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Isolation and molecular characterization of bioactive secondary metabolites from *Callyspongia* spp. associated fungi

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ABSTRACT

Objective: To isolate and characterize the bioactive secondary metabolite from *Callyspongia* spp. associated fungi. **Methods:** *In vitro* antibacterial screening of fungi associated with *Callyspongia* species, collected from south east coast of India, against selected clinical isolates of bacteria were conducted in this study. The extracts showing good antimicrobial activity were subjected to further analysis to identify the active constituents sponge associated fungi (both biomass and filtrate) with five different solvents. The compound responsible for bioactivity was characterized using Fouvier-transform infrared (FT-IR) and gas chromatography-mass spectrometry (GC-MS) instrumental analysis to identify the functional group and compound. The molecular characterization of the elite fungal strains were done by isolating their genomic DNA and amplify the internal transcribed spacer (ITS) region of 5.8srRNA using specific ITS primer. The novelty of the strain was proved by BlastN analysis against non-redundant (NR) database and hence was submitted to GenBank. **Results:** Active compound was Desmethylnomifensine confirmed by GC-MS and the potent fungi was *Aspergillus flavus* GU815344. **Conclusions:** The isolate exhibits a marked antagonistic activity against potential bacterial pathogens thus illuminating the advanced researches in this decade to focus on clinical pharmacology to identify novel therapeutic targets. The present study depicts a promising scenario to focus on *Aspergillus flavus* derived compounds which can be easily scaled up for large biomass production and stable formulation as a drug.

1. Introduction

Marine sponges provide classic examples of microbial-macro faunal partnerships that have been a productive source for the discovery of bioactive compounds[1].Natural products are organic molecules derived from plants, animals, or microorganisms, and represent the starting point for most of the anti-infective and anti-cancer drugs on the market today. Until recently, the majority of natural products have been isolated from terrestrial sources. During the last two decades, however, the rate of discovery of novel compounds has declined significantly, as exemplified by the fact that extracts from soil-derived actinomycetes have yielded unacceptably high numbers of previously described metabolites[2]. Bioactive compounds are frequently associated with marine invertebrates, including sponges, bryozoans, mollusks,and tunicates[3]. More recently, marine microorganisms have been recognized as a productive source of novel secondary metabolites. To date, the majority of natural products of marine bacterial origin have arisen from a small number of taxonomic groups that include *Streptomyces*, *Alteromonas*, *Pseudomonas*, *Vibrio*, *Agrobacterium*, and the cyanobacteria[4,5]. Over the past decade, a consensus has developed among marine natural products chemists and chemical ecologists, who believe that most novel natural products found in extracts of marine invertebrates are synthesized, either in part or in their entirely, by the symbiotic microbes that are intimately associated with these marine metazoans. According to chemical ecologists, secondary metabolites are produced as part of a chemical arsenal designed to deter grazers by imparting toxicity or low palatability to the metazoan host. These marine invertebrate-microbial assemblages also produce toxic compounds to prevent colonization by other non-beneficial microbial species. Interestingly, some of the small molecules isolated from marine metazoans display a striking resemblance to prokaryotic-borne metabolites. It has thus been suggested that the source of many of these bioactive is in fact the symbiotic microorganisms[6].These reviews suggest the enormous potential of sponge derived

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fungi in therapeutic aspects. Hence, this work focuses on exploring the marine niche to isolate and characterize at least one such novel bioactive compounds that can be formulated for clinical purpose.

2. Materials and methods

2.1. Biological material

In the present study sponge sample was collected at a depth of 5-10 m by self-contained underwater breathing apparatus (SCUBA) diving from Kovalam Coast which is situated on the west coast of Kerala about 14 km to the south of Thiruvananthapuram at 8° 23′ N latitude and 76° 57′ E longitude in India. Samples of sponges were collected in sterile polythene bags and transported to the laboratory within minimum possible time to avoid the external microbial contamination and excessive proliferation. All the epiphytic faunas were removed.

2.2. Isolation of fungi and preparation of extract

The sponge sample was washed with sterile water (distilled water : sea water=1 : 1) and ground in a mortar and pestle under aseptic conditions. One mL of the above was mixed with 9 mL of sterile water (distilled water : seawater=1 : 1) to get dilution 10^{-1} aseptically. The serial dilution was repeated till 10^{-6}. From each dilution plating was done in Sabourauds agar by spread plate technique. The plates were then incubated at 27 ℃ for 5 days. After 5 days, the plates were examined and the pure culture was isolated on pure agar plate.

The fungal mycelia were homogenized using sea water. Then the biomass was subjected to an extraction of biologically active components which were carried out with different solvents in the order of increase polarity: hexane, ethyl acetate and methanol by soaking at ambient temperature. The residues (crude extracts) thus obtained were finally dried under rotary vacuum evaporator and screened against two human pathogen bacteria using the agar disk diffusion method.

2.3. Antibacterial assay

Antibacterial activity was carried out against human pathogenic gram negative bacterium [*Escherichia coli* (*E. coli*), ATCC 25922] and gram positive bacterium [*Staphylococcus aureus* (*S. aureus*), ATCC 29213] by agar well diffusion method. Muller Hinton agar plates were prepared and wells were made by using gel puncture. Test culture was swabbed aseptically and inoculated on the surface of the nutrient agar so as to make a lawn. This was allowed for 5 mins for the agar surface to dry before making the wells. One mL of fungal filtrate was mixed with 1 mL of the solvent from which various (15 μL, 25 μL, 50 μL/well) concentrations and loaded in the well using micropipette and one well was loaded with the respective solvent as control. Plates were incubated for 16 to 18 hrs at 37 ℃. The zone of inhibition was observed around the well. This indicates whether the test organism is resistant (no zone of inhibition) or sensitive (clear zone of inhibition).

2.4. Purification and characterization of bioactive metabolites

One mL of fungal extract was mixed with 1 gm of silica gel and loaded on a silica gel column packed with hexane and eluted with hexane and chloroform (9 : 1 to 1 : 9 and 100% chloroform) followed by ethyl acetate and methanol (9 : 1 to 1 : 9 and 100% methanol). The absorbance of eluted fractions was measured in the UV-Visible spectrum. The gas chromatography-mass spectrometry (GC-MS) equipped with Agilent 5975 inert XL MSD was used for analysis of bioactive secondary metabolites. Infra-Red spectroscopy was used to investigate and predict any physiochemical interactions between different components in a formulation using Fouvier-transform infrared (FT-IR). The spectra obtained through those samples were compared and interpreted for the shifting of functional peaks.

2.5. Molecular characterization and Identification of elite fungi by internal transcribed spacer (ITS) sequencing

The fungi were grown in culture in potato dextrose broth at room temperature in the dark for 48 to 72 hours. The genomic DNA was isolated and the ITS region of 5.8sRNA was amplified using primer ITS1: Forward 5′ TCCGTAGGTGAACCTGCGG 3′ and Reverse ITS5 5′ TCCTCCGCTTATTGATATGC 3′[7] and sequenced using automated sequencer.

3. Results

3.1. Isolation of fungi from sponge

The sponge identification was confirmed as *Callyspongia* spp based on spicules morphology. Six different associated fungi like *Aspergilus niger*, *Aspergilus flavus* (*A. flavus*), *Hypocrea lixii*, *Trichoderma hypericum*, *Eurotium amstelodami* and an unidentified fungi were isolated from the sponge.

3.2. Antibacterial activity of the isolated fungi

Ethyl extract of *A. flavus* showed promising result by exhibiting maximum activity against two significant human pathogens *S. aureus* and *E. coli*. The zone of inhibition using *A. flavus* biomass was measured as 31 mm for *E. coli* and 18 mm for *S. aureus*. The fungal filtrate was more effective by producing 42 mm zone of inhibition for *E .coli* and 27 mm for *S. aureus*.

3.3. Characterization of bioactive metabolites

The crude fungal filtrate was separated using column chromatography and fraction 9 showed maximum absorbance. Hence, fraction 9 was subjected to further analysis to identify the bioactive compound. The gas chromatography result of fungal filtrate reveals that the active compound of *A. flavus* are Desmethylnomifensine (27.72%), 2-Butenoic acid (12.29%), 2-Benzoic acid (8.62%) and 7H-Furo furo xanthen-7-one (6.86%).

3.4. FT-IR spectrum

The FT-IR spectrum of *Aspergillus niger* extract in ethyl acetate showed bands at 3 350cm^{-1}, 2 945 cm^{-1}, 2 832 cm^{-1},

2 522 cm^{-1}, 2 227 cm^{-1} and 2 044 cm^{-1} correspond to the stretching vibrations of primary and secondary amines, respectively, while the band seen at 1 660 cm^{-1} corresponds to amide (C=O) (Figure 1). The two bands observed at 1 447 cm^{-1} and 1 115 cm^{-1} can be assigned to the C-N stretching. Vibrations of aromatic and aliphatic amines, respectively.

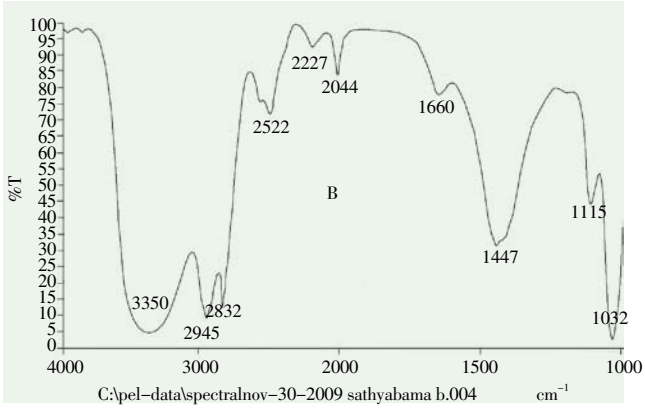

Figure 1. FT-IR spectrum.

3.5. Molecular characterization and identification of elite fungi

Blast search sequence similarity was found against the existing non redundant nucleotide sequence database thus, identifying the fungi as *A. flavus*. The percentage of similarity between the fungi and database suggested it as novel strain. Thus, the novel strain was named as *A. flavus* strain MV5 and made publically available in GenBank with an assigned accession number GU815344 (http://www.ncbi.nlm.nih.gov/nuccore/GU815344.1)

4. Discussion

Ethyl extract of *A. flavus* showed maximum activity. The activity present in the fungal extracts of least diameter of zone inhibition was considered as positive. It was already reported that some marine fungi produced active metabolites secreted from mycelia8 and culture filterate[9,10]. In this study also both culture filtrate and biomass were used and exhibited reasonable antibacterial activity.

The activity present from fungal culture filtrates has been conducted and proven by many studies[9,11, 12]. However, the low activity from the fungal mycelia does not indicate that this fungus does not have any antibacterial activity. The production of active metabolites can be boosted in many ways such as by using other microbes to compete for space and nutrients and the use of different media supplied with different nutrient sources with different physiological parameters such as pH, temperature, salinity and light[13, 14]. On the other hand, extraction of fungal mycelia using the different solvent is one way to determine the actual activity from fungal mycelia. Solvent system serves different functions in the extractions of different compounds in fungus. Crude extracts and purified fractions of *Aspergillus* spp showe of antimicrobial activity. Similarly, several metabolites of the marine isolate, *Aspergillus niger* show antibacterial and antifungal potential which supports the present study.

The isolate exhibits a marked antagonistic activity against potential bacterial pathogens thus illuminating the advanced researches in this decade to focus on clinical pharmacology to identify novel therapeutic targets. Since, the terrestrial environment seems to be exploited thoroughly, marine sources act as replenished source of bioactive compounds that have a diverse spectrum of activity. These results depict a promising scenario to focus on such marine microbe derived compounds which can be easily scaled up for large biomass production and stable formulation as a drug.

Conflict of interest statement

We declare that we have no conflict of interest.

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