

Screening of Bioactive Secondary Metabolites from Sea Sponge (*Clathria Indica*) Against Bacteria Associated with Urinary Tract Infections

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Abstract: The marine sponge *Clathria indica*, collected from Thondi-Palk Strait region of Tamil Nadu, was studied for bacterial antagonistic activity. Sponge species were identified based on specula morphology. Ethyl Acetate extracts yielded a total of 0.8g, 0.12g, 0.01g, 0.13g and 0.17g from 1.5g of sponge crude extracts respectively. The antagonistic activity of crude extracts against bacterial pathogens showed clear inhibition zones against *Pseudomonas sp.*, *Streptococcus sp.* and *Vibrio sp.* The extracted sponge metabolites had growth inhibitory activities against all the three Urinary tract pathogens, *Vibrio sp.*, *Pseudomonas sp.* and *Streptococcus sp.* and Bactericidal activities against two Urinary tract pathogens, *Vibrio sp.* and *Pseudomonas sp.* The partial isolation of DNA was done by using Gel electrophoresis. On the gel the DNA showed one defined band which had a size of 39.360Kb.

Key words: Sea sponge *Clathria indica*; *Vibrio sp.*; *Pseudomonas sp.*; *Streptococcus sp.*;

Antagonistic Activity, Minimum Inhibitory Concentration, Minimum Bactericidal Concentration, Gel electrophoresis.

INTRODUCTION

Marine

The potential of marine life as a source of novel molecules is immense and has been barely investigated. Because of their longer evolutionary history, marine organisms likely possess a greater molecular diversity than do the terrestrial organisms. The intensive research since 1970s has proved that marine organisms are important sources of bioactive secondary metabolites (Y. Le Gal *et al.*, 2005).

The marine resources are nowadays widely studied because of many reasons. One of the reasons is that the oceans cover more than 70% of the world surface and among 36 known living phyla, 34 of them are found in marine environments with more than 300,000 known species of fauna and flora (Pereira *et al.*, 2001). The marine environment contains over 80% of world's plant and animal species (Pereira *et al.*, 2001). In recent years, many bioactive compounds have been extracted from various marine animals like tunicates, sponges, soft corals, bryozoans, sea slugs and marine organisms (Pereira *et al.*, 2001).

Importance of marine

The ocean has been considered as a rich source of compounds possessing novel structures and biological activities (Venkateshvaran, 2001). Biologically active molecules isolated from marine flora and fauna have applications in: - pharmaceuticals, cosmetics, nutritional supplements, enzymes, molecular probes, fine chemicals and agrochemicals (Stempein, 1970).

Important secondary metabolites, including antibiotics, herbicides and growth-promoting substances are produced by several members of the marine microorganisms (Venkateshvaran, 2001).

Sponges

Marine sponges are an important component of benthic communities throughout the world, with regard to its biomass as well as their potential to influence benthic or pelagic processes (Rodkina, 2005). Sponges (phylum Porifera) are among the oldest multicellular animals (Metazoa) and show relatively little differentiation and tissue coordination (Rodkina, 2005). More than 8,000 species of sponges have been described; they inhabit a wide variety of marine and freshwater ecosystems and are found throughout tropical, temperate and Polar Regions (Newman *et al.*, 2004). The most intensively investigated sponges have been those collected from the China Sea, Japan and the West Pacific, followed by those from the Indian Ocean and other regions (Blunt *et al.* 2009). Among marine organisms, the largest number of secondary metabolites isolated since 1965 have come from sponges (Belarbi *et al.*, 2003), and they have been the primary source of biologically active molecules. The main biological activities of those sponge Metabolites have been cytotoxic and antimicrobial while other activities have been limited (Belarbi *et al.*, 2003).

Symbiotic association of bacteria in sponges

Sponges are host organisms for various symbiotic microorganisms such as archaea, bacteria, cyanobacteria and microalgae. Sponges are also sources of a wide variety of useful natural products like cytotoxins, antifouling agents, antibiotics, anti-inflammatory and antiviral compounds (Becerro *et al.*, 1997). Symbiotic microorganisms in sponges can be sources of various natural products, because metabolites previously ascribed to sponges have recently been demonstrated to be biosynthesized by symbionts (Garson, 1994). If a symbiotic microorganism from which some natural products are derived can be cultured, the microorganism could be used in the mass production of the bioactive compounds (Garson, 1994). Sponges (Phylum Porifera) are very fertile host animals for diverse symbiotic microorganisms. Sponges are simple multicellular invertebrates attached to solid substrates in benthic habitats (Rodkina, 2005).

Micro-organisms found in sponges

Various microorganisms have been found in sponges. They include a diverse range of archaea, heterotrophic bacteria, cyanobacteria, green algae, red algae, cryptophytes, dinoflagellates and diatoms (Assmann *et al.*, 2000).

Location of micro-organisms in sponges

The symbionts locate both intra- and extra cellularly, and each symbiotic microorganism seems to have aspecific habitat in the host sponge (Kuramoto *et al.*, 1997). Extracellular symbionts are present on the outer layers of sponges as

exosymbionts, or in the mesohyl as endosymbionts. Intracellular or intranuclear symbionts permanently reside in host cells or nuclei (Kuramoto *et al.*, 1997). In the case of the sponge *Theonella swinhoei*, all populations of symbiotic bacteria are located extracellularly. Bacteria, e.g., *Pseudomonas* sp. and *Aeromonas* sp. inhabit as free-living cells in the mesohyl and/or as intracellular symbionts in sponge *Verongia*. The amount of symbiotic microorganisms residing in sponges varies between host species (Duckworth *et al.*, 2003).

Natural compounds derived from sponges.

Marine microorganisms are good candidates for new pharmaceuticals and bioactive natural products. There is accumulating evidence that demonstrates the involvement of symbiotic microorganisms in the natural products originally attributed to the sponge host (Donia *et al.*, 2003). For example, symbiotic bacterium *Micrococcus* sp. produces diketopiperazines previously ascribed to the host sponge *Tedania ignis*. Another symbiotic bacterium *Vibrio* sp. produces brominated biphenyl ethers formerly attributed to the host sponge *Dysidea* sp. A dinoflagellate *Prorocentrum lima* produces okadaic acid, first isolated from the host sponge *Halichondria okadae*. Symbiotic bacterium *Vibrio* sp. produces an anti-*Bacillus* peptide andrimid that was found in the sponge *Hyatella* sp. extract (Donia *et al.*, 2003).

Bioactivity of sponges

Sponges have different biological activities on different micro-organisms which include;- Antibiotic, Antibacterial, Cytotoxic, Antimicrobial, Somatostatin/ Vasoactive Intestinal Peptide inhibitor, Antileukemic, Antituberculosis, enzyme inhibitor, Phosphatase inhibitor, Antifungal, Insecticidal, Antitumor, Antiparasitic, Immunosuppressive, Na⁺/K⁺-ATPase inhibitor, receptor inhibitor, Neurotoxin, Cardiovascular effector, Topoisomerase II inhibitor, Antiproliferative (Ernesto *et al.*, 2002).

Urinary Tract Infection

UTI describes a condition in which there are micro organisms established and multiplying within the urinary tract. It is most often due to bacteria (95%), but may also include fungal and viral infection (Thomas M. Hooton, 2000). Urinary tract infection (UTI) is defined in various ways by different authors. Stamm *et al* (1997) have defined UTI as an infection which occurs in a patient with anatomically abnormal urinary tract or significant medical or surgical morbidities. Nicolle *et al*, 2002, defined urinary tract infection as that occurring in individuals with functional or structural abnormalities of the genitourinary tract. The largest group of patients with UTI is adult women, because a woman's urethra is short, allowing easy access of bacteria to the urinary bladder. Additionally a woman's urethral opening is near sources of bacteria namely the anus and vagina (Stamm *et al.*, 1993).

Pathogens associated with UTI.

Yildiz *et al* (2004), from their recent study reported that Gram negative organisms were the most common uropathogens causing UTI in the pediatric age group. Peterson and others (2006) reported that *E. coli* was the most common organism causing UTI in the United States. However similar studies in India are very few (Yildiz *et al.*, 2004). The most common pathogens associated with UTI are; *E. coli*,

Pseudomonas aeruginosa, *Klebsiella*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Proteus mirabilis*, *Candida albicans*, *Pneumoniae* (Yildiz *et al.*, 2004).

The objectives of the study were isolate endophytic heterotrophic bacteria in *Clathria indica* species of sea sponges along the East Coast of India, Palk Strait and isolate secondary metabolite from selected THB strains. The antimicrobial activities of the ethyl acetate extracted metabolites, against urinary tract pathogens would be studied in order to gauge the level of bioactivity of the metabolites. The other objective was to type the most promising THB isolates using DNA methods.

MATERIALS AND METHODS

Description of the Study Area

Thondi is a small village situated in the Palk Strait region of Tamil Nadu. The study area lies in the latitude of 99°44'N and 79 10' 45" E (Fig. 1). The rainfalls in Thondi region are mainly due to north east and south west monsoon. Thondi coast has very minimal wave action. Turbidity of the seawater is moderately low and also they are rich in nutrients hence, it serves as a treasure house for valuable marine resources like sea grass, seaweeds, and invertebrates like coelenterates, echinoderms and shell fishes (George *et al.*, 1997). The major occupation of the people is fishing.



Figure 1: Thondi region: Location and study sites. Part named S1 represents the coastline where samples were collected

Collection of Samples

Clathria indica species of Sea sponges were collected from Thondi Park Strait during the monsoon month of January in order to isolate the endophytic THB strains (Fig. 1).

Isolation of Endophytic Organisms

A sample of 1 gm of fresh sea sponge species were aseptically weighed and were washed thrice with sterilized distilled water. The samples were then ground using mortar and pestle after adding 1 ml of sterilized distilled water. Ground samples were serially diluted with sterilized seawater and were spread on Zobell marine agar

medium. The plates were incubated in an inverted position for 24 hrs at 37 ± 2 °C (J. Marine Research N: 42. 1941). The bacterial colonies on the Zobell marine agar medium were counted and the total number of bacterial colonies was expressed as Colony Forming Units (CFU) per gram of sample.

Maintenance of Pure Culture

Based on the morphological differences and color, the different types of THB colonies were selected and then sub cultured to obtain pure cultures and then streaked on Nutrient agar slants and refrigerated.

Isolation of urinary tract associated pathogens

In this study, the mid-stream urine sample from a sick patient was collected from Salem Government Hospital using sterile container.

Isolation Techniques

Spread plate method

One ml urine was serially diluted in sterile tubes with sterile sea water and then 0.1ml of each dilution was spread on nutrient agar and then incubated at room temperature for 4 – 7 days in dark room or in an incubator at 30°C for 7 -10 days. Bacterial culture growth results were then observed.

Maintenance of pure culture

Based on the morphological differences and color, the different types of colonies were chosen. The chosen colonies were streaked on Nutrient agar slants and refrigerated.

Gram staining

Smear was prepared on a clean slide. It was heat fixed by passing through the flame of heat. Two to three drops of crystal violet solution were added to the prepared slide and then allowed to react for two to three minutes and then washed by passing it through running tap water. Two drops of grams iodine solution were added and left for some two minutes to react. The slide was then washed with tap water

Culture

The Media which were used for culturing were: Nutrient Agar, Blood agar, Macconkey Agar, Eosin Methylene Blue Agar, Tetractyl bile salt agar , Christenson's Urea Agar, Cetrinide Agar

The colonies from nutrient agar slants were sub-cultured on blood agar (for *Streptococcus* sp), Macconkey agar (for *Pseudomonas* sp.) and Tetractyl bile salt agar for *Vibrio* sp.

Biochemical tests.

The following were the biochemical tests performed:
Indole test, Methyl Red test, Voges Proskauer test.

Isolation of DNA

The bacteria cells were grown in 500ml of LB broth medium and incubated overnight, later it was centrifuged to obtain a pellet. The pellet was isolated and dissolved in 5ml of TE buffer. (500mM tris at pH 8.0, 50mM EDTA). The cell

suspension was frozen at -20°C for 30 min. To the frozen suspension, was added 0.5ml of 250mM Tris (pH 8.0) and lysozyme (10mg/ml) and the contents thawed at room temp. After thawing, the suspension was again placed on ice for 45 - 50min. 1ml of 0.5% SDS, 50mM Tris(pH 7.5), 0.4M EDTA, 1mg/ml proteinase K was added and incubate in H₂O bath at 50°C for 60 min. After incubation 6ml of phenol (equilibrated with Tris) was used as an extractant and centrifuged at 10,000g for 15min, then the top layer was transferred to a new tube to which was added 0.1 volume of 3M sodium acetate and gently mixed after which 2 volumes of 95% ethanol was added and mixed by inversion. The DNA precipitate was separated and 5ml of 50mM Tris (pH 7.5), 1mM EDTA, and 200µg/ml RNase were added and allowed to dissolve overnight by rocking at 4°C. The extract was gently mixed with equal volume of chloroform and centrifuged at 10,000g for 5 minutes. The sedimented DNA was isolated using a micropipette and stored at 4°C under refrigeration until further use.

Cross Streak Assay

Using a clean sterilized inoculation wire loop, the isolated UTI pathogen (*Vibrio sp.*) was looped and streaks were made on nutrient agar media plate and there after using another sterile inoculation loop take THB strain isolate and streak against the streaks of the UTI pathogen. This was repeated on other nutrient agar plates using the other UTI pathogens (*Pseudomonas sp.* and *Streptococcus sp.*) (Fig. 2) and the results were tabulated (Table 1)



Figure 2: Cross streak Assay against UTI Pathogens 1:*Vibrio sp.*, 2: *Streptococcus sp.*, 3: *Pseudomonas sp.*, S1 – S5 are THB strain isolates

Table 1: Cross Streak Assay Method for Urinary Tract Associated Pathogens.

Strain no.	<i>Vibrio .sp</i>	<i>Pseudomonas.sp</i>	<i>Streptococcus.sp</i>
SS2	+	+	-
SS5	+	+	+
SS ₃	+	+	+
SS9	-	+	+
SS10	-	+	+

SS – Sponge Strain, + = Presence of Activity, - =Absence of activity
Primary Screening

The antagonistic (inhibition) activity of the THB strains was tested by using the cross streak assay method as shown in Fig. 3 above. Single streaks (4-6mm in diameter) of the 3 isolated bacterial strains were made on the surface of Muller Hinton Agar plates.

Secondary Screening

Mass Cultivation

A loopful of each 5 isolated THB strains was further inoculated into separate 1litre conical flask each containing 300ml of nutrient broth and kept at 28°C for 72 hours on a shaker.

Extraction of Extra Cellular Proteins

The mass cultivated broth was filtered by using filter paper. 300ml of filtrate was mixed with 300ml of ethyl acetate in a separating funnel to extract bioactive compounds. After removing the lower aqueous phase, the upper solvent phase was concentrated in a vacuum evaporator at room temperature for 24 hours and a crude extract was obtained. This crude extract was used for further secondary screening studies against human pathogens. (Figure 3)



Figure 3: Isolated Protein of THB strain

Minimum Inhibitory Concentration (MIC)

0.5 ml of various concentration of extracts (31, 62, 125, 250, 500, 1000, 1500, 2000µg) were taken and mixed with 0.5ml of nutrient broth. Nutrient Broth alone served as positive control. Whole setup in duplicate was incubated at 37°C for 48 hours.

Minimum Bactericidal Concentration (MBC)

A bactericidal agent is anything that kills bacteria. The MBC were determined by sub culturing the above (MIC). From each subculture streaking was done onto Nutrient Agar plates using sterile inoculation loop and incubating at 37°C for 24 hours. (Figure 4)

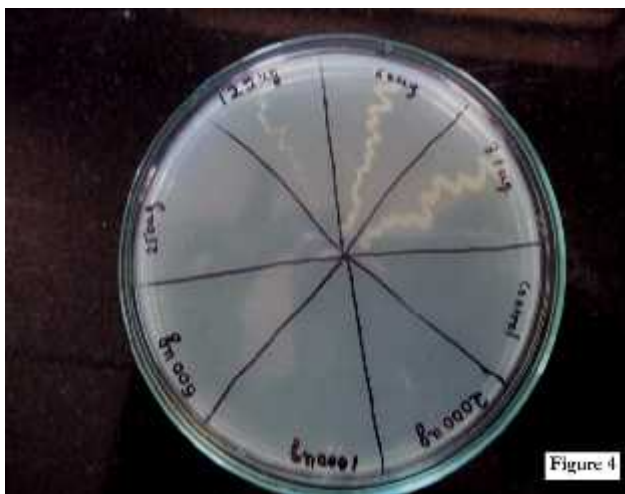


Figure 4: MBC (Minimum Bactericidal Concentration) using sponge strain 5 against vibrio sps; A: 2000µg B: 1000µg C: 500µg D: 250µg E: 125µg F: 63µg G: 31µg C: Control

RESULTS

Five THB strains were isolated from sea sponge species from these strains. Growth of *Vibrio* sp. increased by the reduction of the concentration amount of the THB strains. There was no growth of *Vibrio* sp. in a concentration of 2000µg and 1000µg. Low level of growth was observed on concentration of 500µg and 250µg of sponge strains SS9 and SS10 while medium level of growth of *Vibrio* sp. was observed on concentration of 125µg and 62µg except for SS2. There was high level of growth on concentration of 31µg in all the five sponge strains (Table 2).

Table 2: Minimum Inhibitory Concentration test for antibiotic resistant *Vibrio* Sp

Strain no.	2000µg	1000µg	500µg	250µg	125µg	62µg	31µg
SS2	-	-	-	-	+	++	+++
SS5	-	-	-	+	++	++	+++
SS7	-	-	-	-	++	++	+++
SS9	-	-	+	+	++	+++	+++
SS10	-	-	+	+	+	++	+++

SS – Sponge Strain, + = Low growth, ++ = Medium growth, +++ = High growth, - = Absence of growth

There was no growth of *Pseudomonas* sp. in a concentration of 2000µg, 1000µg and 500µg except on sponge strain No. SS7. Low level of growth was observed on concentration of 250µg of all other sponge strains except on SS2 while medium level of growth of *Pseudomonas* sp. was observed on concentration of 125µg and 62µg. There was high level of growth on concentration of 31µg in all the five sponge strains (Table 3).

Table 3: Minimum Inhibitory Concentration test for antibiotic resistant pseudomonas Sp

Strain No.	2000µg	1000µg	500µg	250µg	125µg	62µg	31µg
SS2	-	-	-	-	++	+++	+++
SS5	-	-	-	+	+	++	+++
SS7	-	-	+	+	++	++	+++
SS9	-	-	-	+	++	+++	+++
SS10	-	-	-	+	+	++	+++

SS – Sponge Strain, + = Low growth, ++ = Medium growth, +++ = High growth, - = Absence of growth

There was no growth of *Streptococcus* sp. in a concentration of 2000µg, 1000µg, 500µg and 250µg except on sponge strain No. SS7 and SS9. Low level of growth was observed on concentration of 125µg of all other sponge strains except on SS7 while medium level of growth of *Streptococcus* sp. was observed on concentration of 125µg and 62µg. There was high level of growth on concentration of 31µg in the sponge strains except on SS10 (Table 3).

Table 4: Minimum Inhibitory Concentration test for antibiotic resistant Streptococcus Sp

Strain No.	2000µg	1000µg	500µg	250µg	125µg	62µg	31µg
SS2	-	-	-	-	+	+++	+++
SS5	-	-	-	-	+	++	+++
SS7	-	-	+	+	++	+++	+++
SS9	-	-	-	+	+	+++	+++
SS10	-	-	-	-	+	+	++

SS – Sponge Strain, + = Low growth, ++ = Medium growth, +++ = High growth, - = Absence of growth

Strain No. SS2 had MIC value of 250µg against all the three UTI pathogens, *Vibrio* sp., *Pseudomonas* sp. and *Streptococcus* sp. (Table 5 and Fig. 5). SS5 showed MIC value of 250µg against *Streptococcus* sp. and MIC value of 500µg against *Vibrio* sp., and *Pseudomonas* sp. (Table 5 and Fig. 5). SS7 showed MIC value of 250µg against *Vibrio* sp. (table 5 and Fig. 5). SS9 showed MIC value of 500µg against *Pseudomonas* sp. and 500µg against *Streptococcus* sp. (Table 5 and Fig. 5). SS10 showed MIC value of 250µg against *Pseudomonas* sp. and 500µg against *Streptococcus* sp. (Table 5 and Fig. 5).

Table 5: Antimicrobial MICs of the strains (Mg/ml)

Strain no.	<i>Vibrio</i> .sp	<i>Pseudomonas</i> .sp	<i>Streptococcus</i> .sp
SS2	250µg	250µg	250µg
SS5	500µg	500µg	250µg
SS7	250µg	—	—
SS9	—	500µg	500µg
SS10	—	500µg	250µg

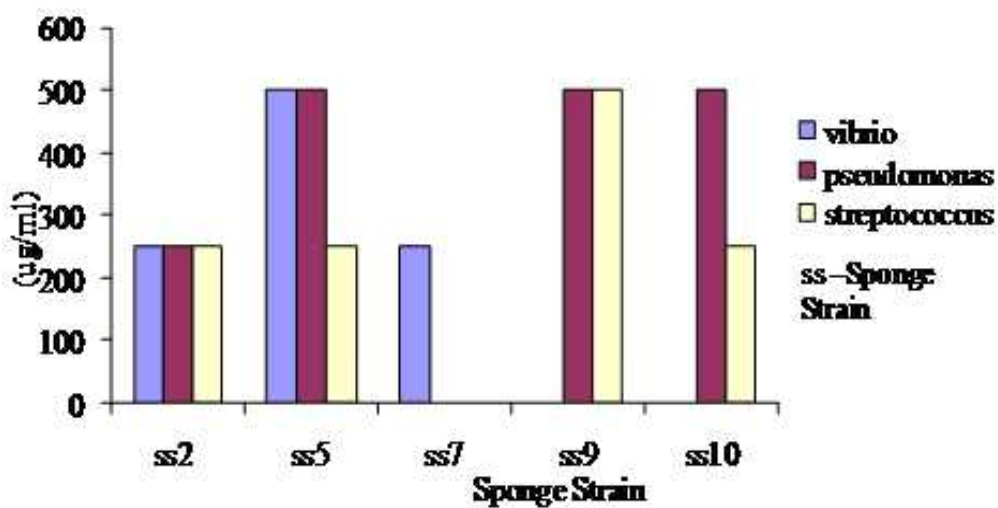


Figure 5: Antimicrobial MICs Sponge Strains in (ug/ml)

The strain No. SS5 which showed a clear MBC value of against *Vibrio* sp. as shown in Fig. 4 above was confirmed gram negative and rod shaped. The SS5 strain DNA had a molecular weight band of 39.360Kb.

DISCUSSION

Many discoveries are still going on about the ocean and its natural composition. The ocean has been considered as a rich source of compounds possessing novel structures and biological activities (Venkateshvaran K., 2001). Sponges are sessile filter feeders that have developed efficient defense mechanisms against foreign invaders such as viruses, bacteria or eukaryotic organisms. The aim of the present work was to study the antagonistic activity of the Indian sponge *Clathria indica* against UTI pathogens; *Pseudomonas* sp., *Streptococcus* sp. and *Vibrio* sp. which were extracted from the mid stream urine of sick patient.

Earlier studies done by [S. Ravichandran et al](#) on *Clathria indica* against 16 human pathogens which include eleven bacteria with four of them being multidrug resistant and five pathogenic fungi. All fractions showed effective antibacterial activity against common and multidrug-resistant *Salmonella typhi* and antifungal activity against *C. albicans* and *C. neoformans*. However, they were ineffective against *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes* and *Staphylococcus aureus* (Dendy, 1889). From his studies crude methanol, chloroform and *n*-butanol were used as the extractant and got five THB strains which were in effective against *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Dendy, 1889). In this recent study we used ethyl acetate as the extractant where sixteen THB strains were extracted of which two of the THB strains were effective against *Pseudomonas* sp. and *Staphylococcus* sp. this proved that ethyl acetate was a better extractant than crude methanol, chloroform and *n*-butanol.

Sundaram Ravikumar performed his MIC activity using Al₂O₃ nanoparticle against *E. coli*, *Pseudomonas* sp. and *Vibrio* sp. He got a result of maximum inhibition at the concentration of 5g/mL against *E. coli*, followed by 10g/mL against *Pseudomonas* sp. and *Vibrio* sp. (Sundaram Ravikumar *et al*, 2007). From our study, results of the antimicrobial MICs of the sponge strains were noted that there was a maximum inhibition concentration of 250µg/ml against *Vibrio* sp., *Pseudomonas* sp. and *Streptococcus* sp. When compared nanoparticles are quiet expensive to sponges which are available freely in the sea, more over from the results it can be directly interpreted that less amount of ethyl acetate extracted THB strain from *Clathria indica* was effective against *Vibrio* sp. and *Pseudomonas* sp. compared to Al₂O₃ nanoparticles.

Biologically active molecules isolated from marine flora and fauna have applications in pharmaceuticals, cosmetics, nutritional supplements, enzymes, molecular probes, fine chemicals and agrochemicals (Stempein M.F., 1970). In our study we isolated THB strains from sea sponges which showed antagonistic activity against *Vibrio* sp. *Pseudomonas*.sp and *Streptococcus*.sp., this shows that these secondary metabolites can be important in the pharmaceuticals sector as the can be used as antibiotics to inhibit the diseases caused by *Vibrio* sp. *Pseudomonas*.sp and *Streptococcus*.sp.

According to different studies we came to understand that there are different discoveries made on various compounds that have inhibitory activity against bacterial antigens like *Vibrio* sp. *Pseudomonas*.sp and *Streptococcus*.sp. Our findings encourages *Clathria indica* associated bacteria to be incorporated in drug development. It is concluded that, the ethyl acetate extract of *Clathria indica* associated bacterial isolates proved as a massive resource to develop potential antibacterial drugs against *Vibrio* sp., *Pseudomonas* sp. and *Streptococcus* sp. This justifies more research to be conducted on the *Clathria indica* associated bacterial extracts to determine their active principles responsible for antibacterial activity.

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