.33±0.33	8.66±0.33	8.33±0.33
Callyspongia fibrosa	TCN-9	8.00±0.00	11.66±0.88	9.00±0.57	12.66±0.66	9.66±0.33	8.00±0.00	0.00±0.00	0.00±0.00
Spirastrella inconstans var. *moeandrina*	TCN-10	12.00±0.57	12.66±0.66	12.33±0.33	10.33±0.33	11.66±0.33	10.33±0.33	0.00±0.00	10.33±0.33
Spongia sp.	TCN-11	7.66±0.33	11.00±0.57	9.33±0.33	8.66±0.66	8.66±0.66	8.33±0.88	9.00±0.57	10.33±0.33
Spirastrella inconstans var. *globosa*	RSM-13	14.00±0.57	17.00±0.57	14.33±0.33	13.66±0.66	14.00±0.57	14.33±0.33	11.00±0.57	11.66±0.33