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SCREENING AND CHARACTERZATION OF ANTIMICROBIAL COMPOUND FROM ENDOPHYTIC FUNGUS *CURVULARIA LUNATA*,ISOLATED FROM *CATHARANTHUS ROSEUS*

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ABSTRACT

Endophytes are microorganisms that reside within the tissues of plants with symbiotic or mutualistic relationship. During the present study, endophytic fungus *Curvularia lunata* was isolated from the symptomless leaves of the *Catharanthus roseus.* The secondary metabolites produced by the endophytic fungus *C.lunata* showed significant antimicrobial activity towards the test organisms. TLC, HPTLC analyses of the extract revealed the presence of terpenoid like compound. FTIR spectra showed characteristic triterpenoid saponin absorptions. UV-Visible spectroscopy showed a sharp peak at 301nm characteristic of terpenoids.

Keywords Antimicrobial activity, Endophytic fungi, *Catharanthus roseus, Curvularia lunata*, terpenoid**.**

INTRODUCTION

Plants have been known to act as host to various endophytic microorganisms known to produce plethora of substances of potential use to modern medicine, agriculture and pharmaceutical industry [1].Endophytes usually have a mutual relationship with their host are relatively unexplored. These endophytes protect plant against diseases [2], increase tolerance against abiotic stress [3].Therefore they are considered as an outstanding source of bioactive natural products [4] thus representing potential leads for the development of new pharmaceutical agents [5].Natural products from fungal endophytes have a broad spectrum of biological activity and they can be grouped in to several categories including alkaloids,

steroids, terpenoids, phenylpropanoids etc.[5]. Microbial source of valuable products are usually easier and more economical to produce. An overview of literature indicated that 51% of bioactive substances isolated from endophytic fungi were previously unknown compared to 38% from soil fungi [6].

Catharanthus roseus has been in use from ancient times for the treatment of blood pressure, diabetes etc., in indian system of medicine as well as in folk-lore medicinal practice [7,8]*C.roseus* is well known for its production several anticancerous alkolids such as vincristine, vindesine, vinblastin etc. Zhang et al [9] and Tung et al [10] have discovered that vincristine is produced by *Fusarium oxysporum*, an endophyte of this host. Hence this plant was selected as a source plant to examine the endophytic fungal population to discover other biologically active compounds made by endophytes of this important medicinal plant.

MATERIALS AND METHODS

Isolation of endophytic fungi

Leaves of *C. roseus* were collected from Tamilnadu Agriculture University Campus Aduthurai, Tamilnadu, India. The healthy leaves were washed in running tap water and processed as follows: Samples were cut into 0.5 cm^2 segments and were surface sterilized by sequential dipping into 0.5% sodium hypochlorite (5min), 70% ethanol (1 min), and rinsed with sterile water, (2 min) for 3 times then allowed to surface-dry under sterile conditions [11]. The surface sterilized explants were placed in a petridish containing potato dextrose agar (PDA) supplemented with 150mg/l Chloramphenicol and incubated at 26^0 C for 2 weeks with 12 h light and 12 h dark condition. The plates were observed daily for growth of fungi. Emerging fungus from leaf segments were isolated and brought into pure culture. The respective fungal growths were transferred to PDA without any antibiotic for colony morphology and further identification.

Identification of culture

Identification of fungal strain was based on colony or hyphal morphology of culture, characteristics of the spores and reproductive structures. For molecular identification, DNA was isolated from fresh mycelium using CTAB method. A region of nuclear rDNA (ITS1 and ITS2) was amplified by PCR using universal primer ITS1 (5'-TCCGTAGGT-GAACCTGCGG-3')andITS4(5'- TCCTCCGCTTATTGATATGC-3') in a thermal cycler, using the following parameters: 1 min initial denaturation at 94°C, followed by 30 cycles of 30 s denaturation at 94°C, 1 min primer annealing at 57°C, 90 s extension at 72°C, and a final

extension period of 10 min at 72°C. Each PCR reaction was electrophoresed on 1.5% agarose minigels (containing 0.5 mg/µl ethidium bromide) for 1 h in Tris-acetate buffer. The PCR products were revealed under UV light. Direct sequencing of the PCR amplicons corresponding to the ITS region of ribosomal DNA (rDNA) was performed through outsourcing (Eurofins Genomics, Bangalore).

Extraction of bioactive compounds

The fresh mycelia (grown on PDA) of representative endophytic fungus was transferred to 250 ml Erlenmeyer flasks containing 100 ml of Czapek Dox Broth. The flasks were incubated at 25ºC ±1^{0}C for 15 days with periodical shaking at 120 rpm. After the incubation period, the cultures were taken out and filtered through filter paper and mesh cloth to remove the mycelia from culture broth. The culture filtrate was extracted with equal volume of ethyl acetate and the solvent phase was reduced under pressure using vacuum evaporator. The residue was stored in eppondorf tubes for subsequent analyses by chromatographic separation and spectrometric analysis [12].

Antimicrobial assay by disc diffusion method

Antimicrobial evaluation was carried out by disc diffusion method. The fungal crude extract was impregnated (20 µl /disc) on to sterile Whatman No 3 discs (6 mm diameter). Penicillin/Ampicillin disc act as a positive control and the solvent ethyl acetate was used as negative control and the antibacterial activity was assayed against, *Bacillus subtilis, Salmonella Paratyphi, Proteus vulgaris, Vibrio cholerae, Staphylococcus aureus, Escherichia coli.* Plates were incubated at 37^o C for 24h, and observed for zone of inhibition.

Instrumental Analyses

Thin-Layer Chromatographic separation

TLC analysis was carried out on 0.1 mm silica gel coated TLC plates (10 x 10 cm MERCK, GERMANY). The crude ethyl acetate extract was separated using toluene: chloroform: methanol (5:8:3; v/v/v). The bioactive compound was visualized by spraying 1 %(w/v) Vanillin in Sulphuric acid and their R_f values were calculated.

High Performance Thin-Layer Chromatographic separation

HPTLC was performed using a CAMAG HPTLC system equipped with a sample applicator Linomat V, TLC scanner III and integration software CAT 4.0. TLC Aluminium sheets (10 x 10 cm) of silica gel were used. The mobile phase of toluene: chloroform: methanol

(5:8:3v/v/v) was used for separation of triterpenoid and detected with vanillin sulphuric acid. The plates were dried at l10 ^{0} C for 5 minutes to enable the spots to develop and scanned at 254 and 366 nm wavelength in UV reflection mode.

UV- visible spectral analysis

The crude ethyl acetate extract was analyzed spectroscopicaly for further confirmation. This was scanned in the wavelength ranging from 200- 400 nm using Shimadzu spectrophotometer.

FT-IR spectroscopic analysis

The IR spectrum of the compound was measured in Shimadzu FT-IR 8000 series instrument. The sample was grounded with IR grade potassium bromide (KBr) pressed into discs under vacuum 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.

RESULTS AND DISCUSSION

Isolation and identification of endophytic fungus

An endophytic fungus was isolated from healthy leaves of *C. roseus* (Fig.1). The fungus was identified based on their morphological features as *Curvularia lunata* by Dr Prameela devi, Indian Agriculture Research Institute New Delhi, India, and assigned to the ITCC I.D.NO: 8428.11. Analysis of the ITS r DNA sequence revealed 99% identity with *C. lunata* (GenBank Accession no: KF727293).

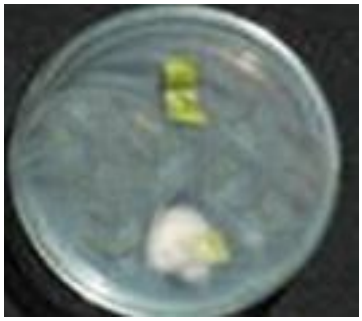

Figure 1. Endophytic fungus *C. lunata* isolated from leaves of *C. roseus.*

Antimicrobial acitivity

The ethyl acetate extract *C.lunata* of culture filtrate was examined for its antimicrobial activity against *B. subtilis, S. paratyphi, P.vulgaris, V. cholerae, S. aureus* and *E. coli.* The ethyl acetate extract showed appreciable antimicrobial activity towards the test organisms (Table 1).Maximum zone of inhibition 17mm, 15mm was observed with *E. coli, S. paratyphi*

respectively. Various researchers demonstrated the antimicrobial, antifungal, antiviral properties of endophytic microorganisms [13, 14, 15, 16].

Table 1. Antibacterial assay of ethyl acetate extract of *C.lunata*

Test Bacteria	Zone of Inhibition (mm)		
	Ethyl acetate extract from *C. lunata*	(Penicillin/Ampicillin)	Control (Ethyl acetate)
Proteus vulgaris	14	10	0
Escherichia coli	17	20	0
Vibrio cholerae	12	13	0
Bacillus subtilis	11	17	0
Salmonella Paratyphi	15	20	0
Staphylococcus aureus	11	19	0

0 -No Zone of Inhibition

TLC and HPTLC analyses

The ethyl acetate extract of fungal culture filtrate separated by Thin Layer Chromatography showed bluish colour spot with an R_f value around 0.61 indicating the presence of terpenoids in the bioactive compound (Fig 2). The ethyl acetate extract of fungal culture filtrate analysed by HPTLC showed bluish black spot appeared at UV 254and 366 nm confirmed the presence of terpenoids in the extract (Fig 3a, b). Chromatograms of sample peak at R_f value of 0.61 was shown Fig.3c. Nithya and Muthumary[17] reported that ethyl acetate extract of an endophytic fungus *Phomopsis* sp. separated on TLC exposed to UV rays showed bluish spot in longer wavelength region with R_f value of 0.61.

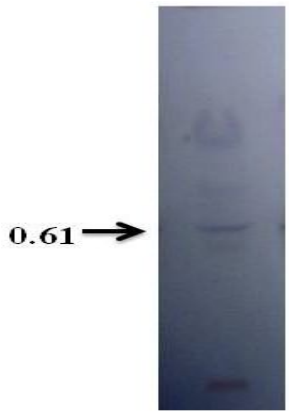

Figure 2.TLC Plate

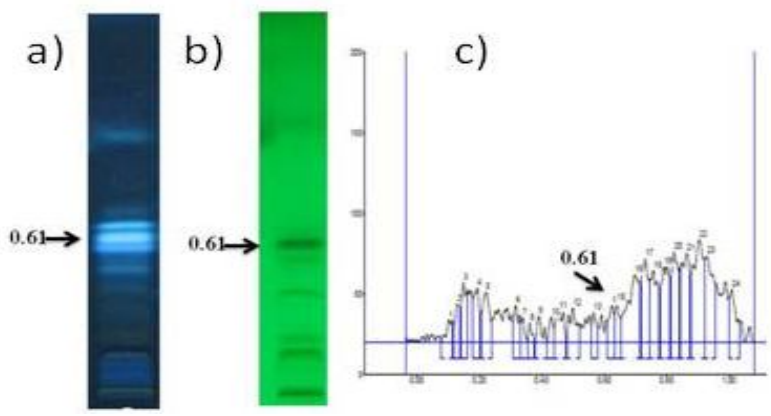

Figure 3a, b, c . This shows separation of compound by HPTLC. a-366nm, b-254nm, c-HPTLC Chromatogram peak. The R_f value found to be 0.61

UV-Visible spectral analysis

UV- visible spectral analysis of the ethyl acetate extract of *C. lunata* showed a sharp peak at 301 nm indicating the presence of terpenoids (Fig 4).

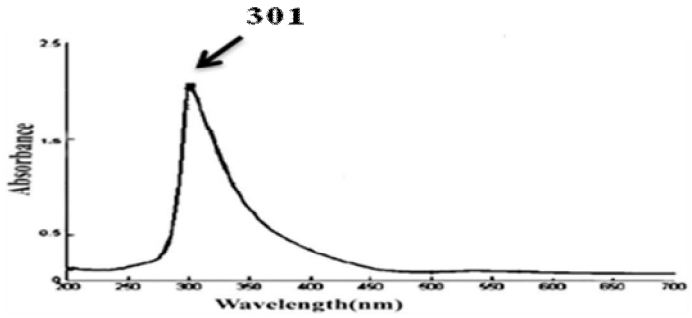

Figure 4.UV- Visible spectrum of ethyl acetate extract of *C.lunata* isolated from *C. roseus.*

FTIR Spectroscopic analysis

The ethyl acetate extract of *C. lunata* subjected to FT-IR spectral analysis showed the characteristics stretching frequencies in the region 3436.44,2989.96,1639.24 due to OH,C=H,C=C stretching. C=O stretching frequency was observed as sharp peak at 1763.68 confirms the presence of lactone ring in the compound (Fig. 5). These oleanane- type triterpenoid saponins are characterized by the C=O infrared absorbance due to the oleanolic acid/ester. Such terpenoid saponins are also like to be bidesmosides [18, 19].The above infrared functional group absorptions characteristic of saponins were cited in literature. [20, 21]Further identification and structure predictions are underway.

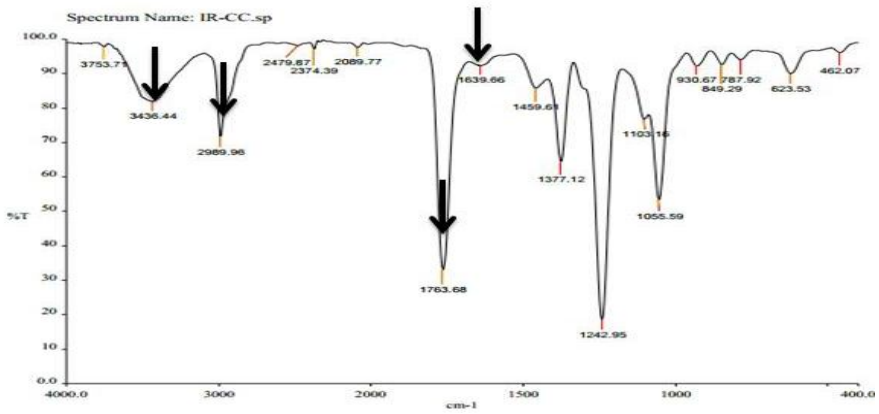

Figure 5.FT-IR Spectrum

CONCLUSION

Endophytes are poorly investigated group of microorganisms that represent an abundant and dependable source of bioactive and chemically novel compounds with potential for exploitation in a wide variety of medical, agriculture and industrial areas.

In this study, an attempt has been made to isolate endophytic fungus from leaves of *C.roseus.* The ethyl acetate extract of isolated endophytic fungus showed antimicrobial activities towards test organisms. Instrumental analyses further indicated the chemical nature of bioactive compounds which may belongs to terpenoids.

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