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Endolichenic Fungi Associated With Pyxinecocoescollected From Arunachal Pradesh And Their Antimicrobial Activities Against Some Test Pathogens

Aparna Choudhury[1], Chayanika Sharma[1], Dildar Hussain[2], Nisha Moshahary[2], Bhagyashree Goswami[2], Dipanjali Devi[1*]
[1,2*]PG Department of Botany, DarrangCollege.

[*]**Corresponding author:**
e-mail: sumidipa13@gmail.com

Abstract
Pyxinecocoes, foliose lichen with recognized medicinal value, contains a wide range of endolichenic fungi that may produce biologically active metabolites. This study focused on isolating and assessing the antimicrobial potential of these fungi from surface-sterilized thalli of P. cocoes. Fungal isolation was carried out on Potato Dextrose Agar (PDA), Malt Extract Agar (MEA), and Water Agar (WA), yielding 47 distinct isolates. Identification was based on macroscopic colony features and microscopic observations. Mycelia sterilia was the most frequently occurring isolate, along with other genera such as Penicillium and Drechslera. Antimicrobial activity was evaluated using the agar cup diffusion method against clinically important pathogens–Candida albicans (MTCC 227), Escherichia coli (MTCC 443), and Staphylococcus aureus (MTCC 737). All isolates exhibited varying levels of inhibition. The results highlight P. cocoes as a reservoir of endolichenic fungi with significant potential for discovering novel therapeutic compounds.
Keywords*-Antimicrobial, foliose, novel, agar cup diffusion method and therapeutic*

INTRODUCTION

Lichens are composite organisms formed through a symbiotic association between the mycobiont (fungal partner) and a photobiont such as green algae or cyanobacteria (Heskenset al., 2012). Recent findings indicate that basidiomycete yeast may act as an additional symbiotic partner within the lichen thallus (Spribilleet al., 2016). Besides these primary partners, lichens host a diverse community of associated microorganisms—including fungi and bacteria—collectively referred to as endobionts (Arnold et al., 2009; Grube and Berg, 2009; Honegger, 2012; Lagarde et al., 2018).Among these, endolichenic fungi represent cryptic microfungi that colonize the internal tissues of healthy lichens in close proximity to the photobiont. These fungi do not produce visible disease symptoms (Lawrey and Diederich, 2003; Arnold et al., 2009) and are typically horizontally transmitted, offering ecological or functional benefits to the host. Most endolichenic fungi belong to the subphylum Pezizomycotina, with multilocus phylogenetic analyses placing them across several orders, including Pleosporales, Xylariales, and Hypocreales (Arnold et al., 2009; U'Ren et al., 2010; Suryanarayanan and Thirunavukkarasu, 2017).

Endolichenic fungi are recognized as prolific producers of secondary metabolites such as steroids, quinones, terpenoids, peptides, xanthones, and sulfur-containing chromenones, many of which possess reported anticancer, antiviral, anticytotoxic, antifungal, antibacterial, and anti-Alzheimer activities (Suryanarayanan and Thirunavukkarasu, 2017; Bioscaet al., 2016; Muggia et al., 2016).Pyxinecocoes, a foliose lichen belonging to the family Caliciaceae (Wedin and Grube, 2012; Crespo et al., 2012), is commonly employed in biomonitoring studies due to its high pollutant-accumulating capacity (Bajpai et al., 2010). Despite its ecological relevance, the antimicrobial potential of endolichenic fungi associated with P. cocoes has not been previously investigated.

The present study aims to isolate endolichenic fungi from healthy thalli of Pyxinecocoes and to evaluate their antimicrobial activity against clinically relevant human pathogens. To our knowledge, this constitutes the first report on the antimicrobial properties of endolichenic fungi derived from P. cocoes.

MATERIALS AND METHODS

Lichen identification

Healthy thalli of Pyxinecocoes were collected from the Tipi region (27.0274° N, 92.6102° E) and the Dahung region (27.2109° N, 92.5067° E) of Western Arunachal Pradesh, both located within the Indo-Burma biodiversity belt. The identification of P. cocoes was carried out based on its morphological and anatomical characteristics. Morphological features were examined using Leica EZ4 and Leica S9i stereozoom microscopes, while anatomical structures were studied under a Leica DM2500 compound microscope. Standard chemical spot tests were performed following the methods of Orange et al. (2001). Thin-layer chromatography (TLC) was conducted in solvent system C (toluene:acetic acid, 85:15 mL) according to Orange et al. (2001). Final

confirmation of the species was made using relevant taxonomic keys and descriptions provided in Awasthi (1991; 2007).

Isolation and identification of endolichenic fungi

The thalli of Pyxinecocoes were surface sterilized following the standard protocol described by Guo et al. (2003). The sterilized thalli were then cut into small sections and air-dried under sterile conditions. These fragments were placed on three different culture media—Potato Dextrose Agar (PDA), Malt Extract Agar (MEA), and Water Agar (WA)—each supplemented with 0.01% streptomycin sulfate to inhibit bacterial contamination. The plates were incubated at 28 ± 2 °C in a BOD incubator until the emergence of endolichenic fungal colonies. Identification of the isolates was based on macroscopic colony morphology and reproductive structures, using standard mycological manuals (Barnett and Hunter, 1998; Gilman, 1971). Pure cultures of the identified fungi were maintained on PDA slants and stored at 4 °C for further analysis.

Endolichenic fungi diversity data analysis

The relative colonization frequency (CF %) of endolichenicspecies was calculated using the same formula as applied to endophytic fungi:

$CF\ \% = (N_{col} / N_t) \times 100$

Where, N_{col}stands for the number of segments colonized by each endolichenic fungal species, and N_t stands for the total number of segments plated (Hata and Futai,1995; Tayung and Jha 2006).

Endolichenic fungi cultivation and secondary metabolites extraction

Endolichenic fungal isolates were initially cultured on Potato Dextrose Agar (PDA) plates to obtain pure and actively growing colonies. The plates were incubated at 28 ± 2 °C for a period of 7–10 days, allowing the fungi to develop distinct mycelial growth suitable for further processing. From these cultures, mycelial discs were carefully excised using a sterile cork borer and transferred into sterile 250 mL Erlenmeyer flasks containing 100 mL of Potato Dextrose Broth (PDB). The inoculated flasks were maintained under static culture conditions at 28 ± 2 °C for 21–30 days, providing adequate time for the fungi to proliferate and synthesize extracellular secondary metabolites within the broth.At the end of the incubation period, the entire culture volume was filtered through Whatman No. 1 filter paper to effectively separate the fungal biomass from the culture filtrate. The clarified filtrate, containing the dissolved metabolites, was subjected to solvent extraction using an equal volume of ethyl acetate. The mixture was vigorously agitated to ensure maximum transfer of metabolites into the organic phase and then allowed to settle for clear phase separation. The ethyl acetate layer was carefully recovered and concentrated to dryness using a rotary evaporator maintained at 40–45 °C to prevent thermal degradation of thermolabile compounds.

The resulting crude extract, containing the secondary metabolites produced by the endolichenic fungi, was collected in sterile vials and stored at 4 °C until further use in antimicrobial screening assays and other bioactivity evaluations.

Determination of Antimicrobial activity

The antimicrobial activity of the endolichenic fungal extracts was evaluated using the agar cup diffusion assay. Three clinically important human pathogens—Candida albicans (MTCC 227), Escherichia coli (MTCC 443), and Staphylococcus aureus (MTCC 737)—were used as test organisms. All strains were obtained from the Institute of Microbial Technology (IMTECH), Chandigarh, and cultured under recommended conditions prior to experimentation. Bacterial test pathogens were aseptically inoculated onto Muller Hinton Agar (MHA) plates, while C. albicanswas inoculated on Sabouraud Dextrose Agar (SDA). Each pathogen was uniformly spread across the surface of the respective media using sterile cotton swabs to ensure even lawn formation. Sterile cork borers (7 mm diameter) were employed to create wells in the agar plates.

The crude secondary metabolites obtained from endolichenic fungi were dissolved in dimethyl sulfoxide (DMSO) to prepare a final concentration of 1 mg/mL. These metabolite solutions were carefully dispensed into the agar wells. The inoculated plates were incubated at 36 ± 1 °C for 24 hours for bacterial pathogens and at 28 ± 1 °C for 48 hours for C. albicans, allowing sufficient time for the metabolites to diffuse and interact with the test organisms.Antimicrobial activity was assessed by measuring the clear zones of inhibition surrounding each well, indicating the sensitivity of the test pathogens to the fungal metabolites

RESULTS

Identification of lichen

Pyxinecocoes (Sw.) Nyl.. Bellara (Bilbao) 5: 108. 1857.

Description: Thallus foliose, corticolous, orbicular to suborbicular, 3–6 cm diam., pale grey, tightly adnate to the substrate; lobes linear, discrete, 0.5–1 mm wide, plane to concave, with diffused pruina mostly in the apical region; maculae marginal and laminal, distinct in the apical region, developing into pseudocyphellae and then into soralia; soralia orbicular, ellipsoid, linear or irregular in outline; soredia farinose to granular; lobes 110–220

µm thick; upper cortex paraplectenchymatous, 12–20 µm thick; medulla white; lower cortex brown to black, paler towards the margin, prosoplectenchymatous, 15–30 µm thick; rhizines ± dense, furcated. Apothecia not seen.

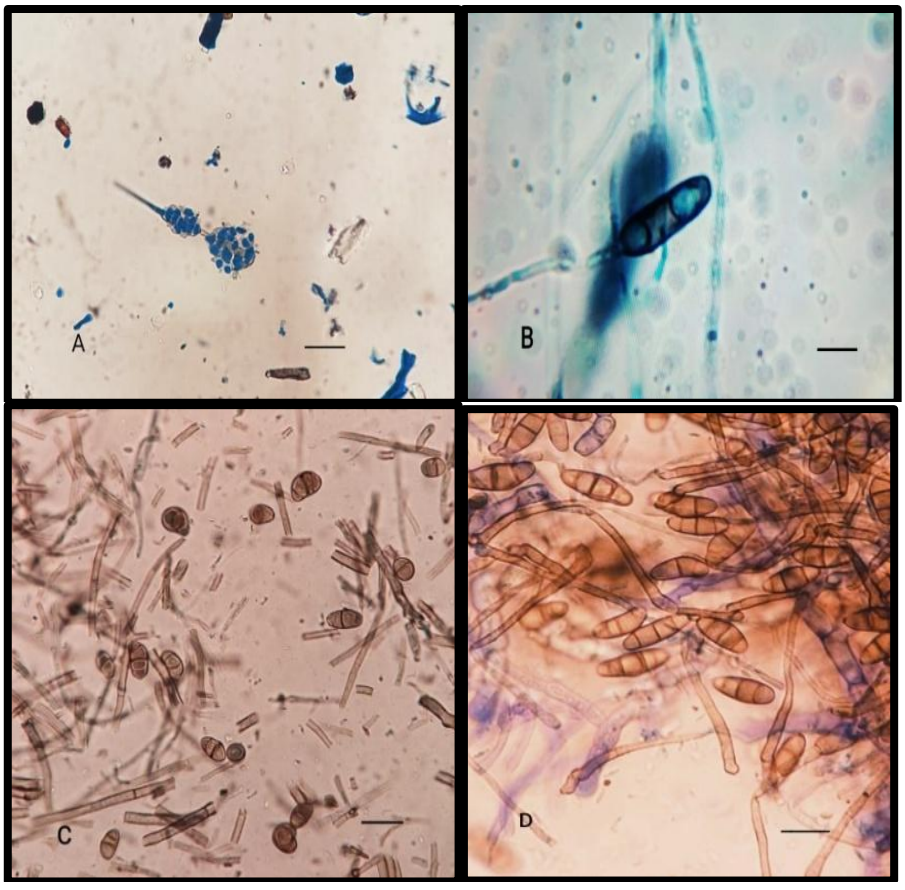
Chemistry: Spot tests: Cortex K−, C−KC−, P−, UV+ yellow; medulla K−, C−KC−, P−; TLC: lichexanthone.

Distribution: India (Assam, Goa, Karnataka, Kerala, Madhya Pradesh, Maharashtra, Manipur, Odisha, Rajasthan, Tamil Nadu, Uttar Pradesh and West Bengal); tropical and subtropical regions of the world[Singh and Sinha,2010](**Fig I**).

Figure I- Figure representing lichen thallus of Pyxinecoccoes (Scale bar =5mm)

Isolation of endolichenic fungi

A total of 47 numbers of isolates belonging to 4 different genera were recovered from surface sterilized lichen fragments of Pyxinecocoes(**Table I, Fig II**).The recovery rate in each of the media viz., PDA, MEA and WA were recorded, in which PDA represents the highest recovery rate from both the sites. The colonization frequency was 58.75%.Thoseendolichenic fungal isolates that did not sporulate in culture were termed as Mycelia sterilia and conventionally classified as Morphotypes. Morphotype 1 represents hyaline mycelium, light whitish green colony, Morphotype 2 is white spreading colony, mycelium thread like, Morphotype 3 represents cream slimy colony, Morphotype 4 represents light brownish colony, myecelium branched, Morphotype 5 represents white colony with septate hyphae and Morphotype 6 represents light orangish colony, appeareance of light orangish round like structures microscopically. Mycelia sterilia showed highest colonizing frequency (43.75 %). Out of the two regions, Dahung represents highest number of endolichenic fungal isolates.



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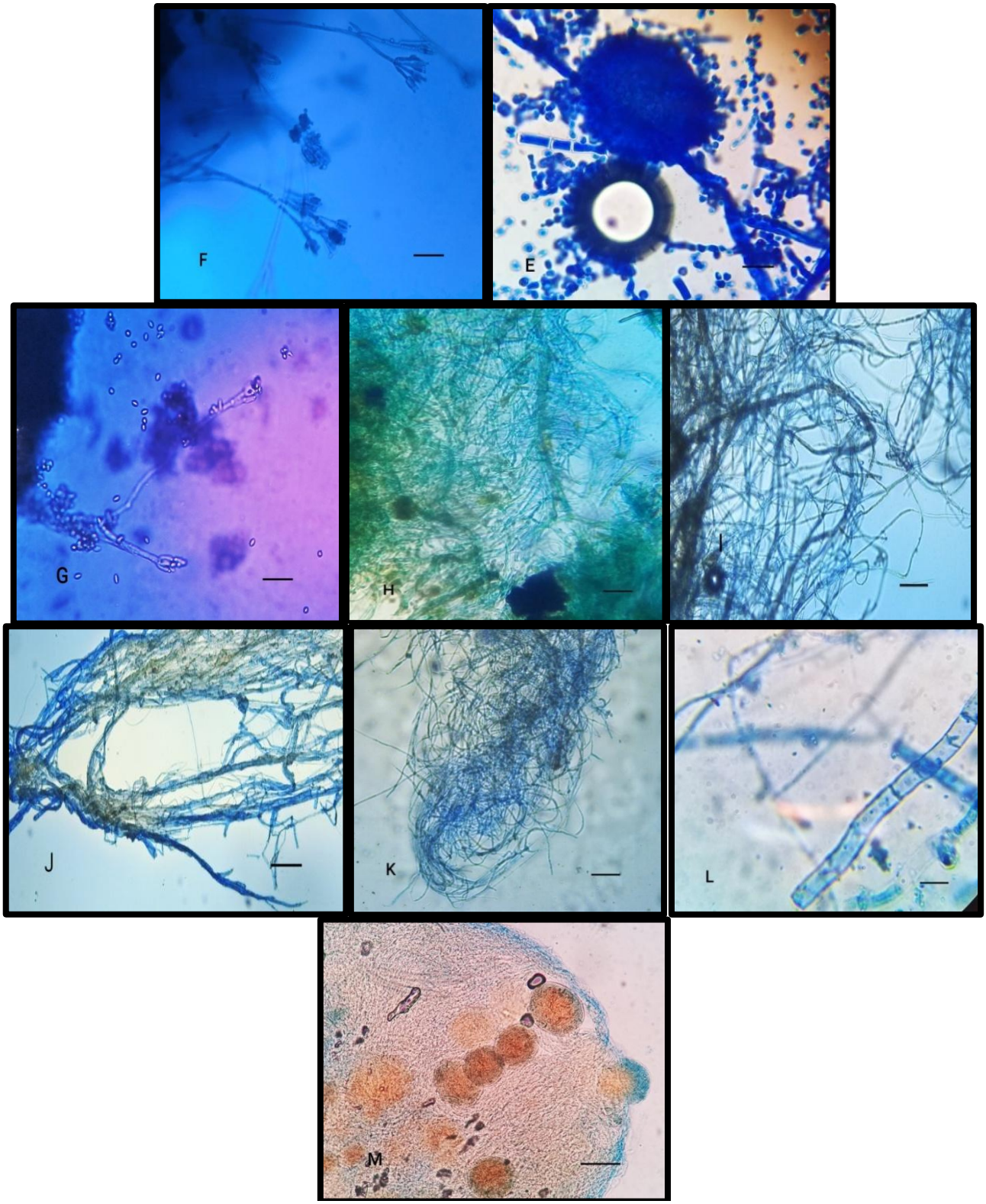

Fig II-Photoplates showing the microscopic view of endolichenic fungi isolated from Pyxinecocoes
A-Alternaria sp., **B-**Dreschlera sp.1, **C-**Dreschlera sp. 2, **D-**Dreschlera sp. 3,
E-Periconia sp., **F-**Penicillium sp. 1, **G-**Penicillium sp. 2, **H-** Morphotype 1,
I- Morphotype 2 ,**J-** Morphotype 3, **K-** Morphotype 4, **L-** Morphotype 5, **M-** Morphotype 6
(Scale bars- A- 30µm, **B-F-** 50 µm, **G-** 30 µm, **H-** 50 µm, **I-**100 µm, **J-**200 µm, **K-**100 µm, **L,M-**50 µm)

Table I- Comparative analysis of endolichenic fungal isolates isolated from Pyxinecoccoes collected from Dahung and Tipi

Endolichenic fungi	Location		Total isolates/80 fragments	CF%
	Dahung	Tipi		
Alternaria sp.	1	-	1	1.25
Dreschlera sp.1	2	-	2	2.5

Dreschlera sp.2	1	-	1	1.25
Dreschlera sp.3	-	1	1	1.25
Morphotype 1	6	-	6	7.5
Morphotype 2	3	-	3	3.75
Morphotype 3	-	4	4	5
Morphotype 4	-	5	5	6.25
Morphotype 5	9	-	9	11.25
Morphotype 6	-	8	8	10
Penicilliumsp.1	-	2	2	2.5
Penicillium sp.2	1	-	1	1.25
Periconia sp.	3	1	4	5
Total	**26**	**21**	**47**	**58.75**

Determination of antimicrobial activity

In this study, the crude extracts obtained from the culture filtrates of endolichenic fungi grown in Potato Dextrose Broth at 28 ± 2 °C demonstrated notable antimicrobial potential against the selected human pathogens. Out of the 47 endolichenic fungal isolates examined, 13 exhibited inhibitory activity against at least one of the test microorganisms (**Table II**). Overall, 69.2% of the active isolates displayed antifungal activity against Candida albicans, indicating a high prevalence of antifungal metabolite production. Additionally, 61.5% of the isolates were effective against both the Gram-positive bacterium Staphylococcus aureus and the Gram-negative bacterium Escherichia coli.

Among all the screened isolates, the two strains belonging to the genus Penicillium demonstrated the strongest and broadest antimicrobial activity, inhibiting the growth of all three test pathogens. This suggests that Penicillium spp. associated with Pyxinecocoes are particularly promising candidates for further investigation of bioactive secondary metabolites.

Table II- Preliminary antimicrobial activities of endolichenic fungal isolates against some pathogens

Endolichenic fungi	**Candida albicans (MTCC 227)**	**Staphylococcus aureus (MTCC 737)**	**Escherichia coli (MTCC 443)**
Alternaria sp.	+	+	-
Dreschlera sp.1	-	++	+
Dreschlera sp.2	-	+	-
Dreschlera sp.3	-	+	+
Morphotype 1	+	-	+
Morphotype 2	+	++	-
Morphotype 3	+	+	-
Morphotype 4	+	-	+
Morphotype 5	+	-	++
Morphotype 6	+	-	+
Penicilliumsp.1	**++**	**+++**	**-**
Penicillium sp.2	**-**	**++**	**+++**
Periconia sp.	++	-	++

(+) indicates zone of inhibition < 15 mm; (++) indicates zone of inhibition > 15 mm; (–) indicates empty cells.

DISCUSSION

Endolichenic fungi have been isolated from many lichen species indicating that they are ubiquitous in nature[Arnold et al., 2009;Triparthi and Joshi 2015,2019; Suryanarayan et al., 2017]. In the present study endolichenic fungi were isolated from Pyxinecoccoes, lichen commonly used in biomonitoring studies as it accumulates heavy metals. The results indicated that Pyxinecoccoes harbors endolichenic fungi and some of the isolates showed promising antimicrobial activity. This showed that this lichen can be explored for endolichenic fungi. To our knowledge, our study is the first report from India on the isolation of endolichenic fungi from Pyxinecocoes, as no further work has been reported till date. Further studies are needed to identify the active compounds produced in order to discover novel drugs which may be helpful to the mankind.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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