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ASSESSMENT OF ANTIBACTERIAL ACTIVITIES OF ENDOPHYTIC FUNGI ISOLATED FROM THE LEAVES OF CASSIA *ALATA*

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ABSTRACT

Endophytes are microorganisms that lives within plants for atleast a part of their life cycle without causing any visible manifestation of disease.plants have long provided mankind with a source of medicinal agents, with natural products once serving as source of all drugs. Fungal endophytes isolated from the leaves of cassia alata shows antibacterial activity against almost all bacterial pathogens. Endophytic fungal pigments may be the better source of the antimicrobial activity of natural products have opened new ways for drug development in the control of antibiotic resistant pathogens.

KEYWORDS: *Cassia alata,* endophytic fungi, antibacterial activity.

1. INTRODUCTION

Endophytes are microorganisms that lives within plants for atleast a part of their life cycle without causing any visible manifestation of disease (Bacon and white, 2000). The term endophyte (Gr. Endon - within; phyton - plant) was first coined by De Bary in 1866.Common endophytes include a variety of bacteria, fungi and actinomycetes and they can be isolated from wild or cultivated crops of either the monocots or dicots. Among the microbial group the most frequently isolated endophytes are fungi (Maroof Ahmed *et al*., 2012). The importance of endophytes had been demonstrated over a long period as a source of pharmaceutical bioactive compounds, as many of endophytes were exposed to produce novel bioactive metabolites such as immunosuppressive drugs, antibacterial, antifungal, antiviral, antitumor, antioxidant, anti-inflammatory, and many related compounds.

Endophytes are well known for the production of various classes of natural products and have been reported to exhibit a broad range of biological activity and are grouped into various categories, which include alkaloids, terpenoids, steroids, lactones, phenolic compounds, quinones and lignans. The emergence of antibiotic resistance in pathogenic microorganisms and prevalence of potent mutated strains, have created an alarming situation for both humans and crop plants. Since, natural bioactive compounds are known to control pathogens, there is an urgent need for continuous and vigorous search for novel natural products from different sources including plants, microorganisms and organisms inhabiting to unique niches (Kumar *et al*., 2016). Endophytes may affect plant litter quality organisms that control litter decomposition and the availability of nutrients in plant communities (Saikkonen *et al*., 2015).

Cassia alata (also known as *Senna alata*) is a shrub belonging to the Fabaceae family, found in tropical areas. Leaves, flowers, and fruits of *C. alata* is used as antidiabetic, anti-inflammatory, analgesic, against digestive problems and infectious diseases (as antibacterial and antifungal agents). *Senna alata* is often called ring worm bush because of it very effective fungicidal property, for treating ring worm and other fungal infection of the skin. A fresh preparation is made every day. Its effective ingredients include the yellow chrysophanic acid. Its laxative effect, due to its anthrequioninone content is also well proved.

MATERIALS AND METHODS

Surface Sterilization

Sodium hypochlorite (NaOCl) was used as sterilization agent. 10% solution of NaOCl in water was used for surface sterilization of the sample.

Collection of leaf samples from *Cassia alata*

Leaves that appeared healthy collected from different branches of *Cassia alata* from Ottapalam, Palakkad district, Kerala. The plant leaves were randomly collected and brought to the laboratory under sterile conditions using sterilized polythene bags. Fresh plant materials were selected for the isolation of endophytic

fungi within two hours of collection to reduce the chance of contamination.

Isolation of endophytic fungi

Isolation of fungal endophytes was carried out with the surface sterilization method. The plant material was first washed with tap water to remove dirt particles; then thoroughly washed with distilled water. The leaves are taken for further sterilization into laminar airflow chamber. The samples were cut into small pieces (approximately 2*1.5 cm) using sterile scissors and forceps, and were dropped into a petriplate containing sterile distilled water.The fragments are then rinsed with 10% NaOCl for 1 minute each. The leaves were again washed in sterile distilled water respectively. The surface sterilized leaves are placed in PDA (Potato dextrose agar) plates supplemented with chloramphenicol (100 g mL^{-1}). All of the plates were incubated at 28°C until growth initiation. The hyphal tips that emerged as a result of incubation were subcultured for purification, and the isolated pure cultures were maintained on PDA for further studies.

Morphological characterization of the isolated endophytes

The endophytic fungi were morphologically characterized according to their microscopic structures and identified by their colony morphology, type of hyphae, kinds of reproductive structures by light microscope. A drop of lacto phenol cotton blue was placed on the center of a clean glass slide. A portion of mycelia mat from fungal colony was transferred into the drop of mounting fluid with the help of flamed and cooled needle. The fungi mycelia mat was gently spread with the help of two needles, so that the mycelia were evenly spreaded. Coverslip was placed over the wet mount preparation without trapping air bubbles. Excess stain was blotted with blotting paper and microscope first by low power objective and then high power objective.

Cultivation and extraction of fungal metabolites

The fungal endophytes isolated from the *Cassia alata* plant were cultured on Potato dextrose broth for liquid fermentation. The mycelial agar plugs were inoculated into a 250 mL Erylen Meyer flask containing 100 mL of Potato dextrose broth at 28°C for 14 days. After incubation, the fermentation broth was filtered to remove the mycelial mass using Whatmann No.1 filter paper which was then screened for antimicrobial activity against selected pathogens.

***In vitro* antimicrobial assay**

The inhibitory effects of endophytic fungal metabolites were tested by using agar well diffusion method on MHA (Mueller-Hinton agar) plates against the 5 human pathogens such as *Staphylococcus* sp, *Pseudomonas* sp, *Klebsiella* sp, *Escherichia coli*, *Salmonella* sp. All the bacterial samples were obtained from Lakshmi Hospital Palakkad. The bacterial cultures were spread on the agar plates then wells were cut on the plates using a sterile well cutter. After cutting the wells the filtered broth containing fungal metabolites were inoculated in the wells. All the plates are then incubated at 37°C for 24 hours for zone production.

FTIR analysis

The Fourier Transform Infrared Spectroscopy (FTIR) analysis was carried out in the mycelial free filtrates of fungal cultures to know the different functional groups present in the fungal extracts. The liquid fungal samples were subjected to FTIR in which the samples were irradiated by a broad spectrum of infra-red light and the level of absorbance at a particular frequency was plotted after Fourier transformation of the data. The resulting spectrum was characteristic of the organic molecule present in the sample. The absorbance was measured at 400-600 nm for the identification and quantification of organic species. Compounds contained in the extracts were identified according to standard infrared chart.

Statistical analysis

The experimental data were processed statistically by analysis of variance in Randomized Block Design.

RESULT

Efficacy of sterilization

The surface sterilization protocol was a critical prerequisite for isolating plant endophtyic bacteria and fungi. This study proved that the surface sterilization protocol combined with the imprint technique was effective in removing epiphytic organisms and that the bacterial and fungal isolated strains can be considered to be true endophytic organisms.

Isolation, screening and preliminary identification of endophytic fungi

A total of two endophytic fungal organisms were isolated from healthy plant leaves of *Cassia alata* and the fungal isolates were identified as *Aspergillus* sp and *Penicillium* sp based on the morphological characters under the light microscope and colony morphology on the growth medium employed.

Aspergillus sp easily grow on potato dextrose agar at 25^{o}C. Colonies starts white to pale yellow but quickly form jet – black conidia; and characteristically has profuse conidiation so that circumferential conidia obscure vesicle. The conidia are spherical, and roughen with maturity. Microscopically, it is identified by its hyaline, septate hyphae. *Penicillium* sp produce greenish colonies and its branching or simple conidiophores supporting phialides in brush – like clusters known as penicillin. It is differentiated from paecilomyces by its phialides lacking long, pointed apical extensions. On microscopic observation septate hyaline hyphae were observed.

Antibacterial screening

The inhibitory effects of endophytic fungal metabolites were tested by using well diffusion method on MHA

(Mueller-Hinton agar) plates against the 5 human pathogens such as *Staphylococcus* sp, *Klebsiella* sp., *Pseudomonas* sp., *Escherichia coli*, *Salmonella* sp. and the results obtained are given in Table 1, Figure 1 and 2.

On the basis of antimicrobial activities of mycelial mass free extracts (broth solution) of fungal endphytes, it was evident that they shows significant antibacterial activity against almost all bacterial pathogens. High dilution of *Aspergillus* sp. and *Penicillium* sp. shows some antibacterial activity against almost all pathogens , which is better than the reference antibiotic disc (Ciprofloxacin) used. Both fungal species showed good antibacterial resistance against all pathogens, even though *Aspergillus* sp. is more effective than *Penicillium* sp. in terms of zone of inhibition produced against pathogens.

FTIR analysis

The FTIR analysis of the broth cultures for *Aspergillus* sp. is given in Figure 3 and the result for *Penicillium* sp. are given in Figure 5.

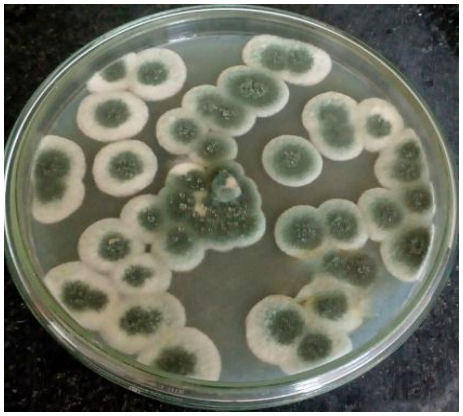
The FTIR analysis of *Aspergillus* sp. broth shows peaks at 3680.18, 3649.32, 3622.32, 3564.45, 3502.73, 3448.72, 3390.86, 2981.95 and 2947.23 indicates the presence of OH group(indicating the presence of alcohol). The peaks at 2364.73 and 2322.29 show the presence of NH group. The peak corresponding to 1913.39 and 1739.79 indicates C=O stretch. The peak at 1647.21 shows NH Stretch, while the peak at 1516.05 shows of C=C group(preferably belonging to a benzene ring).

The FTIR analysis of *Penicillum* sp. broth shows peaks at 3680.18, 3649.32, 3622.32, 3564.45, 3525.88, 2978.09 indicates the presence of OH group. The peak at 2927.94 indicate the presence of OH stretch. The peaks at 2364.73 and 2322.29 indicates NH Stretch. The peak at 1739.79 shows the presence of C=O Stretch. The peak value 1647.21 indicates NH stretch. The peak at 1627.92 indicates C=C stretch. The peak value of 1516.05 indicates the presence of C=C group (preferably belonging to benzene ring).

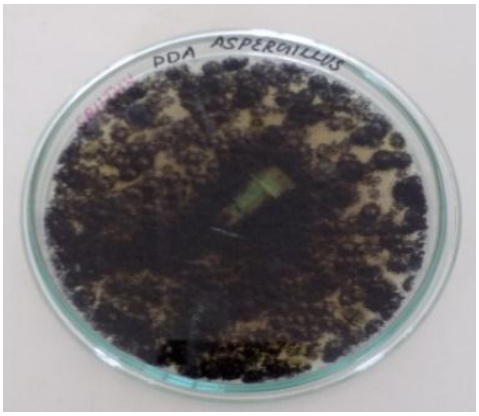
Table 1: Zone Of Inhibition Produced By Endophytic Fungal Strains Against Pathogenic Organisms.

Bacterial pathogens	**Zone of inhibition (in mm)**							
	***Aspergillus* sp.**				***Penicillium* sp.**			
	Dilutions			**Ciprofloxacin**	**Dilutions**			**Ciprofloxacin**
	50µl	**100µl**	**150µl**		**50µl**	**100µl**	**150µl**	
Staphylococcus sp.	15	18	20	15	14	18	21	15
Salmonella sp.	14	16	17	16	15	19	23	16
Klebsiella sp.	17	18	20	10	15	23	24	10
Escherichia coli	13	15	16	8	16	17	18	8
Pseudomonas sp.	9	11	12	22	9	12	13	23

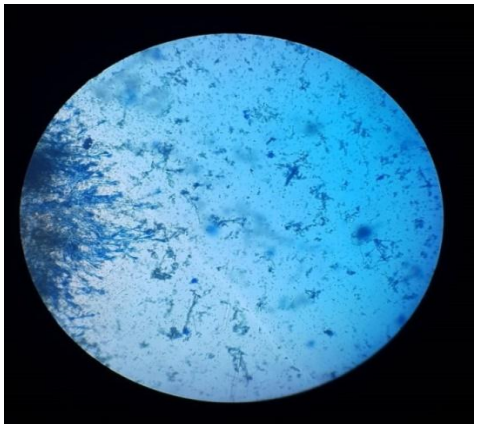
***Cassia alata* plant**



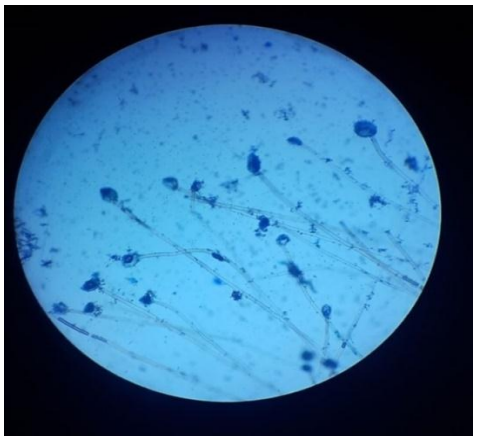
***Penicillium* sp. on PDA**



***Aspergillus* sp. on PDA**



Microscopy- *Penicillium*



Microscopy- *Aspergillus* sp.

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