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BIOACTIVE METABOLITE PRODUCED BY *PHOMOPSIS* SP., AN ENDOPHYTIC FUNGUS IN *ALLAMANDA CATHARTICA* LINN.

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Abstract

Endophytes are microbial entities that live within living tissues of plants. In most cases their relationship with the host plant is symbiotic and probably mutualistic. Many are capable of synthesizing bio-active compounds that can be used by the plant for defense against fungi and bacteria. Some of these compounds have been proven useful for novel drug discovery. By encouraging the endophytes to grow outside the plant in nutrient rich media, it is possible to harvest the bio-active compounds that they produce. In the present investigation we are trying to isolate endophytic fungi from *Allamanda cathartica* (Apocynaceae). The secondary metabolite obtained from the endophytic fungi was found to inhibit the growth of human pathogenic bacteria. The compound was extracted with organic solvents and bioautogram was done to check compound's antibacterial activity. Thin layer chromatogram and various other spectroscopic analyses were done to identify the compound as terpene.

Keywords: Endophytes; *Allamanda*; Apocynaceae; Antibacterial activity; bioautogram; *Phomopsis*

Introduction

Medicinal plants have been recognized as a repository of fungal endophytes with novel metabolites of pharmaceutical importance (Strobel *et al.,* 2004, Wiyakrutta *et al.,* 2004; Kumar *et al.,* 2005, Tejesvi *et al.,* 2007). Endophytes are the chemical synthesizers inside plants (Owen *et al.,* 2004). Many of them are capable of synthesizing bioactive compounds that can be used by plants for defense against pathogens and some of these compounds have been proven useful for novel drug discovery (Guo *et al.,* 2008).The various natural products produced by endophytic fungi possess unique structures and great bioactivities, representing a huge reservoir which offers an enormous potential for exploitation in medicinal, agricultural and industrial uses (Tan and Zou 2001; Zhang *et al.,* 2006). Since natural products are adapted to a specific function in nature, the search for novel secondary metabolites should concentrate on organisms that inhabit novel biotypes. Endophytic fungi inhabit such a biotype. *Allamanda cathartica* Linn. (Apocynaceae), is native of South and Central America. This plant is notable for its medicinal properties; all parts of the plant contain allamandin a toxic iridoid lactone. The roots are used for jaundice, complications with malaria, and enlarged spleen in suriname's traditional medicine. The flower acts as a laxative. Yellow allamanda has antibiotic action against *Staphylococcus* (Nayak *et al.,* 2006). Plumericin and isoplumericin exhibited cytotoxic activity against Madison lung carcinoma (M109)(Abdel-kader *et al.,*1997). This observation prompted us to launch a program aiming to search novel bioactive metabolites from cultures of endophytes colonized inside *A. cathartica*. This is the first attempt to isolate endophytes from this latex bearing plant. Previously endophytic fungi such as *Colletotrichum gloeosporioides* found to produce taxol in least amount (Nithya and Muthumary 2009) and *Phomopsis* sp., found to produce terpene (Nithya and Muthumary 2010) were isolated from *Plumeria acutifolia* a latex bearing plant belongs to Apocynaceae. It was reported that *Phomopsis* sp., found to possess novel secondary metabolites; hence this isolate was used for further studies. The main aim of the study was to isolate endophytes from this latex bearing medicinal plant, then extract bioactive secondary metabolite from it using solvent and then determine their antibacterial activity; terpenoid nature of the compound was confirmed through chromatographic and spectroscopic analysis.

Materials and Methods

Isolation of endophytic fungi

The fungi used in this study are the endophytic fungi isolated from the leaves of medicinal plant *Allamanda cathartica* in Guindy Campus Chennai city, India. The healthy plant tissues were washed in running tap water and processed as follows: Samples were cut into 2mm^2 segments and were surface sterilized by sequentially dipping into 0.5% sodium hypochlorite (2min) and 70% ethanol (2 min), and rinsed with sterile water, then allowed to surface-dry under sterile conditions (Arnold *et al.,* 2000). The

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material was then inoculated on to a petridishes containing PDA (Potato Dextrose Agar) amended with chloramphenicol 150mgl^{-1} medium. The petridishes were sealed using parafilm™ and incubated at 25 ±1°C in a light chamber with 12h light followed by 12h of dark cycles, and checked from the second day for fungal growth. Individual fungal colonies were transferred onto other plates containing PDA. The plates were continuously monitored for spore formation by stereo and light microscopy. The identifications of the endophytic fungi were based on their morphology and the mechanism of spore production using standard monograph.

Extraction of bioactive compound

The Fungus was grown in 4L Erlenmeyer flasks containing 1L of Matsumae *et al.,* (1963) medium containing 6% glucose, 0.5% peptone,0.3% NaCl, and 0.3% $CaCo_3$ then incubated for 20 days at 25°C. After that the culture was filtered through three layers of muslin cloth to remove the mycelia. Then the culture filtrate was extracted thrice with ethylacetate. Then the solvent phase was reduced under pressure using rotary vaccum evaporator at 40°C. The residue was redissolved in methanol for subsequent separation and the crude extract was analyses by chromatographic separation and spectrometric analysis.

Antimicrobial activity by well-diffusion method

Test bacterial samples of *Escherichia coli*, *Pseudomonas* sp., *Klebsilla* sp*., Bacillus subtilis, Streptococcus aureus,* and *Salmonella typhi*, were maintained in nutrient agar slants for further studies. The antimicrobial assay was carried out on 2% nutrient agar medium (Peptone-5 g, Beef Extract-3 g, NaCl-5 g, distilled water-1000 ml, Agar powder-20 g, pH 7.0) which was sterilized and used for the experiment. The antimicrobial activity of the crude extract was performed against test bacteria. One ml of inoculum was swapped on molten nutrient agar plates then using the sterile cork borer 7 mm wells were made in the plates then, 20 µl of crude ethylacetate fraction was loaded, streptomycin act as positive control and the solvent (Ethylacetate) was used act as negative control. After incubation of 24 h at 37° C, the plates were observed for zone of inhibition and measured

Chromatographic separation

The TLC analysis were carried out on merck 0.25 mm silica gel plates, developed in the following solvent system chloroform:methanol (9:1) and their Retention Factor (R_f) values was calculated using the following formula distance traveled by the solute divided by distance traveled by solvent. Then the TLC plates were exposed to UV rays (both shorter and longer wavelength regions) was observed. Then TLC plate was kept in Iodine vapor which is the confirmation test for terpenoids. By spraying 1% vanillin sulphuric acid reagent and heating gently the plate confirms the presence of terpenoids. The compounds antibacterial activity was determined through bioautogram. Bioautogram was performed using 0.25mm (2 × 8 cm) aluminum precoated silica gel plates (Merck). About 15-20 µl of crude was loaded in the plate, dried and run through the solvent system as used in TLC. These loaded plates were placed at the center of the petriplates and a thin layer of nutrient agar medium was poured. On the solidified media bacterial inoculum was swapped and incubated for 24 h at 37° C. After incubation the plates were observed for zone of inhibition and measured (Begue and Kline, 1972).

Spectral Analysis

a) uv-vsible spectral analysis

The sample containing the bioactive compound was analyzed spectroscopically for further confirmation. After chromatography, the area of plate containing active band at R_f of 0.60 was carefully removed by scrapped exhaustively eluting it with methanol. This was scanned in the wavelength ranging from 200 – 700 nm using Beckman DU 40 Spectrophotometer and the characteristic peaks were detected.

b) FT-IR spectroscopic analysis

The IR spectra of the compound were measured on Shimadzu FT-IR 8000 series instrument. Similarly the R_f of 0.60 substance was grounded with IR grade potassium bromide (KBr) (1:10) pressed in to discs under vaccum using spectra lab pelletiser. The IR spectrum was recorded in the region 4000-400 cm^{-1} and the typical stretching frequency of the bioactive substance was recorded for further characterization study.

Results and Discussion

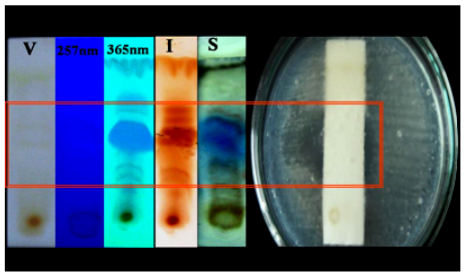
In various studies, was demonstrated that crude extracts from culture broth of endophytic microorganisms displayed antibacterial, antifungal, antiviral, anti-inflammatory and anti-tumor activity (Silva *et al.,* 2007). Therefore, the use of endophytic fungus opens up new areas for biotechnological exploitations, which leads to the isolation and cultivation of these organisms. The aim of the study was to isolate endophytic fungi from *Allamanda cathartica* (Plate 1) and extract a terpenoid compound from *Phomopsis* sp., Qui *et al.,* (2008) reported that the endophytic fungi were abundant in six medicinal plants and the number of endophytic fungi was higher in twigs than in leaves. *Alternaria alternate* and *Phomopsis* sp., were common species in six medicinal plants. Based on the morphology of the mycelial colony as well as the characteristics of the conidia, the endophytic fungus was identified as *Phomopsis* sp., *Phomopsis* sp.,

mycelium immersed, branched, septate, hyaline to pale brown. Conidiomata eustromatic immersed, brown to dark brown. Conidia of two basic types, but in some species with intermediate between the two α – conidia hyaline, fusiform, straight, usually biguttulate (one guttule at each end) but sometimes with more guttules, aseptate; ß- conidia hyaline, filiform, straight or more often hamate, eguttulate, aseptate (Plate 1). Conidia are used for the cultures with the aim to screen secondary metabolite production by this fungus. The fungal extract from culture filtrate was examined for the presence of compound by antimicrobial activity, chromatographic and spectroscopic analyses. The crude ethylacetate fraction was subjected to antimicrobial activity through well diffusion method against six different bacteria the results are presented in (Fig. 1). The compounds were separated by Thin Layer Chromatographic (TLC) with solvents system as shown in Materials and Method. Then TLC plates were with exposed to UV rays (Both Shorter and Longer wavelength regions) it showed bluish spot in longer wavelength region and when it is exposed to Iodine vapour showed a brown spot at R_f 0.60 (Plate2). This shows that the compound was terpenoid in nature. It was further confirmed by spray reagent like vanillin sulphuric acid which gives a blue spot at 0.60 R_f value. The terpenoid compound having the antimicrobial activity was further confirmed with the bioautogram plate, the compound showed a zone of inhibition in the TLC plate at R_f value of 0.60 (Plate 2). Then the compound with the R_f value of 0.60 region was scrapped off and dissolved in methanol, subjected to UV spectroscopic analysis. A sharp peak was observed at λnm of 350nm in the sample which confirmed the presence of terpenoid nuclei (Fig. 2). The bioactive compound obtained through TLC was subjected to FT-IR spectral analysis showed the characteristic stretching frequency at 3391, 2920, and 2849 due to the –CH2 and –CH stretching and C=O stretching frequency was observed as sharp peak at 1704 confirms the presence of lactone ring in the compound (Fig.3). Previously it was reported that all parts of the plant contain allamandin; which is a toxic iridoid lactone. According to Silva *et al.*, (2005), *Phomopsis cassiae* an endophytic fungi in *Cassia spectabilis*, Phomopsilactone was obtained as ethyl acetate fraction; it gives a R_f value of 0.58 and in chloroform methanol, ratio, when viewed under UV light and then with anisaldehyde sulfuric acid reagent. Similar R_f value obtained for *Phomopsis* sp which was extracted with ethyl acetate (0.60) sprayed with vanillin sulphuric acid. Our results were matched with the previous report of Silva *et al.*, (2005) which has an effective antibiotic used as fungicides and anticancer compound known as Phomopsilactone. Triterpenoids of *Combretum imberbe* leaves had a reasonably antibacterial activity on *Staphylococcus aureus*, *Escherichia coli, and Enterococcus faecalis*. It appears that the major antibacterial compounds were isolated but the antibacterial activity of the crude extract was higher than could be extrapolated from the activity of the isolated compounds (Angel *et al.,* 2007). Further intensive studies have to be done for determining the structure of terpenoid compound, whether it belongs to phomopsilactone obtained from *Phomopsis* sp or iridoid lactone obtained from the host plant.

Plate 1. Fungal samples isolated from host plants

Conidial morphology X 40

Plate 2. Confirmation of bactericidal compound through TLC and bioassay guided fractionation



This shows the separation of compounds through different spray reagents V- Visible light, UV 257 & 365 nm, I- Iodine vapor, S- Spray reagent. The R_f value was found to be 0.60

Fig.1. Antibacterial activity for Matsumae medium for *Allamanda cathartica* in Ethylacetate Fraction

S.No	Bacteria	Zone of Inhibition in mm
01.	*Pseudomonas sp*	16±0.32
02.	*Escherichia coli*	15±0.30
03.	*Staphylococcus aureus*	25± 0.50
04.	*Salmonella typhi*	18±0.36
05.	*Klebsilla sp*	16±0.32
06.	*Bacillus subtilis*	25± 0.50

Figure 2: UV-Visible Spectrum of bactericidal substance

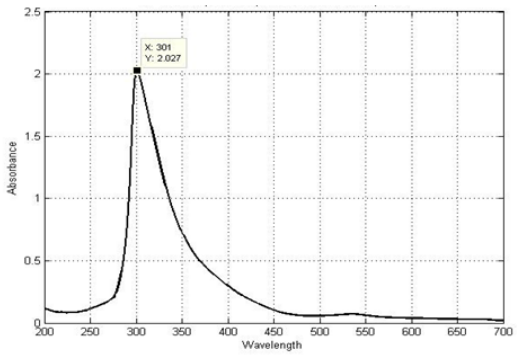


Fig.2. shows the peak at 301nm corresponds to terpenoid nucleus

Figure 3; FT-IR Spectrum of bactericidal substance

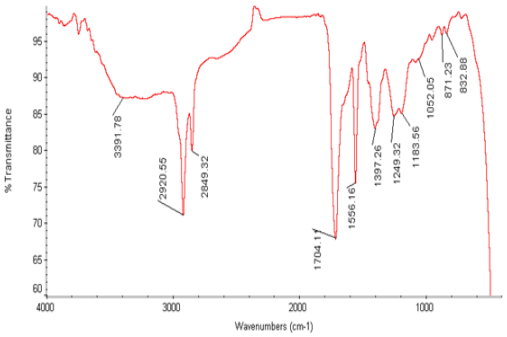


Fig.3 shows the presence of functional groups present in the terpenoid compound.

Conclusion

From this work it is concluded that *Phomopsis* sp. isolated from *Allamanda cathartica* found to contain terpene compound which is having antibacterial activity.

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