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Screening and molecular identification of marine sponges with cytotoxic activities, collected from Gulf of Mannar, Indian Ocean

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The marine environment, a rich source of biological and chemical diversity, offers tremendous scope for discovering novel metabolites with pharmacological activities. Marine invertebrates, especially sponges, have gained more attention in drug discovery programmes since they exhibit unique metabolic and physiological capabilities and an extraordinarily symbiotic association with diverse bacterial communities. The present study focuses on screening the cytotoxic activity of the methanolic extracts of sponges against the Non-Small Cell Lung Cancer (NSCLC) cell line, NCI-H460. In the bioactivity screening, two sponges (IOS-11 and IOS-12) were found with potent cytotoxic activity. The sponge IOS-12 showed almost complete cell growth inhibition (99 %) at 50 µg/ml and 80 % inhibition at 5 µg/ml; whereas IOS-11 showed cell growth inhibition of 96 % and 77 % at 50 µg/ml and at 5 µg/ml, respectively. Amplification of the mitochondrial cytochrome C oxidase subunit I (COI) and subsequent nucleotide analysis enabled the identification of sponge samples, IOS-11 and IOS-12, as *Rhabdastrella globostellata* and *Halichondria* sp., respectively. The study concludes that the two marine sponges, *Rhabdastrella globostellata* and *Halichondria* sp., possess excellent cytotoxic activities and could be considered good candidates for the isolation of anticancer compounds with biomedical applications. The isolation, purification and structural elucidation of the lead molecules from these sponges are ongoing studies, which might yield potential new compounds with promising cytotoxic activities.

[**Keywords**: Cytotoxic activity, Gulf of Mannar, *Halichondria* sp., NCI-H460, *Rhabdastrella globostellata*, Sponges]

Introduction

Marine bioprospecting, also known as marine natural products research, deals with the exploitation and exploration of the rich biological and chemical diversity found among the marine organisms. Marine ecosystems are diverse and dynamic and inhabited by organisms possessing unique chemical compounds. The discovery rate of new bioactive compounds was low for the past few years; however, the rediscovery of known compounds has increased[1]. This enforces bioprospecting of underexplored or unexplored habitats for unique metabolites with desirable bioactivities[2,3]. Exploring the natural product profiles of marine organisms, especially soft-bodied invertebrates and associated microorganisms, has become a hotspot in drug discovery. The increased scientific interest in marine pharmacology led to eight *FDA-approved drugs* of marine origin being on the market and more than 15 compounds at various stages of the drug pipeline[4].

The biological and chemical diversity of the marine environment is unfathomable and therefore is a prodigious resource for discovering new anticancer drugs. Of all the marine forms, soft-bodied marine animals, especially sponges, have gained more attention as they exhibited unique physiological and metabolic capabilities, which ensure survival in draconian environments and render the potential to synthesise compounds with antitumor and other pharmacological activities that may be sporadic in terrestrial organisms. Interestingly, out of the 13 marine natural products currently under clinical trials as new drug candidates, 12 are derived from invertebrates. Providing a greater number of natural products, especially novel pharmacologically active compounds, Porifera remains as the most important phylum[5,6]. Sponges are sessile invertebrates that lack an innate immune system and mechanical defence structures like spines or shells. Therefore, the only method they have been preserving themselves is the chemical means; by producing metabolites that act as a self-defence mechanism. They produce a variety of chemical molecules, including nucleosides, alkaloids, sterols, terpenes,

peroxides, amino acid derivatives, fatty acids and cyclic peptides that have been reported as active compounds with anticancer potential[7]. Many of them, such as alkaloids, are envisaged to be very potent molecules demonstrating toxicity at lower concentrations that had been developed as defence chemical weapons against predation[8]. Among the marine natural products derived from sponges, alkaloids are the most well-known cytotoxic compounds[9] and were recognized as the most isolated bioactive compounds[10].

The Indian Ocean, especially the Gulf of Mannar and Palk Bay, is exceptionally rich in biological diversity. However, the Indian Ocean is largely unexplored for therapeutic and prophylactic compounds. Here, an effort has been made to screen marine sponges collected from the Indian Ocean with cytotoxic properties and identified the potential sponges through molecular taxonomy.

Materials and Methods

Collection of marine sponges

A total of 14 sponges (IOS-1 – IOS-14) (Fig. 1) were collected from the Gulf of Mannar, Indian Ocean, either by skin-diving or snorkeling from depths of 2 – 10 m. Each sample was chopped into small pieces, brought to the laboratory in ice cold condition/ fixed in ethanol, and maintained at -20 °C until extraction. Information on the organism, the place of collection, date, and depth were recorded. A voucher specimen was taken and preserved for species identification and reference. Voucher code numbers of all species are given with photographs. For molecular identification of the sponges, a small portion of the samples was stored in 100 % ethanol at -20 °C till further analysis.

Preparation of crude extract

Extraction of bioactive compounds from sponge samples was done as per the methods described by

Fig. 1 — Image showing the fourteen sponge samples (IOS1 – IOS14) collected from the Gulf of Mannar, Indian Ocean

Conlon[11]. Briefly, 50 to 100 g of sponge tissue was homogenized with approximately 300 ml methanol using a rotor-stator-type homogenizer. The temperature of the homogenate was not allowed to increase above 10 °C by placing the homogenizer container in an ice-cold solution. The homogenate was centrifuged at 10,000 rpm and the supernatant was filtered and collected. The methanol extract was concentrated on a rotary evaporator (Buchi, Rotavapor) under reduced vacuum to yield the crude extract. For cytotoxicity assay, the dried crude extract was redissolved in 10 % dimethyl sulfoxide (DMSO) to obtain a final concentration of 1 mg/ml and subjected to cytotoxicity screening assay on NCI-H460 Non-Small Cell Lung Cancer (NSCLC) cell line using SRB assay.

Screening for potential cytotoxic activity

The cytotoxic effect of the sponge extracts on NSCLC cell line NCI-H460 (NCCS, Pune, India) was checked using sulforhodamine B (SRB) colorimetric assay[12]. The assay was performed in 96-well culture plates and was maintained by RPMI-1640 medium (Himedia, India) supplemented with 10 % heat-inactivated fetal bovine serum (FBS) in a humidified, 5 % (*v*/*v*) CO_2 atmosphere at 37 °C. Aliquots of 190 ml exponentially growing cell suspension were seeded in 96-well plates at a density of 1.9×10^4 cells/well. The crude extracts were maintained at a uniform concentration of 1 mg/ml using sterile 10 % DMSO, and 10-fold serial dilutions were prepared. 10 µl of the diluted extract was added to each well to make a final concentration of 50 µg/ml and 5 µg/ml. Negative control wells were setup by adding 190 µl cell suspension and 10 µl 10 % (vol/vol) DMSO. Assays were performed in triplicate. A no-growth control (day 0) was also maintained by keeping a plate with cell suspension alone in three columns. The plate was incubated at 37 °C in a humidified incubator with 5 % CO_2 until cell attachment was completed (≈ 2 – 3 hrs). The cell monolayer was fixed by gently adding 100 ml cold 30 % (wt/vol) trichloroacetic acid (TCA) sustaining the cell culture supernatant and was incubated for 1 hr at 4 °C. The remaining assay plates were kept for incubation in a CO_2 (5 %) incubator at 37 °C for 72 hr and were subsequently fixed with 100 ml cold 30 % (wt/vol) trichloroacetic acid (TCA) for 1 hr at 4 °C. The plates were washed with slow-running tap water for about four times and were completely air dried.

100 µl of 0.057 % (wt/vol) sulforhodamine B (SRB) prepared in double distilled water with 1 % acetic acid was added to each well and incubated at 28 °C for 30 min. Excess stain was washed out by quickly rinsing the plates with 1 % acetic acid about four times, and then the plates were dried at room temperature. 200 µl of 10 mM Tris base solution (pH 10.5) was added to each well to solubilize the protein-bound dye, and the plate was kept on a gyratory shaker for 10 min. After mixing, the OD was measured using a micro plate reader (Tecan, Switzerland) at 510 nm wavelength and the percentage of cell Growth Inhibition (GI) was calculated[13].

Percentage of growth inhibition = 100 - Percentage of control cell growth

Percentage of control cell growth = [(Mean OD sample – Mean OD day 0)/ (Mean OD negative control – Mean OD day 0)] ×100

Molecular identification of potent sponge species by cytochrome oxidase (COI) gene analysis

In the bioactivity screening, two sponges, IOS-11 and IOS-12, were observed with potent cytotoxic activity against NCI-H460 cell line. To identify these sponges, the mitochondrial cytochrome C oxidase subunit I (COI) gene was amplified by PCR, and the amplicon was sequenced and analysed[14]. Briefly, small pieces of sponges preserved in alcohol were transferred to 1.5 ml MCT and DNA extraction was carried out using PureLink® Invitrogen Genomic DNA Mini Kit (Thermo Fisher Scientific). The concentration and purity of extracted DNA were determined spectrophotometrically. The DNA was used to amplify the target mitochondrial gene Cytochrome Oxidase subunit I (COI) using PCR. PCR amplification of the COI gene was performed using forward primer LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3' and reverse primer HCO2198 5'-TAAACTTCAGGGTG ACCAAAAAATCA-3' as described by Folmer *et al.*[14]. The reaction mixture comprises 5 µL 2X Emerald Amp GT PCR Master Mix (Takara Bio Inc., Japan), 0.4 µL template, and 0.5 µL each of the forward and reverse primers (10X). The PCR amplification reaction was as follows: initial denaturation step at 94 °C for 3 min, followed by 35 – 40 cycles at 94 °C for 30 sec, at 45 °C for 40 sec and at 72 °C for 60 sec, and finally an extension step at 72 °C for 10 min. PCR products were confirmed on 1 % agarose gel *via* electrophoresis. The amplified products were purified by Exosap (Affymetrix, USA) and sequenced for DNA barcoding. Both the sequences, forward and reverse were assembled using

GeneTool TM Lite 1.0[ref. 15] and were submitted to Genbank. The nucleotide sequences of IOS-11, 658 base pairs in length, were aligned using ClustalW on MEGA 11[ref. 16]. A phylogenetic tree was constructed using the neighbour-joining method with the matched COI sequences of closely related sponges from the Genbank database collections.

Results and Discussion

The Indian Ocean, especially the Gulf of Mannar and Palk Bay, is exceptionally rich in biological diversity. So far, 451 species of sponges have been recorded from the Indian waters. Among the 321 species belonging to 129 genera reported in the Gulf of Mannar and Palk Bay region, 257 species belonging to 63 genera were endemic[17]. However, few studies were conducted on the bioprospecting potential of the marine sponges collected from the Indian Ocean. Limna Mol *et al.*[18] reported the antifouling activity of selected marine sponge extracts collected from the Gulf of Mannar. Similar to the present study, the antimicrobial activity of metabolites from bacterial symbionts associated with marine sponges in the coastal area of the Gulf of Mannar was demonstrated by Skariyachan *et al.*[19]. Selvin & Lipton[20] also reported the cytotoxic and antibacterial potential of sponge *Dendrillanigra* collected from the Gulf of Mannar.

In this study, the marine sponges collected from the Gulf of Mannar were examined for cytotoxic activity and the potential sponges were identified at the molecular level by DNA barcoding. Cytotoxic activity screening of the methanolic extracts of 14 sponges resulted in identifying two potential sponges with enhanced cytotoxic activities (Fig. 2). The sponge IOS-12 showed 99 % cell growth inhibition and 80 % cell growth inhibition at 50 µg/ml and 5 µg/ml, respectively. At the same time, IOS-11 showed cell growth inhibition, *i.e.*, 96 % and 77 % when treated with 50 and 5 µg/ml, respectively. Molecular identifications of these two prioritized sponges were performed by amplifying the mitochondrial cytochrome C oxidase subunit I (COI) and subsequent nucleotide analysis.

The mitochondrial COI gene sequence (629 bp) of the IOS-11 showed 99.81 % sequence similarity to *Rhabdastrella globostellata* using BLAST (Fig. 3). The gene sequence was submitted to Genbank with accession no. MN306534. The COI gene sequence analysis of sponge IOS-12 (690 bp) showed 98.33 % similarity to *Halichondria okadai* (Fig. 4). All sequences were submitted to the Barcode of Life Data system version 4.0 (BOLD, http://www.barcodinglife.org, *Rhabdastrella globostellata* GBMNB74091-20 and *Halichondria okadai* GBMNB74092-20).

Sponges have been extraordinarily rich sources of highly bioactive and structurally diverse natural products. Among the eight FDA approved drugs of marine origin, three were isolated from marine

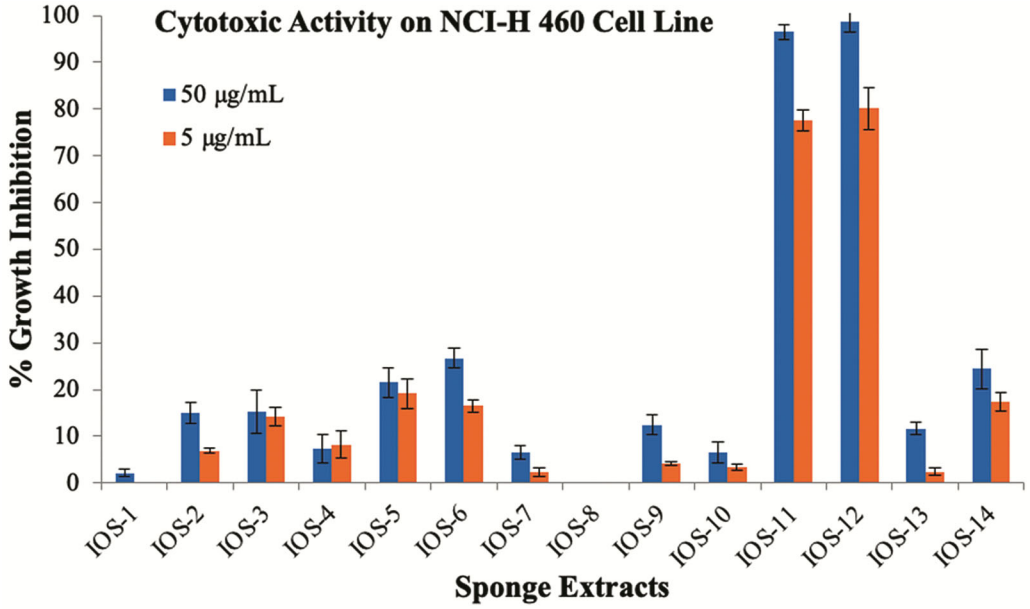

Fig. 2 — A graph showing the cytotoxic activity of 14 sponges against NCIH-460 lung cancer cell line using SRB assay

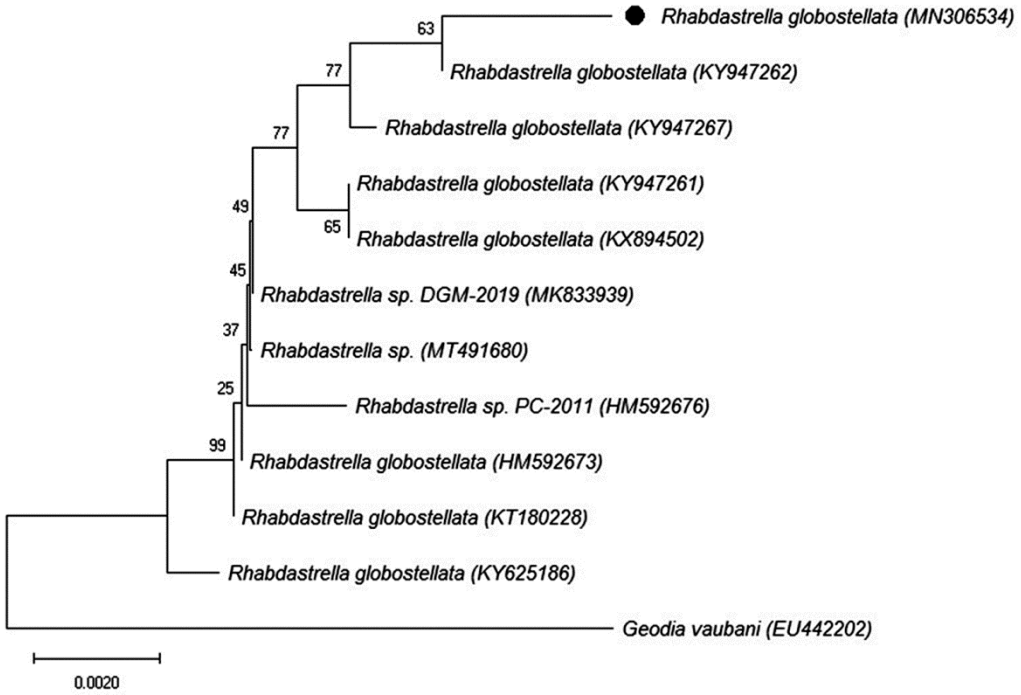

Fig. 3 — Phylogenetic tree of COI sequences of *Rhabdastrella globostellata* (IOS-11) constructed using MEGA11 software. The UPGMA tree constructed was computed using the kimura-2 parameter substitution model with 1000 bootstrap replications

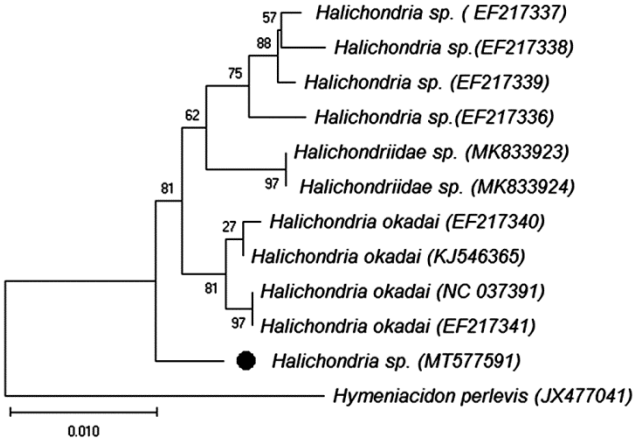

Fig. 4 — Phylogenetic tree of COI sequences of *Halichondria okadai* (IOS-12) constructed using MEGA11 software. The UPGMA tree constructed was computed using kimura-2 parameter substitution model with 1000 bootstrap replications

sponges. They include the well-known anticancer drug Cytosar-U® and antiviral drug Vira-A®, both belonging to nucleoside analogues initially isolated from the Caribbean sponge *Cryptotethya crypta*[21]. Halichondrin B, first isolated from a sponge *Halichondria okadai* is a macrocyclic polyether later developed and marketed as anticancer drug Eribulin mesylate (Halaven®), was found to be highly effective against the metastatic breast cancer[22].

A variety of bioactive compounds, including sesquiterpenes and polyether macrolide, have been extracted from sponges of the order Halichondri. Extracts of two marine sponge species, *Halichondria magniconulosa* and *Halichondria melanadocia* collected from Yucatan peninsula coast exhibited significant cytotoxic activity against human cervix cancer (SiHa) cell lines and hormone-dependent breast cancer (MCF-7)[23].

Recently Raiju *et al*.[24] reported the cytotoxic activity of (6R, 7S)-7-amino-7, 8-dihydro-a-bisabolene isolated from *Halichondria* sp. against HeLa cells. Khalaman *et al*.[25] showed a pronounced cytostatic effect of *Halichondria panicea* extract on tissue explant development of adult male Wistar rats. Haliclona diamine analogues recovered from the Okinawan marine sponge *Halichondria panicea*, along with haliclona diamine and papuamine, exhibited potential antibacterial activity against *Mycobacterium smegmatis*, and some of them showed weak cytotoxicities against the hepatic cancer cell line[26]. Purushothama *et al*.[27] reported the hemolytic activity of methanolic and chloroform-methanol extracts of marine sponge *Halichondria panicea* collected from the Arabian Sea.

The marine sponge genus *Rhabdastrella* is considered an excellent source of potential natural

products. Tasdemir *et al*.[28] reported cytotoxic activity of two terpenoids, Stellettin B and E, isolated from *Rhabdastrella globostellata.* Isomalabaricane analogues, isogeoditin A, 13-(E)-isogeoditin A, 22, 23-dihydrostellettin B, and isogeoditin B were isolated from the marine sponge *Rhabdastrella* aff. *distincta*[29]. Rhabdastrellic acid-A is an isomalabaricane triterpenoid isolated from *Rhabdastrella*, which was shown to inhibit the growth of cancer cell line HCT-116(ref. 30). By blocking the Akt pathway in human cancer cells Rhabdastrellic Acid-A could induce autophagy-associated cell death[31]. It induced caspase-independent cell death in both the cell lines and inhibited proliferation of human cancer cell lines Hep3B and A549. Guo *et al*.[32] suggested that Rhabdastrellic acid-A could inhibit PI3K/Akt pathway and induce caspase-3 dependent-apoptosis in HL-60 human leukemia cells. Diverse cytotoxic activity exhibiting compounds such as isomalabaricane-derived globostelletins A-I[33], jaspolides A-F[34], rhabdastrellic acid-A[31], stellettin B[35] were isolated so far.

In conclusion, the two marine sponges, *Rhabdastrella* sp. and *Halichondria* sp., collected from the Gulf of Mannar (Indian Ocean), were identified to possess excellent cytotoxic activities and could be considered as good candidates for the isolation of anticancer compounds with biomedical applications. The isolation, purification and structural elucidation of the lead molecules from these sponges, etc. are ongoing studies, which might yield potential new compounds with promising cytotoxic activities.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical Statement

Ethical approval for the collection of sponges was sought from Principal Chief Conservator of Forests and Chief Wildlife Warden, Environment and Forest (FR9) Department, Govt. of Tamil Nadu, India (C. No. WL5/50371/2012).

Author Contributions

STP & VJ conceived and designed the research. MTS & MU collected the samples. MTS, MU & DM conducted the experiments. STP, MTS & MU analyzed the data. MTS & STP wrote the manuscript. MU, DM & VJ edited the manuscript. All authors read and approved the manuscript.

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