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SECONDARY METABOLITE FROM *PHOMOPSIS* SP. ISOLATED FROM *PLUMERIA ACUTIFOLIA* POIRET

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Abstract

Plumeria acutifolia Poiret (Apocynaceae) contains eight species of mainly deciduous shrubs and tree, originated from Central America. Some species of this plant are used for the cure of rheumatism, diarrhoea, blennorhea, venereal disease and leprosy. Endophytes are the chemical synthesizers inside plants (Owen et al., 2004). Leaves of *Plumeria acutifolia* were submitted to isolation of endophytic fungi. The strain *Phomopsis* sp was selected for chemical and biological investigation because of the strong antibacterial activity and also rich source of secondary metabolites. Ethylacetate fraction of the fungus found to have terpenoid compound, which was further subjected to Thin layer chromatography (TLC), UV, FT-IR spectroscopic analysis. The terpenoid compound was found to have antibacterial activity whereas the same compound does not show any positive results towards human pathogenic *Candida albicans*. This is the first report of the isolation, cultivation of fungus *Phomopsis* sp and evaluation of antibacterial activity.

Keywords: Apocynaceae, Antibacterial activity, Terpenoids, Endophytes, *Phomopsis*, Coelomycetes

Introduction

Endophytes are microorganisms that reside in the tissues of living plants, are relatively unstudied as potential sources of novel natural products for potential exploitation in medicines, agriculture and industry. Endophytes are known as sources of biologically active secondary metabolites (Strobel *et al.,* 2004, Phongpaichit *et al.,* 2007) 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). Secondary metabolites are low molecular weight compounds and are not required for growth of an organism in pure culture and are produced as an adaptation for specific functions in nature. Secondary metabolites are used in medicines due to their anticancer, antiviral, antidiabetic and immunosuppressant properties etc. Globally, there are at least one million species of endophytic fungi in all plants (Ganley *et al.,* 2004), which can potentially provide a wide variety of structurally unique, bioactive natural products such as alkaloids, benzopyranones, benzoquinones, flavonoids, phenols, steroids, terpenoids, tetralones, xanthones, and others (Tan and Zou 2001).

Plumeria acutifolia Poiret (Apocyanaceae) has their origin from Central America. Some iridoids and triterpenoids obtained from this plant are reported to have algicidal, antibacterial and cytotoxic activity. It has been used in the treatment of several ailments in traditional folklore medicine as a bitter tonic, expectorant, and purgative as well as in the treatment of skin disease. The bark of some species was found to be biologically active as a diuretic, antipsychotic, antitumour agent and as an inhibitor of the human immune deficiency virus type-1(HIV-1(Mahabeer *et al*., 2004). *Plumeria* species have also been investigated in various laboratories for isolation of a variety of iridoides and triterpenoids, which exhibited algicidal, antibacterial and cytotoxic activity. It was already reported that an endophytic *Colletotrichum gloeosporioides* isolated from the same plant was found to produce a diterpenoid compound namely Taxol an anticancer drug by Nithya and Muthumary (2009). Siddiqui *et al.,*(2004) isolated dammarane tri-terpenoids from *Plumeria obtusa*. Sesquiterpene lactone (SLs) are class of secondary metabolites found in several plant families. SLs have been widely studied because of their various biological and therapeutic activities (antiphologistic, spasmolitic, cytotoxic, antihelminthic, sedative, etc.). Sesquiterpene are low-volatility thermolabile compounds with molecular mass generally rainging from 230-500 (Bicchi *et al.,* 1996). *Phomopsis* sp isolated as endophytes found to produce number of secondary metabolites. In the present study isolation and the extraction of terpenoid from this fungus was attempted. The presence of Terpenoid was confirmed through antimicrobial assays, TLC and UV spectroscopic methods. The functional group of terpenoid was determined through FT-IR analysis.

Materials and Methods

Isolation of endophytic fungi

The endophytic fungus isolated from the leaves of medicinal plant *Plumeria acutifolia* in Dry ever green

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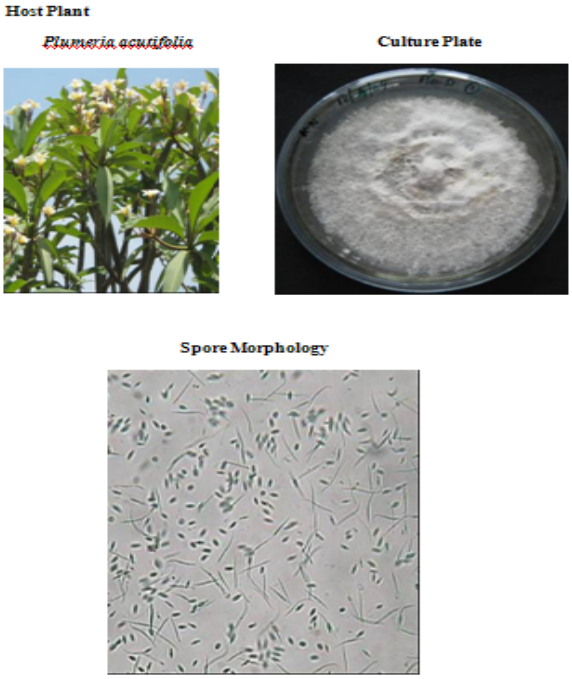
forest, Guindy National Park. The healthy plant tissues were washed in running tap water and processed as follows: Samples were cut into 2mm^2 segments and were surface sterilized by sequentially dipping into 0.5% sodium hypochlorite (2min) and 70% ethanol (2 min), and rinsed with sterile water, then allowed to surface-dry under sterile conditions (Arnold *et al.,* 2000). The material was then inoculated in a petridishes containing PDA (Potato Dextrose Agar) amended with chloramphenicol 150mgl^{-1} concentration. 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 medium containing 1% glucose, 0.5% peptone, 0.2% NaCl, and 3% glycerol Omura (1976) 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, and 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 analyzed by chromatographic separation and spectrometry.

Plate 1. Fungal samples isolated from host plants



Antimicrobial activity by Well-Diffusion method

Test bacterial samples of *Escherichia coli*, *Pseudomonas* sp, *Klebsilla* sp., *Bacillus subtilis, Streptococcus aureus,* and *Salmonella* typhi, and *Candida albicans* were maintained in nutrient agar slants for further studies. The antimicrobial assay was carried out on 2% nutrient agar (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. One ml of inoculum was swapped on nutrient agar plates, then 7 mm wells were made in the plates. 20 µl of crude ethylacetate fraction was loaded, streptomycin was used as positive control and the solvent (Ethylacetate) 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 0.25mm silica gel plates (Merck), developed in the following solvent system chloroform : methanol (9:1) and their Retention Factor (R_f) was calculated using the following formula:

$$R_f = \frac{\text{Distance traveled by the solute}}{\text{Distance traveled by solvent}}$$

Then the TLC plates were exposed to UV rays (both shorter and longer wavelength regions) for derivatization of bioactive compounds. Then TLC plate was kept in Iodine vapor which will be the confirmation test for terpenoids. By spraying 1% vanillin sulphuric acid reagent and heating gently the plate; this will 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-visible 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 0.58 was carefully removed by scrapping exhaustively eluting it with methanol. This was scanned in the wavelength ranging from 200 – 800

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 Rf value at 0.58 was grounded with IR grade potassium bromide (KBr) (1:10). The IR pellet was recorded in the region 4000-400 cm-1 and the typical stretching frequency of the bioactive substance was recorded for further characterization study.

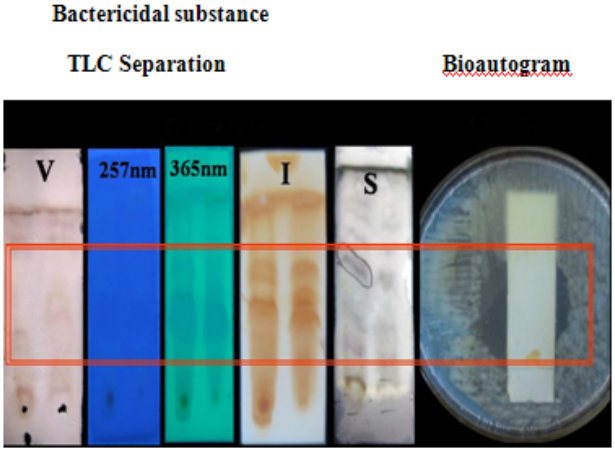
Results and Discussion

In various studies, was demonstrated that the 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 *Plumeria acutifolia* (Plate 1) and extract a terpenoid compound from *Phomopsis* sp. Qui *et al.,* (2008) reported that the endophytic fungi were abundant in medicinal plants and the number of endophytic fungi was higher in twigs than in leaves. *Alternaria alternate* and *Phomopsis* sp were common species in medicinal plants. Based on the morphology of the mycelial colony as well as characteristics of conidia, the endophytic fungus was identified as *Phomopsis* sp. (Plate 1). On Potato Dextrose Agar, the strain produces a luxuriant white to cream-colored and felty aerial mycelium consisting of septate, branched, hyaline, and guttulate hyphae that are 0.8-10 µm in diameter. After 3-4 weeks, the mycelium develops dark brown, spherical, separate or aggregated eustromatic conidiomata. They arise superficial or semi- immersed either from the normal mycelium or from dark brown areas and reach up to 1mm in diameter. The conidiophores are simple or sparingly branched at the base and bear long, tapering phialides that form two types of hyaline and one celled conidia. The macroconidia are filiform, curved or hamate, 15-36 X 1-1.5 µm in size, and eguttulate, while the microconidia are ellipsoidal, mostly attenuated towards the base, 5 – 10 µm in size, and guttulate (often biguttulate). The conidial slime leaking from the conidiomata was creamy white (Sutton 1980). Conidia are used for the cultures with the aim to screen secondary metabolite production by this fungus. The fungal extract was examined for the presence of compound by antimicrobial activity, chromatographic and spectroscopicanalysis.

Table-1. Antibacterial activity of *Phomopsis* from *Plumeria acutifolia* in Ethylacetate fraction

S.No	Bacteria	Zone of Inhibition in mm
01.	*Pseudomonas sp*	11
02.	*Escherichia coli*	12
03.	*Staphylococcus aureus*	24
04.	*Salmonella typhi*	16
05.	*Klebsilla sp*	28
06.	*Bacillus subtilis*	24
07.	*Candida albicans*	-

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 0.58.

Fig.1. UV-Visible Spectrum of crude extracted isolated from *Phomopsis* sp.

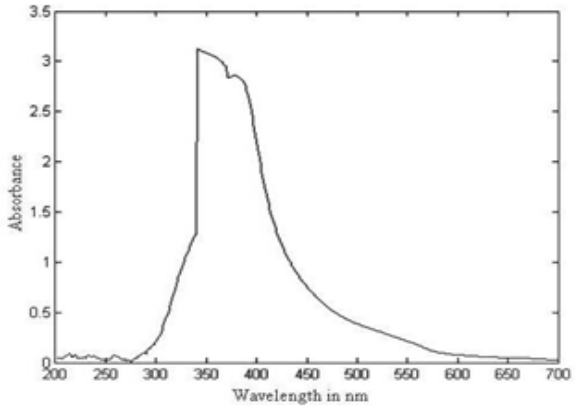


Fig.1. shows the peak at 350 nm corresponds to terpenoid nucleus.

Fig. 2. FT-IR Spectrum of crude extracted isolated from *Phomopsis* sp.

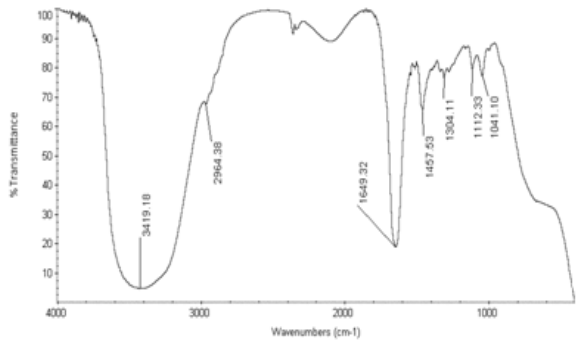


Fig. 2 shows the presence of functional groups present in the terpenoid compound. The crude ethylacetate fraction was subjected to antimicrobial activity through well diffusion method against six different bacteria and one fungus *Candida albicans* the results are presented in Table 1. Thin Layer Chromatographic (TLC) separated the compounds with solvents system as shown in Materials and Method. Then the TLC plates were exposed to UV rays (Both Shorter and Longer wavelength regions) it showed bluish spot in longer wavelength region and when it was exposed to Iodine vapor showed a brown spot at R_f 0.58 (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 0.58 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 0.58 (Plate 2). Then the compound with the R_f value of 0.58 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. 1) similarly, Silva *et al.*, (2005) reported that *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.The bioactive compound obtained through TLC was subjected to FT-IR spectral analysis showed the characteristic stretching frequency at 3419, 2964, due to –OH stretching and C=O stretching frequency was observed as sharp peak at 1649 confirms the presence of conjugated C=O ring which corresponds to the amide in the compound (Fig. 2). A-seco- oleane- type triterpenes from *Phomopsis* sp. (strain HKI0458) isolated from the mangrove plant *Hibiscus tiliaceus* a new report from microbial source by Li *et al.*, (2008). Lin Xiao et al., (2008), reported one new ten-membered lactone from Phomopsis sp. B27, an endophytic fungus of Annaona squamosa L. Terpenoids, Phomopsilactone has an effective antibiotic activity used as fungicides and as anticancer compound. Triterpenoids of *Combretum imberbe* leaves had a reasonably antibacterial activity on *Staphylococcus aureus*, *Escherichia coli and Enterococus 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.

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