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Screening of Secondary metabolites of Endophytic fungi Alternaria alternata Isolated from Microstachys chamaelea (L) Muell.Arg.

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Abstract
Endophytes are the treasure biopotential sources for the active biocompounds which can be used as pharmaceuticals and agrochemicals. In the current study, endophytic fungi Alternaria alternata isolated from Microstachys chamaelea was investigated. Ethyl acetate extract of the endophytic fungal metabolite was screened by Gas Chromatography - Mass Spectrometry technique. The identified biocompounds are Phthalic acid-cyclobutyl tridecyl ester, Phthalic acid-cyclobutyl isobutyl ester, Phthalic acid- 4-bromophenyl octyl ester, Phthalic acid- 4-cyanophenyl nonyl ester, Phthalic acid- 6-ethyl-3-octyl butyl ester, Phthalic acid- cyclobutyl tetradecyl ester, Phthalic acid- 4-nitrophenyl octyl ester, Phthalic acid-cyclobutyl hexyl ester, Phthalic acid-cyclobutyl heptyl ester and Phthalic acid-heptyl pentyl ester. These findings suggest that only Pthalic acid derivative secondary metabolites were found in the isolated Endophytic fungi Alternaria alternata from Microstachys chamaelea. These bioactive compounds could be used in the development of antimicrobial agents.

Keywords: Endophytic fungi, Alternaria alternata, Microstachys chamaelea, Gas Chromatography - Mass Spectrometry (GC-MS Profiling).

Introduction

Endophytes are harmless to their host plants even though they reside within the tissue of the plant. Endophytic microorganisms synthesize bioactive chemicals within the host plant tissues, which is beneficial to the plant [1]. The exploration of new resources by endophytic fungi isolated from plants that have the ability to produce plant-specific compound are gaining attention. The metabolite profiles of endophytes are hopeful products for novel biologically active compounds with great impact on medical and agro field.

For example, the Paclitaxel bioactive compound produced by an endophytic Cladosporium cladosporoides and photinides produced by Pestalotiopsis photiniae from Roystomea regia plant [2] 3-0 methallalatermin, altersolanol A identified by the endophytic fungi Ampelomyces sp. [3]. Since the effects of the endophytic fungi-plant associations could be beneficial to humans, research interest in understanding, explaining and exploiting the plant-microbe relationship has increased in recent years [4]. The quest for new bioactive molecules isolated from fungal endophytes is one of the foci of new advances in biotechnology. Despite this, it is still a tiny percentage of fungal endophytes that have been isolated and studied [5]. In the present study, we carried out the screening of secondary metabolites found in the endophytic fungus Alternaria alternata has valuable therapeautic applications.

Materials and Methods

Isolation and Identification of endophytic Fungi

To isolate endophytic fungi, healthy plant samples were collected from Karigatta hill (12°25′05″N 76°43′17″E) located in the Srirangapatna, Mandya district, Karnataka. Samples placed in sterile zipped bags and carried aseptically to the laboratory. Samples were washed under running tap water and surface sterilization was carried out by immersion in 70% ethanol for 1 min, 5% sodium hypochlorite for 5 min, 70 % ethanol for 1 min and sterile distilled water for 1 min for two times, then allowed to dry under sterile conditions.

The leaf, stem and flower samples were cut into several segments of approx. 5mm diam. then samples were inoculated on water agar media plates and incubated at 28° C in the darkness. Individual fungal colonies were transferred to new PDA medium and this was repeated thrice for fungus purity. The identifications of the endophytic fungi were based on their morphology and mechanism of spore production. The promising fungus was identified by using molecular techniques [6, 7].

Secondary metabolites extractions from selected endophytic fungus
The most active isolate was incubated with potato dextrose broth (PDB) in 500 ml flask under shaking condition (200 rpm) for 7 days. Ethyl acetate was added to the fungus culture and incubated overnight then ethyl acetate phase with metabolites was collected in a conical flask and this was repeated twice followed by evaporation using a Rotary flash evaporator and stored in vials for further use [8].

Gas Chromatography-Mass Spectrometry (GC-MS) analysis
The ethyl acetate fungal extract was subjected to GCMS analysis to analyze various metabolites present in it. The Clarus 680 GC was used in the analysis employed a fused silica column, packed with HP-5MS (5% biphenyl 95% dimethylpolysiloxane, 30 m × 0.25 mm ID × 250µm df) and the components were separated using Helium as carrier gas at a constant flow of 2 ml/min. The injector temperature was set at 280°C during the chromatographic run. Individual peaks of compounds were assigned by comparing their RI (retention indices) and MS (mass spectra) by computer matching against NIST (National Institute of Standards and Technology) library [9].

Results and Discussion:
A total of 12 endophytes were isolated from Microstachys chamaelea. Among them Alternaria alternata exhibited an effective antimicrobial activity against bacterial and fungal strains. Hence, Alternaria alternata was selected for secondary metabolite analysis. As per our literature survey, this is the first report describing the isolation of endophytic fungi associated with Microstachys chamaelea plant.

GC-MS profiling of metabolites from ethyl acetate culture filtrate of Alternaria alternata
In this study ethyl acetate was used as extraction solvent since it is the most efficient method for obtainment of fungal secondary metabolites. The ethyl acetate extract of Alternaria alternata was characterized and identified by GC-MS analysis. The interpretation on mass spectrum GC-MS was conducted using the database of National institute standard and technology (NIST). The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library (figure1).The active principles with their molecular formula, molecular weight, exact mass and NIST numbers are represented in Fig.1 and Table 1.

The GC-MS analysis of endofungal extract revealed that the presence of Phthalic acid-cyclobutyl tridecyl ester, Phthalic acid-cyclobutyl isobutyl ester, Phthalic acid- 4-bromophenyl octyl ester, Phthalic acid- 4-cyanophenyl nonyl ester, Phthalic acid- 6-ethyl-3-octyl butyl ester, Phthalic acid- cyclobutyl tetradecyl ester, Phthalic acid- 4-nitrophenyl octyl ester, Phthalic acid- cyclobutyl hexyl ester, Phthalic acid-cyclobutyl heptyl ester and Phthalic acid-heptyl pentyl ester [Table. 2].

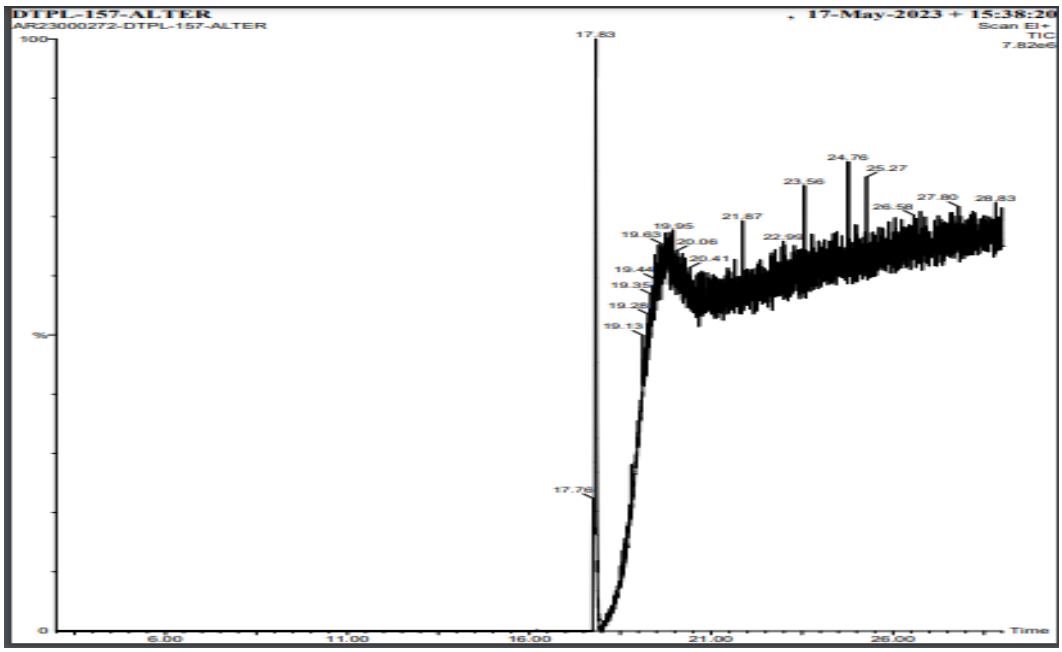
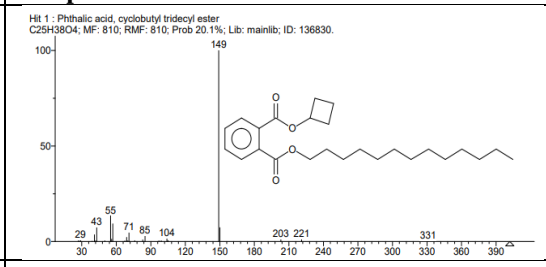
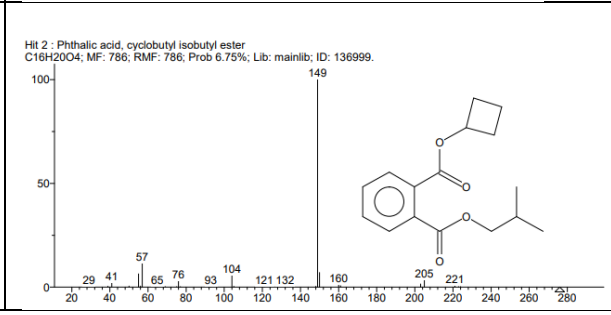


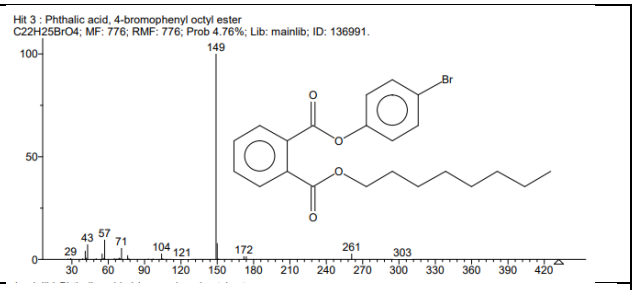
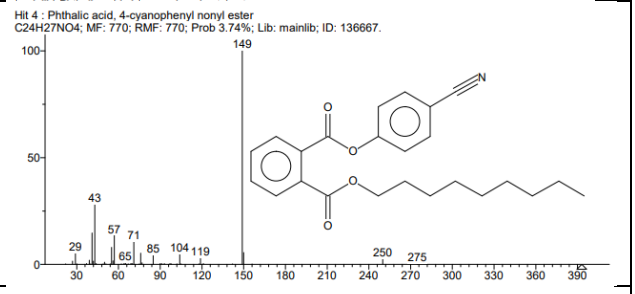
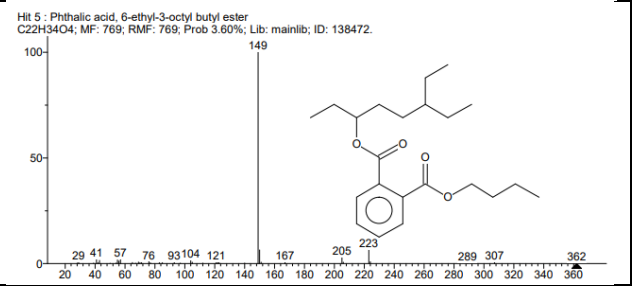
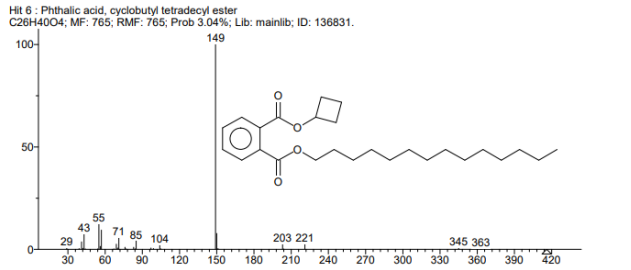
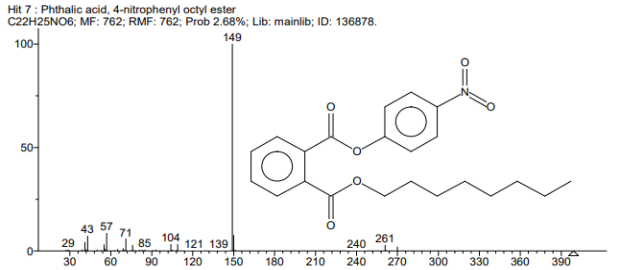
Table 1: Compounds identified in the crude ethyl acetate extract of Alternaria alternata by GC-MS analysis

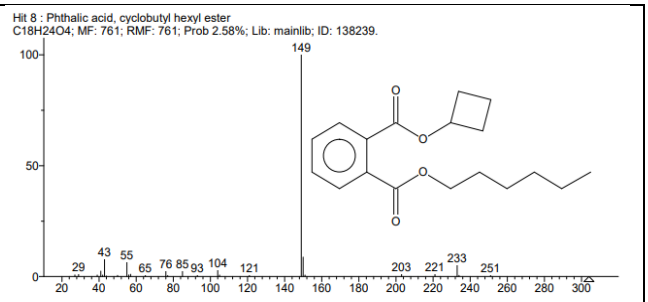
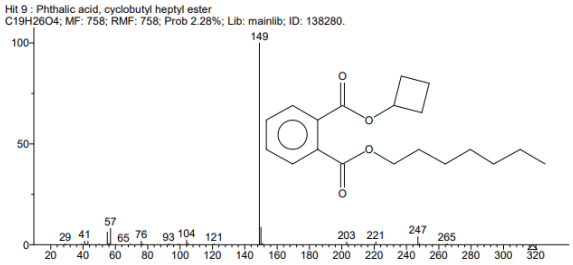
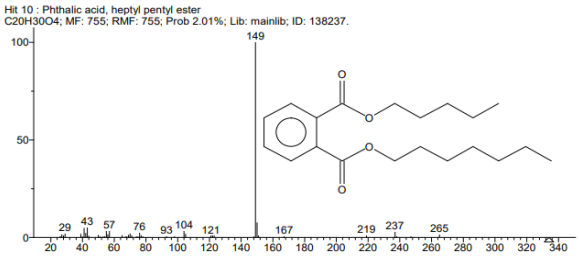
Sl. No	Systemic name	Molecular formula	Molecular weight	ID#	Exact mass	NIST#
1	Phthalic acid-cyclobutyl tridecyl ester	C25H38O4	402	136830	402.27	314908
2	Phthalic acid-cyclobutyl isobutyl ester	C16H20O4	276	136999	276.13	314911
3	Phthalic acid- 4-bromophenyl octyl ester	C22H25BrO4	432	136991	432.093	309815
4	Phthalic acid- 4-cyanophenyl nonyl ester	C24H27NO4	393	136667	393.19	309797
5	Phthalic acid- 6-ethyl-3-octyl butyl ester	C22H34O4	362	138472	362.24	315174
6	Phthalic acid- cyclobutyl tetradecyl ester	C26H40O4	416	136831	416.29	314909
87c8	Phthalic acid- 4-nitrophenyl octyl ester	C22H25NO6	399	136878	399.16	309769
8	Phthalic acid- cyclobutyl hexyl ester	C18H24O4	304	138239	304.16	314901
9	Phthalic acid-cyclobutyl heptyl ester	C19H26O4	318	138280	318.18	314902
10	Phthalic acid-heptyl pentyl ester	C20H30O4	334	138237	334.21	308941

Previous studies have shown that, the Anticancerous bioactive compound taxol identified by Alternaria alternata isolated from Taxodium distichum [10]. In our study from Alternaria alternata identified only pthalic acid derivative secondary metabolites. Hence, it also concludes endophytes use chemical signals to associate with other species, which might be vital for the host's survival and adaptability to varying environmental and biological challenges [11].

Table 2: Mass spectrometric analysis of identified compounds with their structure

Sl no	Name of the compound	Full scan mass spectrometric analysis of compounds with structure
1	Phthalic acid-cyclobutyl tridecyl ester	
2	Phthalic acid-cyclobutyl isobutyl ester	

3	Phthalic acid- 4-bromophenyl octyl ester	
4	Phthalic acid- 4-cyanophenyl nonyl ester	
5	Phthalic acid- 6-ethyl-3-octyl butyl ester	
6	Phthalic acid- cyclobutyl tetradecyl ester	
7	Phthalic acid- 4-nitrophenyl octyl ester	
8	Phthalic acid- cyclobutyl hexyl ester	

		
9	Phthalic acid-cyclobutyl heptyl ester	
10	Phthalic acid-heptyl pentyl ester	

In the early 1990s, about 70 metabolites from Alternaria fungi were reviewed [12,13]. Several reviews on Alternaria phytotoxins have been published over the last few decades [14,15]. In recent years, more and more metabolites with bioactivities from Alternaria fungi have been isolated and structurally characterized.

Conclusion

Plants increase the importance of finding alternative sources of bioactive molecules from eco-friendly endophytic fungi. Present literature revealed a total of ten compounds were identified and investigation to check their antimicrobial activity is under experimentation to determine their potential as an alternative to new biological sources which to solving the current problems faced in agriculture, environmental health and medicine.

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