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RESEARCH ARTICLE

Antimicrobial and antioxidant properties of fungal endophytes associated with *Piper mullesua* Buch.-Ham. Ex D. Don.

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Abstract

Endophytes are microscopic organisms that live inside plants without causing disease. Among them, endophytic fungi are important biological resources known to produce pharmaceutically important metabolites. Several important bioactive metabolites of endophytic fungal origin have been identified and are being considered for large scale production. Their types and quantity vary from plant to plant; thus, they need to be investigated from new plant sources. *Piper mullesua* is a vital medicinal plant frequently used by indigenous populations of Arunachal Pradesh, India but not been examined so far. This investigation aims to identify fungal endophytes from the roots of *P. mullesua* and assess their antimicrobial and antioxidant activities. Total 5 endophytic fungi identified as *Colletotrichum gloeosporioides* (OR136407), *Fusarium oxysporum* (OR136409), *Fusarium solani* (OR136405), *Gliocladiopsis tenuis* (OR136407) and *Penicillium crustosum* (PP706771) were isolated from *P. mullesua* roots. Crude extract of *P. crustosum* and *G. tenuis* exhibited significant antimicrobial and antioxidant activities compared to others. *P. crustosum* was reported to produce a considerably higher zone of inhibition against pathogenic bacteria *Enterococcus faecalis*, *Escherichia coli*, *Proteus vulgaris* and *Staphylococcus aureus* with notably higher antioxidant activity than *G. tenuis.* The antioxidant activity index (AAI) and ferric reducing antioxidant power value were found to be 4.5 ± 0.01 and 936.68 ± 9.9 for *P. crustosum* and 1.02 ± 0.0 and 263.93 ± 10.9 for *G. tenuis* respectively. This is the first report on the antimicrobial and antioxidative properties of *G. tenuis*, highlighting the potential of endophytes as sources of pharmaceutically important metabolites. Bio-prospecting of fungal endophytes, thus, is an important task that may lead to new drug discovery.

Keywords: fungal endophytes; functional metabolites; *Piper mullesua*

Introduction

Plants have been known to establish friendly relationships with several microbes. One such important association is the relationship between plants and endophytes. The term "endophyte" (endo=within, phyton=plant) was coined by De Bary (1). Endophytes are microbes that inhabit the host plant with no signs of disease (2). This association between plants and endophytes is at least 400 million years old (3). Both bacteria and fungi can exist as endophytes, with fungi being more commonly encountered (4). Fungal endophytes are ubiquitous, having been reported in members of all divisions of the plant kingdom (5).

Endophytic fungi are a group of fungal communities that colonize host tissue intracellularly or intercellularly without causing any disease, rather making the host plant resistant to pathogens. An individual plant species may contain at least one or more endophytes, which may vary according to host conditions (6, 7). Endophytic fungi play a crucial role in plant development, their survival, form equilibrium and regulate some defence mechanisms. They secrete a range of bioactive compounds in plant cells when plants are under such stress to incorporate them in specific pathways (8). They secrete stress controlling compounds such as gibberellins, cytokinin, salicylic acid, indole-3-acetic acid etc., enhance plant growth and make many physiological changes to manage such stresses (9).

Currently, the healthcare system is facing serious challenges due to the increasing population, multiple drug resistance and increased prevalence of various diseases like cancer, cardiovascular disorders, diabetes, etc. Hence, biochemical and pharmaceutical industries are investigating new frontiers for therapeutic biomolecules (known or novel). The endophytic fungi associated with medicinal plants have shown primacy in demonstrating the ability to produce novel secondary metabolites such as alkaloids, flavonoids, terpenoids, lignans, coumarins, glycosides, quinones as well as essential steroids (10, 11). These fungi may serve as important natural sources of bioactive metabolites with promising therapeutic properties. Taxol and Podophyllotoxin are essential anticancer drugs, but their production is unsustainable. The Taxol required for one patient is obtained by purging four trees of *Taxus brevifolia* and in the case of Podophyllotoxin, the primary source, *Podophyllum emodi* is an endangered species (12, 13). Antimicrobial resistance (AMR) in pathogens is a serious issue, making treatment and

containment of microbial diseases difficult (14). Fungal endophytes can be utilized to overcome these issues, they have been reported to produce various medicinally functional metabolites (15). Endophytic fungi have been reported to produce Taxol and Podophyllotoxin (16, 17). They have also produced metabolites effective against multi-drug-resistant pathogens (18). Thus, an extensive investigation of fungal endophytes must be conducted to select potential fungal endophytes that can be utilized for the discovery, development and production of important therapeutic compounds in a sustainable way.

Traditional medicine practices widely use medicinal plants to manage various ailments. The World Health Organization (WHO) estimates that the majority of individuals in developing countries rely on medicinal plants for basic healthcare (19). *Piper mullesua* Buch.-Ham. ex D. Don. is an important indigenously used medicinal plant in Arunachal Pradesh, India, used locally by tribal communities, especially Nyishi people residing in the study area for various medicinal purposes. The fruits of *P. mullesua* are used to treat coughs and colds (20). Apart from the fruit, the whole plant has been reported to be used as a remedy for various health-related ailments in traditional Chinese medicine (21). Various essential oils with medicinal value have also been isolated from the roots of different *Piper* species (22). Given the importance of the *P. mullesua* species in indigenous and traditional medicine systems, this study aimed to isolate and investigate fungal endophytes from its roots to assess their antimicrobic and antioxidant potential.

Materials and Methods

Plant material

P. mullesua is a dioecious, erect shrub with creeping runner shoots. In this investigation, plant samples (roots) were acquired from Karsingsa village (27°07'29.8"N; 93°46'35.2"E) in Papum Pare district, Arunachal Pradesh, India. The identification of the plant sample was authenticated by the Botanical Survey of India (BSI), Itanagar. The sample herbarium was submitted to the BSI, Itanagar and the accession number (42668) was procured.

Processing of plant samples and isolation of fungal endophytes

Healthy plants without visible symptoms of disease were selected. The roots of the selected plants were collected and packed in clean plastic bags. Surface sterilization of the root segments was performed by the method of Gond et al. (23), with ethanol (70 %) and sodium hypochlorite (3 %) as surface sterilant. The sterilized root segments were cut into 5–10 mm sections and then plated onto Potato Dextrose Agar (PDA) plates with 60 mg/L of chloramphenicol and streptomycin each. A total of 100 root sections were inoculated onto PDA plates, with 10 sections per plate and incubated for one week at 28 ± 2 °C. Isolation frequency (IF %) was determined using the following formula (24):

$$IF\ \% = \left(\frac{\text{total number of fungi isolated}}{\text{Total number of plant segments examined}} \right) X\ 100 \quad \text{(Eqn. 1)}$$

Colonization frequency (CF %) was estimated by following equation (25):

$$CF\ \% = \left(\frac{Ncol}{Nt} \right) X\ 100 \quad \text{(Eqn. 2)}$$

Where, N_{col} = number of plant segments colonized by a fungus and N_t = number of segments examined.

Identification and phylogenetic analysis of fungal endophytes

Fungal endophytes were initially identified by examining their cultural and micro morphological characteristics (color, size, sporulation, etc.). Microscopic characters were observed by staining the fungal isolates with cotton blue stain and visualizing them under the compound research microscope (Zeiss Axiolab 5). The fungal endophyte genome's Internal transcribed spacer (ITS) region was sequenced for final identification and confirmation.

The gDNA was extracted by the method of Xia et al. (26). A one-week-old fungal mat developed on Sabouraud Dextrose Broth (SDB) was used for gDNA isolation. The extracted gDNA was subjected to PCR amplification using ITS1 and ITS4 DNA primers (27). BIO-RAD T100 PCR device was used to perform the PCR (28). The obtained PCR product was sequenced by the Sanger sequencing method (Eurofins Private Limited, Bengaluru, India).

The sequences of the fungal endophytes were subjected to the BLAST program of the National Centre for Biotechnology Information (NCBI) for identification and then deposited to the GenBank to acquire their accession numbers. The Maximum Parsimony (MP) method was used for Phylogenetic analysis in MEGA (version 11, USA) with a bootstrap value of 1000 (39). Tree modification and annotation was done using Interactive Tree Of Life (iTOL) (30).

Growth of fungal endophytes in different culture media and preliminary antimicrobial screening

Three different agar media, namely Sabouraud Dextrose Agar (SDA), Potato Dextrose Agar (PDA) and Czapek-Dox Agar (CZA), were screened to select the most suitable one for growth of selected fungal endophytes. The growth of the fungal endophytes on three different agar media was evaluated by measuring their colony diameter after 7 days. At the same time, the growth of the fungal endophytes was also assessed by growing them in 3 different liquid media, namely Sabouraud Dextrose Broth (SDB), Potato Dextrose Broth (PDB) and Czapek-Dox Broth (CZB). The fungal endophytes were cultured in 50 mL of broth media for 14 days. After 14 days, the fungal culture was filtered using Whatman no.1 filter paper to separate the fungal mycelium from the broth. The fungal mycelia was dehydrated at 55°C for 24 hours to obtain dry fungal biomass. After separating the fungal mycelia, the remaining liquid medium was extracted using ethyl acetate to obtain crude extract (31). Preliminary antimicrobial screening of the crude extracts obtained from the culture broths was performed by the disc-diffusion method against *Enterococcus faecalis* (MTCC439), *Escherichia coli* (MTCC40), *Proteus vulgaris* (MTCC426) and *Staphylococcus aureus* (MTCC96) (32). The final selection of the medium for large-scale

culturing was based on the assessment of fungal growth in solid media (colony diameter), biomass in broth media (dry weight of mycelia), the amount of crude extract obtained in different broths and the antimicrobial activity of the crude extracts of different broths.

Preparation of fungal crude extracts

Plugs from 7-day old, actively growing cultures of the fungal endophyte were inoculated into 1000 mL of SDB and kept at ambient temperature for 30 days to extract fungal metabolites. After 30 days, the fermentation broth was strained using filter paper and extracted using an equivalent quantity of ethyl acetate. The obtained organic phase was dried using rotary evaporator (IKA RV 10) (31). The dried crude extract was dissolved in ethyl acetate and filtered using Whatman filter paper no.1 to remove insoluble impurities. The liquid crude extract was then dried and kept in the refrigerator at -10°C for further analysis.

Antimicrobial activity of fungal endophytes

The dried crude extract of fungal endophytes was used to test antimicrobial activity. Antimicrobial assays were conducted against four test organisms: *E. faecalis* (MTCC439), *E. coli* (MTCC40), *P. vulgaris* (MTCC426) and *S. aureus* (MTCC96). The disc-diffusion method was used to assay antimicrobial potential (32). Five-millimetre Whatman filter paper No. 1 discs were impregnated with fungal crude extracts (10mg/mL) and air-dried under sterile conditions. The discs were placed onto agar plates seeded with the pathogens (1.5×10^8 CFU) and incubated at 37°C for a day. Bacterial inoculum with 1.5×10^8 CFU was prepared in sterile saline by adjusting the turbidity of the inoculum to that of 0.5 McFarland standard (33). Streptomycin (1 mg/mL) was used as a positive control for this test. After incubation, the antimicrobial activity of all fungal isolates was ascertained by observing and measuring the zone of inhibition (ZOI) around the disc using a measuring scale.

Antioxidant activity of fungal crude extracts

The antioxidative property of the crude extracts of the fungal endophytes were tested via DPPH radical scavenging activity (RSA) and Ferric Reducing Antioxidant Power (FRAP) assays.

DPPH radical scavenging activity

The antioxidant activity of the most active fungal crude extract (from the antimicrobial assay) was assessed using the radical scavenging activity (RSA) method of Kishan et al. (33), with slight modification. 0.1 mM solution of DPPH was concocted for the assay. The test was performed using a 96-well microplate, where the reaction mixture in each well consisted of 10 µL of crude extract, 90 µL of methyl alcohol and 100 µL of DPPH. The microplate was kept in the dark for 60 minutes, after which the absorbance reading was taken using a Thermo Scientific™ Multiskan GO at 517 nanometre. The RSA % was estimated by the formula (34):

$$RSA\ \% = \left(\frac{Ac - As}{Ac}\right) X\ 100 \qquad \text{(Eqn. 3)}$$

Ac and As are the absorbance of the control and the sample, respectively.

The IC_{50} value (the concentration exhibiting 50 % activity) was determined by using different concentrations (125, 62.5, 31.2, 15.6, 7.8, 3.9, 1.9, 0.9 µg mL^{-1}) of the crude extracts. The antioxidant activity index (AAI) was calculated using the following formula (34):

$$AAI = \frac{\text{Final concentration of DPPH (m/L)}}{IC_{50}\ (\mu g\ mL^{-1})} \qquad \text{Eqn. 4}$$

Finally, the antioxidant potential of the crude extract of the fungal endophytes was ascertained as per the AAI value criteria of Scherer and Godoy (34): poor activity if AAI < 0.5, moderate activity if AAI < 1.0, strong activity if AAI < 2.0 and very strong activity if AAI > 2.0.

Ferric reducing antioxidant power

The FRAP assay was performed by the method of Shukla at al. (35) with minor modification. The assay mixture comprised of 20 µL of fungal crude extract and 180 µL of FRAP reagent. The FRAP reagent was concocted by mixing 20 mL of 300 mM sodium acetate buffer (pH 3.6) with 2 mL of 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) and 2 mL of 20 mM ferric chloride. The assay mix was kept in dark for 30 minutes, after which the absorbance was recorded at 593 nm using a Thermo Scientific™ Multiskan GO. A standard curve was generated using varying concentrations of Ferrous sulphate (0-1000 µM). FRAP activity/result was expressed as Ferrous equivalent in µM.

Statistical analysis

One-way analysis of variance (ANOVA), followed by Tukey's HSD at 5 % significance level, was used to analyze the data.

Results

Isolation of fungal endophytes

In all, seven fungal endophytes were obtained from the roots of *P. mullesua* (Fig. 1). Their isolation frequency was calculated to be 7 %. These fungal endophytes were grouped into five morphotype based on their macroscopic and microscopic characters (Fig. 2) (Supplementary Table S1). Each fungal endophyte's colonization frequency (CF %) was calculated (Fig. 3). The CF % of fungal endophyte PM1 was found to be moderately high at 3 %, while it was recorded as lowest for fungal endophytes PM2, PM3, PM4 and PM5 (1 % each).

Identification and phylogenetic analysis of fungal endophytes

The gDNA of each fungal endophyte was extracted and the ITS region was amplified and sequenced (Supplementary Table S2). The acquired sequences were matched with data from the NCBI GenBank database for identification. Accession numbers were obtained by submitting the sequences of the identified fungal endophytes to NCBI GenBank (Table 1). The fungal endophytes were identified as *Fusarium oxysporum* (OR136409), *Fusarium solani* (OR136405), *Penicillium crustosum* (PP706771), *Gliocladiopsis tenuis* (OR136407) and *Colletotrichum gloeosporioides* (OR136407). A phylogenetic tree was created using MEGA (version 11, USA). Sequences of the five fungal endophytes and 25 reference fungi obtained

Fig. 1. Plant sample; (A) *Piper mullesua* plant (B) Root of *P. mullesua.*

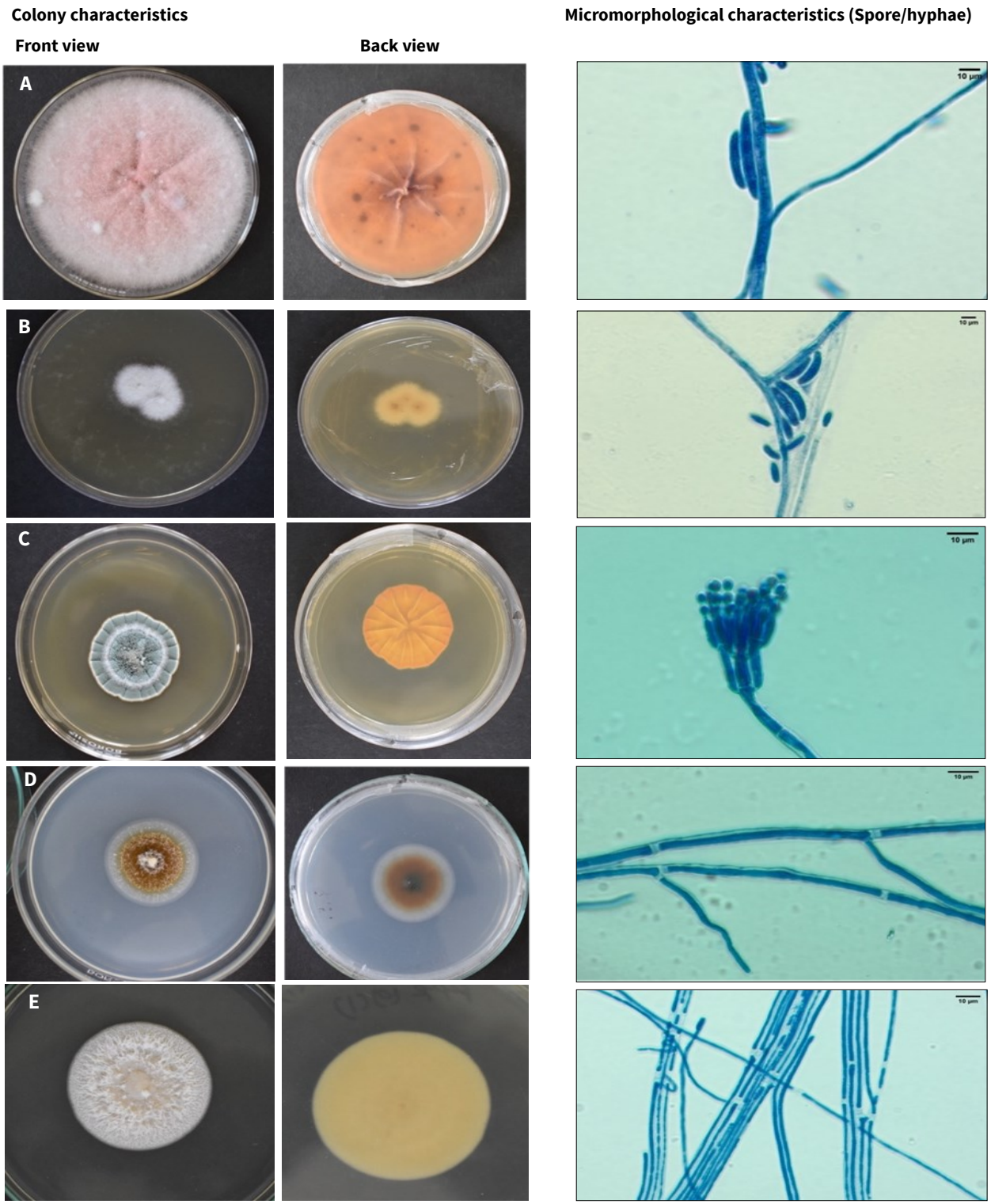

Fig. 2. Colony morphology of the fungal endophytes with micro-morphological characters (Hyphae/spore) (A) *Fusarium oxysporum*, (B) *Fusarium solani*, (C) *Penicillium. crustosum*, (D) *Gliocladiopsis tenuis* and (E) *Colletotrichum gloeosporioides.*

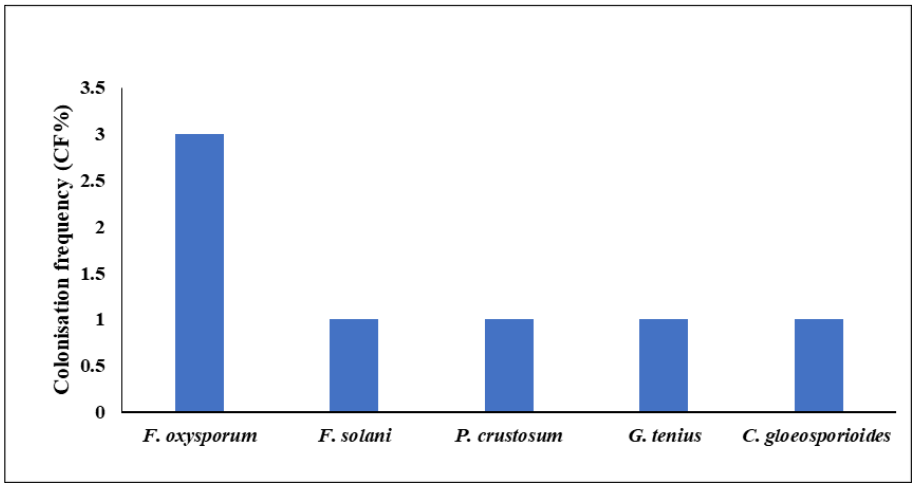

Fig. 3. Colonization frequency of fungal endophytes isolated from *P. mullesua* roots.

Table 1. Fungal endophytes with their GenBank accession number

Isolate code	Top hit taxon	Similarity	Accession number	Identification	No. of isolates
PM1	*Fusarium oxysporum* isolate	100 %	OR136409	*Fusarium oxysporum*	3
PM2	*Fusarium solani* isolate PN17	100 %	OR136405	*Fusarium solani*	1
PM3	*Penicillium crustosum* isolate PA20	100 %	PP706771	*Penicillium crustosum*	1
PM4	*Gliocladiopsis tenuis* isolate 580812	100 %	OR136407	*Gliocladiopsis tenuis*	1
PM5	*Colletotrichum gloeosporioides* strain A800	100 %	OR136408	*Colletotrichum gloeosporioides*	1

from NCBI were used for phylogenetic tree construction (Fig. 4). The tree showed that the fungal endophytes identified using ITS sequencing formed clades with identical or closely related taxa.

Growth of fungal endophytes in different culture media and preliminary antimicrobial screening

All fungal endophytes were cultured in different solid (SDA, PDA and CZA) and liquid (SDB, PDB and CZB) culture media. The colony diameter of fungal endophytes on agar media, fungal biomass, crude extract yield in liquid media and antimicrobial activity of oil extracts from different broths were evaluated to determine the most suitable culture medium. The colony diameter was the largest on SDA for all fungal endophytes (Fig. 5). The dry fungal biomass and crude extract yield were also the highest in SDB for the fungal endophytes (Table 2; Fig. 6). Crude extracts from two fungal endophytes, PM3 (*P. crustosum*) and PM4 (*G. tenuis*), exhibited antimicrobial activity, with the crude extract obtained from SBD being the most active compared to those from PDB and CZB (Table 3). Based on high fungal growth (in terms of diameter and biomass), high crude extract yield and antimicrobial activity of different crude extracts, the SDB medium was selected for further studies.

Antimicrobial activity of the fungal endophyte

Crude extracts of the fungal endophytes were assessed for their antimicrobial activity against four pathogens. The endophytic fungi *P. crustosum* and *G. tenuis* exhibited antimicrobial activity against all the pathogens tested (Fig. 7). At the same time, crude extracts of the other three fungal endophytes (*F. oxysporum*, *F. solani* and *C. gloeosporioides*)

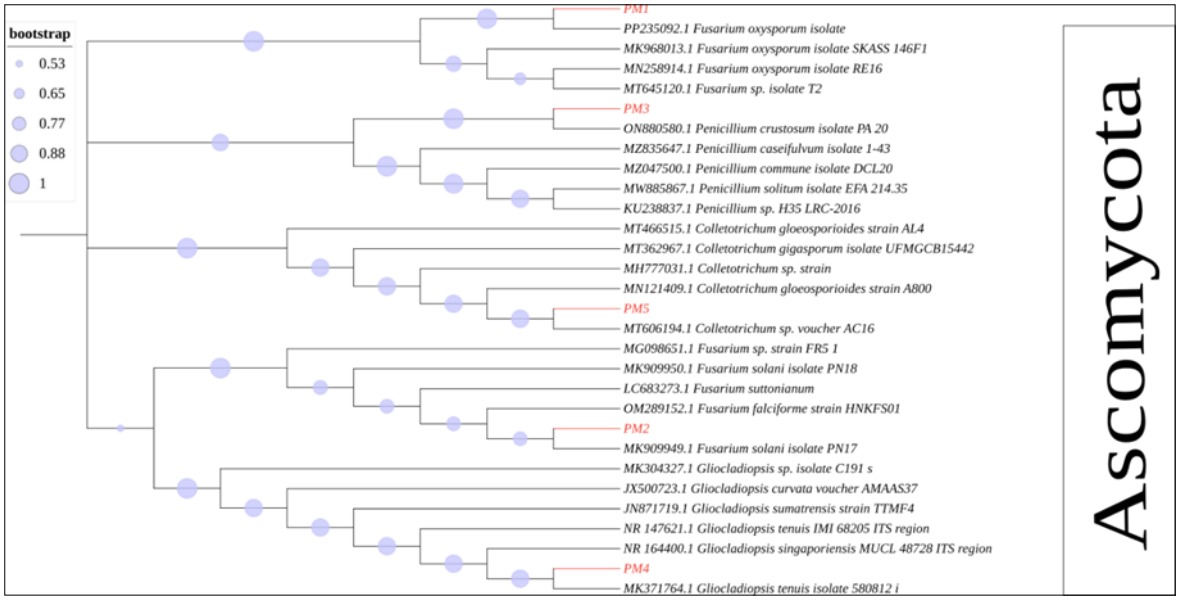

Fig. 4. Phylogenetic tree of isolated fungal endophytes using maximum Parsimony method (1000 Bootstrap replicates).

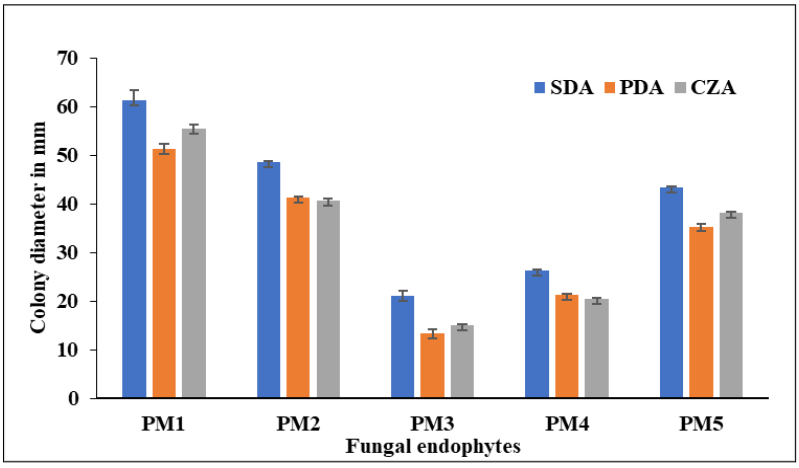

Fig. 5. Colony diameter of fungal endophytes in different agar media.

Table 2. Biomass of fungal endophytes in different liquid media

Culture Media	Weight of dry fungal biomass after 14 days of incubation (in mg)				
	PM1	PM2	PM3	PM4	PM5
Sabouraud Dextrose broth	357 ± 1.7a	221 ± 1.4a	286 ± 1.3a	185 ± 1.5a	194 ± 1.8a
Potato Dextrose broth	89 ± 3.1b	132 ± 3b	64 ± 2.3b	116 ± 0.1.2b	79 ± 1.2b
Czapek-Dox broth	145 ± 2.6c	88 ± 2c	204 ± 3.1c	75 ± 1.6c	82 ± 0.8b

The experiment was performed in triplicates. The values of mean of weight of dry biomass ±standard error is given, statistical analysis was performed by one-way anova followed by Tukeys test. Values followed by same letter in a column is not significantly different at $p<0.05$

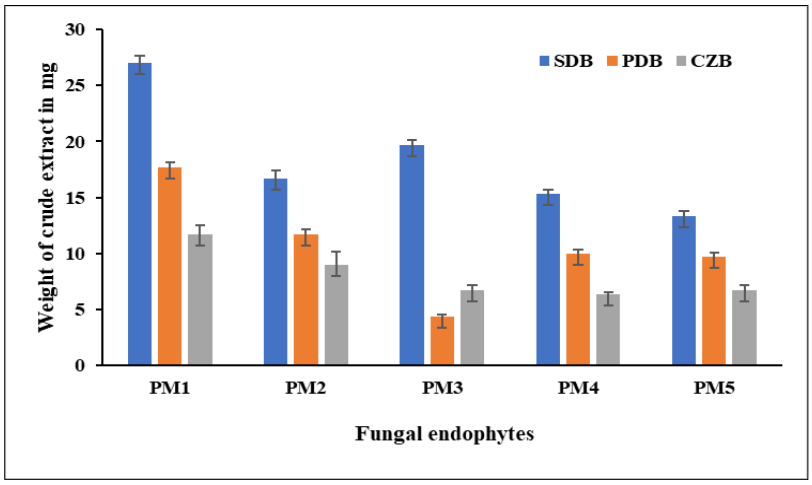

Fig. 6. Weight of dried extract of fungal endophytes in different liquid media.

Table 3. Preliminary screening of antimicrobial activity of fungal endophytes using crude extracts obtained from different liquid media

Crude extract	Pathogens	Fungal endophytes				
		PM1	PM2	PM3	PM4	PM5
SDB extract	***Enterococcus faecilis***	-	-	++	+	-
	Escherichia coli	-	-	++	++	-
	Proteus vulgaris	-	-	++	++	-
	Staphylococcus aureus	-	-	++	+	-
PDB extract	***Enterococcus faecilis***	-	-	-	+	-
	Escherichia coli	-	-	+	+	-
	Proteus vulgaris	-	-	+	+	-
	Staphylococcus aureus	-	-	+	-	-
CZB extract	***Enterococcus faecilis***	-	-	+	-	-
	Escherichia coli	-	-	+	-	-
	Proteus vulgaris	-	-	+	+	-
	Staphylococcus aureus	-	-	+	+	-

++: high activity; +: moderate activity; -: no activity.

failed to show any activity. The ZOI was measured to determine the antimicrobial activity (Table 4). The ZOI against *E. faecalis*, *E. coli*, *P. vulgaris* and *S. aureus* for endophytic fungi *Penicillium crustosum* (PM3) was measured as 13.67 ± 0.33 mm, 12.67 ± 0.33 mm, 14.0 ± 0.57 mm and 13.33 ± 0.33 mm. For *G. tenuis (PM4)*, it was recorded as 8.67 ± 0.33 mm, 10.67 ± 0.33 mm, 10.33 ± 0.33 mm and 9.33 ± 0.33 mm (Table 4). The antimicrobial activity of *P. crustosum* was higher than that of *G. tenuis* against all tested pathogens.

Antioxidant activity of fungal crude extracts

The crude extracts of two fungal endophytes, *P. crustosum* and *G. tenuis*, exhibited antimicrobial activity and were selected for the antioxidant assay.

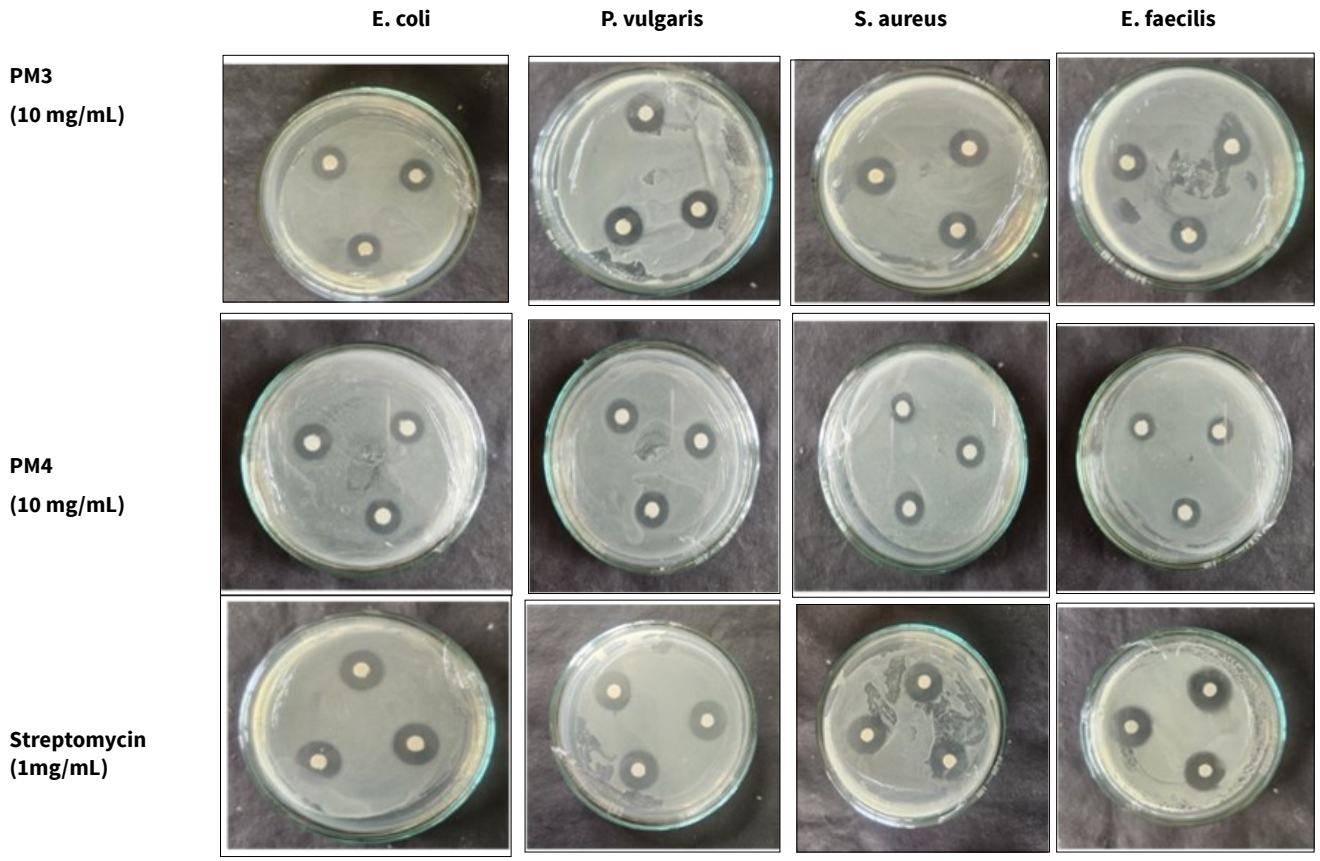

Fig. 7. Zone of inhibition exhibited by the crude extracts of the endophytes *P. Crustosum* (PM3) and *G. tenuis* (PM4).

Table 4. Antimicrobial activity of the crude extracts of fungal endophytes

Fungal endophyte	*Enterococcus faecilis*	*Escherichia coli*	*Proteus vulgaris*	*Staphylococcus aureus*
	Diameter of zone of inhibition in mm			
PM1	0	0	0	0
PM2	0	0	0	0
PM3	13.67 ± 0.33a	12.67 ± 0.33a	14.00 ± 0.57a	13.33 ± 0.33a
PM4	8.67 ± 0.33b	10.67 ± 0.33a	10.33 ± 0.33b	9.33 ± 0.33b
PM5	0	0	0	0
Streptomycin as Control (1mg/mL)	18.00 ± 1.15c	15.67 ± 0.67b	14.67 ± 0.33a	17.67 ± 0.33c

The experiment was performed in triplicates. The values of mean of diameter of ZOI ±standard error is given, statistical analysis was performed by one-way anova followed by Tukeys test. Values followed by same letter in a column is not significantly different at p<0.05.

DPPH radical scavenging activity

Crude extracts of both fungal endophytes displayed considerably high antioxidant activity (Fig. 8). The IC_{50} value for the crude extracts of *P. crustosum* and *G. tenuis* was 8.74 ± 0.02 and 39.23 ± 0.32 µg mL^{-1}, respectively (Table 5) (Supplementary Fig. S1-2). Both P. crustosum and G. tenuis extracts exhibited 'very strong' antioxidative activity. The AAI values of *P. crustosum* and *G. tenuis* extracts were 4.5 ± 0.01 and 1.02 ± 0.0, respectively (Table 5). According to the AAI value criteria, *G. tenuis* exhibited 'strong' antioxidant activity, while *P. crustosum* displayed 'very strong' antioxidant activity.

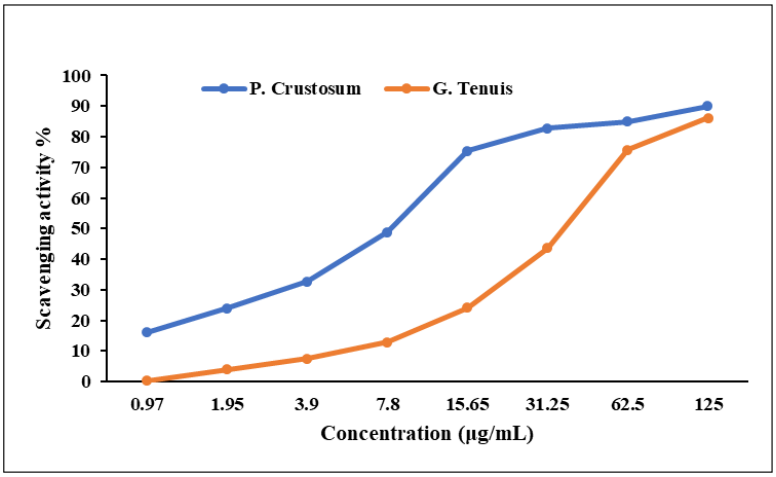

Fig. 8. Antioxidant activity of two fungal endophytes.

Thus, the crude extract of *P. crustosum* was more potent than that of *G. tenuis*.

FRAP assay

In the FRAP assay, crude extracts of the two fungal endophytes, *P. crustosum* and *G. tenuis* exhibited reducing ability (Table 5). The FRAP value was determined using the ferrous sulphate standard curve (Fig. 9). The FRAP value was found to be 936.68 ± 9 µM FE(II) equivalent for *P. crustosum* and 263.93 ± 10.9 9 µM FE(II) equivalent for *G. tenuis.*

Discussion

This is the first account of fungal endophytes isolated and investigated from the *P. mullesua* plant (Fig. 1). In this study, seven fungal endophytes were recovered from the roots of *P. mullesua*, resulting in a low isolation frequency of 7 %. A similar trend of low isolation frequency of fungal endophytes from other floras' roots has been previously observed (29). The CF % of *F. oxysporum* was found to be the highest (3 %), with the rest having comparatively lower CF % (1 % each) (Fig. 3). *F. oxysporum* has also been reported as a dominant fungal endophyte in the roots of other floras (36, 37). All seven isolated fungal endophytes in this investigation belonged to the taxonomic group Ascomycota (Fig. 4). Fungal endophytes are predominantly comprised of members of the Ascomycota group (38).

In this study, the SDA medium was found to be most suitable for the growth of all fungal endophyte strains and exhibited increased growth in terms of colony diameter (Fig. 5). SDA has previously been reported to support more significant mycelial growth (colony diameter) than PDA and CZA. SDA was the most suitable growth medium for *Aspergillus* sp. in two previous studies (39, 40). The SD broth medium also resulted in high mycelial biomass and high crude extract yield in all fungal endophytes (Table 2; Fig. 6). Similarly, high mycelial biomass for fungal endophyte *Xylaria* sp. was observed in SDB compared to PDB and CZB (41). Extracts of two fungal endophytes, *P. crustosum* and *G. tenuis*, exhibited antimicrobial activity, with the maximum activity observed for the crude extracts of SDB (Table 3). Based on the results of this study, the SD medium was selected for further culturing and analysis.

Bioprospecting is identifying diverse bioactive compounds of biological origin with potential for commercialization. Bioprospecting of fungal endophytes from medicinal plants has shown numerous promising pharmacological applications. Their functional metabolites have been reported to exhibit bactericidal, antimycotic, antioxidative, antiviral and antineoplastic properties (15, 42). The development of AMR in pathogens is a serious global issue, making the treatment of diseases difficult (14). Thus, exploring naturally produced biomolecules for novel drug development is crucial and fungal endophytes can be effectively utilized. In this study, two fungal endophytes, *P. crustosum* and *G. tenuis*, were active against all four test pathogens (Fig. 7). *P. crustosum* was more potent than *G. tenuis* (Table 4) among the two. *P. crustosum* has previously been reported to produce metabolites exhibiting antimicrobial properties, such as viridicatin and crustane (43, 44).

Free radicals are chemical entities that can exist independently with one or more unpaired electrons,

Table 6. Classification of antioxidant compounds based on IC_{50} value (48)

IC_{50} value (µg/mL)	Antioxidant activity
< 50	Very strong
50-100	Strong
101-250	Medium
250-500	Weak
> 500	No activity

Table 5. Antioxidant activity of the crude extracts of fungal endophytes

Fungal endophyte	IC_{50} value (µg/mL)	AAI	FRAP value µM FE(II) equivalent
P. crustosum	8.74 ± 0.02a	4.5 ± 0.01a	936.68 ± 9.9a
G. tenuis	39.23 ± 0.32b	1.02 ± 0.0b	263.93 ± 10.9b

Poor activity if AAI < 0.5, moderate activity if AAI < 1.0, strong activity if AAI < 2.0 and very strong activity if AAI > 2.0. The experiment was performed in triplicates. The mean values of the IC50 and AAI with standard error is given and statistical analysis was performed by one-way anova followed by Tukeys test. Values followed by same letter in a column is not significantly different at p<0.05.

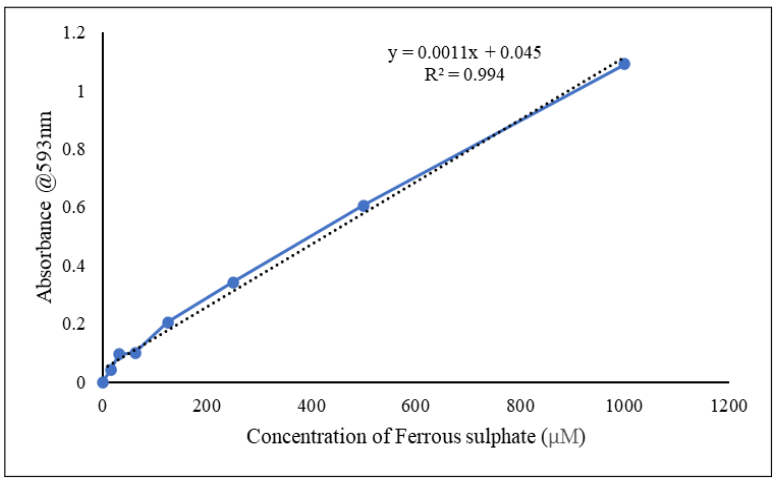

Fig. 9. Ferrous sulphate standard curve.

capable of causing oxidative damage by modifying biomolecules such as DNA, lipids and proteins (45). Antioxidants are essential compounds that neutralize free radicals. Fungal endophytes produce metabolites with strong antioxidant activity (42). Bioactive crude extracts of the two fungal endophytes, *P. crustosum* and *G. tenuis*, exhibited high antioxidant activity (Fig. 8). *P. crustosum* has been reported to possess antioxidative activity (46). Penifuranone A, a novel alkaloid with strong antioxidant activity, was isolated from crude extracts of *P. crustosum* by Jia et al. (47). Based on IC_{50} value criteria proposed by Kusumawati et al. (48), both *P. crustosum* and *G. tenuis* exhibited 'very strong' antioxidant activity according to the criteria (Table 6). A lower IC_{50} value denotes stronger antioxidant activity; thus, the antioxidant activity displayed by *P. crustosum* is stronger than that of *G. tenuis* (Table 5). Although the IC_{50} value varies with different concentrations of DPPH, the AAI value remains constant (34); therefore, in addition to the IC_{50} value, the AAI value was also determined to express antioxidant activity. According to the AAI value (Table 5), *P. crustosum* exhibited 'very strong' antioxidant activity, while *G. tenuis* exhibited 'strong' antioxidant activity. In the FRAP assay, the antioxidative property of a compound is tested by its ability to reduce Fe(III)-TPTZ to Fe(II)-TPTZ (49). A high FRAP value indicates higher reducing/antioxidant potential. In this study, crude extracts of *P. crustosum* were found to have a higher FRAP value than *G. tenuis* (Table 5). Thus, *P. crustosum* has a more significant reducing/ antioxidant potential than *G. tenuis.* In both the test (DPPH RSA and FRAP assay), extracts of *P. crustosum* were found to have higher antioxidant potential than *G. tenuis.*

The fungal endophyte *G. tenuis* isolated in this study showed comparatively lower antimicrobial and antioxidant activities. However, this is the first report of bioactivity (both antimicrobial and antioxidant) for this particular fungal species.

Conclusion

P. mullesua, an indigenous medicinal plant of Arunachal Pradesh, India, was investigated for its association with fungal endophytes and metabolites of biological significance. Seven fungal endophytes were isolated from the roots of *P. mullesua* and grouped into five morphotypes. They were identified as *Colletotrichum gloeosporioides* (OR136407), *Fusarium oxysporum* (OR136409), *Fusarium solani* (OR136405), *Gliocladiopsis tenuis* (OR136407) and *Penicillium crustosum* (PP706771). Among them, crude extract of *two fungal isolates, P. crustosum* and *G. tenuis,* exhibited significant antimicrobial and antioxidant properties. *P. crustosum* exhibited higher antimicrobial and antioxidant potential than that of *G. tenuis.* The crude extract of *P. crustosum* produced a larger zone of inhibition and exhibited higher AAI and FRAP values than *G. tenuis.* This is the first report of *G. tenuis* exhibiting such bioactivities. Fungal endophytes are important biological resources for producing pharmaceutically significant metabolites; however, further analysis may determine the specific compounds responsible for the activities. Due to their culturable nature, fungal endophytes can be utilized as sustainable sources of bioactive compounds. Therefore, bioprospecting of fungal endophytes might lead to the discovery of metabolites with pharmaceutical significance.

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Authors' contributions

CM performed the experiments, analysed the data and participated in the writing, reviewing and proofreading the research article. KS analysed the data and participated in writing, reviewing and proofreading the research article.

All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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