Journal of the Marine Biological Association of the United Kingdom

cambridge.org/mbi

Isolation, identification, and antibacterial activity of endophytic fungus from *Acanthus ilicifolius*

V. P. Sruthi[1,2,3], K. R. Sreenath[1], T. G. Sumithra[1], V. N. Anusree[1] and P. Sainamole Kurian[3]

[1]Marine Biodiversity and Environment Management Division, ICAR-Central Marine Fisheries Research Institute (CMFRI), Kochi, Kerala, India; [2]Department of Ocean Science & Technology, Kerala University of Fisheries & Ocean Studies (KUFOS), Panangad, Kerala 682 506, India and [3]Department of Plant Pathology, Kerala Agricultural University (KAU), Vellanikkara, Thrissur, Kerala, India

Research Article

Cite this article: Sruthi VP, Sreenath KR, Sumithra TG, Anusree VN, Sainamole Kurian P (2024). Isolation, identification, and antibacterial activity of endophytic fungus from *Acanthus ilicifolius*. *Journal of the Marine Biological Association of the United Kingdom* **104**, e41, 1–10. https://doi.org/10.1017/S0025315424000286

Received: 14 July 2023
Accepted: 18 February 2024

Keywords:
antibacterial activity; endophytic fungi; mangrove fungi; mangrove-associated fungi; phylogeny

Corresponding author:
K. R. Sreenath;
Email: sreenath.ramanathan@icar.gov.in

Abstract

Antimicrobial-resistant bacteria pose serious public health risks, necessitating bioprospecting for novel antimicrobial drugs. The endophytic fungi of the mangrove ecosystem are hotspots for discovering new bioactive chemical compounds. In this context, an investigation was designed to determine the isolation of the major endophytic fungi inhabiting the leaves of *Acanthus ilicifolius*, a mangrove plant with a long history of traditional use in the Chinese and Indian medical systems. Based on the morphological characterizations and molecular analysis of internal transcribed spacer rDNA sequence data, the study identified three unique endophytic fungal species, namely, *Periconia macrospinosa*, *Coprinopsis cinerea*, and *Alternaria* sp. Of these, *P. macrospinosa* was identified as the most dominant one, with the highest relative frequency (35.22%). The antibacterial activity of *P. macrospinosa* isolate (CMFRI/fPM-01) was evaluated by the well and diffusion method against six human pathogens, *viz.*, *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*. The results demonstrated a high and wide spectrum of antimicrobial action of the isolate against all the tested human pathogens, with no significant difference ($P > 0.05$) in the activity between the pathogens. The antibacterial activity was further confirmed by determining the fungal culture supernatant's minimum inhibitory concentration and minimum bactericidal concentration. Although the studied fungi are known from other sources, this is the first report of *P. macrospinosa* and *C. cinerea* as endophytes in *A. ilicifolius* leaves. The outcomes also showed that the *P. macrospinosa* isolate could be used to discover effective antibacterial drugs against various human diseases.

Introduction

Novel agents displaying antimicrobial activity are urgently needed to tackle the public health hazards posed by the global spread of antimicrobial-resistant bacterial pathogens (Miethke *et al.*, 2021). Bioprospecting studies of endophytic microbes for antimicrobial activity are fundamental for discovering novel human antimicrobial agents (Strobel and Long, 1998; Strobel, 2002; Strobel and Daisy, 2003). Around the world, investigations are being carried out to explore endophytic fungi of various plants to discover new, potentially valuable secondary metabolites (Tiwari and Bae, 2022). Among different plants, mangrove plants are attractive biodiversity hotspots for prospecting novel bioactive compounds, including those having potential medicinal applications (Cadamuro *et al.*, 2021). As endophytic fungi of plants can produce similar biologically active constituents similar to their hosts (Khan *et al.*, 2017), mangrove-associated fungi have received great attention in the past two decades. The mangrove ecosystem is a significant source of novel fungal strains, constituting the second-largest ecological category of marine fungi (Li *et al.*, 2008). Accordingly, worldwide, several mangrove species, such as *Avicennia officinalis*, *Avicennia marina*, *Acanthus ilicifolius*, *Aegiceras corniculatum*, *Arthrocnemum indicum*, *Bruguiera gymnorrhiza*, *Ceriops decandra*, *Excoecaria agallocha*, *Kandelia candel*, *Lumnitzera racemosa*, *Rhizophora mucronata*, *Rhizophora apiculata*, *Sesuvium portulacastrum*, *Suaeda fruticosa*, *Suaeda maritima*, and *Sonneratia caseolaris* have been investigated for their endophytic fungus relationships (Kumaresan and Suryanarayanan, 2001; Ananda and Sridhar, 2002; Chi *et al.*, 2019; Yanti and Anwarrudin, 2021). These studies revealed that each mangrove plant's dominant endophytic fungi species were specific, although many common endophytes existed in several hosts (Kumaresan and Suryanarayanan, 2001). Furthermore, the fungi in mangrove plants are shown as a mixture of soil, marine, and freshwater fungi (Ananda and Sridhar, 2002). Chi *et al.* (2019) and Yanthi and Anwarrudin (2021) investigated the potential antimicrobial and anti-inflammatory activities of six fungal species isolated from *A. ilicifolius*, namely, *Corynespora cassiicola*, *Phellinus noxius*, *Xylaria* sp., *Geotrichum* sp., *Humicola* sp. and *Aspergillus* sp. The findings suggested that endophytic fungi associated with *A. ilicifolius* leaves and stems could be a source of several novel active substances.

India's mangroves are well recognized for their remarkable biodiversity and spatial spread. India's mangroves comprise a total area of 4740 km^2, representing ~3% of the world's vegetated area (FSI, 2019). On the west coast of India, the mangroves of Kerala constitute one of the most critical ecosystems (Sreelekshmi *et al.*, 2021). *A. ilicifolius*, belonging to Acanthaceae, is one of the most common mangrove species in Kerala (Sreelekshmi *et al.*, 2021) and is widely utilized in traditional medicine for a variety of medicinal uses (Ragavan *et al.*, 2015; Saranya *et al.*, 2015). It has been established that this plant is an abundant source of alkaloids, long-chain alcohols, flavonoid glycosides, benzoxazinoid glucosides, megastigmane glucosides, triterpenes, triterpenoid saponins, and steroids, especially stigmasterol (Bandaranayake, 2002; Bai *et al.*, 2014). The plant showed several medicinal properties, *viz.*, antioxidant, hepatoprotective, anticarcinogenic, cytotoxic, antimicrobial, anti-inflammatory, anti-ulcer, and antibacterial activities (Zhang *et al.*, 2005), pointing out the high opportunity for finding endophytic fungi with high bioactivities. *Phomopsis* sp. and *Colletotrichum* sp. are reported as the dominant fungal endophytes of *A. ilicifolius* (Suryanarayanan and Kumaresan, 2000; Chaeprasert *et al.*, 2010). Further, three independent studies in Indonesia, Thailand, and Taiwan demonstrated that endophytic fungi affiliated with *A. ilicifolius* possess strong antimicrobial actions (Chaeprasert *et al.*, 2010; Chi *et al.*, 2019; Yanti and Anwarrudin, 2021). The endophytic fungus of *A. ilicifolius* from the genera *Geotricum*, *Humicola*, and *Aspergillus* exhibited a high *in vitro* antagonistic activity against harmful bacteria such as *Escherichia coli* and *Staphylococcus aureus* (Yanti and Anwarrudin, 2021). Chi *et al.* (2019) revealed that endophytic fungi from *C. cassiicola* and *Xylaria* sp. have potent antimicrobial activity against *Bacillus subtilis*, *S. aureus*, and *E. coli*. According to Chaeprasert *et al.* (2010), *Xylaria* sp. found in the leaves of *A. ilicifolius* from Thailand possesses extensive antibacterial activities. These results imply that a range of biologically active endophytic fungi may exist in *A. ilicifolius*. However, only a few studies have identified biologically active endophytic fungi associated with the Indian mangrove trees (Ananda and Sridhar, 2002; Maria and Sridhar, 2003; Priyadharshini *et al.*, 2015). In this context, the present study analyses the cultural diversity of the fungal endophyte assemblage on the healthy leaves of *A. ilicifolius* established on the mangrove habitat of the west coast of India, with an ultimate aim to elucidate their potential antimicrobial applications.

Materials and methods

Sample collection

The leaves of two apparently healthy mature trees of *A. ilicifolius* (Figure 1A) were collected from Thevara (9.924348705°N, 76.28840469°E) on the south coast of Kerala, India, during the pre-monsoon season (April and May 2021). The physicochemical parameters at the sampling site were recorded as follows: salinity: 25 ppt, temperature: 31°C, pH: 7.8, and dissolved oxygen: 2.8 mg l^{-1}. The samples were placed in sterile sampling bags, placed in clean thermocol boxes with ice packs, brought to the lab (Cha *et al.*, 2021), and analysed within 24 h of collection.

Isolation and enumeration of endophytic fungi

The trituration procedure was used to prepare the samples to isolate endophytic fungus (Kloepper *et al.*, 1999). The leaves were sequentially washed in 70% ethanol for 1 min and 4% sodium hypochlorite solution for 1 min to sanitize the surface. The leaves were then rinsed thrice in distilled sterile water. To test the efficacy of the surface disinfection step, the disinfected samples were cultured on

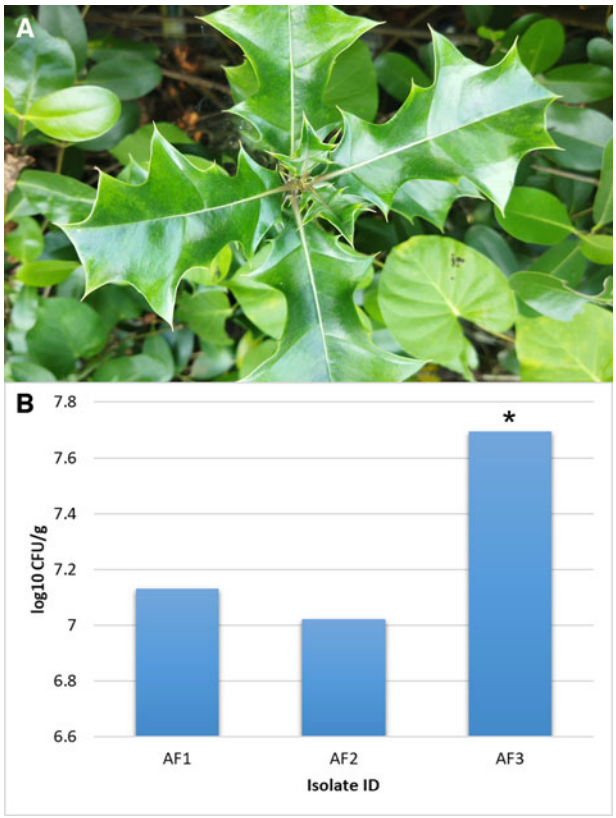

Figure 1. Mangrove leaves in the study and results of endophytic fungi enumeration: (A) leaves of *A. ilicifolius* used in the study and (B) results of endophytic fungi enumeration. Observed means of log CFU ± SD per g of leaves are given. *Indicates significant difference at $P < 0.05$.

Martin's Rose Bengal streptomycin agar plates, followed by incubation for 2 weeks at 28°C to confirm the absence of epiphytes. The surface-disinfected leaf samples were then triturated using a mortar and pestle with sterile potassium phosphate buffer. Serial ten-fold dilutions of the triturate were prepared, and each dilution was spread plated in duplicates onto Martin Rose Bengal streptomycin agar medium. All plates were incubated at 28°C for 48 h. The total viable count was calculated by multiplying with the dilution factor and expressed as the number of colony-forming units (CFU) per g. Using potato dextrose agar (PDA) media, the cultivated fungal colonies were subcultured and purified for subsequent identification and activity testing. Further, the PDA plates with pure isolates were photographed. The glycerol stocks were prepared in a 30% glycerol solution and preserved at the Microbial Culture Collection, Fish Microbiology Lab, ICAR-Central Marine Fisheries Research Institute, Kochi.

Morphological characterization

Endophytic fungal isolates were grown in pure culture on PDA media, and the colony characters such as colour, texture, etc., were studied. Morphological characters were studied by observing the slide culture (Harris, 1986) of fungi under a microscope. The septation of hyphae, conidiophore, and conidia characters were observed, and photomicrographs were recorded. Established taxonomic keys (Kohlmeyer and Kohlmeyer, 1979; Kohlmeyer, 1984; Kohlmeyer and Volkmann, 1991; Jones *et al.*, 2009) were used to identify the isolates.

Molecular characterization

Fresh axenic cultures were cultivated on PDA media in the dark for 7 days at 28°C to extract genomic DNA. Fungal hyphae

https://doi.org/10.1017/S0025315424000286 Published online by Cambridge University Press

Table 1. Details of human pathogenic bacteria used in the study

Sl. no.	Bacteria	Strain ID	Source	Bacterial density in OD_{600} adjusted to 0.1, culture suspension (CFU ml^{-1})
1	*V. cholerae*	MTCC 15025	Microbial Type Culture Collection (MTCC), Chandigarh	2.7×10^7
2	*V. parahaemolyticus*	CMFRI/VP-07	Marine Microbial Culture Collection (MMCC), Central Marine Fisheries Research Institute (CMFRI), Kochi	1.5×10^7
3	*V. vulnificus*	CMFRI/VP-02	MMCC, CMFRI, Kochi	9×10^7
4	*S. aureus*	CMFRI/SA-01	MMCC, CMFRI, Kochi	3.05×10^7
5	*K. pneumoniae*	CMFRI/KlP-01	MMCC, CMFRI, Kochi	2×10^7
6	*E. coli*	ATCC 35218	HiMedia, India	3.5×10^7

(100 mg) were scraped from the agar plate surface and ground in liquid nitrogen. Following that, fungal DNA was collected using the NucleoSpin® Plant II Kit according to the manufacturer's protocol (Macherey-Nagel GmbH & Co., Düren, Germany). The internal transcribed spacer (ITS) sequences were then amplified by polymerase chain reaction using the primers ITS1 and ITS4 (White *et al.*, 1990) (ITS1: 5′-TCCGTAGGTGAACCTGCGG-3′; ITS4: 5′-TCCTCCGCTTATTGATATGC-3′). The initial denaturation was conducted at 98°C for 30 s, followed by 40 reaction cycles (denaturation at 98°C for 30 s, annealing at 58°C for 10 s, extension at 72°C for 15 s), and final extension at 72°C for 60 s The amplicons were then sequenced in both directions at the Regional Facility for DNA Fingerprinting, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram. The sequence data from forward and reverse sequencing reactions were edited and compiled using Editseq (DNASTAR, Lasergene, Madison, WI, USA). Next, we BLAST-ed (with default settings) the nucleotide sequence to check for similar sequences in the GenBank database at the National Center for Biotechnology Information (NCBI). The results of molecular characterization were then compared with those of morphological characterization for the final identification. The representative gene sequences of each distinct endophyte species were then deposited in GenBank and allocated accession numbers.

In vitro antimicrobial assay

The fungal isolate representing the most dominant species in the leaves of *A. ilicifolius* was only used for the downstream experiments. The antibacterial activity was tested against a panel of six indicator human pathogens (Table 1) by the well diffusion method (Taye *et al.*, 2011) and disc diffusion method (Mabrouk *et al.*, 2014). Briefly, 18 h old cultures of the indicator pathogens were centrifuged, and the pellet was resuspended by adjusting the optical density of the suspension test culture to 0.1 at 600 nM. Each indicator strain's resuspension was applied separately on Mueller–Hinton agar (HiMedia, Mumbai, India) to create a lawn culture. Subsequently, for the well diffusion method, the wells were prepared on each plate, and 100 µl of supernatant of 7 day old fungal culture was transferred to the wells. Simultaneously, the disc diffusion method was also applied, for which 50 µl of supernatant of fungal culture was added to 10 mm sterile disc (HiMedia, Mumbai, India) and allowed to dry.

Table 2. Morphological characteristics of the isolates

Isolate ID/characteristics	AFE-1 (*C. cinerea*)	AFE-2 (*Alternaria* sp.)	AFE-3 (*P. macrospinosa*)
Colony colour during initial growth (initial 7 days)	White in colour (Figure 2A)	White in colour (Figure 3A)	White in colour (Figure 4A)
Colony colour after 14 days	Black (Figure 2B)	Grey to dark-black (Figure 3B)	Dark brown to black (Figure 4B)
Colony diameter after 7 days (mm)	3–5	3–6	2–4
Colony diameter after 14 days (mm)	6–8	8–10	**7**–9
Hyphae	Branched, septate (Figure 2C)	Dichotomous branching, smooth, septate (Figure 3C)	Dichotomous branching, smooth, septate (Figure 4C)
Conidiophores	Arising directly from the stipe (Figure 2C)	Head of conidiophore showing the chain of conidia (Figure 3C)	Macronematous conidiophores formed after 7 days on PDA media, head of conidiophore showing the chain of conidia, immediately below the head bearing shorter branches or stipes (Figure 4C)
Conidiogenous cells	Apically bearing a cluster of conidiogenous hyphae (Figure 2C)	Arising mostly directly from the stipe (Figure 3C)	Arising mostly directly from the stipe, spherical, smooth-walled (Figure 4D)
Conidia	Cylindrical conidia (Figure 2C)	Conidia in chains, often of more than three, most conidia with apical beak, club-shaped, produced long chains; multiseptate with both transverse and longitudinal septa (Figure 3C)	Bearing simple or branched chains of conidia, well-defined conidial heads, mature conidiophores with head of verruculose conidia (Figure 4D)

https://doi.org/10.1017/S0025315424000286 Published online by Cambridge University Press

Discs containing the supernatant were placed on the surface of the medium containing the lawn culture of each pathogen. The plates (both well and disc diffusion) were incubated for 2 days at 37°C. For positive cases, the inhibitory zone developed after the incubation period was registered and measured. To validate the antimicrobial activity, the test was repeated thrice for positive instances. The mean zone diameter was then measured for the study. Further, to confirm the results of the disc and well diffusion methods the antibacterial efficacy in terms of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against the indicator pathogens was determined by the microtitre plate method (Marshall *et al.*, 1996; Sharma *et al.*, 2021). In brief, the fungal culture supernatant mentioned above for the disc and well diffusion methods was used as the stock. Then, two-fold serial dilutions of the stock were prepared in Mueller–Hinton broth, and 100 µl of each dilution was added to polystyrene sterile flat-bottom 96-well plates in duplicates. All the wells were then inoculated with 100 µl of bacterial suspension (10^6 CFU ml^{-1}) followed by incubation at 37°C for 24 h. The wells containing bacterial inoculum without fungal supernatant served as the control. At the end of incubation, the lowest concentration of supernatant that did not show visible bacterial growth was noted as MIC. Subsequently, 50 µl of suspension from each well was cultured onto Mueller–Hinton agar plates and incubated at 37°C for 24 h. The plates were inspected for bacterial growth after incubation, and the lowest concentration showing no bacterial growth on agar plates was noted as MBC.

Phylogenetic analysis

The evolutionary relationship of the most dominant endophytic fungal isolate was inferred through phylogenetic analysis using the sequences representing 18S rRNA gene partial sequence, 5.8S rRNA gene, 28S rRNA gene partial sequence, ITS1, and ITS2. Initially, a 551 bp-sized sequence representing the partial sequences of the 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, and 28S rRNA gene of the fungus was obtained by editing and compiling the sequences obtained from forward and reverse sequencing reactions using Editseq (DNASTAR, Lasergene, Madison, WI, USA). Then, using CLUSTALW, multiple sequence alignment of this sequence, corresponding sequences of other fungi of the same family (Periconiaceae), and two representatives from the nearest family, *viz.*, Massarinaceae was then performed (Altschul, 1997). Molecular Evolutionary Genetics Analysis, version 10 (MEGA 10), was then employed to perform a phylogenetic analysis on the aligned data (Kumar *et al.*, 2018). The Kimura two-parameter model (Nei and Kumar, 2000), which had the lowest Bayesian information criterion score was used to calculate the evolutionary distances. Following that, the phylogenetic tree was built using the neighbour-joining method with 1000 bootstrap replications (Saitou and Nei, 1987), and the gamma distribution

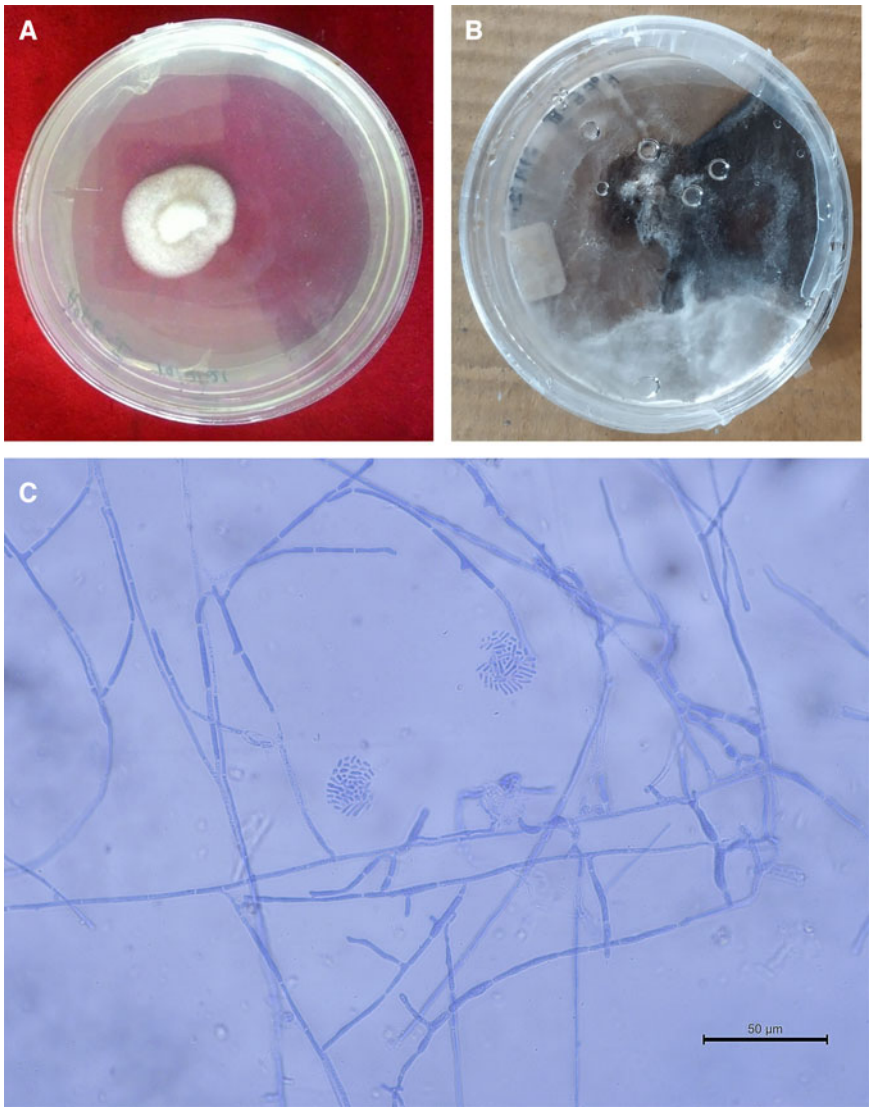

Figure 2. Morphological characteristics of *C. cinerea*: (A) colony morphology during initial growth, (B) colony morphology after 14 days of growth, and (C) microscopic morphology after lactophenol cotton blue staining.

https://doi.org/10.1017/S0025315424000286 Published online by Cambridge University Press

with five discrete categories was employed to model the variations in evolutionary rates among sites. For the purpose of rooting, the corresponding sequence from an uncultivated Agaricomycetes was used (GenBank accession number: GQ268657.1).

Data analysis

Data on the enumeration of endophytic fungi were recorded as the log colony-forming units per g of leaf tissue (log CFU ± SD). The inhibition zone diameter (the difference between the total zone diameter and the bacterial colony/disc diameter) of the three-triplicate testing (mean ± SD) was used to express the antimicrobial assay results. The colony counts and the inhibition zone diameters (antibacterial tests) were compared using one-way analysis of variance (ANOVA), with a P-value of <0.05 set to indicate a significant difference. Tukey's test was employed for post-hoc analysis following one-way ANOVA (SPSS software program, ver. 16). The relative frequency (R_F) of each distinct fungal isolate was then estimated as the ratio of the number of isolates of a particular taxon (n) to the total number of isolates (N) [$(n/N) \times 100$] (Du *et al.*, 2020). The zone of inhibition diameter was used to score each isolate against each pathogen (Sumithra *et al.*, 2019). High (>10 mm), moderate (5–10 mm), low (1–4 mm), and no inhibition activities were given scores of 4, 3, 2, 1, and 0, respectively.

Results

Isolation and enumeration of endophytic fungi

Three different endophytic fungi, designated AFE-1, AFE-2, and AFE-3, were successfully isolated from the leaf segments of *A. ilicifolius*. The results of the enumeration of these fungi are depicted in Figure 1B. A statistical method was used to test the overall significant difference ($P < 0.05$) between the $\log_{10}$ CFU values of different fungal isolates, with significantly higher counts of AFE-3 compared to the other two isolates. In brief, the results showed that the most predominant fungus was AFE-3, with an average $\log_{10}$ CFU g^{-1} of leaf tissue of 7.70 ± 0.06.

Identification of the isolates

The morphological characteristics of the three fungal isolates on PDA media are depicted in Table 2 and Figures 2–4. During molecular characterization, an amplicon of 484, 439, and 546 bp was obtained from AFE-1, AFE-2, and AFE-3 isolates, respectively. GenBank accession numbers ON897769, ON898017, and OM085665 were assigned to the submitted sequencing data derived from the three fungal isolates. In total, three different fungus species belonging to the genera *Alternaria* sp., *Coprinopsis* sp., and *Periconia* sp. were identified (Table 3). A combined analysis of the morphological and molecular characteristics classified the

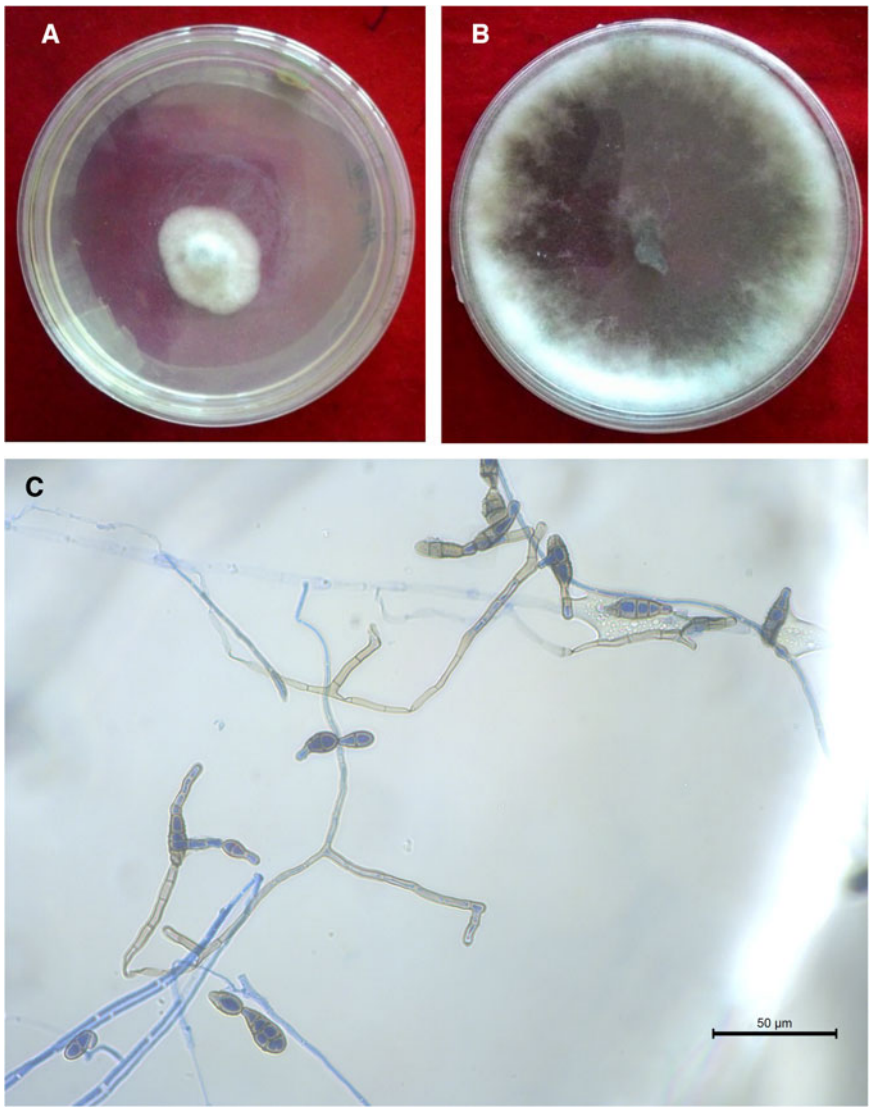

Figure 3. Morphological characteristics of *Alternaria* sp.: (A) colony morphology during initial growth, (B) colony morphology after 14 days of growth, and (C) microscopic morphology after lactophenol cotton blue staining.

https://doi.org/10.1017/S0025315424000286 Published online by Cambridge University Press

first two fungi as *Periconia macrospinosa* (strain: CMFRI/fPM-01) and *Coprinopsis cinerea* (strain: CMFRI/fCC-01). The third fungus (strain: CMFRI/fAl-01) could not be classified to the species level. The R_F of each unique fungal isolate was calculated as 35.22, 32.64, and 32.13% for CMFRI/fPM-01, CMFRI/fCC-01, and CMFRI/fAl-01, respectively.

In vitro *antimicrobial assay*

The antibacterial activity of the fungal isolate of the most prevalent species in *A. ilicifolius* leaves was investigated. The results are shown in Figure 5. In essence, the *P. macrospinosa* strain CMFRI/fPM-01 demonstrated a broad-spectrum antibiotic efficacy against all indicator pathogens tested. There was no significant difference in the antibacterial activity against the tested pathogens ($P > 0.05$). More importantly, the fungus scored 4, indicating high inhibitory activity against various pathogens. MIC and MBC values of the culture supernatant against the tested pathogens were 1:8 and 1:2 dilutions, respectively

Phylogenetic analysis

The outcome of the phylogenetic analysis of the promising fungal isolate AFE-3 with potential antibacterial action is shown in Figure 6. Based on the derived phylogenetic tree, the Periconiaceae family of the Pleosporales order is distinguished from the Massarineae as a sister taxon with a distinct lineage. There were two well-separated subclades in the family 'Periconiaceae' clade (order: Pleosporales). The first subclade contained *P. macrospinosa*, *Periconia igniaria*, *Periconia epilithographicola*, *Periconia variicolor*, *Periconia homothallica*, *Periconia digitata*, *Periconia elaeidis*, *Periconia cyperacearum*, and *Periconia psuedodigitata*. The strains belonging to *Periconia echinochloae*, *Periconia pseudobyssoides*, and *Periconia byssoides* formed a different subclade (II). The isolate CMFRI/fPM-01 was clustered in the first subclade with close similarity to *P. macrospinosa* strains.

Discussion

Among different plants, mangrove plants are the most attractive biodiversity hotspots for prospecting novel bioactive compounds (Cadamuro *et al.*, 2021). *A. ilicifolius* is a widely used mangrove species for various medicinal purposes, especially in the field of conventional medicines (Ragavan *et al.*, 2015; Saranya *et al.*, 2015). The previous limited studies on *A. ilicifolius* (Chaeprasert *et al.*, 2010; Chi *et al.*, 2019; Yanti and Anwarrudin, 2021) suggested that *A. ilicifolius* may possess a wide variety of biologically active endophytic fungi. In this context, the cultural diversity of the endophytic fungal assemblage on the healthy leaves of *A. ilicifolius* established on the mangrove habitat of the west coast of India was explored to elucidate their possible antimicrobial applications.

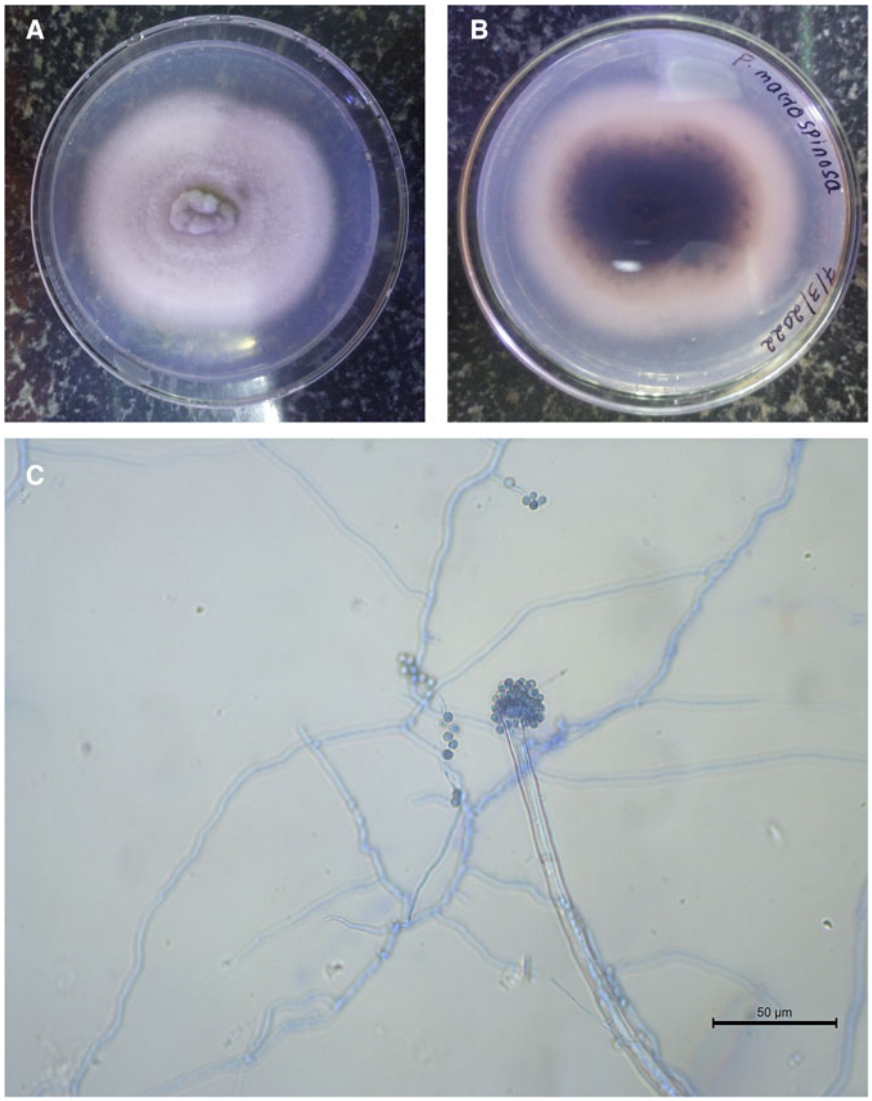

Figure 4. Morphological characteristics of *P. macrospinosa*: (A) colony morphology during initial growth, (B) colony morphology after 14 days of growth and (C) microscopic morphology after lactophenol cotton blue staining.

https://doi.org/10.1017/S0025315424000286 Published online by Cambridge University Press

Table 3. Composition of endophytic fungi from the leaves of *A. ilicifolius*

Sl. no.	Culture code	Relative frequency (%)	Phylum	Class	Order	Family	Genus	Species	Strain ID
1	AFE-1	32.64	Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	*Coprinopsis*	*C. cinerea*	CMFRI/fCC-01
2	AFE-2	32.13	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	*Alternaria*	*Alternaria* sp.	CMFRI/fAl-01
3	AFE-3	35.22	Ascomycota	Dothideomycetes	Pleosporales	Lophiostomataceae	*Periconia*	*P. macrospinosa*	CMFRI/fPM-01

Only three taxonomically distinct endophytic fungi could be identified from the leaves of *A. ilicifolius* in the current investigation. The diversity of endophytic fungi in the plants relates to the host species and their environment. The number of distinct endophytic fungal species identified from the mangrove plants varies among the studies. According to Petrini (1986) and Rajagopal *et al.* (2018), only one or a small number of endophytic fungi dominate within a single host species due to inter-competition. The same might be a reason for the low diversity of the fungal endophytes obtained in the present research. In accordance with our results, Rodrigues and Samuels (1999) and Rajagopal *et al.* (2020) recovered only 13 and six endophytic fungal species from *Spondias mombin* and *Eichhornia crassipes*, respectively. Similarly, Mao *et al.* (2021) could isolate only 15 different endophytic fungi from the branches and fruits of *Eucalyptus exserta*, using PDA media. Wibowo *et al.* (2016) isolated 20 compounds from the mangrove-associated fungus *Pseudolagarobasidium acaciicola*, originally isolated from the *B. gymnorrhiza* tree. The work underscored the importance of mangrove fungi as a rich source of novel bioactive compounds. Because most endophytic fungi cannot be cultured on artificial media and PDA media is not always an optimal medium, the three species in this study may represent only a subset of the total endophytic fungi found in *A. ilicifolius*. Arnold and Lutzoni (2007) recommended using 2% malt extract agar to encourage the growth of the most diversity of fungi, which has to be explored in future studies. Bosshard (2011) recommended an incubation period of 2–4 weeks to recover the maximum possible diverse fungi from the samples; accordingly, a shorter incubation period might be another reason for the observed low diversity in the present study. Further, Yu *et al.* (2021) showed that the diversity of foliar endophytes in plants varies significantly with age and is shown to be spread out and insignificant on young leaves. Accordingly, using older leaves, diverse isolation media, prolonged incubation, trying other isolation methods, such as surface sterilization method and application of high-throughput sequencing, are recommended to isolate more diverse endophytic fungi from *A. ilicifolius* or to confirm the low diversity; these are the future perspectives of the present study.

The three fungal isolates of the present study were identified as *C. cinerea*, *Alternaria* sp., and *P. macrospinosa* belonging to the phylum Basidiomycota, Ascomycota, and Ascomycota, respectively. The results support the hypothesis of Du *et al.* (2020) that all endophytic fungi belong to either *Ascomycetes* or *Basidiomycetes*. Also, Mishra *et al.* (2016) and Pecoraro *et al.* (2018) reported that *Ascomycetes* are the most prevalent members of endophytic fungal communities isolated using traditional separation techniques, as in agreement with our findings. Nevertheless, the diversity analysis of endophytes based on culture-dependent methodologies underestimates the actual diversity, and many isolates could still be identified by the culture-independent methods (Wang *et al.*, 2015). Our study identified the dominant endophytes on the healthy leaves of *A. ilicifolius* in the mangrove ecosystem of the west coast of India, which will be helpful in screening and setting the groundwork for their application. It is important to mention that none of the species identified in the present study has been previously reported as the endophyte of *A. ilicifolius* leaves. Among the three species identified in the current research, *Alternaria* sp. is already described as a dominant endophytic fungal species of different plants (Rashmi *et al.*, 2019) and has been isolated from other mangrove species such as *A. marina*, *Sonneratia alba*, *Myoporum bontioides*, and *Rhizophora annamalayana* (Kjer, 2009; Elavarasi *et al.*, 2014; Wang *et al.*, 2014; Liu *et al.*, 2016).

Similarly, *C. cinerea* has been identified in *Eugenia jambolana* leaf tissue (Rashmi *et al.*, 2019) but not from any mangrove species. However, other fungal species of the same genus *Coprinopsis*,

https://doi.org/10.1017/S0025315424000286 Published online by Cambridge University Press

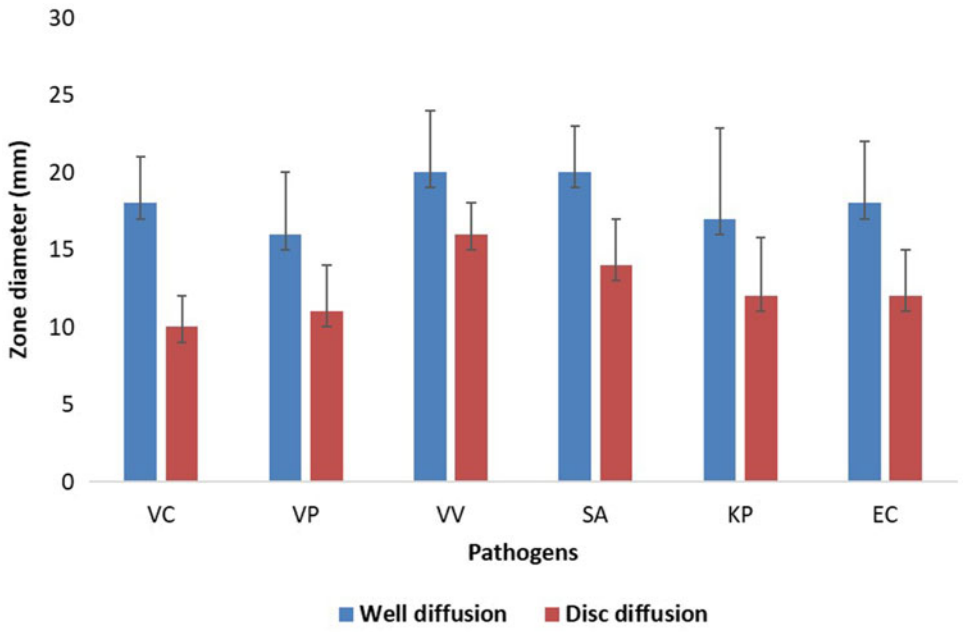

Figure 5. Antibacterial activity of *P. macrospinosa* (CMFRI/fPM-01). VC, *V. cholerae*; VP, *V. parahaemolyticus*; VV, *V. vulnificus*; KP, *K. pneumoniae*; SA, *S. aureus*; EC, *E. coli*.

have been reported in certain mangrove plants (Li *et al.*, 2016; Devadatha *et al.*, 2021). Similarly, *P. macrospinosa* and other *Periconia* spp. are reported as the endophytes of the leaves of *Acer truncatum* and different plants, respectively (Rashmi *et al.*, 2019). Further, other *Periconia* spp. have been reported as the endophytes of certain mangrove plants (Costa *et al.*, 2012; Hamzah *et al.*, 2018). However, the study forms the first report of *P. macrospinosa* and *C. cinerea* as endophytes in any mangrove plant species. It was also interesting to note that the common endophytes of mangrove plants such as *Aspergillus* and *Penicillium* could not be obtained in this study (Costa *et al.*, 2012; Salini, 2014).

The fungal isolate representing the most dominant species in the leaves of *A. ilicifolius*, *P. macrospinosa* (strain: CMFRI/fPM-01), was then evaluated for possible antimicrobial activity. Surprisingly, *P. macrospinosa* showed antibacterial properties against all the tested indicator pathogens, displaying a broad-spectrum antibacterial efficacy. More importantly, the fungus scored 4, indicating the solid inhibitory action against several pathogens. These findings concur with the report of Azhari and Supratman (2021), identifying *Periconia* spp. as an exciting resource for natural product research. The secondary metabolites of this genus have been found to have strong antimicrobial, cytotoxic, anti-HIV, and anti-inflammatory properties, including terpenoids, polyketides, cytochalasin, meroterpene, macrolides, macropshelides, aromatic compounds, and carbohydrate derivatives (Azhari and Supratman, 2021). The results are exciting as the spectrum of the antimicrobial action includes three ESKAPE pathogens (*S. aureus*, *Klebsiella pneumoniae*, and *E. coli*), which cause the vast majority of nosocomial infections and have been shown to exhibit rising rates of antibiotic resistance and pathogenicity (Mulani *et al.*, 2019). It is worth mentioning that the usage of *E. coli* ATCC 35218 in the study, which is the quality control organism for β-lactam–β-lactamase inhibitor compounds, further signifies the antimicrobial action of *P. macrospinosa*.

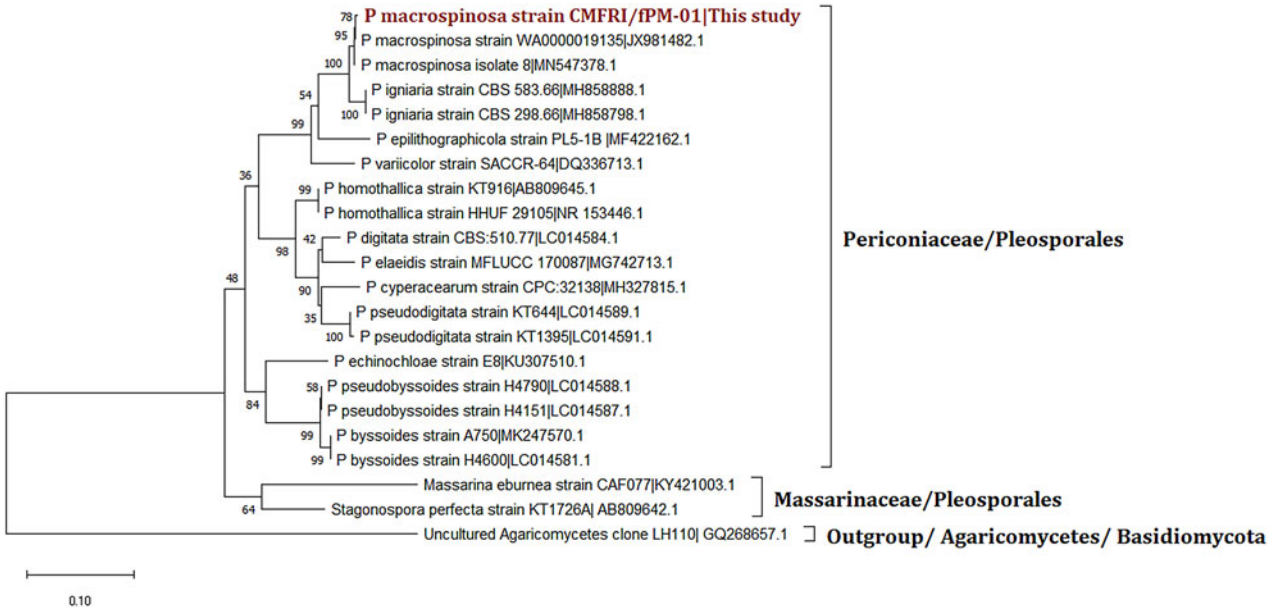

Figure 6. Phylogenetic analysis of *P. macrospinosa* strain CMFRI/fPM-01 based on ITS sequence.

https://doi.org/10.1017/S0025315424000286 Published online by Cambridge University Press

Conclusion

The present study demonstrated the cultural diversity of endophytic fungal assemblages on the healthy leaves of *A. ilicifolius* established in the mangrove habitat of the west coast of India. The study has identified the lesser-known genera *Alternaria*, *Coprinopsis*, and *Periconia* as endophytic members of the *A. ilicifolius* leaf fungal community. More importantly, this study is the first report of *P. macrospinosa* and *C. cinerea* as endophytes in mangrove plant species. The results also demonstrated the high and wide spectrum of antimicrobial action of the isolated *P. macrospinosa* against human pathogens, including ESKAPE pathogens. The isolate could serve as a potential resource for developing novel antimicrobial drugs, which could aid in the fight against antibiotic-resistant bacteria. Future studies shall be carried out using different media and high-throughput sequencing to isolate more endophytic fungi from the same plant or to confirm the low diversity found in this study. Also, further efforts are warranted to adequately characterize the isolated organism's biotechnological potential.

Acknowledgements. The authors are grateful for the facilities provided by the Director, ICAR-CMFRI, and Vice-Chancellor, KUFOS. The authors also thank the Vice Chancellor, KAU, for the support extended during the study.

Data availability statement. The authors confirm that the data supporting the findings of this study are available within the article. All the sequences generated were submitted to NCBI under unique accession numbers (ON897769, ON898017, and OM085665).

Author's contribution. All authors contributed to the study's conception and design. Conceptualization was done by KR Sreenath and TG Sumithra. Material preparation, data collection, and analysis were performed by P. Sainamole Kurian, VN Anusree, and VP Sruthi. The first draft of the manuscript was written by VP Sruthi and all authors contributed to the previous versions of the manuscript. All authors read and approved the final manuscript.

Financial support. The lead author received Kerala e-Grantz as a research fellowship during the study.

Competing interests. None

Ethical Approval. No ethical approval of research ethics committee was required to accomplish the goals of this study because no live animals were used for the experiments.

References

Altschul SF (1997) *Theoretical and Computational Methods in Genome Research*. New York: Plenum Press, pp. 1–14.

Ananda K and Sridhar KR (2002) Diversity of endophytic fungi in the roots of mangrove species on the west coast of India. *Canadian Journal of Microbiology* **48**, 871–878. https://doi.org/10.1139/w02-080

Arnold AE and Lutzoni F (2007) Diversity and host range of foliar fungal endophytes: are tropical leaves biodiversity hotspots? *Ecology* **88**, 541–549.

Azhari A and Supratman U (2021) The chemistry and pharmacology of fungal genus *Periconia*: a review. *Scientia Pharmaceutica* **89**, 34. https://doi.org/10.3390/scipharm89030034

Bai S, Seasotiya L, Malik A, Bharti P and Dalal S (2014) GC-MS analysis of chloroform extract of *Acacia nilotica* L. leaves. *Journal of Pharmacognosy and Phytochemistry* **2**, 79–82.

Bandaranayake WM (2002) Bioactivities, bioactive compounds and chemical constituents of mangrove plants. *Wetlands Ecology and Management* **10**, 421–452. https://doi.org/10.1023/A:1021397624349

Bosshard PP (2011) Incubation of fungal cultures: how long is long enough? *Mycoses* **54**, e539–e545.

Cadamuro RD, da Silveira Bastos IMA, Silva IT, da Cruz ACC, Robl D, Sandjo LP, Alves Jr S, Lorenzo JM, Rodríguez-Lázaro D, Treichel H and Steindel M (2021) Bioactive compounds from mangrove endophytic fungus and their uses for microorganism control. *Journal of Fungi* **7**, 455. https://doi.org/10.3390/jof7060455

Cha H-J, Chiang MWL, Guo S-Y, Lin S-M and Pang K-L (2021) Culturable fungal community of *Pterocladiella capillacea* in Keelung, Taiwan: effects of surface sterilization method and isolation medium. *Journal of Fungi* **7**, 651. https://doi.org/10.3390/jof7080651

Chaeprasert S, Piapukiew J, Whalley AJ and Sihanonth P (2010) Endophytic fungi from mangrove plant species of Thailand: their antimicrobial and anticancer potentials. *Botanica Marina* **53**, 555–564. https://doi.org/10.1515/bot.2010.074

Chi WC, Pang KL, Chen WL, Wang G and Lee TH (2019) Antimicrobial and iNOS inhibitory activities of the endophytic fungi isolated from the mangrove plant *A. ilicifolius* var. *xiamenensis*.*Botanica Marina* **60**, 1–8. https://doi.org/10.1186/s40529-019-0252-3

Costa IP, Maia LC and Cavalcanti MA (2012) Diversity of leaf endophytic fungi in mangrove plants of northeast Brazil. *Brazilian Journal of Microbiology* **43**, 1165–1173. https://doi.org/10.1590/S1517-83822012000300044

Devadatha B, Jones EBG, Pang KL, Abdel-Wahab MA, Hyde KD, Sakayaroj J, Bahkali AH, Calabon MS, Sarma VV, Sutreong S and Zhang SN (2021) Occurrence and geographical distribution of mangrove fungi. *Fungal Diversity* **106**, 137–227. https://doi.org/10.1007/s13225-020-00468-0

Du W, Yao Z, Li J, Sun C, Xia J, Wang B, Shi D and Ren L (2020) Diversity and antimicrobial activity of endophytic fungi isolated from *Securinega suffruticosa* in the Yellow River Delta. *PLoS ONE* **15**, e0229589. https://doi.org/10.1371/journal.pone.0229589

Elavarasi A, Peninal S, Rathna GS and Kalaiselvam M (2014) Studies on antimicrobial compounds isolated from mangrove endophytic fungi. *The World Journal of Pharmaceutical Sciences* **3**, 734–744.

FSI, Forest Survey of India (2019) *India State of Forest Report*. Uttarakhand, India: Forest Survey of India.

Hamzah TNT, Lee SY, Hidayat A, Terhem R, Faridah-Hanum I and Mohamed R (2018) Diversity and characterization of endophytic fungi isolated from the tropical mangrove species, *R. mucronata*, and identification of potential antagonists against the soil-borne fungus, *Fusarium solani*. *Frontiers in Microbiology* **9**, 1707. https://doi.org/10.3389/fmicb.2018.01707

Harris JL (1986) Modified method for fungal slide culture. *Journal of Clinical Microbiology* **24**, 460–461.

Jones EB, Sakayaroj J, Suetrong S, Somrithipol S and Pang KL (2009) Classification of marine Ascomycota, anamorphic taxa and Basidiomycota. *Fungal Diversity* **35**, 187. http://dx.doi.org/10.1007/s13225-015-0339-4

Khan AL, Gilani SA, Waqas M, Al-Hosni K, Al-Khiziri S, Kim YH, Ali L, Kang SM, Asaf S, Shahzad R and Hussain J (2017) Endophytes from medicinal plants and their potential for producing indole acetic acid, improving seed germination and mitigating oxidative stress. *Journal of Zhejiang University-Science B* **18**, 125–137. https://doi.org/10.1631/jzus.B1500271

Kjer J (2009) New natural products from endophytic fungi from mangrove plants – structure elucidation and biological screening (PhD thesis). Faculty of Mathematics and Natural Sciences, Heinrich Heine University, Dusseldorf.

Kloepper JW, Hallmann J and Rodroa R (1999) Chitin-mediated changes in bacterial communities of the soil, rhizosphere and within roots of cotton in relation to nematode control. *Soil Biology and Biochemistry* **31**, 551–560.

Kohlmeyer J (1984) Tropical marine fungi. *Marine Ecology* **5**, 329–378.

Kohlmeyer J and Kohlmeyer E (1979) *Marine Mycology. The Higher Fungi*. New York, London: Academic Press.

Kohlmeyer J and Volkmann-Kohlmeyer B (1991) Illustrated key to the filamentous higher marine fungi. *Botanica Marina* **34**, 1–35.

Kumar S, Stecher G, Li M, Knyaz C and Tamura K (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution* **35**, 1547–1549. https://doi.org/10.1093/molbev/msy096

Kumaresan V and Suryanarayanan TS (2001) Occurrence and distribution of endophytic fungi in a mangrove community. *Mycological Research* **105**, 1388–1391. https://doi.org/10.1017/S0953756201004841

Li DL, Li XM, Li LT, Dang HY, Proksch P and Wang BG (2008) Benzaldehyde derivatives from *Eurotium rubrum*, an endophytic fungus derived from the mangrove plant *Hibiscus tiliaceus*. *Chemical and Pharmaceutical Bulletin* **56**, 1282–1285. https://doi.org/10.1248/cpb.56.1282

Li JL, Sun X, Chen L and Guo LD (2016) Community structure of endophytic fungi of four mangrove species in Southern China. *Mycology* **7**, 180–190. https://doi.org/10.1080/21501203.2016.1258439

Liu Z, Chen Y, Chen S, Liu Y, Lu Y, Chen D, Lin Y, Huang X and She Z (2016) Aspterpenacids A and B, two sesterterpenoids from a mangrove

endophytic fungus *Aspergillus terreus* H010. *Organic Letters* **18**, 1406–1409. https://doi.org/10.1021/acs.orglett.6b00336

Mabrouk AM, Effat BA, Sadek ZI, Tawfiek NF, Hassan ZMR and Magdoub MN (2014) Antibacterial activity of some Lactic acid Bacteria isolated from Egyptian Dairy Products. *International Journal of ChemTech Research* **6**, 1139–1150.

Mao Z, Zhang W, Wu C, Feng H, Peng Y, Shahid H, Cui Z, Ding P and Shan T (2021) Diversity and antibacterial activity of fungal endophytes from *Eucalyptus exserta*. *BMC Microbiology* **21**, 1–12. https://doi.org/10.1186/s12866-021-02229-8

Maria GL and Sridhar KR (2003) Diversity of filamentous fungi on woody litter of five mangrove plant species from the southwest coast of India. *Fungal Diversity* **14**, 109–126.

Marshall SA, Jones RN, Wanger A, Washington JA, Doern GV, Leber A and Haugen TH (1996) Proposed MIC quality control guidelines for national committee for clinical laboratory standards susceptibility tests using seven veterinary antimicrobial agents: ceftiofur, enrofloxacin, florfenicol, penicillin g-novobiocin, pirlimycin, premafloxacin, and spectinomycin. *Journal of Clinical Microbiology* **34**, 2027–2029.

Miethke M, Pieroni M, Weber T, Brönstrup M, Hammann P, Halby L, Arimondo PB, Glaser P, Aigle B, Bode HB and Moreira R (2021) Towards the sustainable discovery and development of new antibiotics. *Nature Reviews Chemistry* **5**, 726–749. https://doi.org/10.1038/s41570-021-00313-1

Mishra VK, Singh G, Passari AK, Yadav MK, Gupta VK and Singh BP (2016) Distribution and antimicrobial potential of endophytic fungi associated with ethnomedicinal plant *Melastoma malabathricum* L. *Journal of Environmental Biology* **37**, 229–237. PMID: 27097442.

Mulani MS, Kamble EE, Kumkar SN, Tawre MS and Pardesi KR (2019) Emerging strategies to combat ESKAPE pathogens in the era of antimicrobial resistance: a review. *Frontiers in Microbiology* **10**, 539. https://doi.org/10.3389/fmicb.2019.00539

Nei M and Kumar S (2000) *Molecular Evolution and Phylogenetics*. New York: Oxford University Press.

Pecoraro L, Caruso T, Cai L, Gupta K and Liu Z (2018) Fungal networks and orchid distribution: new insights from above- and below-ground analyses of fungal communities. *IMA Fungus* **9**, 1–11. https://doi.org/10.5598/imafungus.2018.09.01.01

Petrini O (1986) Taxonomy of endophytic fungi of aerial plant tissues. In Fokkema NJ and van den Huevel J (eds), *Microbiology of the Phyllosphere*. Cambridge, UK: Cambridge University Press, pp. 175–187.

Priyadharshini R, Ambikapathy V and Panneerselvam A (2015) Isolation and identification of endophytic fungi from *A. ilicifolius* in Muthupet mangroves. *International Journal of Scientific Research* **4**, 169–171. https://www.doi.org/10.36106/ijsr

Ragavan P, Saxena A, Mohan PM, Jayaraj RS and Ravichandran K (2015) Taxonomy and distribution of species of the genus *Acanthus* (Acanthaceae) in mangroves of the Andaman and Nicobar Islands, India. *Biodiversitas: Journal of Biological Diversity* **16**, 225–237. https://doi.org/10.13057/biodiv/d160218

Rajagopal K, Meenashree B, Binika D, Joshila D, Tulsi PS, Arulmathi R, Kathiravan G and Tuwar A (2018) Mycodiversity and biotechnological potential of endophytic fungi isolated from hydrophytes. *Current Research in Environmental & Applied Mycology* **8**, 172–182. https://doi.org/10.5943/cream/8/2/2

Rajagopal K, Sasiprabha P, Sabarish S, Arulmathi R, Meenashree B, Kathiravan G and Kathireshan AK (2020) Enumeration of endophytic fungi from *Eichhornia crassipes* root and preliminary screening for tannase enzyme production. Asian Journal of Microbiology, *Biotechnology and Environmental Sciences* **22**, 329–333.

Rashmi M, Kushveer JS and Sarma VV (2019) A worldwide list of endophytic fungi with notes on ecology and diversity. *Mycosphere* **10**, 798–1079. https://doi.org/10.5943/mycosphere/10/1/19

Rodrigues KF and Samuels GJ (1999) Fungal endophytes of *Spondias mombin* leaves in Brazil. *An International Journal on Biochemistry, Physiology, Genetics, Morphology, and Ecology of Microorganisms* **39**, 131–135.

Saitou N and Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**, 406–425.

Salini G (2014) Pharmacological profile of mangrove endophytes – a review. *International Journal of Pharmacy and Pharmaceutical Sciences* **7**, 6–15.

Saranya A, Ramanathan T, Kesavanarayanan KS and Adam A (2015) Traditional medicinal uses, chemical constituents and biological activities of a mangrove plant, *A. ilicifolius* Linn.: a brief review. *American–Eurasian Journal of Agricultural & Environmental Sciences* **15**, 243–250. http://www.idosi.org/aejaes/jaes15(2)15/17

Sharma SRK, Sumithra TG, Suja G, Azhar S, Sanil NK, Ashok Kumar K and Patil PK (2021) Evaluation of biosafety and tissue residue of oxytetracycline in juvenile snubnose pompano, *Trachinotus blochii* along with in vitro efficacy against fish pathogens. *Aquaculture* **533**, 736184.

Sreelekshmi S, Veettil BK, Nandan SB and Harikrishnan M (2021) Mangrove forests along the coastline of Kerala, southern India: current status and future prospects. *Regional Studies in Marine Science* **41**, 101573. https://10.0.3.248/j.rsma.2020.101573

Strobel GA (2002) Rainforest endophytes and bioactive products. *Critical Reviews in Biotechnology* **22**, 315–333. https://doi.org/10.1080/07388550290789531

Strobel G and Daisy B (2003) Bioprospecting for microbial endophytes and their natural products. *Microbiology and Molecular Biology Reviews* **67**, 491–502. https://doi.org/10.1128/MMBR.67.4.491-502.2003

Strobel GA and Long DM (1998) Endophytic microbes embody pharmaceutical potential. *ASM News* **64**, 263–268.

Sumithra TG, Reshma KJ, Christo JP, Anusree VN, Drisya D, Kishor TG, Revathi DN and Sanil NK (2019) A glimpse towards cultivable hemolymph microbiota of marine crabs: untapped resource for aquatic probiotics/antibacterial agents. *Aquaculture* **501**, 119–127. https://doi.org/10.1016/j

Suryanarayanan TS and Kumaresan V (2000) Endophytic fungi of some halophytes from an estuarine mangrove forest. *Mycological Research* **104**, 1465–1467.

Taye B, Giday M, Animut A and Seid J (2011) Antibacterial activities of selected medicinal plants in traditional treatment of human wounds in Ethiopia. *Asian Pacific Journal of Tropical Biomedicine* **1**, 370–375. https://doi.org/10.1016/S2221-1691(11)60082-8

Tiwari P and Bae H (2022) Endophytic fungi: key insights, emerging prospects, and challenges in natural product drug discovery. *Microorganisms* **10**, 360. https://doi.org/10.3390/microorganisms10020360

Wang J, Cox DG, Ding W, Huang G, Lin Y and Li C (2014) Three new resveratrol derivatives from the mangrove endophytic fungus *Alternaria* sp. *Marine Drugs* **12**, 2840–2850. https://doi.org/10.3390/md12052840

Wang C, García-Fernández D, Mas A and Esteve-Zarzoso B (2015) Fungal diversity in grape must and wine fermentation assessed by massive sequencing, quantitative PCR and DGGE. *Frontiers in Microbiology* **6**, 1156. https://doi.org/10.3389/fmicb.2015.01156

White TJ, Bruns T, Lee S and Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In Innis MA, Gelfand DH, Sninsky JJ and White TJ (eds), *PCR Protocols: A Guide to Method and Applications*. New York: Academic Press, pp. 315–322.

Wibowo M, Prachyawarakorn V, Aree T, Mahidol C, Ruchirawat S and Kittakoop P (2016) Cytotoxic sesquiterpenes from the endophytic fungus *Pseudolagarobasidium acaciicola*. *Phytochemistry* **122**, 126–138.

Yanti NA and Anwarrudin S (2021) Antibacterial activity of fungi endophytic isolated from leaves the mangrove *A. ilicifolius*. *Journal of Physics: Conference Series* **1899**, 012015.

Yu Z, Ding H, Shen K, Bu F, Newcombe G and Liu H (2021) Foliar endophytes in trees varying greatly in age. *European Journal of Plant Pathology* **160**, 375–384.

Zhang Y, Vareed SK and Nair MG (2005) Human tumor cell growth inhibition by nontoxic anthocyanidins, the pigments in fruits and vegetables. *Life Sciences* **76**, 1465–1472. https://doi.org/10.1016/j.lfs.2004.08.025