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Antibacterial activity of endophytic fungus isolated from *Cyathodium cavernarum* Kunze ex Lehm.

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Abstract

Bryophytes are a group of non-vascular plants that include mosses, liverworts and hornworts. They are indeed the third largest group in the plant kingdom but have not received much attention with only pint-size research work going on due to their small size and difficulty in taxa identification. Many bryophytes form a mutualistic relationship with other microorganisms. These endophytes are capable of promoting the growth as well as the production of several metabolites that offer resistance to the host. *Cyathodium* is a fluorescently green liverwort found in wet and shady areas. The present study deals with isolation and characterisation of endophytic fungi from *Cyathodium* and evaluating their antibacterial activity against 4 bacterial species, *Proteus vulgaris*, *Bacillus subtilis*, *Listeria monocytogenes* and *Xanthomonas campestris*.

Key words : Bryophytes, Endophytes, *Cyathodium*, Antibacterial activity.

Bryophytes constitute the second largest group of land dwelling plants[20]. India being a megadiverse country represents about 17.27 % of global bryophyte diversity. Of the 2504 species reported in India about 642 species are endemic[7]. Being cryptogamic they are less familiar to the common people and are not being widely used due to their small size and less biomass[14]. The life cycle of bryophytes is characterised by a dominant gametophytic phase which is the most evolved haploid phase in the plant kingdom[29]. These organisms exhibit multifunctional properties rendering them suitable for heavy metal biomonitoring, facilitors of nitrogen fixation and enhances soil structure and fertility[13, 25]. Phytochemical studies indicate bryophytes to be a rich source of diverse chemicals[19]. They possess several potential compounds with bioactivities such as alkaloid, terpenoids, flavonoids, glycosides, carbohydrates, lipid, polyphenols, organic acids, sugar alcohols, fatty acids, aliphatic compounds, acetogenins, phenyl- quinones, aromatic and phenolic substances etc[2, 23].

Plant tissues are most probably inhabited by endophytic microbes especially fungi and bacteria. Almost all vascular plants are hosts for several endophytes. Similar to other plants, fungi have been found in bryophytes as commensals, endophytes, saprophytes and parasites[8]. Melanized non sporulating fungal endophytes were isolated from *Riccia* sps, one of the most commonly occurring liverwort[3]. The members of Aneuraceae are known to harbour many basidiomycetous fungi[5,9,10,18,22,24]. Because endophytic microbes can directly produce secondary metabolites that strengthen plants' tolerance to biotic and abiotic stresses, they aid in the growth of their host plant. Furthermore, they can biosynthesize medically significant substances that were believed to be produced solely by the host organism[28].

Widely found in tropical regions, the complex thalloid liverwort, *Cyathodium* is a member of the monotypic family Cyathodiaceae[16,17]. They can thrive in a wide range of habitats such as soil, rocks, tree barks, concrete walls etc[6]. The present study deals with isolation of endophytic fungi from *Cyathodium cavernarum* thallus and culturing the fungus in Czapek- Dox liquid media to assess the antibacterial activity of intracellular and extracellular extract.

Collection and identification of the specimen :

The plant was collected from Peruva, Kottayam district. The thallus along with soil was collected. It was cleaned with water to remove adhering soil particles. Tax level identification were carried out using standard literature.

Detection of endophytes :

The plant was killed and fixed using FAA. The thallus was cleared by boiling in 10% KOH solution, followed by washing in 5% HCl and then with water. The specimen were stained with trypan blue in lactophenol. The thallus after maceration was scanned for fungal hyphae using compound microscope.

Isolation and culturing of fungal endophyte:

For the isolation of endophyte, the gametophyte was surface sterilised with 0.5% sodium hypochlorite for 3 minutes, followed by washing with sterile distilled water. The thallus was then placed over sterile potato dextrose agar medium. The plates were incubated at 30 °C for 14 days. Fungal mycelium developed at the point of inoculation was selected and subcultured on to fresh potato dextrose media.

Fermentative production of metabolite:

A loop full of mycelium of the isolated fungus was inoculated on to 100 ml sterile Czapek-Dox medium taken in 250 ml conical flask. The flasks were incubated at 30 °C for 7 days without agitation. After the incubation period, the mycelium was harvested by filtration through Whatman No 1 filter paper. The filtrate represent the extracellular metabolite and was stored at 4 °C. The collected mycelia were ground in 10 ml phosphate buffer and centrifuged. The supernatant forms the intracellular metabolite and was stored at 4 °C.

Antibacterial activity :

Disc diffusion agar method was

employed to detect antibacterial activity. The bacterial strains used for the assay were *Proteus vulgaris*, *Listeria monocytogenes*, *Xanthomonas campestris* and *Bacillus subtilis*. The bacterial strains were cultured in 5.0 ml nutrient broth solution and incubated for 16 hours at 30 °C. Bacteria at their log phase were swabbed aseptically onto sterile nutrient agar plates so as to create bacterial lawn. Sterile discs dipped in extracts (both extra cellular and intra cellular), after drying were placed on the bacterial lawn. Erythromycin was used as the positive control. For incubation, the plates were kept in inverted position for 24 hrs and screened for zone of inhibition. All experiments were conducted in triplicates.

The gametophytic thallus was delicate, green and fluorescent, without any distinct midrib (Figure 1). Anatomically the thallus had a large photosynthetic region, divided in to uniseriate chambers. Chambers opened out through simple air pores. Upper epidermis with teeth shaped cells. Assimilatory filaments were absent. Storage region was narrow and composed of parenchyma cells. Lower epidermis had smooth walled and tuberculated

Figure 1. Gametophytic thallus in substratum

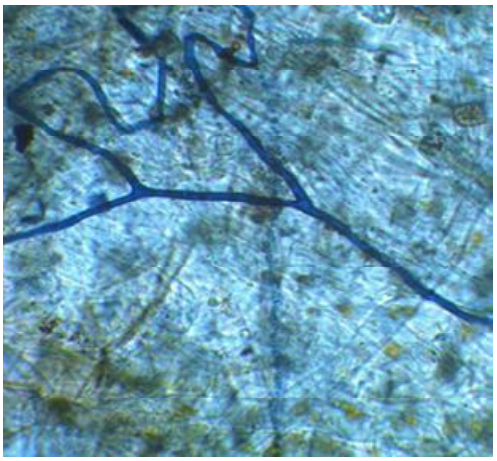

Figure 2- Septate fungal endophyte in thallus of *Cyathodium*

rhizoids. Filamentous green scales were seen towards apex of the thallus. Spores baculate spinose. Based on these features, the taxa was identified as *Cyathodium cavernarum* Kunze.

Microscopic observation after staining with trypan blue revealed fungal mycelium in the thallus. The fungal hyphae were extracellular and branched. Hyphae readily absorbed the stain and appeared blue (Figure 2). Septate were seen in all the filaments indicating the fungus to be septate endophyte. Colonization percentage was found to be 85 specifying the widespread occurrence of hyphae in bryophyte thallus. No microsclerotia formation was visible in the preparation. The connection between bryophytes and fungi has been subjected to numerous investigations. The fungi- bryophyte interaction may be parasitic, saprophytic or symbiotic. Major leafy liverwort families such as Lepidoziaceae, Cephaloziaceae, Calypogeiaceae and Cephaloziellaceae can act as host for *Rhizoscyphus ericae*, a member of Ascomycotina[11,12].

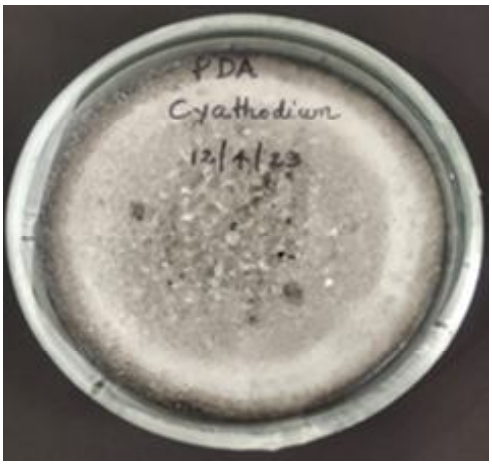

Figure 3. Septate endophyte isolated from *Cyathodium* in PDA plate

Fungal mycelium were visible in potato dextrose agar plates within a week of inoculation. The mycelium grew radially and covered the agar surface with 2 weeks of incubation (Figure 3). No spores were seen. Mycelium on microscopic observation was similar to that found in the bryophyte thallus.

The fungal extracts, both extracellular and intracellular, showed antibacterial activity against all the tested bacterial strains (Table 1). The extract was active against both Gram Positive and Gram Negative bacteria (Figure 4-7). Among Gram positive bacteria, the extract was more effective against *Bacillus subtilis*. Against Gram negative bacteria, extract had more pronounced activity against *Proteus vulgaris*. Both extra and intracellular extract showed antibacterial activity and extracellular extract showed slightly more activity than the intracellular extract.

Table-1. Antibacterial activity of extracellular and intracellular extract of the endophytic fungi

Extract	Zone of inhibition in mm			
	Proteus vulgaris	*Listeria monocytogenes*	*Xanthomonas campestris*	*Bacillus subtilis*
Intracellular	23 ± 1	21 ± 2	17 ± 2	25 ± 0
Extracellular	25 ± 1	23 ± 1	18 ± 3	26 ± 1
Erythromycin	0	26 ± 1	17 ± 0	28 ± 1

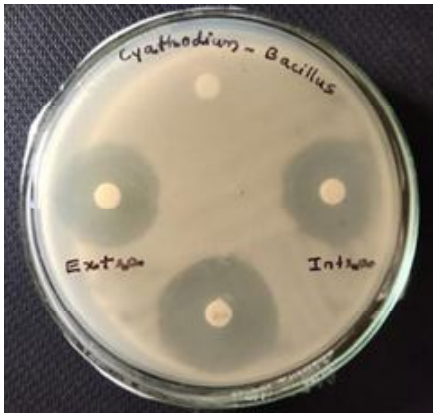

Figure 4. Antibacterial activity against *B. subtilis*

Figure 5- Antibacterial activity against *X. campestris*

Figure 6- Antibacterial activity against *P. vulgaris*

Figure 7- Antibacterial activity against *L. monocytogenes*

Several studies revealing the antibacterial activity of bryophytes are known to date. Methanol extract of *Notothyals indica* have antibacterial activity against 10 bacterial strains including *Bacillus subtilis*[4]. Singh *et al*.[26] have reported antibacterial activity of the liverworts *Plagiochasma appendiculatum, Conocephalum conicum* and mosses *Bryum argenteum* and *Mnium marginatum*. The antibacterial efficacy of *Atrichum undulatum*, *Rhytidiadelphus squarrosus*, *Rhodobryum roseum* and *Frullania dilata* has been confirmed against *Staphylococcus aureus*[15]. Mathew and Mathew[19] reported antibacterial effect of methanol extract of *Pterobryopsis pilifolia*. There are several possibilities that the antibacterial potential of bryophytes arises from the endophytes or by the plant itself. Even though reports on the antimicrobial potential of endophytes from higher plants are plentiful, only little attention was given by the researchers on the potential of bryoendophytes. Several antibacterial compounds are reported from liverwort endophytes. One among them was ent-homocyclopiamine B, isolated from *Penicillium concentricum* an endophytic fungi of *Trichocolea tomentella*[1]. Phytochemical analysis of *Smardaea,* an endophytic fungi from *Ceratodon purpureus,* has yielded isopimarene- type diterpenoids, representing the first and only reported instance of terpenoid production by bryoendophytes[27]. The moss *Schistidium antarctici*, native to Antarctica has been known to harbour a fungal endophyte, *Mortierella alpina* with potent antioxidant and antibacterial activity[21]. In the present study, the endophyte isolated from *C. cavernarum* showed antibacterial activity. Thus harbouring a potential fungi that can prevent bacterial growth may be an adaptation of the bryophyte. Further work include axenic culture of *C. cavernarum*, identification of the fungus and purification and characterization of active principles from the extract.

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