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Antibacterial Properties of Endophytic Fungi Isolated from selected species of *Selaginella*

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Abstract

The endophytes from plants are important resources for discovery of natural products and bioactive compounds having the potential biotechnological applications in agriculture, industry, medicine, and allied sectors. Endophytes have been sorted out from all the parts of the medicinal plants such as root, stem, leaves, fruits, flowers, bark, scales, resin canals, and even from meristem. The Medicinal plants which are known to be used centuries as a substitute of medicine are a precious source for bioprospecting endophytes. The endophytic fungi such as *Sporotrichum*, *Aspergillus sp.*, *Clladosporium*, *Penicillium* sp., *Cephalosporium* and other NSF have been reported from the four selaginella species namely *Selaginella willdenowii, S. plana, S. dixitii*and *S. inaequalifolia.* Some of these endophytic fungi have antimicrobial activity against some pathogenic bacteria *Staphylococcus aureus*. The fungi extracted from *Selaginella plana, S. wildenowii* and *S. dixitii* have shown antimicrobial activity against *Staphylococcus aureus.*

1. Introduction

The term Endophytes [Greek: "Endo"-within, "phyton"-plant] was first coined by de Bary (1886). An endophyte is an endosymbiont (a bacterium or fungus) that lives within a plant for at least part of its life cycle without causing apparent disease or symptoms. Endophytes are ubiquitous and have been found in all species of plants. Endophyte with the plant varies from mutualistic or symbiotic to antagonistic or slightly pathogenic (Moustafa & Mahmoud, 2010).

Endophytic fungi are polyphyletic microorganisms that inhibit plant tissues without inciting disease symptoms, and eventually establish mutualistic associations with their host plants (Pandian *et al*., 2011). The intrinsic nature of the interactions among and between endophytes and host plants and pests, which are mediated by such compounds, is an area open to future discoveries (Subramani & Aalbersberg, 2012). The elucidation of such connections can not only enhance the understanding of evolution of complex defence mechanisms in plants and their associated organisms, but also help to exploit the latter for a sustained production of a few valuable compounds to be used in biotechnologies (Subramanian, 1983; Ramarao & Manoharachary, 1990; Wilson *et al*., 1991).

Plants especially perennials are colonised by many types of endophytic microorganismswhich live inside plant tissues either throughout their life cycles without causing visible damage or morphological changes in their hosts (Jalgaonwala *et al*., 2011) These microorganisms include both fungi and bacteria and usually co-exist with pathogens (Jorgensen *et al*., 2015).According to their colonizing behaviours, endophytic micro flora can be sorted into facultative

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endophytes colonise plants and certain stages of their life cycles, but they may also reside outside the plant at other stages to form an association with the immediate rhizosphere soil of host plants (Khan *et al*., 2011).

Pteridophytes are known as the oldest vascular plant creature on earth and constitute a major group of vascular plants. Pteridophytes position, known as intermediate between the lower plants and higher plants has made the whole group interesting for research & study. Pteridophytes groups have a long connection on our planet and were known as far back as 380 million years ago. Pteridophytes are also known for their medicinal value for more than 200 years (Strobel & Daisy, 2003). The Selaginellaceae are mostly distributed in tropical and warm regions, worldwide. Economic importance includes cultivated ornamentals and local medicinal plants. This family is distinguished fromLycopodiaceaeby having scale-leaves bearing a ligule and by having spores of two types. *Selaginella* occurs mostly in the tropical regions of the world, with a handful of species to be found in the arctic-alpine zones of both hemispheres (Punja, 1997).

The present study mainly aims to isolate and identify the endophytic fungi from selected species of *Selaginella* and also check its antimicrobial properties.

2. Materials and Methods

2.1 Sample collection

The plant samples of *Selaginella plana*, *S. wildenowii, S. dixitii* and *S. ineaqualifolia* were collected from Bio resource nature park KFRI sub centre at Nilambur in Malappuram district. Four species are collected during the period of November 2023-January-2024, mature apparently healthy leaves of each species were randomly collected and placed separately in sterile polythene bags and samples were brought to the laboratory and stored in refrigerator.

2.1.2 Sterilization of glassware and chemicals

Media and glass wares including petri dishes were sterilized in an autoclave at 15 PSI pressure of 121^{0}C temperature for 30 min. Petri dishes were hot sterilized in a hot air oven at 160° C for 3 hrs.

2.1.3 Preparation of plant material

The leaf samples of *Selaginella* species for the isolation were first cleaned 2 to 3 times under running tap water. Surface disinfection was performed by sequentially rinsing leaves with 70% ethanol for 2 minutes then again rinse using distilled water for 2 to 3 times. The plant samples were dried between the folds of sterile filter paper.

2.1.4 Preparation of potato dextrose agar medium (PDA)

Preparation of potato dextrose agar medium prepared by using HiMedia ready medium of 3.9 g of potato dextrose agar powder weighed and transferred into a 250 ml conical flask and then dissolved with 150 ml of distilled. The media was homogenized by agitating and then sterilized by autoclaving at 121°C for 15 minutes, after that medium aseptically poured into sterile petri plates and allowed to gel.

2.1.5 Isolation of fungal Endophytes

After proper drying the surface sterilized plant sample leaves were cut into small pieces (2mm) and each pieces were placed on potato dextrose agar medium supplemented with streptopencillin (50mg/L). Five

sterilized segments were placed on each PDA plate after inoculation the plates were sealed with Clingfilm and incubate at 25°C for 5 to 7 days (extended to two weeks) under appropriate light and dark 24 hour cycle condition to promote the growth of endophytes and they were regularly monitor for fungal growth each endophytic culture were checked for purity and transferred to freshly prepared PDA plates.

2.1.6 **Identification of fungal Endophytes**

After the incubation, the plates were continuously monitored for mycelial growth and spore formation. The colonies were subculture into PDA medium and were stained with lactophenol cotton blue and examined under a light microscope. The isolated fungi were identified on the basis of colony morphology, mycelium, fruiting-body, spore shape and size by referring standard manuals (Subramanian, 1983; Wang *et al*., 2013; Ibrahim *et al*., 2016).

2.1.7 Extraction of fungal extract in liquid culture method

The fungal endophytes were mass cultivated on potato dextrose broth by placing agar disc of actively growing pure culture in flasks containing 150 ml of culture medium. The flasks were incubated at room temperature for 3 weeks with periodical shaking. After the incubation period, the cultures were taken out and filtered through sterile Whatsmann No.1 filter paper to remove the mycelial mats. Mycelial mats were kept in a hot air oven for drying.

The crude extracts were obtained by organic solvent extraction using ethyl acetate. The fermented medium obtained was mixed well by placing it in a shaker for 10 minutes. The supernatant was transferred to a separating funnel to which was added the same volume of crude ethyl acetate. The funnel was strongly agitated and the separation of the phases occurred by polarity difference. This process was repeated twice. Ethyl acetate solution containing the fungal metabolites was concentrated in a rotary vacuum evaporator at 40°C and the material obtained from the evaporation was suspended in Dimethyl sulfoxide (DMSO) and stored at 4°C. The mycelial mat extract was prepared using methanol. The mycelium dried in the oven was powdered to paste by mixing it with methanol. And this was then separated using a filter paper, so that the filtrate was separated from mycelial paste. It was heated in water bath at 60°C for 2 hours and evaporated to dryness using a rotary flash evaporator. The dried residue was dissolved in DMSO and stored at 4°C.

To prepare aqueous extract, 100 ml of fungal culture filtrate was reduced to 50 ml by evaporating in a water bath at 60°C.

2.1.8 **Antimicrobial activity of endophytic fungal extract**

2.2. Selection of test organisms

Two common human pathogenic bacteria and eleven plant fungal pathogens were used to evaluate the antimicrobial activity of endophytic crude extracts. All the test pathogens were obtained from the KFRI Sub Centre Nilambur. Gram negative *Klebsiellapneumonia* and Gram positive*Staphylococcus aureus* were the bacterial pathogens the selected microbial cultures were subcultured in

agar slants (Nutrient agar slants were used for bacterial culture)

2.2.1**Antibacterial activity**

Endophytic fungal extracts were screened for their antibacterial activity with two human pathogenic microorganisms. Antimicrobial activity was determined by the Disc diffusion method. In the Disc diffusion method, sterilized filter paper discs (5mm diameter) were dipped into 25µl of each extracts such as ethyl acetate extract, mycelial mat extract, aqueous culture filtrate, a negative control and a positive control. These discs were placed on to the lawn of 24 hour old cultures of test organisms. The magnitude of the antimicrobial activity was assessed by the diameter of inhibition zones relative to those of positive and negative controls. Tetracycline was used as positive control. The plates were incubated at 37°C for 24 hours and the zone of inhibition was measured and compared with the control. Three replicates were maintained in each case.

2.2.2**Antibacterial activity of Endophytic fungi**

Screening of selected endophytic fungal isolates such as *Aspergillus* sp., *Penicillium* sp., *Cladosporium, Sporotrichum, Cephalosporium* and eleven non sporulating funguses to determine the antibacterial activity was done by disc diffusion method against two human pathogenic bacterial strains *Staphylococcus aureus, Klebsiella pneumonia.* In disc diffusion method, *Aspergillus* sp. showed promising antibacterial activity against staphylococcus aureus. Crude extracts of *Aspergillus* sp. showed maximum zone of inhibition (11.3±0.8 mm) against staphylococcus. Crude extracts of *Penicillium* sp. and three non sporulating fungi showed slight amount of antibacterial activity against *S. aureus*, while *Cladosporium*, *Sporotrichum* and *Cephalosporium* showed no antibacterial activity against any of the pathogens. In the present study, leaves of four *Selaginella*sps. were taken for the isolation of endophytes. Altogether 20 endophytic fungal strains were obtained from these plants.

3. Results and Discussion

Endophytic fungi were isolated from apparently healthy, symptomless leaf segments of four *Selaginella*sps. namely *Selaginella plana, S. willdenowii, S. dixitii and S. ineaqualifolia*followed by the proper surface sterilization. A total of seventeen Endophytic fungal species were isolated from the leaf segments. The total seventeen species of endophytic fungi were isolated from four different *Selaginella*sps. Among them six of them were identified as *Aspergillus niger, A. versicolor, Sporotrichum, Cephalosporium, Penicillium sp. and Cladosporium* on the basis of colony morphology, mycelium, fruiting-body, spore shape and size by referring standard manuals.

Among seventeen endophytic fungal species, eight species are isolated from *S. plana,* 2 from *S. dixitii*, 4 from *S. willdenowii* and 3 from *S. ineaqualifolia* and 11 of them are NSF. *Penicillium* sp. and *Aspergillus* sp. showed maximum of antibacterial activity against *Staphylococcus aureus* and the other 3 NSF (NSF1, NSF2 and NSF3) showed moderate levels of antibacterial activity. Endophytic fungi have been the unexplored source of natural products that may be exploited

as huge repertoire of unparallell chemical and structural diversity with potent biological activity (Raghukumar. 1986; Wang *et al*., 2011 & Sajitha *et al*., 2013; Sudha *et al*., 2016).

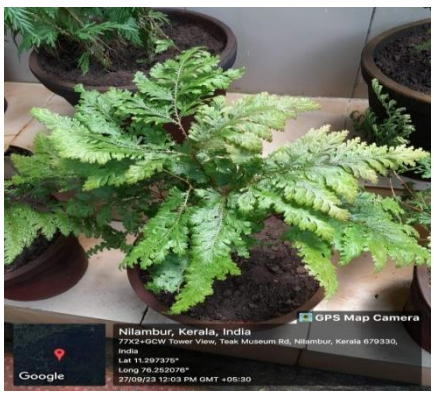
Endophytes produce secondary metabolites, which are biosynthetically derived from primary metabolite by genetically controlled method, enzymatic catalysed reaction that lead to formation of complex compounds (natural product). Generally, production and yield of these compounds are limited as they depend upon physiological and developmental stages of fungus.To circumvent this problems here an attempt has been made to optimize the cultural and physiological conditions in order to enhance the production of biomass, yield of products and their bioactivity.Crude extract yield and zone of inhibition, which show the conformity with the earlier studies (Taghavi *et al*., 2009; Supaphon *et al*., 2013; Thambugala*et al*., 2020). Some other studies also reported PDB as the best medium for the growth and enhanced production of secondary metabolites from *Aspergillus terreus* (Raghukumar *et al*., 1992; Wang *et al*., 2013).In the minimum fungicidal concentration test conducted in this study, *Selaginella plana* extract showed an effect against *Candida albicans* at 100% concentration. This result was proven by the lack of colonies that proliferated on the agar media. This might be because *Selaginella plana* contains secondary metabolites such as flavonoids, tannins and saponins (Hillson. 1977; Wilson *et al*., 1991; Pathak *et al*., 1993).

Plate-1 (A-D): Images of selected *Selaginella* species for the study

A- Habit of *Selaginella wildenowii*

B- Habit of *Selaginella plana*

C - Habit of *Selaginella dixitii*



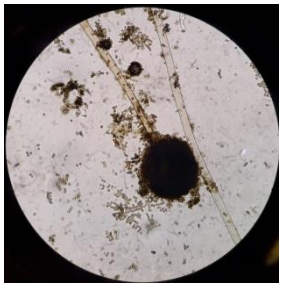
D - Habit of *Selaginella inaequalifolia*

Table-1 Endophytic fungi isolated from the selected specis of *Selaginella*

Sl. No	Endophytic Fungi Isolates	Morphological charecters
1.	*Aspergillusniger*	Hyphae are septate and hyaline. Conidial heads are black, radiate, with a tendency to split into loose columns at maturity. Conidia are globose to subglobose
2.	*Aspergillus versicolar*	Colonies can vary greatly in color, starting as white and changing to yellow, orange, and green with pink or flesh hues intermixed as they mature.
3.	*Sporotrichum*sp.	The fungal growth in the colonies is characterized by branched septate hyphae which produce small distinct asexual spores known as conidia.
4.	*Cephalosporium* sp.	Long slender phialides, and conidia are cylindrical or ellipsoidal, formed in slimy bundles at the tips of the phialides. Lower microscopy shows pin-head spore ball formation.
5.	*Pencillium*sp.	chains of single-celled conidia are produced in basipetal succession from a specialisedconidiogenous cell called a phialide
6.	*Cladosporium*sp.	septate dark hyphae, erect and pigmented conidiophores, and conidia.
7.	NSF-1	White in color. margin fimbriate
8.	NSF-2	White in color. margin fimbriate
9.	NSF-3	White in color. margin fimbriate
10.	NSF-4	White in color. margin fimbriate
11.	NSF-5	White in color. margin fimbriate
12.	NSF-6	White in color. margin fimbriate
13.	NSF-7	White in color. margin fimbriate
14.	NSF -8	White in color. margin fimbriate
15.	NSF -9	White in color. margin fimbriate
16.	NSF- 10	White in color. margin fimbriate
17.	NSF-11	White in color. margin fimbriate

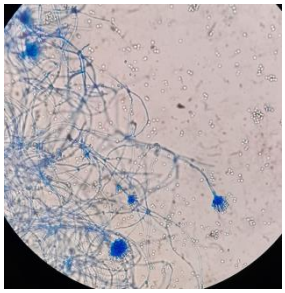
Fig. 1: Colony of fungi in *Selaginella plana*with respect to the edophyticfungal taxa

1.1 Colony of *Aspergillus niger* on PDA



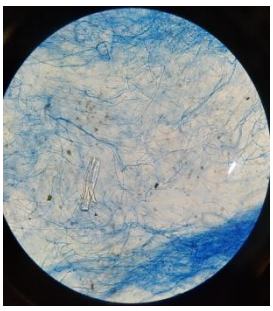
1.2 Conidia of *A. niger*

1.3 Colony of *Aspergillus versicolar on PDA*



1.4 Conidia of *A. versicolar*

1.5 Colony of NSF on PDA

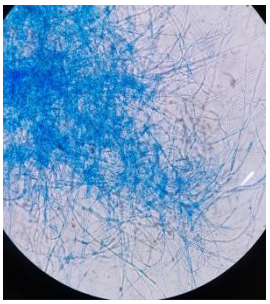


1.6 Microscopic view of NSF

1.9 Colony of NSF on PDA

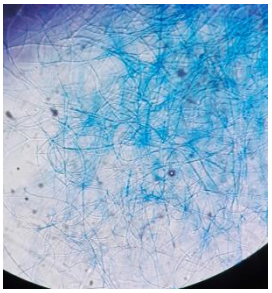
1.10 - Microscopic view of NSF

1.11 Colony of NSF on PDA



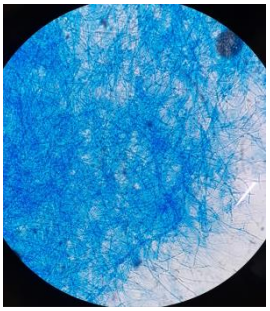
1.12 Microscopic view of NSF

1.13 Colony of NSF on PDA



1.14 Microscopic view of NSF

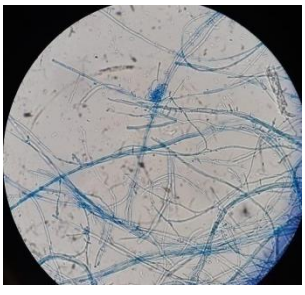
1.15 Colony of NSF on PDA



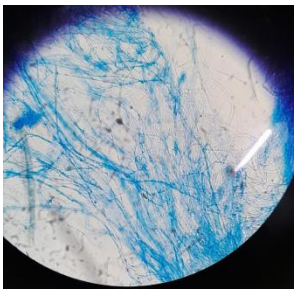
1.16 Microscopic view of NSF

Fig. 2: Colony of fungi in *Selaginella wildenowii* with respect to the edophytic fungi

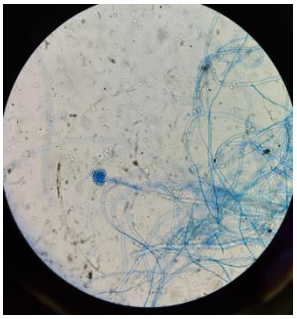
2.1 Colony of *Cladosporium* on PDA



2.2 Conidia of *Cladosporium*

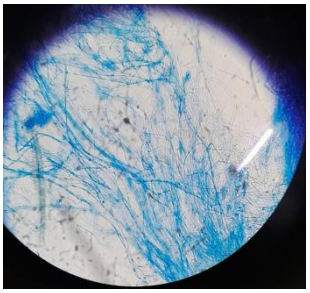


2.3 Colony of NSF on PDA **2.4 Microscpic view of NSF**

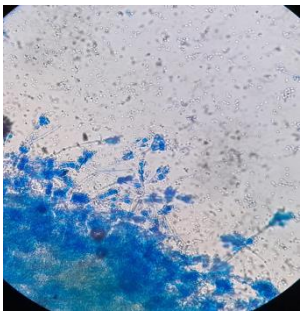


2.6 Colony of *Cephalosporium*on PDA **2.7 Conidia of *Cephalosporium***

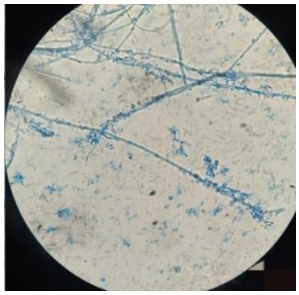
Fig. 3: Colony of fungi in *Selaginella inaequafolia* with respect to the edophytic fungi



3.1 Colony of nonsporing fungus on PDA **3.2 Microscopic view of NSF**

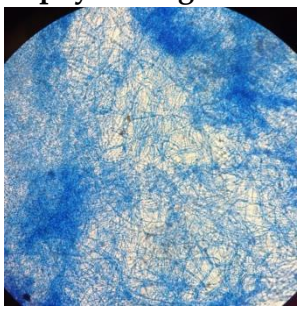


3.4 Colony of *Pencilum* sp. *on PDA* **3.5 Conidia of *Pencilum* sp.**



3.6 Colony of*Sporotrichum* on PDA **3.7 Conidia of *Sporotrichum***

Fig. 4: Colony of fungi in *Selaginella dixittii* with respect to the edophytic fungi



4.1 Colony of NSF on PDA **4.2 Microscopic view of NSF**

Fig. 5: Antibacterial activity shown by Endophytic fungus against *S. aureus*

Fig.5.1 Antibacterial activity of *Aspergillus niger* against *S. aureus*

Fig. 5.2 Antimicrobial activity of *Pencillium* sp. against *S. aureus*

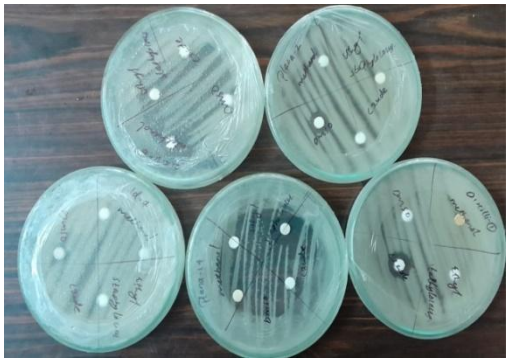


Fig. 5.3 Antibacterial activity of NSF against *S. aureus*

Endophytes have proved to be the promising sources of biologically active products which are of interest for specific medicinal applications. Recent investigations have been intensified by the potential of endophytic fungal strains as biocontrol agents. Recently, research groups have identified more than hundreds of endophytic isolates from South Indian medicinal plants that showed promising activity against several pathogens (Kalyanasundaram *et al.*, 2015). Today, more research has been focused on endophytic fungi isolated from various plants and their antimicrobial activity. Many such studies on endophytic fungi, their metabolites and their role in plant metabolism were carried out by several researchers. These studies on antimicrobial activity are to be carried out for finding their role in plant defence mechanisms (Gautam *et al.*, 2013). In the present investigation, crude extracts of *Aspergillus* sp. showed highest antibacterial activity against bacterial pathogens. Crude extracts of *Penicillium* sp. also showed a great level of antibacterial activity. The 2 of non sporulating fungi showed moderate level of antibacterial activity against the *Staphylococcus aureus*.

4.Conclusion

The present study reveals that the endophytic fungi isolated from four Selaginella species showed the presence of potent antimicrobial activity. Thus endophytes from these four *Selaginella* species are a remarkable natural source of biologically active metabolites. The endophytic fungi isolated from the selected Selaginella species plants are *Aspergillus* sp., *Penicillium* sp., *Cephalosporium*, *Sporotrichum*, *Cladosporium* and twelve NSF. Further research needs to be conducted to determine the bioactive compound that has a role in inhibiting the growth of these pathogenic microbes.

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