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In-vitro antibacterial screening of ethyl acetate extract endophytic fungi isolated from *Phyllanthus amarus* (Schum & Thonn) against pathogenic bacterial strains.

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Abstract:

Phyllanthus amarus (Schum & Thonn) is predominantly one of the herbs in waste lands thoroughout the plains of India. The leaves are used in traditional medicine. In the present investigation, the isolation and identification of endophytic fungi of *Phyllanthus amarus* was carried out. Ethyl acetate extract from the midrib and lamina of *Phyllanthus amarus* were investigated for their antibacterial activity. In the present investigation, the antibacterial screening of ethylacetate extract endophytic fungi from *Phyllanthus amarus* was carried out. The crude and column purified culture extract of *Gleosporium* sp. were active against Gram positive (*Staphylococcus aureus, Streptococcus pneumoniae*) and Gram negative bacteria (*Salmonella typhi, E. coli*) pathogenic bacterial strains.

Key words:

Keywords: *Phyllantus amarus, Gleosporium* sp., endophytic fungi, antibacterial activity.

Introduction:

The endophytic fungi are of biotechnological importance as new pharmaceutical compounds, secondary metabolites, agents of biological control and other useful characteristics could be found by further exploration of endophytes. There is a general call for new antibiotics, chemotherapeutic agents and agrochemicals that are highly effective, posses low toxicity, and will have a minor environmental impact. Endophytic fungi are relatively unexplored producers of metabolites useful to pharmaceutical and agricultural industries [1].

Different endophytic fungal species are found in different parts of a plant; this represents an adaption mechanism of endophyte against micro ecology and physiology of host plant. Various studies demonstrated that endophytic fungi produce secondary metabolites such as enzyme and growth hormone which are useful for treatment of various diseases. These bioactive compounds demonstrated potent anti-bacterial, anti-arthritis, anti-cancer activity as well as immunosuppressive activity [2].

Endophytic fungi are known to have mutulaistic relations to their hosts, often protecting plants against herbivory, insect attack of tissue invading pathogens[3] and in some instances the endophyte may survive as a latent pathogen, causing or quiescent infections for a long period and symptoms only when physiological or ecological conditions favours virulence[4] .

Phyllanthus has been used in ayurvedic medicine for over 2000 years and has a wide number of traditional uses. The World Health Organization has compiled more than 20,000 medicinal plants used in different parts of the world. Among the medicinal plants more than one hundred botanicals have larger potential for commercial exploitation and could be marketed in the world drug markets. *P.amarus*, commonly known as Keelaneli (Tamil), Bhuiaonal (Hindi) belonging to the family Euphorbiaceae occupies a prime position among the commercially cultivated medicinal plants. It has a long history of folk use in drug industry for the treatment of dropsy, urinogenital problems, dystentry, diabetes, skin ulcer, dyspepsia, fever, asthma, bronchial infections, tumour and Hepatitis B. Though *Phyllanthus* is bestowed with several medicinal principles to cure several human disorders, the crop is susceptible to a number of devastating diseases that affects the quality and quantity of the medicinal principles. With the rise in demand for plant based drugs in global market, commercial cultivation of *Phyallanthus* has gained momentum during the last decade. This has led to the occurrence of stem blight, which caused complete failure of the crop. Since *Phyllanthus* is an export oriented medicinal herb, the current research on the etiology and management of stem blight will be a boon to the forming community[5].

Traditional healers claim that some medicinal plants are more efficient to treat infectious diseases than synthetic antibiotics. It is necessary to evaluate, in a scientific base, the potential use of folk medicine for the treatment of infectious diseases produced by common pathogen. They can also be a possible source for new potent antibiotics to which pathogen strains are not resistant[6]. This forced scientists to search for new antimicrobial substances from various medicinal plants. In this study *In-vitro* antibacterial screening of ethyl acetate extract endophytic fungi isolated from *Phyllanthus amarus* against pathogenic bacterial strains.

Materials and Methods:

Collection of leaf samples

The *Phyllanthus amarus* leaves samples were collected from Anna Herbal Garden, Tamilnadu. The samples were transported in closed sterile polythene bags and processed within 24 hours collection.

Test bacterial strains

The pathogenic bacterial strains such as *Salmonella typhi, E. coli, Staphylococcus aureus* and *Streptococcus pnumoniae* were received from Department of Microbiology, Madras University.

Surface sterilization

The leaves were thoroughly washed in running tap water. Then the leaf segments were surface sterilized by immersion in 70% ethanol for five seconds, followed by treatment in 4% sodium hypochloride for 90 seconds and finally rinsed in sterile distilled water for 10 seconds[7] .

Isolation of endophytic fungi

The surface sterilized leaf segments were evenly spaced in Petri dishes containing Potato Dextrose Agar (PDA) medium amended with 10 mg of chloromphenicol. The Petri dishes incubated at 26 ± 1ºC in a light chamber[8] and monitored every day for the growth of endophytic fungal colonies from leaf segments. The hyphal tips, which grew out from leaf segments were isolated and brought into pure culture. The isolated endophytic fungi were identified down to species level using standard manuals and monographs[9,10] .

Data Analysis

The colonization frequency of each endophyte species was calculated by the method of [11].

$$CF\% = \frac{\text{The number of colonized segments}}{\text{Total number of segments observed.}} \times 100$$

Extraction of bioactive compounds

The selected endophytic fungi *Gleosporium* sp. were grown in Czapek's broth for 48 hrs and incubated for 21 days at 120 rpm. The extract was separated using filtration procedure (Whatman No 1). Ethyl acetate was added in culture filtrate and the compounds were separated using separating flask and concentrated in rotary vacuum evaporator. The dry semi solid residue was redissolved in ethylacetate for further use[12] .

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Antibacterial activity

The concentrate crude extract *Gleosporium* sp was then impregnated (80 µl/disc) on to sterile Whatman 6 mm diameter disc and the antibacterial activity was assayed against *Salmonella typhi, E. coli, Staphylococcus aureus* and *Streptococcus pnumoniae* following the disc diffusion assay[13]. The assay was carried out in triplicate. Control plates with solvents were also maintained separately. The zone of inhibition was measured from the edge of the disc to the clear zone in millimeter.

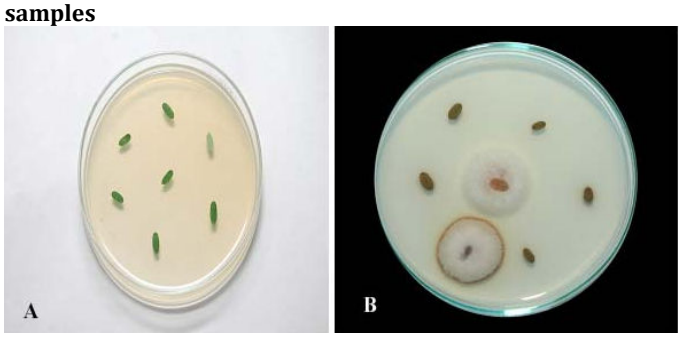
Column chromatography purification

The active *Gleosporium* sp extracts were then column fractionated using normal phase silica gel chromatography employing a step gradient (hexane 100%; hexane 75%: ethyl acetate 25%; hexane 50%: ethyl acetate 50%; hexane 25%: ethyl acetate 75%; ethyl acetate 100). The fractions were concentrated and used for antibacterial assay was carried out to find out the active fraction[12].

Results and discussion:

Fungal endophytes are microfungi that colonize living tissues of plants without producing any apparaent symptoms or injury . They are a largely unexplored component of biodiversity especially in the tropics. Tropical plant are expected to support a high diversity of endophytes and few of them have been screened for endophytic presence[14]. The surface sterilized leaf segments were plated on PDA medium amended with chloramphenicol and incubated at 30±1° C for 2 weeks (Figure 1A). The fungi grow out from tissues were brought into pure culture and identified(Figure 1B).

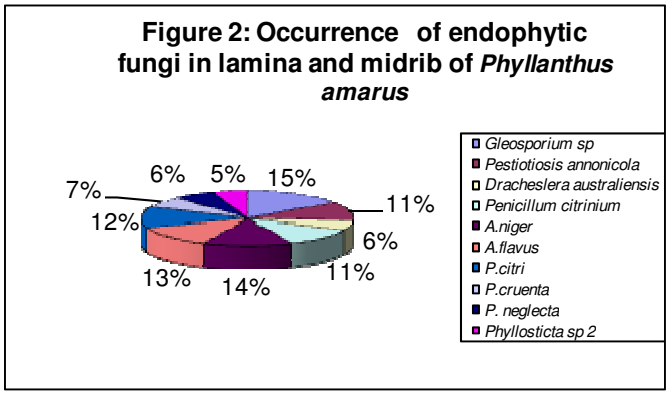
Figure 1: **Endophytic fungus from *Phyllanthus amarus* leaf samples**



A- At the time of inoculation; B- After incubation.

The occurrence and distribution of endophytic fungi from leaves of *Phyllanthus amarus* results are presented in ***(Figure 2)***.[15] observed that the dry tropical forest had much less endophytic diversity compared to wet tropical forest.

Figure 2: **Occurrence of endophytic fungi in lamina and midrib of Phyllanthus amarus**

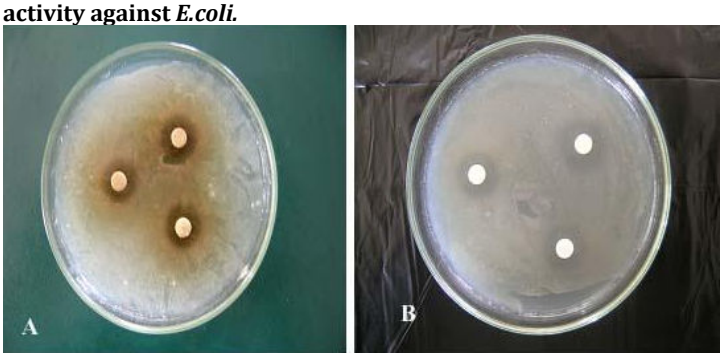


In the present investigation fungal endophytes distribution of medicinal herbs were studied. A total of 10 fungal endophytes were obtained from *Phyllanthus amarus* plant. Out of 10 fungal endophytes, 5 hypomycetes and 5 coelomycetes were isolated as endophytes from the leaves of *Phyllanthus amarus* .

Gleosporium sp. is dominant in *Phyllanthus amarus* plant studied. Among the hypomycetes and coelomycetes the *Gleosporium* sp. showed maximum colonization frequency 6.8% (Figure 2). The more number of endophytes could be recovered from the midrib region than the lamina. The more intensive samplings are necessary to clarify the fungal assemblages of the leaves and branches, as in traditional practice, the local population used mostly the extract from the leaves of the plants [16] .

Crude and purified extracts of *Pestalotiopsis* sp. also showed a wide spectrum of anti-microbial activity in the current study. Endophytic *Pestalotiopsis* sp. are known to generate antifungal, antioxidant and anticancer metabolites[17,18,19] . In this study *Gleosporium* sp. demonstrated a selective antibacterial activity against Gram positive bacteria (*Staphylococcus aureus, Streptococcus pneumoniae*) and Gram negative bacteria (*Salmonella typhi, E.coli*) (Plate 4). The *Gleosporium* sp. crude extracts could inhibit the bacteria. 26µg/ml inhibited Gram positive bacteria (*Staphylococcus aureus* and *Streptococcus pneumoniae*), 76µg/ml inhibited Gram negative bacteria (*Salmonella typhi* and *E.coli*). The *E.coli* showed higher antibacterial activity on ethyl acetate crude extracts of *Gleosporium* sp. against column purified compound (Figure 3A and B).

Figure 3: **Crude and column purified compound antibacterial activity against *E.coli.***



A- Crude extract; B- Column purified fraction (EA 20%: H 80%).

The column purified different fraction of *Gleosporium* sp. showed Antibacterial activity. The *Escherichia coli* showed maximum activity (4.5±0.20) in (75% Hexane: 25% Ethyl acetate) than *Salmonella typhi* (3.1±0.18), *Staphylococcus aureus* (3.4±0.10) and *Streptococcus pneumoniae* (2.2±0.26) (Table 1). Crude extract and purified fractions of *Aspergillus* spp. Showed a wide spectrum of anti-microbial activity. Similarly, several metabolites of the marine isolate, *Aspergillus niger* showed anti-bacterial and antifungal potential [20] .

Table 1: **Antibacterial activity of *Gleosporium* sp. column purified different fractions**

S.No	Pathogenic bacterial strains	Zone of inhibition (mm) (mean ± SD) n = 5 Experiments				
		H 100 %	H:EA 75%:25 %	H:EA 50%:50 %	H:EA 25%:75 %	EA 100%
1	*Escherichia coli*	-	4.5±0.20	2.1±0.22	2.0±0.13	1.6±0.12
2	*Salmonella typhi*	-	3.1±0.18	1.6±0.13	1.4±0.53	2.4±0.16
3	*Staphylococcus aureus*	-	3.4±0.10	1.7±0.52	T	1.3±0.14
4	*Streptococcus pneumoniae*	-	2.2±0.26	1.2±0.11	1.2±0.22	2.1±0.22

EA- Ethyl Acetate; H- Hexane; T- Trace.

Conclusion:

In the current study, the endophytic fungi isolated from the leaves of *Phyllanthus amarus* showed promising antibacterial activity against the gram positive and gram negative bacteria. Endophytic fungi are a 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, agricultural and industrial areas. Hence, the isolation of endophytic fungi from medicinal plants for any bioactive compound may facilitate the product discovery process.

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