Endophytic Fungi Isolated from the Marine Sponges as a Source of Potential Bioactive Compounds

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Abstract

The marine environment with its vast biodiversity is a rich source of natural products and sponges which harbor several microbes are valuable sources with immense pharmaceutical potential. Three marine sponges, T. anhelans, M. arenaria, C. fibrosa which are relatively common along the east and west coasts of India were collected from Vizhinjam and Kovalam in Kerala. In the present study, 19 endophytic fungi were isolated from these marine sponges using Sabouraud Dextrose Agar Medium. Phylogenetic identity of these isolates was done by sequencing the internal transcribed spacer (ITS) region of the fungus. Bioactivity potential such as anticancer, antioxidant and anti-inflammatory activities of these isolates were evaluated using suitable bioassays. Fungi identified as A. sp. MCCF 103, A. sp. MCCF 111, A. sp. MCCF 114, P. sp. MCCF 115 and A. sp. MCCF 119 exhibited significant cytotoxic activity on NCI-H460 lung cancer cells lines, while one isolate identified as A. sp. MCCF 102 exhibited better anti-inflammatory and antioxidant activities. The current study proposes sponge-associated fungi are excellent source of bioactive molecules, which is least explored till date. Further studies on purification and structural characterization of lead compounds are continuing.

Keywords: Endophytic fungi, Marine sponge, Bioactive compounds, Anti-inflammatory, Aspergillus

1. Introduction

Historically microbial natural products have been in the mainstay of drug discovery programme. However, the high rediscovery rate led to a reduction in resources applied across the pharmaceutical industry and a corresponding decline in discoveries of novel natural products. To avoid this redundancy in discovery programme, scientific community started exploring least studied geographical regions such as deep-sea, polar ocean and other specialized ecological environments including the endophytic microorganisms associated with marine invertebrates as a novel source of drug candidates (Pathom-aree et al., 2006; Bredholt et al., 2008; Simmons et al., 2008).

Marine organisms such as sponges, bryozoans, tunicates etc. are well established sources of many unique natural products. Among these marine forms, sponges are a prolific source of diverse secondary metabolites (Faulkner, 2002; Blunt et al., 2005; Laport et al., 2009; Hertiani et al., 2010; Proksch et al., 2010). However, the putative role of the microorganisms associated with these sedentary organisms in the synthesis of such bioactive metabolites have been proved at many instances (Simmons et al., 2008). Marine sponge associated fungi are one such group which have been reported to be a crucial and invaluable source of novel therapeutic agents possessing several bioactive properties including free radical scavenging activity, neurotogenic activity, anticancer activity and kinase inhibition etc. The exploration of fungal metabolites has significantly increased after marine fungi, especially sponge-associated fungi have been reported to produce structurally unique bioactive compounds (Hasan et al., 2015; Imhoff 2016). Several alkaloids, terpenoids, polyketides, peptides and other molecules have been identified from marine fungi with remarkable bioactive properties (Xu et al., 2019). In the present study, fungi were isolated from marine sponges collected from Arabian Sea and were evaluated for different bioactivities such as free radical scavenging activity, cytotoxic activity and anti-inflammatory activity.

2. Materials and Methods

Sponge Collection and Identification

Three marine sponges were collected from rocky shore regions of Vizhinjam and Kovalam Thiruvananthapuram, Southwest coast of India. The collected samples were immediately brought to lab in cold container under sterile conditions and stored at 20 °C till further analysis. A portion of the sponge sample was preserved in 70 % ethanol for the purpose of identification which was done based on the morphological features such as colour, shape, skeletal features and spicle types.

Isolation of sponge-associated fungi

Isolation of sponge associated fungi were done as per Kjer et al. (2010). Briefly, the sponge tissue was cut into small pieces (1 cm x 1 cm approximately) and washed repeatedly with sterile sea water to remove all adhered debris. Surface sterilization of sponges was done by dipping in 70 % ethanol for 60 -120s followed by repeated washing in sterile seawater and further dried with sterile cotton cloth to stop sterilization. The sterilized pieces of sponge tissue were aseptically placed on a petridish containing Sabouraud Dextrose Agar (SDA) media (HiMedia, India) prepared in 30 ppt seawater and the plates were incubated for 7 days at 28° C and observed for fungal growth around
the sponge implant. The fungal isolates (19 strains outgrown from these tissues were isolated, purified and stored for further studies. All nineteen isolates were deposited in the Microbial Culture Collection repository of National Centre for Aquatic Animal Health, Cochin University of Science and Technology, India and assigned with culture identification accession numbers.

**Molecular identification of the fungal strains**

The taxonomic identification of the fungal strains was done by PCR amplification of Internal Transcribed Spacer (ITS) region of fungal genome followed by sequencing. Genomic DNA was isolated using Pure Link Genomic DNA Kit (Invitrogen) and PCR amplification of the ITS region was performed using the universal primers ITS 1 (5´TCC GTA GGT GAA CCT GCGG 3´) and ITS 4 (5´-TCC TAT TAT GAT GAA TAT CAC GTG G-3´) as described by Chomczynski et al. (1997). The PCR product was sequenced by Sanger sequencing method using ABI3730 XI platform (Applied Biosystems, USA) at Eurofins IT Solutions India Pvt Ltd., Bangalore. Nucleotide sequence data were analyzed using BLAST and aligned using Clustal W on MEGA 6 (Tamura et al. 2013). The ITS sequences of closely related fungi were retrieved from GenBank, and their similarity to the current isolate was assessed at the nucleotide level. Phylogenetic trees were constructed separately for all 19 isolates using the Neighbour-Joining (NJ) method with boot strap values based on 1000 replicates.

**Fermentation and crude extract preparation**

Secondary metabolites of all 19 fungal isolates were extracted separately and used for bioactivity studies. The fungal strains were inoculated into 500 mL Erlenmeyer flasks containing 250 mL Sabouraud Dextrose Agar (SDA) medium each and incubated at room temperature for 21 days. The fermentation was brought to an end by adding 250 mL of ethyl acetate to the culture flask and was kept overnight in order to decrease the number of spores which will become airborne once the flask is opened. The metabolites from each fungus were extracted with ethyl acetate (2x250 ml) as described by Kjer et al. (2010). The extract obtained was dried in a rotary evaporator under reduced vacuum and stored in -20°C for further analysis.

**Screening of fungal extracts for cytotoxic activity using NCI-H460 cells**

The cytotoxicity of the crude extract of fungi was evaluated using Sulforhodamine-B (SRB) colorimetric assay on 96 well culture plates as described by Skehan et al. (1990). Non-Small-Cell Human Lung Cancer (NSCLC) cell line NCI-H460 obtained from National Centre for Cell Science (NCCS, Pune, India) was maintained in RPMI-1640 (HiMedia, India) supplemented with 10% Fetal Bovine Serum (FBS) (HiMedia, India) at 37°C in a carbon dioxide incubator (5% carbon dioxide). Aliquots of 190 µl cell suspension with a density of 1.9x10^6 cells/ml were pipetted into 96 well microtiter plates. The fungal crude extracts were diluted to 1 mg/ml with sterile deionized water (with 10% DMSO) and 10 µl of the extracts were added to each well so as to get a final concentration of 50 µg/ml of crude extract in each well. Control wells were inoculated with 190 µl cell suspension and 10 µl of 10 % DMSO. All assays were performed in triplicates. After 72 h incubation at 37°C in CO2 incubator, the cells were fixed with ice cold 30% trichloroacetic acid (TCA) and incubated at 4°C for another 1 hour. The plates were gently washed and air dried at room temperature. To each well a total of 100 µl of 0.057 % (w/v) SRB prepared in 1 % acetic acid was added and left at room temperature for 30 min. Unbound stain was removed by washing with 1% v/v acetic acid and the plates were air dried. To dissolve the cell bound dye, 200 µl of 10 mM Tris base solution (pH 10.5) was added to each well and the plate was shaken on a gyratory shaker for 10 min. Optical density (OD) was read at 510 nm in a microplate reader (TECAN). Percentage of cell-growth inhibition (GI) was calculated as per (Vichai and Kiritkara 2006).

\[
\text{% growth} = \frac{\text{mean OD}_{\text{sample}} - \text{mean OD}_{\text{neg control}}}{\text{mean OD}_{\text{neg control}} - \text{mean OD}_{\text{day0}}} \times 100
\]

% growth inhibition = 100 - % of control cell growth

**ABTS cation free radical scavenging activity**

The ABTS assay is an index of free radical scavenging potential of a compound based on the ability of the substances to scavenge 2,2-azinobis radical cation. The radical cation was prepared by mixing the stock solutions of 7 mM 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 2.45 mM potassium persulphate (Arnao et al., 2001). The stock solutions of ABTS and potassium persulphate were mixed in equal quantities (1/1 v/v) and incubated for 12-16 h at room temperature in the dark for preparing the working solution. For measuring the antioxidant activity of the fungal extracts, the ABTS solution was diluted with methanol in order to obtain absorbance of 0.7 ± 0.01 units at 734nm. 4.85 mL of diluted ABTS was added to 0.15 mL of diluted samples and the absorbance was taken after 5 minutes. Activity was evaluated by comparing with a control which was prepared by mixing 4.85 mL of diluted ABTS and 0.15 mL of 45% methanol. The experiment was done in triplicates and the average was calculated. The capability to scavenge the ABTS radical was calculated using the following equation: ABTS scavenging effect (%) = \( [(\text{Ac} - \text{At})/\text{Ac}] \times 100 \).

**Screening of fungal isolates for anti-inflammatory activity using RAW 264.7 cells**

RAW 264.7 cell line was purchased from National Centre for Cell Science repository (NCCS), Pune, India. Cell lines were maintained in Dulbecco’s Modified Essential Medium (DMEM) supplemented with 10 % Fetal Bovine Serum (FBS), Penicillin (100 U/mL) and Streptomycin (100 µg/mL). The cells were incubated in a carbon dioxide incubator at 5 % CO2 and were subcultured every 3 days. To evaluate the anti-inflammatory activity of the fungal isolates, RAW 264.7 cells were pre-incubated with LPS for 24 h and the quantity of nitrite accumulated in culture medium was measured as an indicator of NO production based on Griess reaction. RAW 264.7 cells were seeded at a density of 2-3 x 10^4 cells/well in 96 well plates and incubated for 24 h at 37 °C and 5 % CO2. The crude extracts of fungi were diluted with DMSO so as to get a final concentration of 50 µg/mL and 0.5% DMSO in each
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well. The mixture was incubated for 1 hour. Further, the cells were stimulated for NO production with 1µg/mL of Lipopolysaccharide (LPS) (Sigma, USA); and after 24 hrs of incubation, concentration of nitrite was determined in cell culture media using Griess Reagent (Sigma, USA) as per Kim et al. (1999). For this, 100 µL of cell culture medium was added to an equal volume of Griess reagent and incubated for 10 min. The absorbance of the compound formed by the diazotization of nitrite with sulphanilamide and further coupling with N-(1-Naphthyl) ethylenediamine dichloride was read at 540 nm by using a microplate reader (TECAN). Wells without extract served as the control. All experiments were done in triplicates.

3. Results and Discussion

Marine sponges are well known to host a large community of microorganisms, which comprise a significant percentage (up to 50–60%) of the host biomass (Wang et al., 2006). The role of these diverse microbes in sponge biology varies from source of nutrition to mutualistic symbiosis (Lee et al., 2001; Hentschel et al., 2003; Hill et al., 2004). Marine sponge associated fungi produce a wide range of secondary metabolites with unique structures and activities (Holler et al., 2000). In the present study, sponge associated fungi were isolated from three marine sponges collected from Vizhinjam and Kovalam were identified as Tedania anhelans, Myxilla arenaria and Callyspongia fibrosa and their bioactive potentials were evaluated (Fig.1.). Nineteen morphologically different fungi (1-19) were isolated from these sponges. Fig.2. shows the growth of fungi from the sponge implant.

Molecular identifications of these 19 fungi were done based on the genome sequence of ITS region (Table 1). Among the nineteen fungal isolates, 9 belonged to the Aspergillus genus, 7 belonged to genus Penicillium, one each to the genera Lasiodiplodia, Schizophyllum and Monascus. All the sequences were deposited in GenBank database with accessions number as detailed in Table 1.

One of the most prominent bioactivities observed in the present study was cytotoxic activity of few sponge associated fungi. In the present work, screening for cytotoxic activity of the fungi on NCI-H460 cells using SRB assay indicated significant growth inhibition (＞95%) by Aspergillus sp. MCCF 103, Aspergillus sp. MCCF 111, Aspergillus sp. MCCF 114, Penicillium sp. MCCF 115 and Aspergillus sp. MCCF 119 (Fig.3.). These five fungi can be thus considered as potential isolates with anticancer activity. Among the isolates, Aspergillus tamarii MCCF 102 (31 %), Penicillium sp. MCCF 117 (21 %) and Penicillium solitum MCCF 109 (7.9 %) exhibited a lower percentage of growth inhibition. However, the extracts of Lasiodiplodia sp. MCCF 104, Aspergillus sp. MCCF 105 Penicillium sp. MCCF 106, Schizophyllum sp. MCCF 107, Aspergillus sp. MCCF 108, Aspergillus sp. MCCF 112, Penicillium sp. MCCF 113 and Monascus sp. MCCF 118 did not show any notable cytotoxic activity against NCI-H460 cells. A number of secondary metabolites from marine fungi have been reported from various sources which are known to possess a range of bioactivities such as antibacterial, antiviral and anticancer agents (Gomes et al., 2015). Several secondary metabolites such as alkaloids, terpenoids, xanthones, sterols, diphenyl ether and anthraquinones with potent anticancer properties were reported from Aspergillus sp. (Wang et al., 2018). Son et al., (2012) reported anticancer activity of phenolic bisabolane sesquiterpenoid dimers, disydonols A and C isolated form marine-derived Aspergillus sp. obtained from the sponge Xestospongia testudinaria. Fractionation of the ethyl acetate extract of a static culture of Aspergillus niger from a Mediterranean sponge Axinella damicornis yielded one new secondary metabolite: 3,3-bicoumarin bicoumanigrin which exhibited moderate inhibitory effects on the growth of leukemia and carcinoma cell lines using incorporation of 3H-thymidine as a marker (Hiort et al., 2004). Another study reported Aspergillus
versicolor isolated from a marine sponge Petrosia sp. to exhibit strong anticancer activity against several cell lines (A-549, SK-OV-3, SK-MEL-2, XF-498, and HCT-15) with IC₅₀ values ranging from 1.22 to 4.61 µg/mL (Lee et al., 2010) which confirms the pivotal role of sponge associated fungi in cytotoxic studies.

Other than cytotoxic activities, anti-inflammatory and anti-oxidant activities of marine sponge associated fungi have been explored by several researchers (Xu et al., 2019). Studies have reported that free radical initiated reactions play a significant role in several degenerative or pathological events in human being (Abdel-Monem et al., 2013). Free radicals are capable of initiating a wide range of toxic oxidative reactions triggering inflammatory responses. Therefore, bioprospecting for anti-oxidative compounds which are capable of eliminating oxidative stress thereby functioning as anti-inflammatory molecules are of high demand as drug leads. Fungi have emerged as a new source of antioxidants in the form of their secondary metabolites which often act by scavenging the free radical species (Arora and Chandra, 2010). Several in vitro assays have been used to assess the antioxidant potentiality of compounds, of which the ABTS assay was used in the present study. The assay utilizes ABTS stable free radical with the characteristic absorption at 734 nm to determine the radical scavenging effect of fungal extracts. Our results showed that Aspergillus sp. MCCF 112 exhibited maximum free radical scavenging activity (84.3 %), thus confirming its antioxidant potentiality. This was followed by Penicillium sp. MCCF 106 (83.3 %) and Aspergillus tamarii MCCF 102 (81.25 %) (Fig. 4).

Nitric Oxide (NO) has been known as an important molecule which regulates biological activities in vascular, neural and immune systems (Moncada et al., 1992). Excessive release of NO is correlated with the progression of neurodegeneration and the molecules which are able to inhibit or downregulate nitrite release can be considered as anti-inflammatory agents. To determine the inhibitory effect of crude fungal extracts on Nitric Oxide (NO) production, RAW 264.7 cells were stimulated with LPS.
### Table 1. List of Sponge associated marine fungi and their culture morphology

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Sponge</th>
<th>Associated Fungi</th>
<th>Nearest Curated Type Strain &amp; Percent Identity</th>
<th>GenBank Accession no.</th>
<th>Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Callyspongia fibrosa</em></td>
<td><em>Aspergillus tamarii</em></td>
<td><em>Aspergillus tamarii</em> -100%</td>
<td>MF402928</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Aspergillus</em> sp. MCCF 103</td>
<td><em>Aspergillus niger</em> -100%</td>
<td>MT913569</td>
<td></td>
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<tr>
<td>2.</td>
<td></td>
<td><em>Lasiodiplodia</em> sp. MCCF 104</td>
<td><em>Lasiodiplodia pseudotheobromae</em> -99.81%</td>
<td>MT913570</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td></td>
<td><em>Aspergillus</em> sp. MCCF 105</td>
<td><em>Aspergillus flavus</em> -100%</td>
<td>MT913571</td>
<td></td>
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<tr>
<td>4.</td>
<td></td>
<td><em>Penicillium</em> sp. MCCF 106</td>
<td><em>Penicillium hispanicum</em> -100%</td>
<td>MT913572</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td></td>
<td><em>Schizophyllum</em> sp. MCCF 107</td>
<td><em>Schizophyllum commune</em> -99.84%</td>
<td>MT913573</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td></td>
<td><em>Aspergillus</em> sp. MCCF 108</td>
<td><em>Aspergillus flavus</em> -99.84%</td>
<td>MT913574</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td></td>
<td><em>Penicillium</em> sp. MCCF 109</td>
<td><em>Penicillium solitum</em> -100%</td>
<td>MT913575</td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td></td>
<td><em>Penicillium</em> sp. MCCF 110</td>
<td><em>Penicillium cavernicola</em> -99.66%</td>
<td>MT913576</td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td><em>Tedania anhelans</em></td>
<td><em>Penicillium</em> sp.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Species</td>
<td>Strain</td>
<td>Percentage</td>
<td>Accession Number</td>
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</tr>
<tr>
<td>10.</td>
<td><em>Tedania anhelans</em></td>
<td>Aspergillus sp. MCCF 111</td>
<td>Aspergillus nomius -100%</td>
<td>MT913577</td>
<td></td>
</tr>
<tr>
<td>11.</td>
<td></td>
<td>Aspergillus sp. MCCF 112</td>
<td>Aspergillus tamarii -100%</td>
<td>MT913578</td>
<td></td>
</tr>
<tr>
<td>12.</td>
<td></td>
<td>Penicillium sp. MCCF 113</td>
<td>Penicillium cavernicola 100%</td>
<td>MT913579</td>
<td></td>
</tr>
<tr>
<td>13.</td>
<td></td>
<td>Aspergillus sp. MCCF 114</td>
<td>Aspergillus penicillioides -100%</td>
<td>MT913580</td>
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<td>14.</td>
<td></td>
<td>Penicillium sp. MCCF 115</td>
<td>Penicillium citrinum -100%</td>
<td>MT913581</td>
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<tr>
<td>15.</td>
<td></td>
<td>Penicillium sp. MCCF 116</td>
<td>Penicillium cavernicola -99.83%</td>
<td>MT913582</td>
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<tr>
<td>16.</td>
<td></td>
<td>Penicillium sp. MCCF 117</td>
<td>Penicillium cavernicola -100%</td>
<td>MT913583</td>
<td></td>
</tr>
<tr>
<td>17.</td>
<td></td>
<td>Monascus sp. MCCF 118</td>
<td>Monascus fumeus -99.82%</td>
<td>MT913584</td>
<td></td>
</tr>
<tr>
<td>19.</td>
<td></td>
<td>Aspergillus sp. MCCF 120</td>
<td>Aspergillus flavus -100%</td>
<td>MT913586</td>
<td></td>
</tr>
</tbody>
</table>
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Fig. 5. Effect of crude ethyl acetate fungal extracts on LPS-induced NO production in RAW 264.7 cells

for 24 h. The production of NO in the culture medium was evaluated by quantifying the level of nitrite in the culture medium. The nitrite level in the control well increased to 3.65 µM from the basal level (1 µM) in RAW 264.7 cells after LPS stimulation (Fig.5.). However, LPS induced increase of NO was significantly inhibited by the extracts of Aspergillus tamarii MCCF 102 to 1 µM, Aspergillus sp. MCCF 119 and Aspergillus sp. MCCF 120 to 2 µM. (Fig.5). Although Aspergillus sp. MCCF 119 and Aspergillus sp. MCCF 120 inhibited NO production to 2 µM, they also exhibited higher cytotoxic activity with 97 % and 74 % cell growth inhibition in SRB assay. Hence these isolates may not be ideal candidates for anti-inflammatory studies as they possess higher cytotoxic activities. Hence Aspergillus tamarii MCCF 102 which exhibited significant radical scavenging (81.25 %) and anti-inflammatory activities while exhibiting least cytotoxicity (31.16 %) has been considered as a promising candidate for further anti-inflammatory studies.

A wide range of marine derived fungi have the potential to produce diverse chemical structures with anti-inflammatory properties. Several anti-inflammatory compounds including alkaloids (Gu et al., 2018), terpenoids (Wu et al., 2018), polyketides (Lee et al., 2013) and peptides (Liu et al., 2018) have been widely reported from fungi. A recent study has reported a novel linear aliphatic alcohol, (3(E),7E)-4,8-di-methyl-undecane-3,7-diene-1,11-diol purified from the coral-associated fungus Aspergillus terreus that exhibited considerable 'NO production inhibitory activity' with IC_{50} values ranging from 17.45 to 29.34 µM (Liu et al., 2018). Another study reported two hexylitaconic acid derivatives separated from the ethyl acetate extract of the fungal strain Penicillium sp. (J05B-3-F-1) colonizing a sponge belonging to Stelletta sp. to be weakly inhibiting the production of IL-1β at the concentration of 200 µM (Li et al., 2011). Trichodermanone C, isolated from the marine fungal strain A12 of Trichoderma citrinoviride associated with the green algae Cladophora sp. was evaluated to show strong inhibitory effect on nitrite levels in LPS-stimulated J774A.1 macrophages (Marra et al., 2018). The results of the bioactive studies in this paper suggest that endophytic fungi isolated from sponges are a remarkable and promising source of potent secondary metabolites with anti-inflammatory activities which warrant further study for the isolation and characterization of the compound(s).

4. Conclusion

In conclusion, in this study, we demonstrated that sponge associated endophytic fungi are a rich source of bioactive compounds. The isolation and characterization of lead compounds responsible for these activities needs to be further investigated. However, this study will pave way for more ground breaking research from unexplored marine organisms and increase the potential to expand the health beneficial value in the pharmaceutical industry.

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5. References


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