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Research Article

ANTAGONISTIC ACTIVITIES OF CELL FREE ETHYL ACETATE EXTRACTS OF MARINE SPONGE DERIVED *ASPERGILLUS MELLEUS* MP3

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ABSTRACT

Objective: This piece of work aims to determine the antagonistic potential of *Aspergillus melleus* MP3 fungi from marine sponge.

Methods: Marine sponge extract was used to isolate fungi on selective medium and molecular identification was performed using ITS sequencing. Fungal mycelium was extracted with solvents of varying polarity and their antibacterial activity was checked against various human pathogenic bacteria using the agar disk diffusion method. Ethyl acetate extracts showed promising results. The bioactive metabolites were purified on TLC and analysed using GC-MS. The mechanism of action of bioactive metabolite was found by estimating the amount of protein present in four potent human pathogens. The proteins were visualised by SDS-PAGE to identify the molecular weight. The cytotoxic ability of the fungi was assessed by MTT assay on HEP2 carcinoma cell line**.** Structure of the metabolites were analysed using XRD and ^{1}H NMR and ^{13}C NMR.

Result: Molecular data revealed the identity of the fungi as *Aspergillus melleus* MP3 and novelty of the sequence lead to its deposition in GENBANK with Accession number HQ 449678.3. Varying concentrations of the fungal filtrate was analysed to identify IC_{50} . The IC_{50} 0f *the Aspergillus melleus* MP3 extract on HEP2 cell line was found to be 6.25 µg. The appearance of a multiple band on SDS-PAGE further suggests the proteins to be multimeric.

Conclusion: The present study of screening bioactive secondary metabolites revealed that *Aspergillus terreus* as a source for the production of three effective metabolites.

Keywords: Marine Sponge derived Fungi, Aspergillus, Bioactive metabolites, Anti bacterial activity.

INTRODUCTION

The marine invertebrate life adapted to extreme environment represents our greatest yet barely investigated resource of new natural products. Because of their longer evolutionary history, marine invertebrates are more likely to possess a greater molecular diversity than their terrestrial counterparts. The rapid growth in the chemistry of marine organisms over the last 15 years has led to the discovery of large number of new structures, many of which have no precedence among structures of terrestrial origin and possess previously unknown pharmacological and toxicological properties [32]. Nearly all forms of marine life have attracted the attention of natural product chemists, with reports on secondary metabolites from sponges dominating by number [5]. Among metazoans, sponges are the most primitive having lived for 700-800 million years and accumulated diverse array of pharmacologically active chemicals [20]. Sponges providing potential drugs against many major life threatening diseases are an emerging and less explored area of research. The mechanism of how, where and when these metabolites are produced in sponges is still very unclear. Recent studies on marine natural product biosynthesis indicate that many bioactive compounds previously found in marine animals and plants are in fact produced or metabolized by associated microorganisms [36, 31, 3, 19, 29]. Sponges are not an exception as many of the chemicals of sponge origin are actually produced by associated microorganisms in their tissues [17]. Some researchers are of the view that marine invertebrates like sponges have evolved chemical defence mechanisms against other invading organisms through production of these secondary metabolites. Such metabolites isolated from sponges have displayed potent anti-cancer, anti-microbial, anti-fungal or anti-inflammatory and other pharmacological activities [39,40,29,7,13,16,11,21] .

As already indicated, sponges produce toxins and other compounds to repel and deter predators [26,38] compete for space with other sessile species[28,42] and for communication and protection against infection. Of the investigated marine sponge species, >10% has exhibited cytotoxic activity [41] suggesting production of potential medicinal compounds. Potentially therapeutic compounds identified in sponges include anticancer agents, immunomodulators and antifouling molecules. Although many bioactives have been discovered in sponges 10,37,[37,25,10,24,27,8,33,30] only a few of these compounds have been scientifically scrutinised under US-FDA regulations and recommended for commercialization . Concentrations of the desired bioactives in sponges are generally low, e.g. 0.4% of dry weight, but concentrations as high as 12% have also been recorded for some metabolites [35]. The endosymbiotic microbes are the true source of sponge metabolites [29]. The diversity of secondary metabolites recorded in sponges during the last fifty years truly make them a chemical goldmine [18].The antibiotics and other secondary metabolites isolated from exotic organisms such as marine sponges may be an alternate source to replace the existing drugs having severe side effects and will play an important role in future combating drug resistant bacteria commonly encountered in many disease situations.

MATERIALS AND METHODS

Sponge samples were collected by scuba during scientific expedition in a rocky slope at water deeper than 20 m from the coastal water of 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., Specimens were cleaned and stored at -20°C until used in extraction. The sponge sample was washed with sterile water (distilled water: sea water; 1:1) and ground in a mortar and pestle under aseptic conditions. Serial dilution was performed and from each dilution, plating was done in Sabourauds agar by spread plate technique. The plates were then incubated at 27°c for 5 days. After 5 days, the plates were examined and the pure culture was isolated on pure agar plate. 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. 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: 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. Agar diffusion assay is used widely to determine the antibacterial activity of crude extract.TLC is used to separate the compound present in the crude extract. 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. In order to study the antitumor activity of a new drug, the cytotoxicity concentration of the extract was studied by MTT assay.

RESULTS AND DISCUSSION

In the present study, the 10^{-5} dilution of the sponge sample yielded fungal isolate that was characterised by pure culture on plate (**Fig. 1**)visualize the morphological features of the fungi.

Fig. 1: Pure culture of *Aspergillus melleus* strain MP3

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 melleu*s. The percentage of similarity between the fungi and database suggests it as novel strain. Thus, the novel strain was named as ***Aspergillus melleus* strain MP3** and made publically available in GenBank with an assigned accession number **HQ 449678.3.**

The fungi *Aspergillus melleus* MP3 was extracted in three solvents of varying polarity (Butanol, Chloroform, Ethyl Aceteate). Ethyl Acetate provided promising results compared to the other solvents. The optimum concentration of ethyl acetate producing maximum inhibition of the pathogen was analyzed using the well diffusion assay for low and High concentration of the potent solvent on potential human pathogens. Higher concentration of ethyl acetate provides a better inhibition activity compared to their low concentration counterpart. The fungal extract subjected to TLC separation revealed the presence of two bioactive metabolites which was visualised in UV short range spectrum -254nm (Fig 4.Iodine Chamber)

Fig. 4: Iodine chamber

The TLC band was eluted and the bioactive metabolites in the elutant responsible for the anti bacterial activity were characterized using GC-MS(Fig 5). The chromatogram revelas the presence of different functional groups in the elutant and the solvent. The elutant is identified as **Lucenin-2** with Chemical formula C_{27} H_{30} O_{16} with molecular weight 610 Da.. The other elutant is identified as **6-Ethyloct – 3-yl- 2- ethylhexyl ester** with Chemical formula C_{26} H_{42} O_4 with molecular weight 418 Da.

The bioactivity of metabolites is visualised as yellow band after spraying MTT (Fig 6.) . The cytotoxic ability of the fungi was assessed by MTT assay on HEP2 carcinoma cell line. Varying concentrations of the fungal filtrate was analysed to identify IC_{50} . The IC_{50} 0f the Aspergillus terreus MP1 extract on HEP2 cell line was found to be 6.25 µg.

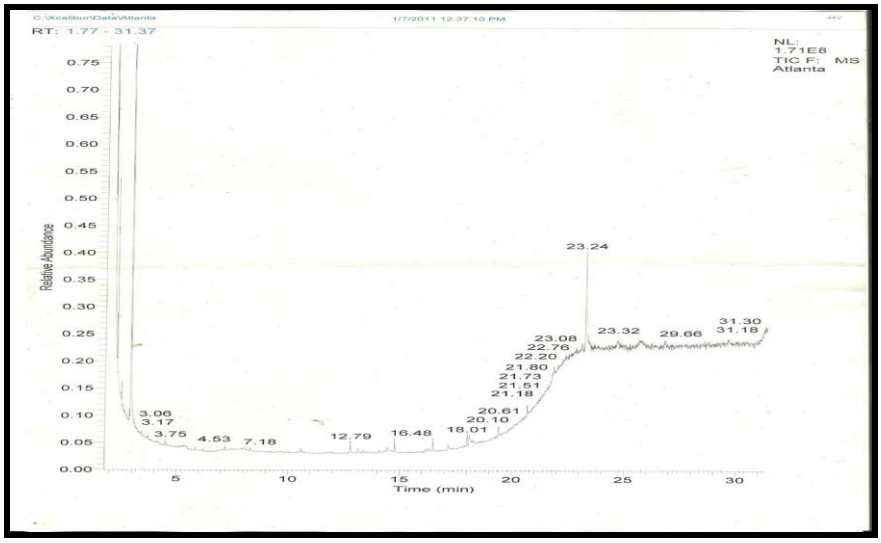

Fig. 5: GC MS Chromatogram of *Aspergillus melleus* strain MP3

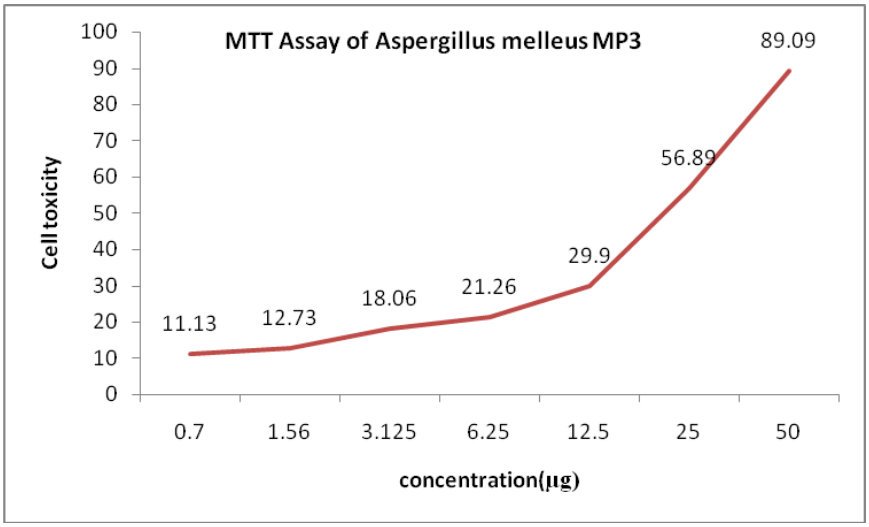

Fig. 6 : Graph showing cytotoxicity of MTT assay of *Aspergillus melleus* strain MP3

The present study of screening bioactive secondary metabolites revealed that *Aspergillus terreus* as a source for the production of three effective metabolites. These metabolites can be further exploited for the biotechnological applications in medicine and agriculture. Sponge (or microbe)-derived substances span a wide range of chemical classes (e.g., terpenoids, alkaloids, peptides and polyketides) with an equally variety of biotechnologically relevant properties (e.g., antibacterial, antifungal, antiviral and antiprotozoal). [9,29,34]. The attention of natural product chemists and pharmaceutical companies, at present, is focused firmly on anticancer drugs, with several promising sponge-derived substances in clinical and preclinical trials. Cultivation of sponge-associated microorganisms that produce bioactive substances is the most direct method for large-scale production of these chemicals [15] and cultivation approaches are widely practiced among those targeting bioactive substances [6,12]. There are numerous examples of the production of biologically active substances by sponge-derived microbial isolates. We have previously characterized the same in *Aspergillus flavus* [22] and *Aspergillus ochraceus* MP2 fungi [23]. A recent review suggests two strategies, the first uses a wide range of media in an effort to grow as many different sponge-associated microbes as possible [14]. Since growth under different culture conditions may influence which metabolites are produced, the use of many different media and conditions should help to maximize the chemical diversity from a given microorganism [15]. An alternative, a more targeted approach, is to go after specific microbial groups with proven track records in the production of bioactive substances.

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