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Biochemical analysis and structure elucidation of α- Campholene aldehyde from marine sponge-derived *Aspergillus terreus* MP1

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ABSTRACT

Non destructive by-catch collection of Sponges from south Indian coastal belts yielded four novel strains of Aspergillus species; among which Aspergillus terreus MP1 strain yielded interesting bioactivity against potent human pathogens and cancer cell lines. The chemical moieties responsible for the bioactivity were identified using GC-MS analysis as 15-methyl, tricycle[6.5.2(13,14).0(7,15)]pentadeca-1,3,5,7,9,11,13-heptene with molecular formulaC16H14 with molecular weight 206 Da, 3,4-Dihydro-2H-1,5-(3-t-butyl)benzodioxepine with molecular formulaC13H18O2 with molecular weight 206 Da, 2,6-dimethyl-N-(2-methyl-α-phenylbenzyl)aniline with molecular formulaC22H23N with molecular weight 301 Da as α-Campholene aldehyde with Chemical formula C10 H16 O with molecular weight 152 Da. α-Campholene aldehyde has an already known 3D structure whereas the other three compounds are novel .

Keywords : Marine Sponge, *Aspergillus terreus* MP1, Bioactive metabolites, structure elucidation.

INTRODUCTION

Marine-derived fungi are potent rich source of structurally novel natural products possessing a broad spectrum of biological activities (Smetanina et al., 2007; Blunt et al., 2006; Pivkin et al., 2006 and Somei et al., 2005). The increasing number of secondary metabolites isolated from marine-derived fungi proves that they are a rich source of bioactive compounds with therapeutic potential. To ensure their survival in their competition with other organisms, marine fungi are dependent on the production of secondary metabolites. This has been proven by the increasing number of substances isolated from marine fungi with antibacterial, antifungal, and cytotoxic

activities (Faulkner,2000;Faulkner,2001). Marine microorganisms have proven to be a promising source for the production of novel antibiotic, anti tumor, and anti inflammatory agents. The marine fungi particularly those associated with marine alga, sponge, invertebrates, and sediments appear to be a rich source for secondary metabolites (Belofsky et al., 1999). About 70-80% of the secondary metabolites that have been isolated from marine fungi are biologically active. Among marine sources, sponges act as important resource for biologically active substances, which are useful to develop pharmaceuticals, agrochemicals and biochemical reagents and their lead compounds. The origin of these biologically active substances is recently thought to be the metabolites produced by the microorganisms associated with the sponges than by the sponge themselves. Sponges act as shelters ; not only for macroorganisms, such as worms, brittlestars, shrimp, crabs, etc., but also for a variety of microorganisms, such as bacteria, fungi, and microalgae, which live in the canals, between cells, and even inside the cell. Because of their particular living conditions, salinity, nutrition, higher pressure, temperature variations, competition with bacteria, viruses and other fungi, they may have developed specific secondary metabolic pathways compared with terrestrial fungi (Liberra and Lindequist, 1995).

MATERIALS AND METHODS

Collection of Sponge and isolation of fungi:

Specimens were collected by SCUBA diving using hammer and chisel from Gulf of Mannar, located at 215 kms from kanyakumari district, in the narrow strip of peninsular land along the south east coast of Tamilnadu state. The sponge sample was washed with sterile water (distilled water: sea water; 1:1) and serial dilution was performed; plating was done on Sabourauds agar by spread plate technique. The plates were then incubated at 27^{o}C for 5 days and examined for pure culture isolate(s) on agar plates.

Molecular characterization and Identification of elite fungi by 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 TO 5’ TCCGTAGGTGAACCTGCGG 3’ and primer ITS5 5’ TCCTCCGCTTATTGATATGC 3’ [7] and sequenced using automated sequencer.

Extraction of metabolites using elite solvent:

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 solvents in the order of increase polarity: choloroform, butanol and ethyl acetate by soaking at ambient temperature. The crude extracts obtained were dried under rotary vacuum evaporator and screened for anti-bacterial activity by the agar well diffusion method as described by Schillinger and Lucke (1989) and Broth Dilution assay described by Ericsson et al., 1971.

MTT ASSAY :

MTT assay was used to investigate invitro tumor activity of ethyl acetate extract against Human laryngeal carcinoma cell line and VERO cell lines according to the previously described method by Mosmann et al (1983).

Bioactive metabolites separation using TLC:
TLC is used to separate the compound present in the crude extract. The separation of the compound also depends on the usage of the solvent. The concentration (1mg per ml) of the drug was plotted on the TLC plate and dried. It was then run with different solvent ratio the spots were identified both in the uv light and in the iodine chamber. The RF value was calculated using the formula

Rf value=Distance travelled by the solute / Distance travelled by the solvent

Gas Chromatography-Mass spectrometry (GCMS) Analysis
The crude extract was quantified using gas chromatograph (GCMS-Shimadzu) equipped with a DB-5 ms column (mm inner diameter 0.25 mm, length 30.0m, film thickness 0.25µm) mass spectrometer (ion source 200º C, RI70eV) programmed at 40-650 ºC with a rate of 4ºC/min. Injector temperature was 280 ºC ; carrier gas was He(20 psi), column flow rate was 1.4ml/min, injection mode–split.

RESULTS

Isolation of fungi:
In the present study, the 10^{-5} dilution of the sponge sample yielded four different isolates. The characterization and analysis was performed for Isolate3. Pure culture of Isolate3 (Fig 1a) was obtained and SEM micrograph (Fig 1b) , lactophenol cotton blue stained image (Fig 1c) was taken to visualize the morphological features of the fungi.

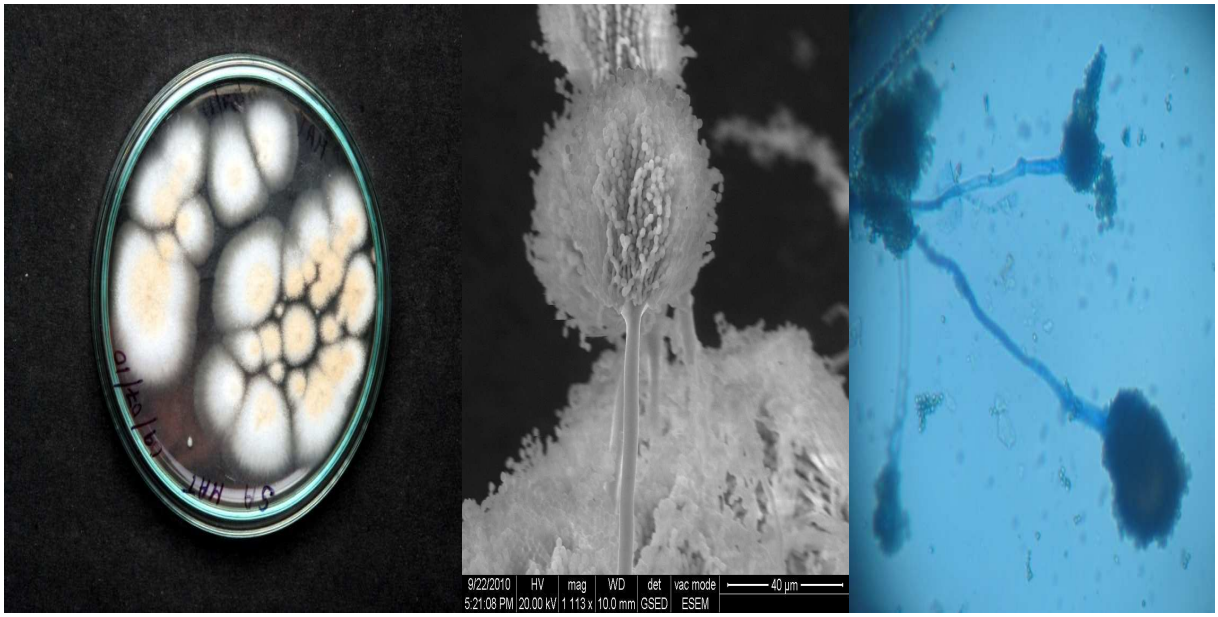


Fig 1a.Pure culture of Isolate3 **Fig 1b. SEM micrograph of Isolate3.** **Fig 1c. microscopic image of Isolate 3**

Molecular characterization and Identification of elite fungi:
The ITS region is now perhaps the most widely sequenced DNA region in fungi, It is most useful for molecular systematics at the species level, and even within species. In the present study, the DNA was isolated from the Isolate1 and the ITS region of 5.8s rRNA was amplified using

specific primers ITS 1 and ITS4 and sequence was determined using automated sequencers. Blast search sequence similarity was found against the existing non redundant nucleotide sequence database thus, identifying the fungi as ***Aspergillus terreus***. The percentage of similarity between the fungi and database suggests it as novel strain. Thus, the novel strain was named as ***Aspergillus terreus*** **strain MP1** and made publically available in GenBank with an assigned accession number **HQ449678**.

Anti-Microbial Assay: Well Diffusion Assay

The fungi ***Aspergillus terreus*** MP1 was extracted in three solvents of varying polarity (Butanol, Chloroform and Ethyl Acetate). Three human pathogens namely *Klebsiella pneumonia* ATCC 15380,*Staphylococcus aureus* ATCC 25923, *Pseudomonasaeruginosa* ATCC 27853 were used to check the anti-microbial activity. Their zone of inhibition in varying concentrations of the sample is given below

Table 1: Zone of inhibition of *Aspergillus terreus* MP1 filtrate in different solvents.

Pathogen	Butanol				Chloroform				Ethyl acetate			
	25µl	50µl	75µl	100µl	25µl	50µl	75µl	100µl	25µl	50µl	75µl	100µl
Pseudomonas	-	-	-	-	-	-	-	-	12	15	18	19
Klebsiella	-	-	-	-	-	-	-	-	11	12	13	-
S.aureus	-	-	-	-	11	13	12	-	13	12	24	12
Control	-	-	-	-	-	-	-	-	-	-	-	-

Ethyl Acetate provided promising results compared to the other solvents. Therefore, the optimum concentration of ethyl acetate producing maximum inhibition of the pathogen was analyzed using the same well diffusion assay for both Low concentration (25-100µl) and High concentration (250-1000µl) of the solvent. Their zone of inhibition is as follows

Table 2:Well Diffusion Assay -Standardization (Ethyl Acetate – Low Concentration)

Concentration (µl)	*Klebsiella (mm)*	*Pseudomonas (mm)*	*Staphylococcus (mm)*	*Micrococcus (mm)*
25	10	-	-	-
50	-	-	-	-
75	14	12	-	15
100	-	-	-	-

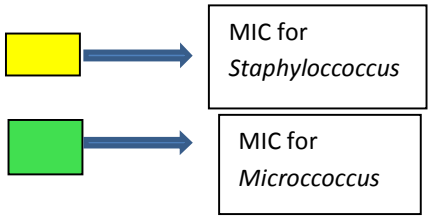
Table 3:Well Diffusion Assay -Standardization (Ethyl Acetate – High concentration)

Concentration (µl)	*Klebsiella (mm)*	*Pseudomonas (mm)*	*Staphylococcus (mm)*	*Micrococcus (mm)*
250	-	-	20	13
500	11	-	20	17
750	14	-	19	18
1000	14	-	22	19

Higher concentration of ethyl acetate provides a better inhibition activity compared to their low concentration counterpart. The MIC of the elite solvent was standardized for two pathogens Staphylococcus and Micrococcus using **Broth dilution assay.**

Table 4: Percentage of viable cells of Staphylococcus and Micrococcus in varying concentrations of Ethyl acetate.

Concentration (µl)	% OF Viable cells of *Staphylococcus*	% of viable cells of *Micrococcus*
100	8%	7%
200	13%	13%
300	16%	17%
400	27%	18%
500	28%	28%
600	30%	35%
700	46%	46%
800	**52%**	46%
900	71%	**54%**
1000	75%	61%



MTT ASSAY:

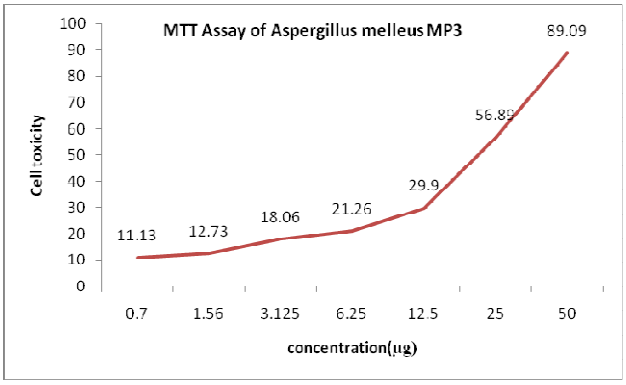


Fig 2 : MTT Assay of *Aspergillus terreus* MP1 on HEP2 cell lines

Anti-cancer potential of *Aspergillus terreus* MP1 strain was evaluated by MTT assay on VERO and HEP2 cell lines. Metabolites showed IC 50 against Hep-2 in low concentration 22.5µg and IC 50 against VERO cell line in 250ug; thus proving it effective against cell lines leaving the normal cells unaffected.

Bioactive metabolites separation using TLC and identification using GCMS :

The fungal extract subjected to TLC separation revealed the presence of four bioactive metabolites which was visualized in and Iodine chamber **(Fig. 3)**.The TLC band was eluted and the bioactive metabolites in the eluant responsible for the bio activity were characterized using GC-MS. The chromatogram (**Fig.4**) reveals the presence of different functional groups in the eluant and the solvent and they are identified as 15-methyl, tricycle[6.5.2(13,14).0(7,15)]pentadeca-1,3,5,7,9,11,13-heptene with molecular formulaC16H14

with molecular weight 206 Da, 3,4-Dihydro-2H-1,5-(3-t-butyl)benzodioxepine with molecular formulaC13H18O2 with molecular weight 206 Da, 2,6-dimethyl-N-(2-methyl-α-phenylbenzyl)aniline with molecular formulaC22H23N with molecular weight 301 Da as α-Campholene aldehyde with Chemical formula C10 H16 O with molecular weight 152 Da. Structure of α- Campholene aldehyde is depicted in **Fig.5.**

Fig 3: Compound separation on TLC

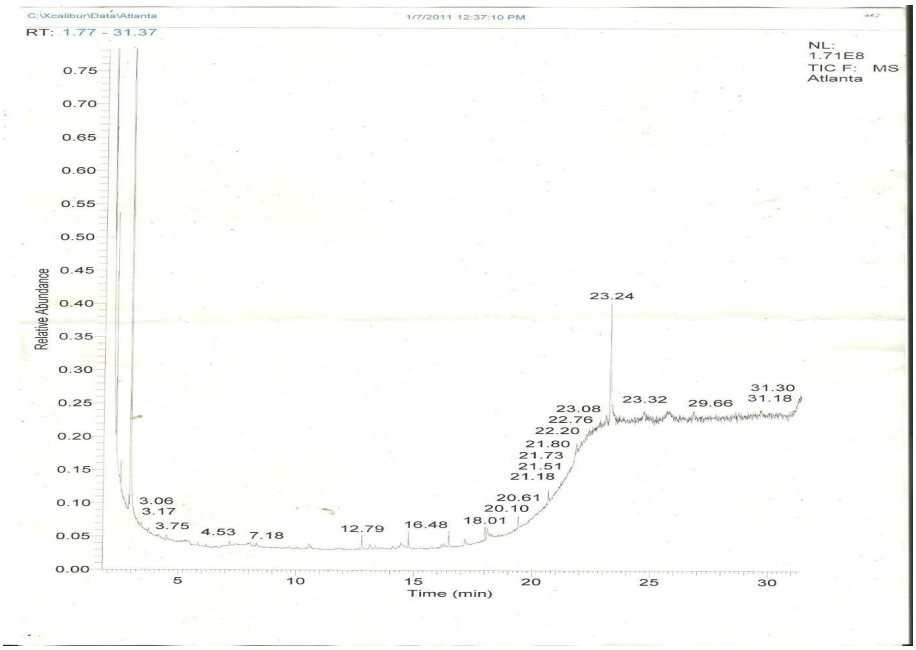


Fig 4: GC-MS chromatogram of *Aspergillus terreus* MP1 filtrate

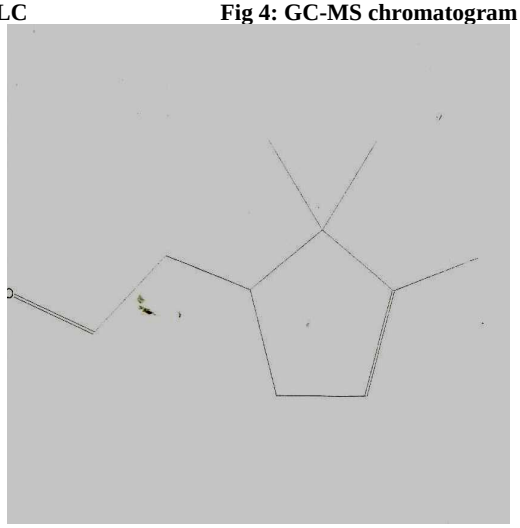


Fig 5: Structure of α- Campholene aldehyde

DISCUSSION

Marine sponge associated fungi were isolated aseptically .The isolated fungal strains were confirmed by isolating the DNA and sequencing using ITS Primer 1 and 4. The sequence

obtained was subjected to BLAST to compare with library data base sequence. The BLAST result showed that the isolated fungal strain was novel and the sequence was submitted to Gen Bank using the tool Sequin in NCBI. The accession number was given as HQ 449678 *Aspergillus terreus* MP1 was isolated from marine origin and completely characterized. The bioactive metabolites were analyzed using different solvents of varying polarity. The active compounds were separated on chromatographic column and characterized using analytical techniques. The isolation of four bioactive compounds from *Aspergillus terreus* MP1 is reported here. The antimicrobial activities of the isolated pure compounds were reported. The isolated compounds showed significant antimicrobial activity against potential human pathogens. The cytotoxic ability of the fungi was assessed by MTT assay on HEP2 carcinoma cell line. Varying concentrations of the fungal filtrate was analyzed to identify IC_{50} . The IC_{50} 0f the *Aspergillus terreus* MP1 extract on HEP2 cell line was found to be 22.5 µg.

Ethyl acetate extract of *Aspergillus terreus* MP1 showed promising effect on various pathogens. The concentrated extract of ethyl acetate was subjected to Thin Layer Chromatography to separate the metabolites. TLC gave distinct bands that were visualized under iodine chamber. The eluants were analysed and identified using GC-MS (Shimadzu instrument). The Gas Chromatography result reveals the presence of different functional groups in the eluant that is responsible for their bioactivity.

CONCLUSION

Fungi are one of the most significant groups of organisms to be exploited for drug discovery purposes. Especially Fungi Imperfecti has provided mankind with numerous different bioactive secondary metabolites such as β-lactam antibiotics, griseofulvin, cyclosporine A or lovastatin. In agreement with their related terrestrial fungi, marine fungi are producers of a high variety of biologically active secondary metabolites. In recent years, marine fungi have been explored more intensely to obtain novel and biologically active compounds. However, compared to marine sponges and bacteria, marine fungi are still less explored. In the search for biologically active natural products the present study deals with the isolation and identification of marine fungi, screening strategies for bioactives production, isolation and structure elucidation as well as pharmacological investigation of the isolated compounds.

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