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RESEARCH ARTICLE

ANTIMICROBIAL ACTIVITY AND GC-MS ANALYSIS OF *ASPERGILLUS CLAVATUS*

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ABSTRACT

The culture filtrate of *Aspergillus clavatus* isolated from mangrove environment grown in corn meal broth was extracted and antimicrobial potential was tested. And filtrates were extracted with four different solvents, such as ethanol, ethyl ether, ethyl acetate and methanol. Their antimicrobial potential was tested against eight pathogenic bacteria and two fungi. The antimicrobial activities were evaluated by agar well diffusion method. The results of the screening revealed the strongest antibacterial and antifungal activities by the ethyl acetate extract followed by methanol, ethanol and ethyl ether. Crude extract showed the weakest inhibition. *A.clavatus* showed minimum inhibitory activity against *Salmonella typhi, Salmonella paratyphi* B and one fungal pathogen, *Cryptococcus neoformans*. But, the extracts showed maximum activity of inhibition against the other bacterial pathogens such as *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Klepsiella pneumoniae, Proteus vulgaris,* and *Candida albicans*. Influence of nutrients and physico-chemical parameters on the production of antimicrobial compounds was investigated so as to find out the optimum culture condition for maximum antimicrobial activity was performed. Based on this investigation, corn meal liquid medium, 9 days of incubation, 30°C temperature, pH 8, salinity 3% and the dextrose as carbon source were considered to be the optimum conditions for the maximum production of antimicrobial compounds, *in vitro* condition. Chromatogram of GC-MS analysis of the extract of *A.clavatus* showed the presence of 5 sets of major peaks and the components corresponding to the peaks were determined.

INTRODUCTION

The marine environment is a rich source of both biological and chemical diversity, where it has been reported that oceans contain nearly 300,000 described species, representing only a small percentage of the total number of species that have to be discovered. Almost all forms of life in the marine environment have been investigated for the possible occurrence of useful natural products content [7]. A lot of structurally and pharmacologically important substances have been isolated with novel antimicrobial, antitumor and anti-inflammatory properties [23, 28, 2, 17, and 18]. Thus, it is obvious that the marine microorganisms are the source of important natural products. Marine fungi have proven to be a rich and promising source of novel anticancer, antibacterial, antiplasmodial, anti-inflammatory and antiviral agents [1, 5, and 25]. Many of these fungi have been proven to be rich source of structurally novel and biologically active secondary metabolites, which are emerging as a significant new chemical resource for drug discovery [3]. The productions of these unique secondary metabolites by marine fungi are possibly because of adaptation to a very distinct set of environmental pressures. Hence the study of secondary metabolites from fungal population receives special attention. Mangrove fungi are the second largest group among marine fungi in the marine environment [14, 20]. With respect to fungal diversity, the mangrove is the best–studied habitat and most attention has been devoted to the wood–inhabiting fungi [21]. In India, particularly in Muthupet mangrove environs, the available information on the microfungi is very little and no substantial work has been done on antimicrobial compounds producing ability of such fungi. Keeping these points in mind and recognizing the significance of fungi as a source of novel bioactive compounds, the present study was planned to screen the antitimicrobial activity of *Aspergillus clavatus* isolated from mangrove environment and analyze its bioactive compounds.

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MATERIALS AND METHODS

Determination of Antimicrobial spectrum of fungi

Aspergillus clavatus culture was inoculated into a 500ml conical flask containing 200ml of 50% v/v seawater corn meal liquid media for 7 days at 28 ± 2° C. After incubation, the mycelia were separated by using filter paper, and the filtrates were subjected through Millipore filter (0.2µm). The cell free culture filtrates were vortexed, centrifuged at 20,000xg and the upper layer was collected and used as crude for antimicrobial activity.

Antimicrobial activity of different solvents extracts of fungi

To derive crude compound from the cultured broth, the fungi was cultured in 5 flasks of 200ml 50% Sea Water Corn Meal broth. The broth after incubation, the cell free filtration were extracted with different solvents viz. ethanol, ethyl acetate, ethyl ether and methanol and used for antimicrobial activity.

Antibacterial spectrum

In vitro antibacterial susceptibility tests were performed against *Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Salmonella typhi, Salmonella paratyphi* B*, Staphylococcus aureus,* and *Streptococcus epidermidis,* by agar diffusion well method. Inoculums of the pathogen for the assay were prepared in nutrient broth. One ml of the broth inoculum was mixed with medium poured into the Petri plates and allowed for solidification. After solidification 6mm diameter duplicate well was made with the help of a sterile cork borer in the medium. In each well 100µl of the filtrate was poured. All the plates were incubated for 24 – 48 hours at room temperature and the zone of inhibition was recorded.

Antifungal spectrum

Antifungal activity of the cell free culture filtrates of *Aspergillus clavatus,* was determined against *Candida albicans* and *Cryptococcus neoformans* using the agar diffusion well method. Potato Dextrose agar (PDA) medium was used for this assay method. PDA medium mixed with pathogenic fungal suspension was poured on to the Petri plate allowed to solidify and 6mm diameter well was made. The extract was poured in to each well and the plates were incubated at 28±2^{o} C for 24 to 48 hours. The zone of inhibition was measured from the duplicate plates.

Optimization of antimicrobial compounds production

Antimicrobial compound production of *Aspergillus clavatus* was carried out in different liquid media, and altering one of the following parameters at a time in corn meal broth. The parameters altered were incubation period, temperature, pH, and salinity and carbon sources.

Effect of different media

To find out suitable media for the optimum production of bioactive metabolites production six different liquid media namely, Corn meal medium (Corn meal 17g; Dextrose 20g; Peptone 20g; 50% Seawater l000ml), Czapek-dox medium (Sodium nitrate 2g; Dipotassium hydrogen sulphate 1g; Magnesium sulphate 0.5g; Ferrous sulphate 0.01g; Sucrose 30g; 50% Seawater 1000ml), Malt extract medium (Malt extract 30g; Dipotassium hydrogen phosphate 1g; Ammonium chloride 1g; Citric acid 15ml; 50% Seawater 1000ml), Potato dextrose medium (Potato 200g; Dextrose 20g; 50% Seawater 1000ml), Rose Bengal medium (Dextrose 10g; yeast extract 0.5g; Potassium dihydrogen phosphate 0.5g; Magnesium sulphate 0.5g; Peptone 0.5g; Rose Bengal dye 0.05g; 50% Seawater 1000ml) and Sabouraud's medium (Peptone 10g; Dextrose 20g; 50% Seawater 1000ml) were prepared and inoculated the fungal cultures in separate 500ml conical flasks and incubated at 28±2^{o} C for 10 days. After incubation, the culture filtrates were analyzed for antimicrobial activity by agar diffusion well method against pathogenic bacteria such as *Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Salmonella typhi, S. paratyphi* B*, Staphylococcus aureus, Streptococcus epidermidis* and fungi *Candida albicans* and *Cryptococcus neoformans*.

Effect of incubation periods

Conical flasks with corn meal broth were inoculated with fungal culture and incubated for 15 days in stationary condition at 28±2^{o} C. filtrates drawn in 3, 6, 9, 12 and 15 days old cultures were subjected to antimicrobial activity by well diffusion method against pathogenic bacteria and fungi.

Effect of temperature

The fungi were inoculated in corn meal broth and incubated at 15, 20, 25, 30, 35, and 40^{o} C temperature. After 9 days incubation, the culture filtrates were analyzed for antimicrobial activity by agar diffusion well method.

Effect of pH

The initial pH of the corn meal broth was adjusted to 5, 6, 7, 8 and 9 in separate conical flasks with 0.1 N NaOH and 0.1 N HCl. All the flasks were inoculated with fungi and incubated at 30^{o} C for 9 days. After incubation the filtrates were tested against pathogenic bacteria and fungi.

Table: 1 Compounds in culture filtrate of *Aspergillus clavatus* identified based on GC-MS analysis and their characterization

S.No	Retention Time (min)	Name of the compound	Molecular Formula	Molecular Weight	Peak Area (%)	Compound Nature	Activity
1.	4.13	Glycerin	$C_3H_8O_3$	92	22.36	Alcohol	Antimicrobial Preservative
2.	6.16	Thymine	$C_5H_6N_2O_2$	126	14.73	DNA Base	No activity reported
3.	7.59	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	144	25.82	Pigment fraction	No activity reported
4.	20.92	Glycyl-L-proline	$C_7H_{12}N_2O_3$	172	14.44	Peptide compound	Antimicrobial
5.	25.79	Undecanoic acid	$C_{11}H_{22}O_2$	186	22.65	Fatty acid	No activity reported

Effect of salinity

Corn meal broth with different salinity concentration viz. 0%. 1%, 2%, 3%, 4% and 5% was prepared by adding NaCl. The fungi were inoculated and incubated at 30° C for 9 days. The filtrates were drawn and tested against the pathogenic microbes.

Effect of carbon sources

Corn meal broth was prepared replacing the carbon source (dextrose), with galactose, lactose, maltose, and sucrose (20g/l) separately. Broths with different carbon sources (pH 8; salinity 3%) incubated at 30° C for a period of 9 days. After incubation, the filtrates were tested against the microbes.

Gas Chromatography and Mass Spectrometry analysis of crude compounds

The GC-MS analysis was carried out using a Clarus 500 Perkin-Elmer (Auto system XL) Gas Chromatograph equipped and coupled to a Mass detector Turbo mass gold- Perkin Elmer Turbomass 5.1 spectrometer with an Elite-1 (100%Dimethyl poly siloxane), 30 m x 0.25 mm ID x 1µm df capillary column. The instrument was set to an initial temperature of 110 °C, and maintained at this temperature for 2 min. At the end of this period the oven temperature was rose up to 280°C, at the rate of an increase of 5°C/min, and maintained for 9 min. Injection port temperature was ensured as 250°C and Helium flow rate as 1 ml/min. The ionization voltage was 70eV. The samples were injected in split mode as 10:1. Mass spectral scan range was set at 45-450(m/z). Using computer searches on a NIST Ver.2.1 MS data library and comparing the spectrum obtained through GC-MS the compounds present in the crude sample were identified.

RESULTS AND DISCUSSION

Crude extraction of *Aspergillus clavatus* showed least antimicrobial activity (4mm to 11mm) while ethyl acetate extractions of fungus showed higher antimicrobial activity (11mm to 23mm) followed by methanol (9mm to 16mm), ethyl ether (6mm to 15mm) and ethanol (8mm to 13mm), against different pathogens. The maximum inhibition was observed with ethyl acetate extract of *A.clavatus* against *S.aureus* (23mm) followed by *B.subtilis* (19mm), *E.coli* (17mm), *S.epidermidis* (17mm), *C.albicans* (17mm), *P.vulgaris* (16mm), *K.pneumoniae* (15mm), *C.neoformans* (15mm), *S.paratyphi* B (14mm) and *S.typhi* (11mm). The other solvent extracts showed minimum to moderate inhibition activity against these pathogens when compared with ethyl acetate extract (Fig:1). All the tested bacteria and fungi were inhibited by *A. clavatus. S. aureus* was most sensitive to it. Anti-bacterial and anti-fungal potential of the genus *Aspergillus* sp. proved [4, 16, and 26] and particularly in *A. clavatus* [12, 27, and 15] are in agreement to this. *A.clavatus* species of fungi grown on Corn meal liquid medium and extracted with ethyl acetate showed good antimicrobial activity against the test pathogens among the six media tested. *A.clavatus* grown on Malt Extract, Rose Bengal and Sabourauds liquid media did not show any inhibition against *K.pnemoniae.* Likewise, *A.clavatus,* did not inhibit *S.typhi* when grown on Sabourauds broth (Fig: 2). Depending on the environmental conditions, different fungal species varied in the synthesis of metabolites and relatively maximum quantities of compounds were produced under optimized conditions [9]. The composition of culture medium has an important role on the production of secondary metabolites by microorganisms. Even though corn meal media was used for the isolation of fungi, further standardization of media showed that corn meal media could be used as a basal medium, for higher antimicrobial compound production. The antimicrobial activity was slightly increased after 3 days of incubation. The activity attained the maximum and stable or slightly varied from 9 to 15 days of incubation depending on the pathogens. Thus the incubation of 9 days was observed to be optimum for maximum antimicrobial metabolite production. The antimicrobial activities remain static or slightly decreased after 9 days of incubation. Maximum antimicrobial activity was recorded after the fungus reached its stationary phase (Fig: 2). This shows that the fungi produce metabolites even after attaining stationary phase. The same trend in case of *Gliocladium* sp. [29] is in agreement with the present study. The incubation of 9 to 10 days was observed to be optimum for maximum biomass and bioactive metabolite production in the case of endophytic fungus, *Fusarium* sp. [11].

The temperature may affect the nutritional uptake and the metabolism. The fungi exhibited growth in the broad temperature range, from 5 to 40° C. They failed to grow at 50° C. The antimicrobial activity showed the peak level when the filtrate obtained by using the fungi at 30° C and falls on either side the fungi. The filtrates prepared growing the fungi at 30° C had their highest antimicrobial activity against all the pathogens. Though the fungi spelled a little growth at 5° C, the filtrates obtained this temperature had no antimicrobial activity (Fig: 2). Similarly Vahidi [31] also reported that the optimum condition for the production of antimicrobial compounds by *Qudemansiella* spp. was situated between 28° C and 30.6° C. An exponential growth pattern was observed at temperatures between 10° C to 30° C, while little growth at 5° C and ceased at 50° C. Studies proved that low temperature may cease the metabolic activity of the fungus and high temperature kills the cell of the fungus. However, in some cases, the optimum temperature requirement for growth and metabolite production is one and the same [8].

The pH of the culture media is one of the determining factors for the metabolism and then for the biosynthesis of secondary metabolites. The pH is related to permeability characteristics of the cell wall and thus either affect the ion uptake or loss of the nutrients from the medium [13]. The fungi exhibited growth in corn meal liquid media at different pH values such as 5, 6, 7, 8 and 9. The filtrate prepared growing the fungi at pH 8 had their highest antimicrobial activity. The culture grown on neutral pH also showed antimicrobial activity (Fig: 2). Evidently Furtado et al. [9] reported that a larger number of antimicrobial compounds were produced by *Aspergillus* at pH 7.5. Observation on growth and production of antimicrobial compounds at neutral pH [24] also lends support to the present study. The fungi exhibited growth in different salinity range from 0% to 5%. The antimicrobial activity showed the peak at the filtrate obtained by growing the fungi at 3% NaCl concentration. Although the growth was observed in the same medium without NaCl, filtrates obtained at this salinity had no antimicrobial activity (Fig: 2). This leads to conclude that the metabolite profiles expressed by fungi are sometimes dependent on the salinity of the media as stated [22]. The

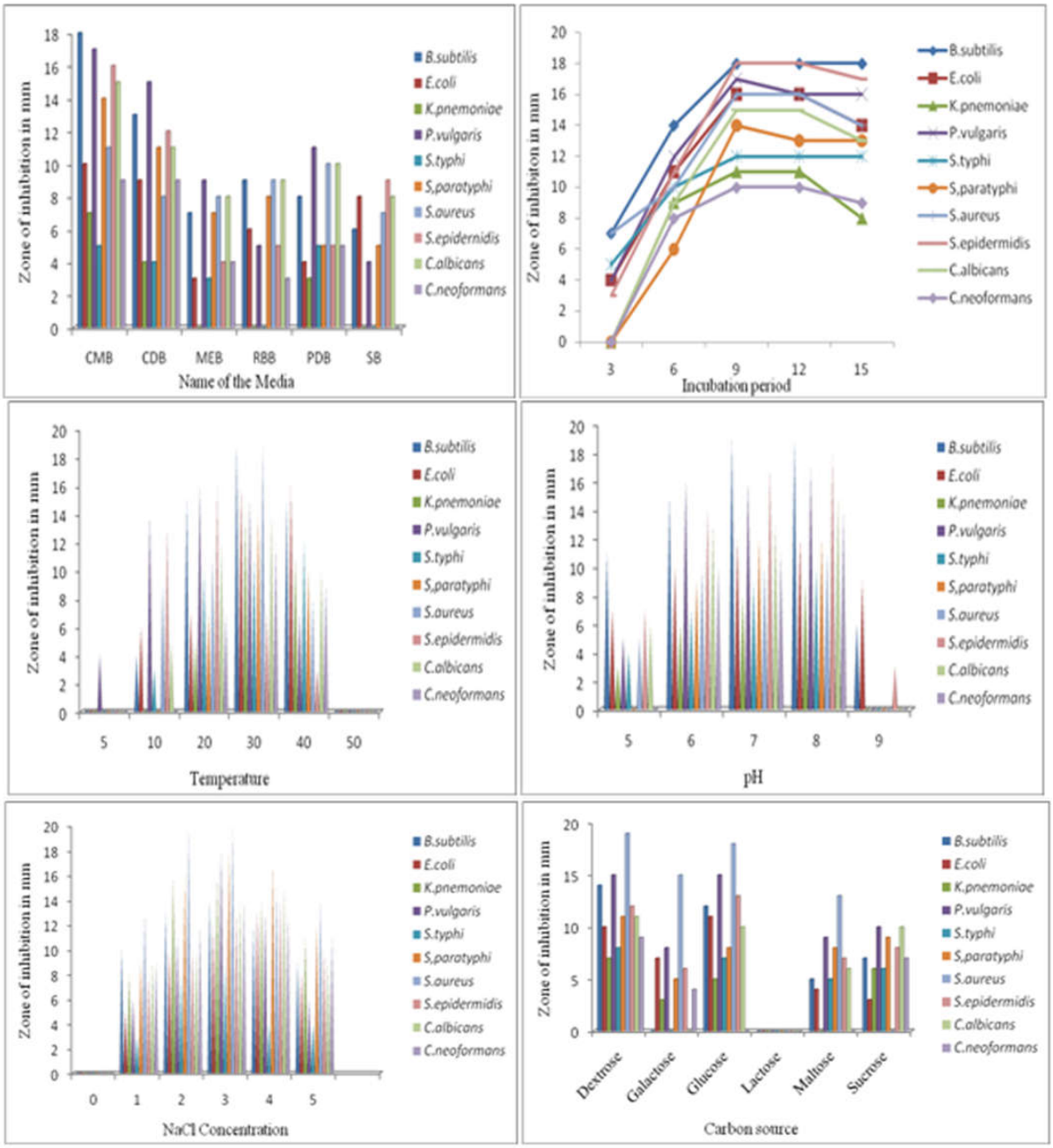

Fig: 2 Antimicrobial efficacy of ethyl acetate extract of *A.clavatus* in different conditions

maximum antimicrobial activity was showed, when the filtrate prepared growing the fungi at dextrose as a carbon source. Though the *A.clavatus* fungi spelled a little growth on maltose and sucrose, the filtrates obtained at these carbon sources had no antimicrobial activity. *A. clavatus* did not show antimicrobial activity when the fungus was grown on lactose amended media (Fig: 2). The fungus grew faster in a high nitrogen medium while exhibiting higher bioactivity in a high carbon medium [19]. The influence of dextrose supplement in the medium for the maximum production of antimicrobial tances by all the fungi could be corroborated with the findings of Turner [30] who stated that the simple carbohydrates like glucose and dextrose through metabolic pathway affect the accumulation of intermediates, which lead to the production of primary as well as secondary metabolites in addition to CO_2, water and energy. In the present study, the compounds present in the culture filtrate of *A.clavatus* was analyzed by using GC-MS. Ethyl acetate was used as a solvent for the separation of antimicrobial compounds present in the fungi. The ethyl acetate culture filtrates of *A.clavatus* exhibited 5 compounds belonged to different categories, which were identified by comparing GC-MS data with those given in library. Chromatograms of the peaks were determined as follows. The first set of peaks were determined to be glycerine at t_R=4.13 min and covered with 22.36% of spectral area. The second set of peaks indicated to be thymine (t_R=6.16 min) with 14.73% of peak area, following this an area of 25.82% covered in the mass spectrum by 4H-pyron- 4-one, 2, 3-dihydro-3, 5-dihydroxy 6-methyl at t_R=7.5min. The next set of peaks considered to be 14.44% of glycyl-L-proline (t_R=20.92 min).

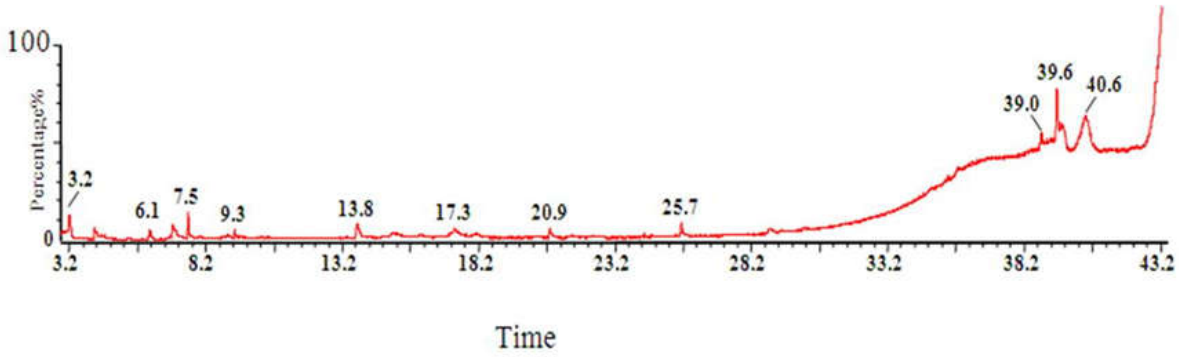

Fig: 3 Mass spectrums of compounds in culture filtrate of *Aspergillus clavatus* identified based on GC-MS analysis

Lastly a peak corresponding to undecanoic acid was determined at t_R=25.79 min according to 22.65% of spectrum (Fig: 3; Table: 1) Among them, 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-, undecanoic acid and glycerin were the major constituents. Thymine also found as one of the compound. A peptide compound glycyl-L-proline was present in the culture filtrates. So far, several peptide compounds with antimicrobial activity have been obtained from microorganisms such as psychrophilin D, a nitropeptide isolated from *Penicillium algidum* [6] and trichodermamide B, a dipeptide from *Trichoderma virens* [10]. This study suggested that marine microorganisms especially mangrove fungi play an important role in inhibition of pathogenic microorganisms due to its secondary metabolite. These metabolites can be used as drug for human pathogenic microorganism. Therefore, it's important to understand the marine derived fungi in ecological system and also research for biotechnology.

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