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RESEARCH ARTICLE

Metabolite profiling and bioactivity assessment of diverse endophytic fungi from the endangered plant, *Nilgirianthus ciliatus*

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Abstract

Endophytic fungi are potential sources of bioactive compounds with therapeutic properties. This study investigated the fungal endophytes associated with *Nilgirianthus ciliatus*, an endangered medicinal plant, to discover its secondary metabolites and bioactivities. Molecular analysis revealed the prominent species to be *Aspergillus niger, Didymella* sp., *Trichoderma viride*, *Bipolaris zeicola* and *Nigrospora sphaerica*. Alkaloids, flavonoids, phenolics, terpenes and saponins were detected in ethyl acetate extracts employing phytochemical screening. *Didymella* sp. has showed the highest level of antioxidant activity, demonstrating strong DPPH radical scavenging and reduction capability. *T. viride* had strong antibacterial action against *Klebsiella pneumoniae* and *Escherichia coli*, meanwhile *Didymella* sp. and *N. sphaerica* were most effective against *E. coli*. GC-MS analysis uncovered many bioactive chemicals, including trans-farnesol and pentadecanoic acid, which are renowned for their antibacterial and antioxidant properties. These findings highlight the presence of the rich variety of diverse endophytic fungi harboring such medicinal plants, which offer promising applications in medicine, biotechnology and agriculture as sources of novel bioactive compounds. Further exploration and characterization of these strains could unlock valuable sustainable resources for various industries.

Keywords

antibacterial; antioxidant; didymellanosine; endophytic fungi; *Nilgirianthus ciliatus*

Introduction

The potential uses of endophytic fungi and their metabolites in industry, agriculture and medicine have drawn a lot of attention to the study of these organisms in recent years. *Nilgirianthus ciliatus*, a fragrant plant from the Western Ghats that is internationally threatened and has numerous uses in Ayurveda, is one such plant of interest. There are many medical uses for Sahacharadi Thailam, a well-known Ayurvedic medication prepared from this plant (1). Despite the increasing focus on the bioactive components of *N. ciliatus*, there remain notable gaps in our understanding of its endophytes and their contributions to the plant's medicinal properties. Recent phytochemical screenings of *N. ciliatus* have identified several bioactive compounds, including stigmasterol, lupeol and betulin, which exhibit promising antioxidant, antibacterial and anticancer activities (2) . The significant potential of *N. ciliatus* in the creation of nanoparticles and their prospective uses as an antibiotic and

pesticide have been brought to light by recent investigations. When *N. ciliatus* leaf extracts were used to create selenium nanoparticles (SeNPs), they showed impressive inhibitory effects against a variety of diseases and pests (3). However, the degree to which endophytes contribute to the therapeutic features of *N. ciliatus*, particularly in the context of its specific environmental conditions and genetic makeup, remains largely uncharacterized. This gap in knowledge is crucial, as the interaction between endophytes and their host can considerably influence the accumulation of bioactive chemicals (4).

Numerous studies have demonstrated that endophytic fungi can maintain the health of host plants by competing with or killing different pathogenic bacteria, as well as by producing bioactive secondary metabolites that complement or mimic the activities of their host counterparts (5). Host plants provide endophytic fungi with the nutrients needed for survival and endophytic fungi reciprocate the host plants by promoting their growth, development and evolution in various ways (6)**.** These endophytes are one of the most inventive classes which produce secondary metabolites, many of which play a crucial role in alleviating human diseases. The medicinal property of the host plants is shaped by the host genotype, environment and endophytic fungal genotype affecting the host plant (7).

Endophytes have been found to play a crucial role in the sustainable biosynthesis of pharmaceutically important secondary metabolites as well as playing a vital role in plant growth, health and development in addition to guarding the plants against several biotic and abiotic stresses (8). Endophytic fungi, living within healthy plant tissues and organs, are vital sources of natural bioactive products and new microbial resources (9). They produce a variety of bioactive secondary metabolites, including alkaloids, polysaccharides, polyketones, terpenes, sterols, anthraquinones, flavonoids, xanthines, phenols, furandione and cyclic peptides (10). This study employs a methodical approach to investigate the endophytic population within *N. ciliatus* and its implications to enhance the plant's medicinal value, consequently contributing to the long-term production of bioactive chemicals for various applications. Although the therapeutic potential of *N. ciliatus* is widely documented, the involvement of its endophytic fungus in increasing its therapeutic value through the generation of bioactive metabolites has received little attention. This study aims to provide a thorough knowledge of these interactions, laying the groundwork for future research and applications in medicine, agriculture and biotechnology.

Materials and Methods

Field study & collection of samples

N.ciliatus is indigenous to the Southern Western Ghats of India and occurs naturally in a temperature range of 20°C to 35°C during the year. In India, it is distributed mostly in the states of Kerala, Karnataka and Tamil Nadu etc. The Transdisciplinary University (TDU) in Bengaluru has conserved this endangered plant through *in vitro* nodal culture. Mature and healthy whole plants of *N.ciliatus* were carefully collected in sterile bags from TDU.

Methodology for isolation and identification of endophytic fungi

Plant samples collected from TDU were washed and explants (including root, stem, leaf and flower) were surface sterilized by three washes with 70% ethanol for 30-45 s and 2% Sodium hypochlorite (NaOCl) solution for 30 to 60 s. On blot-drying, explants were inoculated on Sabouraud Dextrose Agar (SDA) media augmented with 1mg/ml of ampicillin to inhibit bacterial growth. 100 µl of the last wash water was spread plated onto control SDA plates to check for any contamination. All the culture plates were incubated in the dark at 27°C for 6 days to facilitate the growth and isolation of endophytic fungi (11), which were then subcultured to obtain pure cultures.

Morphological identification of endophytic fungi

The morphological features were identified by lactophenol cotton blue staining by observing fungal hyphae. The slide culture method was done using Sabouraud dextrose agar (SDA) to facilitate the observation of hyphae and sporangia in the fungal mycelia (12).

Molecular identification of endophytic fungi

DNA Isolation and Processing: DNA was isolated from the pure cultures of all the five endophytes and its quality was evaluated on a 1.0% agarose gel, revealing a single band of high-molecular-weight DNA. The ITS region fragment was then amplified by PCR, producing a single discrete PCR amplicon band of approximately 600 bp when resolved on agarose gel. This PCR amplicon was purified to remove contaminants (13). Forward and reverse DNA sequencing reactions of the PCR amplicon were performed using ITS1 and ITS4 primers with the BDT v3.1 Cycle sequencing kit on an ABI 3730xl Genetic Analyzer. A consensus sequence of the PCR amplicon was generated from the forward and reverse sequence data using aligner software.

BLAST analysis and phylogenetic construction

The ITS region sequence was then used to perform a BLAST search against the NCBI GenBank database. Based on the maximum identity score, the top ten sequences were selected and aligned using the ClustalW multiple alignment software program. Finally, a distance matrix and phylogenetic tree were constructed using MEGA 10 (14).

Metabolite isolation via solvent extraction

The fresh mycelia from the pure culture of endophytic fungi were inoculated in Sabouraud dextrose broth and incubated at 25°± 2°C in a rotary shaker for 10-15 days. The fungal biomass was separated by filtration through Whatman No. 1 filter paper. Solvent extraction of all five fungal endophytes was performed using a 1:1 solution of ethyl acetate: chloroform and separated using a separating funnel. The crude fungal extracts obtained were then refrigerated at 4°C until they were used for antioxidant and antibacterial assays (15).

Qualitative tests

Concentrated residues from the ethyl acetate extracts were used for the qualitative detection of secondary metabolites, including alkaloids, flavonoids, saponins, phenols, tannins and terpenoids as per the standard protocols (15).

Quantitative test for total phenolic content

To determine the total phenolic content, a mixture was prepared by combining 1.8 ml of Folin-Ciocalteu Reagent with 40 µl of the sample. This mixture was then diluted with distilled water and allowed to rest at 25°± 2°. After 5 minutes, a 7.5% sodium carbonate solution was added and the mixture was stirred and incubated at 25°C for 30 minutes in the dark. The absorbance was measured at 765 nm using a UV-Vis Spectrophotometer, with gallic acid serving as the calibration standard. The phenolic contents were expressed as milligrams of gallic acid equivalents (GAE) per gram of the sample, following the method described by (16).

Estimation of total flavonoid content

For the total flavonoid content, the modified Aluminum chloride colorimetric method was used (17). Briefly, 50 µl of methanolic extract was mixed with 150 µl of 80% methanol, 10 µl of 10% $AlCl_3$ and 10 µl of 1M Sodium acetate. The mixture was then incubated at 25°± 2°C for 45 minutes and the absorbance was taken at 415 nm.

Analysis of bioactive compounds using GC–MS chromatogram

The fungal ethyl acetate extract was subjected to Gas Chromatography-Mass Spectrometry (GC-MS) with model, SHIMADZU GCMS-QP2010 SE (Column: Rtx-5ms 30 m × 0.25 mm I.D., 0.25 µm). The instrument was properly connected and powered, with the GC column and inlet system set up. The GCMS solution Ver. 2.6 software was initialized. Samples were prepared according to analysis requirements (1 mg/mL sample diluted in hexane). A method was created in the GCMS solution software, setting parameters such as injection volume, temperature program and ionization mode. Samples were injected into the GC and mass spectra and chromatograms were acquired. The instrument was monitored throughout the run. The acquired data were analyzed using the software, with peaks identified and the compounds quantified and the reports were compared with the library data (18).

Antioxidant activity assays

The antioxidant potential of the endophytic fungal extracts was analyzed using two distinct assays *viz.* the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay and the reducing power assay. Each experiment was conducted in triplicate and the mean values were determined.

DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging activity: To assess the antioxidant activity of test samples, the change in the optical density of DPPH radicals was assessed. 0.1 mmol DPPH solution in 95% methanol was prepared. The fungal extracts were diluted to a concentration of 1 mg/mL with methanol and 0.1 mmol DPPH solution was made in 95% methanol. Subsequently, 1 mL of the 0.1 mmol DPPH solution was added to the test sample and incubated for 30 minutes at 25°± 2°C in darkness. After this incubation period, the absorbance was measured at 517 nm (15). The resulting free radical scavenging activity was expressed as a percentage as follows:

Inhibition of DPPH radical (%) = [(control absorbance - extract absorbance)/(control absorbance)]×100 (Eqn. 1)

Reducing power assay: The reductive potential of the extracts was tested using a modification of the method proposed by Chung et al.2014. In this procedure, different extracts and a standard solution (1 mg/mL) were mixed with methanol (1 mL) containing phosphate buffer (2.5 mL, 0.2 mol/L, pH 6.6) and potassium ferricyanide (2.5 mL, 1% w/v). After centrifugation for 10 minutes at 3000 rpm, the upper layer of the solution (2.5 mL) was combined with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1% w/v). The absorbance of the resulting mixture was measured at 700 nm using a spectrophotometer. A higher absorbance value indicated greater reductive potential. All experiments were conducted in triplicate and the results were reported as mean ± standard deviation (19).

Screening endophytic fungi for antibacterial properties

The antibacterial properties of endophytic fungi were evaluated against four pathogenic bacteria, including two gram-positive strains [*Enterococcus faecalis* (MTCC No. 439) and *Staphylococcus aureus* (MTCC No. 3160)] and two gram-negative strains [*Escherichia coli* (MTCC No. 443) and *Klebsiella pneumoniae* (MTCC No. 190)] using a slightly modified Agar well diffusion method. Fungal extracts were dissolved in Dimethyl Sulphoxide (DMSO) to prepare stock solutions at a concentration of 5 mg/ml. Overnight grown cultures of bacteria were spread plated on Mueller Hinton Agar medium. Wells of approximately 4 mm diameter were created and filled with varying concentrations of the endophytic fungal extract. Ampicillin and DMSO served as positive and negative controls, respectively. After 24 hours of incubation at 37 °C, the results were documented as the zones of inhibition in millimeters (20).

Determination of Minimum Inhibitory Concentration (MIC): Extracts demonstrating antimicrobial properties were further used to find out the minimum inhibitory concentration (MIC). This was achieved using the broth dilution method, where ethyl acetate extracts obtained from different endophytic fungi (5 mg/ml) were reconstituted in DMSO. Each dilution was then added into overnight grown bacterial cultures (*K. pneumoniae*, *E. coli, S. aureus and E. faecalis*) and serially diluted and incubated at 37 °C for 24 hours. Bacterial growth was monitored using a spectrophotometer at 620 nm. The MIC value was identified as the lowest concentration at which no turbidity was observed. All measurements were performed in triplicate (21).

Determination of Minimum Bactericidal concentration (MBC): From the tubes showing no visible growth (no turbidity), 50 µl of the culture was plated onto agar media. The plates were incubated at 37°C for 24 hours. After incubation, colonies on the plates were counted. The Minimum Bactericidal Concentration (MBC) was determined as the lowest concentration of the extract which resulted in a 99.9% reduction in colony-forming units (CFU) compared to the initial inoculum. The ratio of MBC/MIC was calculated to identify where the fungal extract is bacteriostatic or bactericidal.

Statistical analysis

Statistical analyses were conducted using one-way analysis of variance (ANOVA), followed by Duncan's Multiple Range Test

(DMRT), utilizing SPSS software version 29.0.2.0 for Windows. Results were presented as mean ± standard deviation (SD) for total phenol, total flavonoid, DPPH and ferric-reducing antioxidant power (FRAP) assays across all groups. A p-value of <0.05 was considered statistically significant.

Results

Isolation and identification of endophytic fungi

In our study, endophytic fungi were isolated from various plant parts of *N. ciliatus* and five of them were studied in detail as they showed significant bioactivity. The colony characteristics of the five fungal strains grown in SDA are depicted in Fig. 1. Molecular characterization via 18S rRNA analysis confirmed their taxonomic identity. The sequences were subsequently submitted to GenBank to obtain the accession numbers for all five fungal endophytes *viz. Aspergillus niger* (PQ187659), *Trichoderma viride* (PQ187660), *Didymella* sp (PQ187661), *Bipolaris zeicola* (PQ187677), *Nigrospora sphaerica* (PQ187680).

Phytochemical screening

Phytochemical analysis of the crude fungal extract revealed the presence of alkaloids, phenols, tannins, amino acids, carbohydrates, saponins, terpenes, flavonoids and sterols as described in Table 1. These chemical constituents are responsible for the different biological activities of the extracts. This information additionally highlights the medicinal prospects of these extracts owing to the ample amount of these bioactive compounds.

Total phenolic and flavonoid content

There was a wide range in the total phenolic concentration in the endophytic fungal extracts. The values varied from 0.684 to 1.139 mg GAE/g of dry weight. The highest concentration of phenols was observed in the extract of *Didymella* sp. 1.139 ± 0.02 followed by *Bipolaris zeicola* and *Nigrospora sphaerica* (1.094 ± 0.02 and 1.044 ± 0.19). *Trichoderma viride* and *Aspergillus niger* showed relatively less concentration of phenols *i.e.* 0.928 ±0.15 and 0.684 ±0.02 respectively.

The Total Flavonoid Content (TFC) of the tested fungal strains was within a range of 0.927 to 1.317 mg Quercetin Equivalents (QE) per gram of dry weight. Among the five strains, the endophytic fungus *Trichoderma viride* exhibited the highest TFC, registering at 1.317±0.04 mg QE/g of dry weight. This was closely followed by *Bipolaris zeicola* and *Didymella* sp., which demonstrated concentrations of 1.289±0.05 and 1.195 ±0.03 mg QE/g of dry weight, respectively. Meanwhile, *Nigrospora sphaerica* and *Aspergillus niger* displayed moderate flavonoid concentrations, with values reaching up to 1.012 ±0.1 and 0.927 ±0.07 mg QE/g of dry weight, respectively. These observations enhance our comprehension of the phenolic and flavonoid production abilities of these fungal strains.

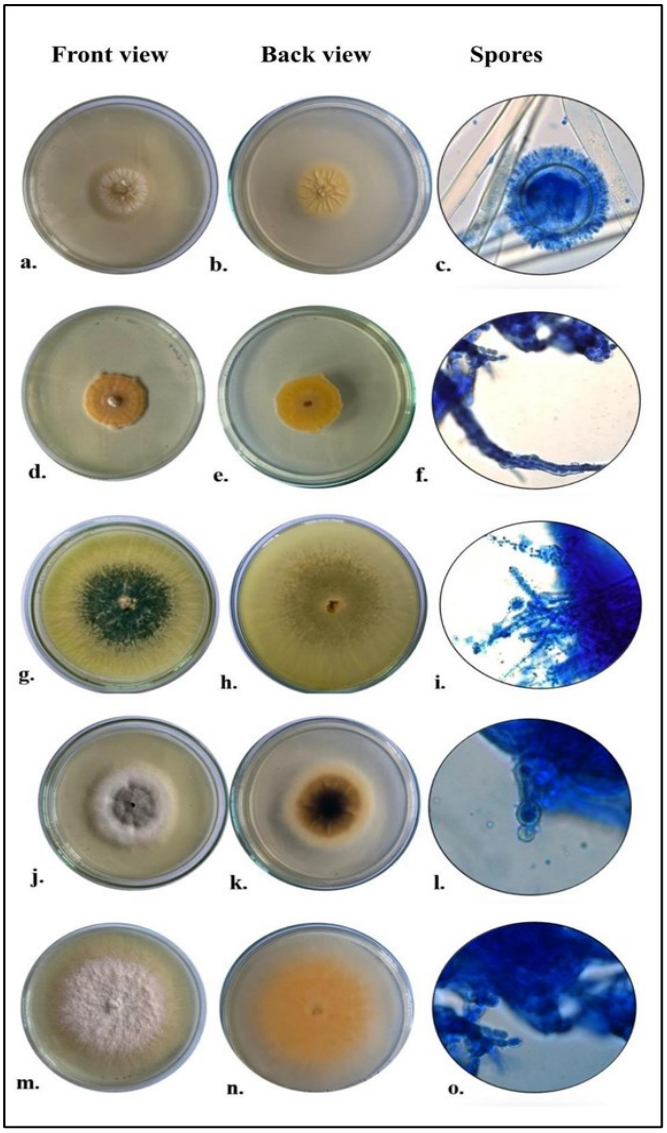

Fig. 1. Colony morphology and hyphal features of pure cultures of endophytic fungi isolated from *Nilgirianthus ciliatus* grown in SDA (a-c. *Aspergillus niger*; d-f. *Didymella* sp.; g- i. *Trichoderma viride*; j-l. *Bipolaris zeicola*; m- o. *Nigrospora sphaerica*).

GC-MS studies and bioactivity analyses of secondary metabolites

All five fungal extracts were analysed using GC-MS to detect important secondary metabolites. The bioactivity of the prominent chemical constituents of the ethyl acetate extracts of the endophytic fungi isolated from *Nilgirianthus ciliatus* are depicted in table 2. *Didymella* sp. produced distinct bioactive substances with antibacterial and anticancer qualities, such as didymellanosine, ascomylactam D and E and derivatives of 3,3-diindolylmethane (DIM). *Trichoderma viride* extract included isosorbide and cholesta-8,24-dien-3-ol, which have antioxidant and anti-inflammatory properties. *Nigrospora sphaerica* produces trans-farnesol, a potent antibacterial and quorum sensing inhibitor. *Bipolaris zeicola* was high in isoaromadendrene epoxide, a compound with significant antifungal properties. *Aspergillus niger* extract comprised

Table 1. Screening for secondary metabolites in the ethyl acetate extract of endophytic fungi of *Nilgirianthus ciliatus*

Fungal endophyte	Alkaloids	Flavonoids	Phenols	Saponins	Tannins	Terpenoids
Aspergillus niger	+	+	+	+	-	+
Didymella sp.	+	+	+	-	-	+
Trichoderma viride	+	+	+	-	+	+
Bipolaris zeicola	+	+	+	-	-	+
Nigrospora sphaerica.	-	+	+	+	-	+

Table 2. Bioactivity of the prominent chemical constituents of the ethyl acetate extracts of the endophytic fungi isolated from *Nilgirianthus ciliatus*

Compound name	Chemical formula	Bioactivity	References
Aspergillus niger			
Docosanoic acid, ethyl ester	$C_{24}H_{48}O_2$	Skin barrier repair and anti-inflammatory	(22)
1-Hexacosanol	$C_{26}H_{54}O$	Antimicrobial and antioxidant	(23)
Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	Antibacterial and larvicidal	(24)
Gamolenic acid	$C_{18}H_{30}O_2$	Anti-inflammatory and for dermatological applications	(25)
***Didymella* sp**			
9,12-Octadecadienoyl chloride	$C_{18}H_{31}ClO$	Repellent of mosquitoes and biting midges	(26)
Pseudo sarsasapogenin	$C_{28}H_{44}O_3$	Potential in drug discovery for steroid derivatives	(27)
Pentadecanoic acid	$C_{15}H_{30}O_2$	Protects cardiometabolic, immune and liver health; activates AMPK; inhibits mTOR	(28)
1-Undecene	$C_{11}H_{22}$	Inhibits mycelial growth of mushrooms, antimicrobial activity	(26)
9-Octadecenal	$C_{18}H_{34}O$	Antioxidant properties	(29)
Heptasiloxane	$C_{16}H_{48}O_6Si_7$	Larvicidal activity against mosquitoes	(30)
Trichoderma viride			
Oleic Acid	$C_{18}H_{34}O_2$	Anti-inflammatory, antioxidant and potential therapeutic agent for various conditions	(31)
2,5-Hexanediol	$C_6H_{14}O_2$	Suppresses chromatin motion and hyper-condenses chromatin in human cells	(32)
9-Octadecenal	$C_{18}H_{34}O$	Antioxidant	(29)
Trans-Z-.alpha.-Bisabolene epoxide	$C_{15}H_{24}O$	Potential use in agriculture as a biologically active natural product	(33)
Bipolaris zeicola			
2-tert-Butyl-4,6-bis(3,5-di-tert-butyl-4-hydroxy benzyl)phenol	$C_{40}H_{58}O_3$	Antioxidant	(34)
Malonic acid, dihydroxy-, diisobutyl ester	$C_{11}H_{20}O_6$	Competitive inhibitor of succinate dehydrogenase	(35)
Threitol, 2-O-heptyl-	$C_{11}H_{24}O_4$	Fungal signal promoting colonization	(36)
Sorbitol	$C_6H_{14}O_6$	Laxative, diuretic	(37)
Cyclooctyne	C_8H_{12}	Bioorthogonal reagent	(38)
Nigrospora sphaerica			
trans-farnesol	$C_{15}H_{26}O$	Antimicrobial	(39)
Oleic Acid	$C_{18}H_{34}O_2$	Anti-inflammatory, anticancer	(31)
Nonadecanoic acid	$C_{19}H_{38}O_2$	Antibacterial	(40)
1,2-benzenedicarboxylic acid, bis(2-methylpropyl) ester	$C_{16}H_{22}O_4$	Chemopreventive	(41)
n-capric acid isopropyl ester	$C_{13}H_{26}O_2$	Antimicrobial	(42)

bioactive lipids such gamolenic acid and E-11-hexadecenoic acid ethyl ester. These findings emphasise the numerous and pharmacologically relevant metabolites found in N. ciliatus' endophytic fungus, emphasising their potential as sources of new therapeutics.

Antioxidant activity

The antioxidant activity of the five fungal extracts were evaluated using two methods, yielding results ranging from 40% to 80%. *Didymella* sp. exhibited the highest antioxidant activity, with robust DPPH radical scavenging and strong reducing properties. Notably, extracts with higher antioxidant activity have higher total phenolic content, as measured by the Folin-Ciocalteu assay.

DPPH radical scavenging activity: DPPH radicals are stable, nitrogen-centered free radicals that produce a violet color in ethanol solution. When a substrate capable of donating a hydrogen atom is added to the DPPH solution, it is reduced to a yellow-colored product known as diphenylpicryl hydrazine. In this assay, the antioxidant nature of fungal extracts was evident by a color transition from purple to yellow. Among the samples tested, *Didymella* sp showed the highest activity of 77.83±1.2% and *B. zeicola* and *T. viride* exhibited robust antioxidant capacities of 68.08 ±1 and 69.07 ± 0.55 respectively. These results highlight the varying antioxidant potential of these samples as shown in fig. 2.

Reducing power assay: In the reducing power assay, the ability of compounds in the fungal extracts, to reduce Fe^{3+} to Fe^{2+} was checked (Fig.3). *T. viride and Didymella* sp exhibited higher absorbance values, indicating their strong reductive potential

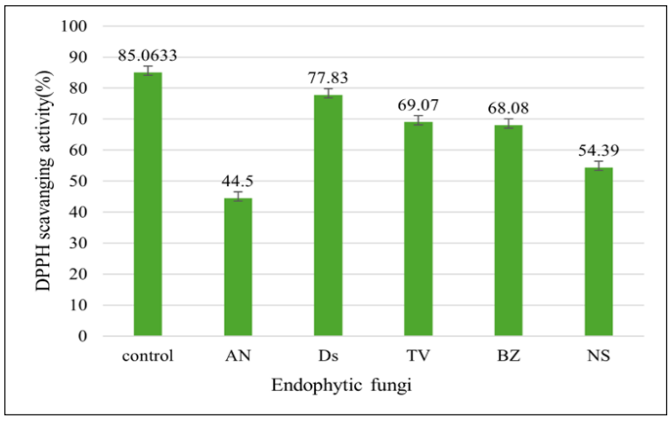

Fig. 2. DPPH radical scavenging activity of *Nilgirianthus ciliatus* endophytic fungal ethyl acetate extracts (AN- *Aspergillus niger*, Ds- *Didymella* sp, TV- *Trichoderma viride*, BZ- *Bipolaris zeicola*, NS- *Nigrospora sphaerica*).

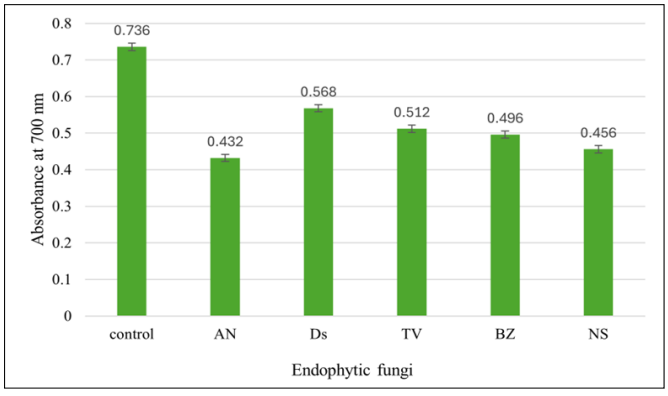

Fig. 3. Ferric-reducing antioxidant power (FRAP) activity of ethyl acetate extracts of the endophytic fungi of *Nilgirianthus ciliatus* (AN- *Aspergillus niger*, Ds - *Didymella* sp, TV - *Trichoderma viride*, BZ - *Bipolaris zeicola*, NS - *Nigrospora sphaerica*).

and electron-donating capacity for neutralizing free radicals. In contrast, *B. zeicola* and *N. sphaerica* demonstrated moderate reducing activity, while *A. niger* exhibited the least activity among all the fungal extracts. These measurements were performed in triplicates.

Screening endophytic fungi for antibacterial properties

When the endophytic fungal isolate extracts were checked for their antibacterial activity against two Gram positive bacteria - [*Enterococcus faecalis* (MTCC439) and *Staphylococcus aureus* (MTCC3160)] and two gram-negative bacteria [*Escherichia coli* (MTCC 443) and *Klebsiella pneumoniae* (MTCC 190)], *Didymella* sp. exhibited the highest inhibition (17 mm) against *E. coli*, followed by *Trichoderma viride* (14 mm) against *K. pneumoniae* as shown in Table 3. Minimum Inhibitory Concentration (MIC) values of the ethyl acetate extracts of the endophytic fungi isolated from *Nilgirianthus ciliatus* are depicted in table 4. The MIC values against *K. pneumoniae* ranged from 0.06 to 0.5 mg/ml, with *Bipolaris zeicola* having the lowest MIC (62.5 µg/ml), indicating resilient potency. *T. viride* had a MIC of 0.25 mg/ml for both *K. pneumoniae* and *E. coli*, whereas *Didymella* sp. and *Nigrospora sphaerica* had a MIC of 0.125 mg/ml for *E. coli*. Colony counts revealed greater resistance against *K. pneumoniae* and *E. coli*, implying that the extracts were more effective against Gram-negative bacteria. The MBC/MIC ratio for all extracts was less than 4, demonstrating their bactericidal properties.

Discussion

Nilgirianthus ciliatus is a shrub native to the Western Ghats with a lot of medicinal properties attributed to it, especially in curing diseases of the nervous and muscular systems. Since it depicts a plethora of bioactivity, it could be harboring a lot of microbial endophytes, which may play a role in imparting these properties. Hence the present study was intended to isolate and analyze the fungal endophytic flora colonizing this much sought-after medicinal plant.

After the isolation and initial screening of *N. ciliatus* for bioactive fungal endophytes, five were chosen for further studies. The highest concentration of phenolics was seen in the extract of *Didymella* sp., followed by *B. zeicola* and *N. sphaerica*. The results obtained from our research align with recent studies that have reported a high amount of phenolic content in certain groups of endophytic fungi. Interestingly, *T. viride*, which exhibited a moderate concentration of phenols, showed the highest TFC. This specifies that the biosynthesis of phenolic compounds and flavonoids may not be directly correlated. Different fungal strains may contain distinct metabolic pathways, benefitting the production of one type of compound over the other (43). Current research has shown that endophytic fungi are a significant source of natural products with remarkable bioactivities. The secondary metabolites of these fungi have been found to contain numerous antioxidant molecules (9) (44).

B. zeicola and *Didymella* sp. showed elevated levels of flavonoids, indicating their promise as suppliers of these important substances. There have been reports of a variety of plant pathogenic and saprobic species associated with a wide range of hosts in *Didymella sp*. (45). This aligns with recent research that has found multiple antioxidant compounds, such as flavonoids, in the secondary metabolites of endophytic fungi (46). *Aspergillus niger* showed moderate levels of phenols and flavonoids. This supports prior studies which have found a large variety of bioactive compounds in *A. niger*(47). The examination of bioactive compounds in endophytic fungi demonstrates a wide variety of substances such as alkaloids, phenols, tannins, amino acids, carbohydrates, saponins, terpenes, flavonoids and sterols (43). . *A. niger* is recognized for synthesizing various secondary metabolites that have been linked to diverse biological functions (47). On the other hand, *Trichoderma viride* is famous for its biocontrol abilities(44). The findings of our report align with these previous studies, suggesting the potential of *A. niger*, *Didymella* sp. and *T. viride* as sources of bioactive compounds. Our report's results are in agreement with prior studies, indicating that *A. niger, Didymella* sp. and *T. viride* have the potential to be used as sources of bioactive compounds. Positive outcomes were demonstrated for the existence of alkaloids, phenols, tannins, amino acids, carbohydrates, saponins, terpenes, flavonoids and sterols. This highlights the need for more research and understanding of these fungal strains for their possible uses in multiple sectors such as pharmaceuticals, agriculture and food.

Table 3. Antibacterial activity of ethyl acetate extracts of the endophytic fungi of *Nilgirianthus ciliatus*

Endophytic fungi	Zone of inhibition (in mm)					
	E. faecalis	*S. aureus*	*E. coli*	*K. pneumoniae*	Ampicillin	DMSO
Aspergillus niger	10	10	12	11	19	-
***Didymella* sp.**	11	13	17	10	18	-
Trichoderma viride	9	14	11	12	19	-
Bipolaris zeicola	11	15	13	12	19	-
Nigrospora sphaerica	9	10	11	10	17	-

Table 4. Minimum Inhibitory Concentration (MIC) of the ethyl acetate extracts of the endophytic fungi isolated from *Nilgirianthus ciliatus*

Endophytic fungi	Minimum Inhibitory Concentration (MIC)(mg/mL) of extract							
	E. faecalis		*S. aureus*		*E. coli*		*K. pneumoniae*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Aspergillus niger	0.25	0.5	0.25	1	0.25	0.5	0.25	0.5
***Didymella* sp.**	0.25	0.5	0.125	0.25	0.125	0.25	0.5	1
Trichoderma viride	0.5	1	0.5	1	0.25	0.5	0.25	0.5
Bipolaris zeicola	0.25	0.5	0.12	0.25	0.25	0.5	0.0625	0.125
Nigrospora sphaerica	0.5	1	0.25	1.0	0.125	0.25	0.25	0.5

Research into the antioxidant properties of the five fungal extracts, using two different methods, revealed a range of 40 to 80%. This aligns with recent studies finding multiple antioxidant molecules in the secondary metabolites of endophytic fungi (48). Moreover, it was noted that the fungal extracts with antioxidant properties also had a significant amount of overall phenols. Our research indicates that *B. zeicola* displays powerful antioxidant and antibacterial properties; important bioactive compounds discovered were isoaromadendrene epoxide and 9-octadecen-12-ynoic acid methyl ester. The findings align with recent studies on various *Bipolaris* species, which are recognized for their wide range of biological effects. Analyzing the mitochondrial genome of *B. zeicola* improves our knowledge of its genetic characteristics. Furthermore, substances such as 2-tert-butyl-4,6-bis(3,5-di-tert-butyl-4-hydroxy benzyl)phenol, isoaromadendrene epoxide, 9-octadecen-12-ynoic acid methyl ester and hexahydrofuro[3,2-b]furan-3,6-diol were detected. These possess antioxidant, anti-inflammatory, antimicrobial, anticancer and neuroprotective qualities, showcasing their potential for therapy (49). This was established utilizing a test reliant on the Folin-Ciocalteau reagent. The alteration in correlation hints at a possible link between the phenolic content and antioxidant activity of the extracts (50). *Didymella* sp. showed the highest DPPH radical scavenging activity of 77.83±1.2%; its GC-MS analysis identified compounds such as didymellanosine and ascomylactam D and E. Didymellanosine, isolated from Didymella sp. IEA-3B.1 showed strong activity against lymphoma and leukemia cell lines (45). This suggests that *Didymella* sp. has a high capacity for neutralizing free radicals (51). *Nigrospora sphaerica* showed moderate levels of antioxidants and had important antibacterial effects. Compounds such as trans-farnesol and oleic acid were identified through GC-MS analysis. Prior research has shown the antimicrobial capabilities of *N. sphaerica*, including bioactive substances like phomalactone which have a wide-ranging antimicrobial effect. Furthermore, *N. sphaerica* found in *Dillenia indica* exhibits notable antioxidant and antimicrobial characteristics(52).

During the reducing power test, *T. viride* and *Didymella* sp. both showed significant ability to reduce and donate electrons to counteract free radicals. Specifically, *T. viride* has been found to possess notable antioxidant characteristics. Trichoderma is a group of fungi that has a variety of uses in farming. In 2022, a review emphasized the characteristics and functions of secondary metabolites found in *T. harzianum*. This species is recognized for its production of diverse bioactive secondary metabolites, which are valuable for developing novel herbicides and antibiotics (53). Multicomponent microbial inoculants with *Trichoderma* were discovered to help control pathogens and promote plant growth (54). These studies indicate that *Trichoderma* species not only efficiently manage plant pathogens but also positively influence seed germination, seedling emergence and overall plant growth. In our research, *T. viride* displayed a 14mm zone of inhibition against *K. pneumoniae* and a MIC of 0.25 $mg.ml^{-1}$ against both *K. pneumoniae* and *E. coli*, highlighting its antibacterial effectiveness. Another endophytic fungus, *Didymella* sp., showed antimicrobial properties. Previous research on this endophytic fungus derived from the medicinal plant *Zanthoxylum simulans* showed that *Didymella* was among the six significant fungal genera with antimicrobial properties (55). Our research found that the ethyl acetate extract of *Didymella* sp. demonstrated a significant 17 mm zone of inhibition against *E.coli* and an MIC of 0.125 mg.ml-1 against *E.coli* and *S.aureus*, suggesting its promise as a supplier of antibacterial substances. *A. niger* is a famous type of fungus that has been researched for its capability to create bioactive substances. In 2023, a research paper identified six substances from *A. niger*, with some displaying potential as antibiotics against *Staphylococcus aureus* and *Bacillus subtilis*(18). Another study in 2024 reported the antioxidant and antibacterial activities of endophytic fungi isolated from the roots of *Taxus wallichiana*, including *Aspergillus* sp. (56). In our study, *Aspergillus niger* showed the least activity towards almost all the strains at 0.5 $mg.ml^{-1}$ concentration, suggesting that its antibacterial potential may vary depending on the specific strain and extraction method used.

The present study could thus reveal the endophyte richness of the endangered plant *Nilgirianthus ciliatus*. The five prominent endophytic fungi *viz. Trichoderma viride, Didymella* sp*., Bipolaris zeicola, Nigrospora sphaerica* and *Aspergillus niger*, isolated from this plant, has multiple bioactivities including the demonstrated antioxidant and antibacterial activities. These findings emphasize the importance of exploring endophytic fungi as a source of new antimicrobial compounds.

Conclusion

Our investigation on the diverse endophytic fungi isolated from *N. ciliatus* has demonstrated substantial bioactive potential with *Didymella* sp. exhibiting the highest phenolic content and DPPH radical scavenging activity and *Trichoderma viride* showing the highest total flavonoid content and significant reducing power. The results align with recent research on similar endophytes, highlighting endophytic fungi as promising sources of novel bioactive compounds. Since, *N. cilautus* from which these endophytes were isolated has been implicated in multidimensional roles in curing diseases of the neuromuscular system, more detailed studies on the effect of these fungal metabolites on the proteins involved in these diseases should be analyzed in future. A comprehensive characterization of these bioactive compounds and elucidation of their modes of action will throw more light on ways to make use of these bioresources. Additionally, understanding the biotechnological applications of these endophytic fungi, such as the development of novel antimicrobial agents and natural antioxidants, could be a significant advancement in both medical and agricultural fields.

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Authors' contributions

SS conceptualized the study and PP performed the isolation and characterization, including bioactivity studies. SS and PP wrote and edited the manuscript.

Compliance with ethical standards

Conflict of interest: The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this research article.

Ethical issues: None

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