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Research Article

GC–MS ANALYSIS OF ANTIFUNGAL COMPOUNDS DERIVED FROM SOIL ACTINOBACTERIA

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ABSTRACT

This study was aimed to evaluate the antifungal property of actinobacterial strain isolated from rhizosheric soil and its compounds analysis through GC-MS. A total of 16 Actinobacterial strains were isolated and screened on the basis of their efficiency to inhibit the growth of fungal pathogen *Fusarium oxysporum.* The secondary metabolites from the active strain (*Streptomyces werraensis* KBR01) were extracted using different organic solvents and subjected to disc diffusion test to check their efficacy against fungal pathogen. The presence of active compounds was confirmed by gas chromatography-mass spectrometry (GC-MS) analysis which revealed the presence of different types of compounds like fatty acid methyl ester (6,9,12-Octadecatrienoic acid, methyl ester, 36.02%), long chain alkanes (Dodecane, 29.47%), fatty alcohol (2- pentadecanol, 13.72%), piperazinedione (2,5-Piperazinedione, 3,6-bis(2-methylpropyl), 8.28%), esters (dodecyl acrylate, 8.29%) and pyrrolizidine (Pyrrolo[1,2- a]pyrazine-1,4-dione, Hexahydro-, 2.8% and Pyrrolo [1,2-a]pyrazine-1,4-dione, Hexahydro-3-(2-methylpropyl), 1.4%). Presence of many antimicrobial compounds in the crude extract of *Streptomyces werraensis* KBR01 advocates the antimicrobial potency of this strain.

Keywords: *Streptomyces*, GC-MS, Antimicrobial, Organic solvents, Rhizospheric soil.

INTRODUCTION

Bioactive compounds produced by microorganisms exhibit pharmacological or toxicological effects in other organisms. Actinobacteria have been considered as one of the significant groups of microorganisms as they represent a broad range of valuable and prominent sources of pharmaceutically active metabolites. They are recognized as prolific producers of novel antimicrobial agents[1] and therefore are much important in the field of pharmaceutical industries and agriculture. A large number of antimicrobial compounds have been discovered from actinobacteria by screening natural habitat such as soils and water bodies[2,3].

Actinobacteria are the diverse group of gram positive bacteria that show substrate and aerial mycelium growth. They are aerobic, spore forming bacteria and have high G+C (>55%) content in their DNA. These bacteria have high mechanisms of survival in unfavourable environments[4]. They have been identified as one of the prominent group of soil microorganisms which differ with soil type, pH, geographical location and climatic condition[5].

Among actinobacteria the genus *Streptomyces* is of commercial interest due to their unique capacity to produce novel metabolites. The vast majority of the secondary metabolic compounds are derived from the single genus Streptomyces. It is estimated this bacteria synthesizes more than 7,000 metabolites[6].

The demand for new antibiotics keeps on growing due to the rapid emerging of multiple antibiotic resistant pathogens. To overcome the problem of these multidrug resistance pathogens more sources of antimicrobial agents need to be studied which can be of better potential and activity than the previously discovered agents. The present study is focused on the efficacy of antifungal secondary metabolite derived from actinobacteria against pathogenic fungus *Fusarium oxysporum.*

MATERIALS AND METHODS

Isolation of Actinobacteria

Soil samples were collected from diseased supressed rhizospheric soil of healthy and young leguminous plants cultivated at local farmers' field in Haridwar, Uttarakhand, India One gram of the soil sample was serially diluted up to 10^{-6} dilution and 0.1ml aliquot from 10^{-4} to 10^{-6} dilutions were spread on Actinomycetes isolation agar (AIA) supplemented with 100 µg/ml Nystatin[7] and incubated at 28±2°C for two weeks. After an incubation period, hard, rough and pin-point colonies were picked up and maintained on ISP 2 medium (Yeast extract malt extract agar medium).

Antagonistic Assay

Dual culture technique[8] was followed to determine antagonistic activity of isolated strains against fungal pathogen *Fusarium oxysporum*. Agar block (5 mm dia) from 5 days old fungal culture containing mycelia was placed in the centre of the CDA (Czapek Dox agar) plate and one loopful culture of each actinobacterial isolate was spotted 2 cm apart from the pathogen and incubated at 28±2 ºC for 7 days. The percent inhibition was recorded by using given below formula:

$$PI = [(C-T)/C]/100$$

where PI = Percent inhibition, C = Radial growth of fungus in control and T = Radial growth of fungus in dual culture

Extraction of Antifungal Metabolites

For the extraction of antifungal metabolites actinobacterial isolate was grown in selected production medium with optimal culture conditions. Biomass was separated from production medium by centrifugation at 11000 rpm for 15 min. Crude antifungal compound produced in culture was extracted through manual shaking with equal volume of organic solvents in a separating funnel. A range of extraction solvents was tested, including hexane, chloroform, diethyl ether, n-butanol and ethyl acetate. The organic extracts were concentrated by rotatory evaporation and the concentrated products were regarded as crude samples and kept at -20ºC[9].

Efficacy of Different Solvent Extracts

Disc Diffusion Assay

To check effectiveness of different solvents crude extracts in growth suppression of *Fusarium oxysporum*, complete dried extracts (10 mg) were dissolved in 1 ml of dimethyl sulfoxide (DMSO), and 20µl samples were loaded on to sterile filter paper discs. The loaded filter paper discs were placed on fungal challenged plates and incubated at 28ºC. Filter paper discs loaded with DMSO served as negative control. After incubation plates were observed for appearance of zone of inhibition. The susceptibility of the fungal pathogen was also checked against antifungal disc of fluconazole and amphotericin B using same method[10].

GC-MS Analysis

Identification of the chemical compounds present in isolate KBR01 was analysed using Gas chromatography mass spectrometry analysis (GC-MS)[11]. GC-MS analysis of the ethyl extracts was carried out using a mass spectrometer system (AccuTOF GCV) with mass range 10 to 2000 amu and mass resolution 6000 equipped with gas-chromatograph (Agilent: 7890) - capillary column (0.25 mm thickness and 30 m in length). The peaks were identified by matching the mass spectra with the National Institute of Standards and Technology (NIST, USA) library.

Identification of the Actinobacterial Strain

The actinobacterial isolate exhibiting high antifungal activity against *F. oxysporum* was identified by morphological, physiological and molecular characterizations. For molecular characterization, genomic DNA of the isolate was extracted following Green and Sambrook[12] and 16S rRNA amplification was carried out using universal eubacterial primers 27F 5′ AGAGTTTGATCMTGGCTCAG 3 and 1492 R′ TACGGYTACCTTGTTACGACTT 3′. All the sequences were compared with 16S rRNA gene sequences available in the GenBank databases of NCBI by BLASTn search. Phylogenetic analysis was performed using MEGA7[13]. Accession number was obtained after submitting the 16S rDNA sequence to GenBank Database.

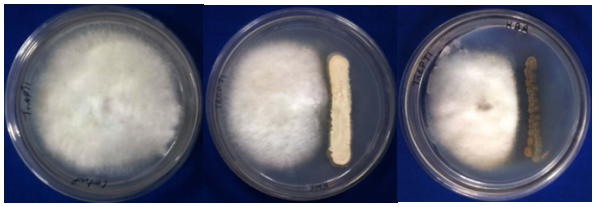
RESULTS

Isolation of Actinobacteria

A large number of actinobacterial isolates were isolated, evaluated and characterized from the rhizosphere of leguminous plants. Based on preliminary investigations and predominance on culture media sixteen actinobacterial isolates were selected for further studies. Colonies of these were appeared with a tough leathery or chalky texture and branching filamentous with or without aerial mycelia.

Antagonistic Assay

Among all the sixteen isolates only two actinobacteria isolates KWC01 and KBR01 showed antifungal activity against *F. oxysporum.* Maximum inhibition was observed with isolate KBR01 by 64.28% followed by KWC01 by 45.23% after 7 days of incubation (Figure1).



(a) (b) (c)

Figure 1: Antagonistic assay (a) Control (b) KWC01 and (c) KBR01

Extraction of Antifungal Metabolites

The antifungal metabolites were extracted from the culture filtrate of the actinobacterial isolate KBR01 using different organic solvents (hexane, chloroform, diethyl ether, n-butanol and ethyl acetate) and studied for the bio efficacy of each solvent extract against fungal pathogen. It was found that ethyl acetate extract was most effective for fungal pathogen growth suppression with inhibition zone of 15± 0.32 mm followed by n- butanol, chloroform and hexane with zone of inhibition 12±0.43 mm, 10±0.67 mm and 9±0.35 mm respectively (Figure 2). Whereas no zone of inhibition was observed using diethyl ether extract. Test fungus showed resistance against Fluconazole and Amphotericin B. Since ethyl acetate extract was found to be most effective for fungal growth suppression it was further subjected to GC-MS analysis.

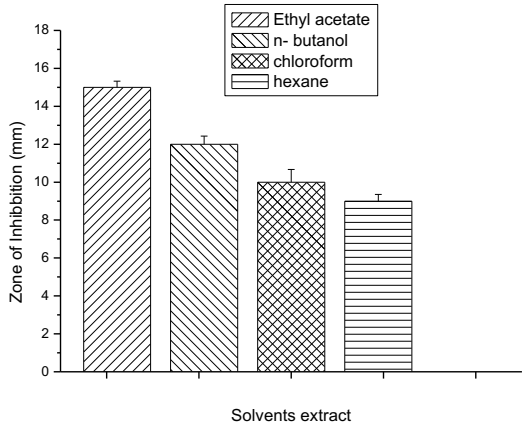


Figure 2: Effect of different solvent extracts on fungal growth inhibition

GC-MS Analysis

The compounds present in the antifungal crude extract of the selected actinobacterial isolate were identified on the basis of the comparison of their mass spectra with those of NIST mass spectral library as well as on comparison of their retention indices either with those of authentic compounds or with literature

values. 7 major compounds were detected in the extract (Figure 3), their molecular weight, retention time and antimicrobial property etc. are given in table 1. It was observed that the crude antifungal metabolite contains different types of compounds like fatty acid methyl ester (6,9,12-Octadecatrienoic acid, methyl ester, 36.02%), long chain alkanes (Dodecane, 29.47%), fatty alcohol (1- pentadecanol, 13.72%), piperazinedione (2,5-Piperazinedione, 3,6-bis(2-methylpropyl), 8.28%), esters (dodecyl acrylate, 8.29%) and pyrrolizidine (Pyrrolo [1,2-a]pyrazine-1,4-dione, Hexahydro-, 2.8% and Pyrrolo[1,2-a]pyrazine-1,4-dione, Hexahydro-3-(2-methylpropyl), 1.4%). The peak area of the compound is directly proportional to its quantity in the extract.

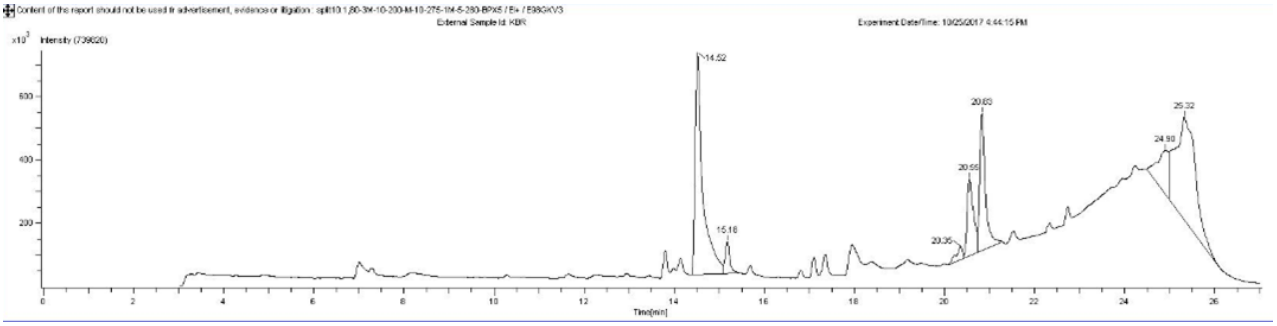


Figure 3: GC–MS chromatogram of the ethyl extract of *Streptomyces werraensis* KBR01.

Table 1: Compounds identified in the ethyl acetate extract by GC-MS

Peak	Time (Min.)	Peak area	Compounds	Compound Nature	Molecular Weight	Property	Reference
1	14.52	29.47	Dodecane	Long chain alkane	170.34	Antimicrobial	[14]
2	15.18	2.8	Pyrrolo[1,2- a]pyrazine-1,4-dione, Hexahydro-	Pyrrolizidine	154	Antioxidant	[15]
3	20.35	1.4	Pyrrolo[1,2-a]pyrazine-1,4-dione, Hexahydro-3-(2-methylpropyl)	Pyrrolizidine	210	Antimicrobial	[16]
4	20.55	8.28	2,5-Piperazinedione, 3,6-bis(2-methylpropyl)	Piperazinedione	226	Unknown	
5	20.83	13.72	2- Pentadecanol	Fatty alcohol	228	unknown	
6	24.90	8.29	Dodecyl acrylate	Ester	240	Antibacterial	[17]
7	25.32	36.02	6,9,12-Octadecatrienoic acid, methyl ester	fatty acid methyl ester	292.45	Antimicrobial	[18]

Identification of the Actinobacterial Strain

The actinobacterial isolate KBR01 was non-motile, Gram-positive, and filamentous. It was identified by 16 S rRNA sequence homology as a strain of *Streptomyces werraensis*. The 16 S rRNA sequence of the isolate showed 99% identity with *Streptomyces werraensis* strain NRRL B-5317. The 16S rRNA sequence of the isolate was submitted in the GenBank under the accession number KY655215.

DISCUSSION

In the present study, the antifungal activity of *Streptomyces werraensis* KBR01 to inhibit drug resistant fungal pathogen *Fusarium oxysporum* has been studied and the results indicate that this actinobacteria can be used to control infections caused by this fungus. Earlier studies by Khamna and Yokota (2009)[9], Nandhini et al., (2015)[14] and Manimaran, *et al*., (2015)[16] also reported antimicrobial activity of actinobacteria *Streptomyce*s sp. GC-MS study revealed the presence of many antimicrobial compounds such as Dodecane[14], Pyrrolo[1,2-a]pyrazine-1,4-dione, Hexahydro-3-(2-methylpropyl)[16], Dodecyl acrylate[17], 6,9,12-Octadecatrienoic acid and methyl ester[18] in the crude extract of *Streptomyces werraensis* KBR01.

CONCLUSION

Streptomyces werraensis KBR01 showed good antifungal activity against *Fusarium oxysporum.* Moreover, the crude extract of *Streptomyces werraensis* KBR01 was found to possess various antimicrobial compounds in its crude extract which supports this strain to be an efficient antimicrobial agent.

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