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ISOLATION, SCREENING, AND ANTIBACTERIAL ACTIVITY OF ENDOPHYTIC FUNGI ASSOCIATED WITH *Acanthus ilicifolius* L. IN INHIBITING THE GROWTH OF *Methicillin-Resistant Staphylococcus aureus* (MRSA)

Isolasi, Penapisan, dan Aktivitas Antibakteri Kapang Endofit yang Berasosiasi dengan *Acanthus ilicifolius* L. dalam Menghambat Pertumbuhan *Methicillin-Resistant Staphylococcus Aureus* (MRSA)

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ABSTRACT

This study examines the phytochemical composition and antibacterial activity of endophytic fungi linked to the mangrove plant *A. ilicifolius* against methicillin-resistant *Staphylococcus aureus* (MRSA) in order to determine their bioprospecting potential. The surface-sterilized root, stem, and leaf tissues of *A. ilicifolius* yielded endophytic fungi. The workflow consisted of screening for viable isolates, culture under controlled circumstances, and molecular identification of the Internal Transcribed Spacer (ITS) section of fungal rDNA. Antibacterial activity was determined using agar well diffusion tests after extraction with methanol, ethyl acetate, and n-hexane solvent fractions. A total of 31 endophytic fungal isolates were recovered: 9 from roots, 15 from stems, and 7 from leaves. Among these, 5 isolates from roots (16.1%), 8 from stems (25.8%), and 5 from leaves (16.1%) exhibited inhibitory effects against MRSA. Isolate AK5, derived from root tissue, demonstrated the highest antibacterial activity and was molecularly identified as *Chaetomium globosum* strain NW 24 (Accession No. MN326469.1). The isolate revealed optimal growth at pH 5–6, with the maximum wet mycelial biomass (29.73 g) achieved on day 24 under shaker incubation. The methanol and ethyl acetate fractions had a considerably greater anti-MRSA efficacy than the n-hexane fraction. Phytochemical analysis of the methanolic extract indicated the presence of several bioactive components, including phenolics, tannins, flavonoids, saponins, alkaloids, and terpenoids, indicating that these constituents contribute to the reported antibacterial effects. Overall, our findings highlight the potential of *A. ilicifolius*-derived endophytic fungi as alternate sources of bioactive compounds for treating antibiotic-resistant infections, specifically MRSA.

Keywords: *Acanthus ilicifolius, Biocontrol, Chaetomium globosum NW24, Endophytic fungus, MRSA*

ABSTRAK

Studi ini mengkaji profil fitokimia dan aktivitas antibakteri jamur endofit yang terkait dengan tanaman bakau *A. ilicifolius* terhadap *Staphylococcus aureus* resisten methicillin (MRSA) untuk

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menentukan potensi bioprospeksinya. Jaringan akar, batang, dan daun *A. ilicifolius* yang disterilkan permukaannya menghasilkan jamur endofit. Alur kerja terdiri dari penapisan isolat yang viabel, kultur dalam kondisi terkendali, dan identifikasi molekuler bagian Internal Transcribed Spacer (ITS) dari rDNA jamur. Aktivitas antibakteri ditentukan menggunakan uji difusi sumur agar setelah ekstraksi dengan fraksi pelarut metanol, etil asetat, dan n-heksana. Sebanyak 31 isolat jamur endofit diperoleh: 9 dari akar, 15 dari batang, dan 7 dari daun. Di antara ini, 5 isolat dari akar (16,1%), 8 dari batang (25,8%), dan 5 dari daun (16,1%) menunjukkan efek penghambatan terhadap MRSA. Isolat AK5, yang berasal dari jaringan akar, menunjukkan aktivitas antibakteri tertinggi dan diidentifikasi secara molekuler sebagai *Chaetomium globosum* NW 24 (Nomor Aksesi MN326469.1). Isolat ini menunjukkan pertumbuhan optimal pada pH 5–6, dengan biomassa miselium basah maksimum (29,73 g) dicapai pada hari ke-24 di bawah inkubasi shaker. Fraksi metanol dan etil asetat memiliki efikasi anti-MRSA yang secara signifikan lebih besar daripada fraksi n-heksana. Analisis fitokimia ekstrak metanol menunjukkan adanya beberapa komponen bioaktif, termasuk fenolik, tanin, flavonoid, saponin, alkaloid, dan terpenoid, yang menunjukkan bahwa komponen-komponen ini berkontribusi terhadap efek antibakteri yang dilaporkan. Secara keseluruhan, temuan kami menyoroti potensi jamur endofit turunan *A. ilicifolius* sebagai sumber alternatif senyawa bioaktif untuk mengobati infeksi yang resisten antibiotik, khususnya MRSA.

Kata Kunci: *Acanthus ilicifolius, Biokontrol, Chaetomium globosum NW24, Jamur endofit, MRSA*

INTRODUCTION

Antibiotic misuse is severely discouraged since it provides little to no therapeutic benefit and contributes considerably to the evolution of antimicrobial resistance (AMR) (Laws et al., 2019; Nadimpalli et al., 2020; Nobrega et al., 2020). AMR is now regarded to be among of the greatest pressing healthcare issues globally (Brown et al., 2017; Prestinaci et al., 2015). Methicillin-resistant *Staphylococcus aureus* (MRSA) has been connected to higher morbidity and mortality as a result of antimicrobial resistance (AMR) and is now acknowledged as a significant cause of hospital-acquired and community-associated infections (Santosaningsih et al., 2019; Sulis et al., 2023). Despite MRSA prevalence varies by country and geographical location, Asia is one of the continents with the highest recorded incidence worldwide.

In Indonesia, the incidence of MRSA across diagnostic infections caused by *S. aureus* has been estimated to be over 28% (Mendes et al., 2013), with 4.3% typically found in postpartum surgery patients (Winarto et al., 2014). This bacterium is resistant to antibiotics that are often used in human and animal therapy (Mulligan et al., 1993). MRSA prevalence and morphological traits have also been found in retail broiler chicken meat (Kim et al., 2018) and carcass samples taken from commercial poultry slaughter facilities (Masimen et al., 2022; Moon et al., 2015). In addition to contaminating meat, *S. aureus* can infect chicken bones and joints, contributing to avian staphylococcosis and creating a zoonotic concern by potentially causing pneumonia in humans (Prenafeta et al., 2014; Szafraniec et al., 2022). One of the most widely adopted approaches in biotechnology is the exploration of microbial species and genera that remain under-investigated (Sadrati et al., 2023).

Endophytic fungi are among the least investigated microbial species, but they are gaining scientific attention due to their extraordinary diversity of bioactive chemicals and ability to generate new therapeutic drugs (Sadrati et al., 2023). As a result, studying endophytic fungi linked with certain host plants is regarded as a vital step in developing plant-derived secondary metabolites for biological and pharmacological uses (Hashem et al., 2023; Hussein et al., 2024). Bioactive compounds generated by endophytic fungi that inhabit mangrove ecosystems have been the subject of an expanding body of study (Awashank et al., 2024; K.-W. Wang et al., 2014).

The second biggest category of marine-derived fungal species are symbiotic fungi found in mangroves. These fungi are known to biosynthesize a variety of bioactive compounds, such as terpenoids, chromones, coumarins, polyketides, alkaloids, and peptides (Deshmukh et al., 2018). *Cladosporium anthropophilum* was isolated from the mangrove species *Avicennia marina* (Forssk.) in Blanakan, Subang Regency, West Java, and inhibited the growth of *S. aureus* ATCC 29213 and *Vibrio harveyi* ATCC 5339 (Mulyani et al., 2024). Similarly, 37 endophytic fungal isolates collected from mangrove plants in Wat Asokaram, Taiban Subdistrict, Samut Prakan Province, Thailand, showed in vitro antagonistic activity against five phytopathogenic species (Sopalun et al., 2021). Awashank et al. (2024) found that *A. stellatus* strain LM-03 had a significant cytotoxic impact on MCF-7 breast cancer cells, with an IC_{50} value of 33.24 µg/mL. With MICs of 125 and 250 µg/mL, respectively, *Ceriops tagal*-derived endophytic isolates inhibited *S. aureus* ATCC 6538 and *S. epidermidis* ATCC 12228 (Putra et al., 2023). Our prior investigation appropriately isolated and evaluated 10 endophytic fungal strains from brown macroalgae (Phaeophyceae) obtained from the coastal waters of Sanur Beach, Bali, as potential biocontrol strategies against ESBL-producing *Escherichia coli* (Wiradana et al., 2024).

The current study, which builds on earlier research, examines the antibacterial properties of endophytic fungi that were isolated from the mangrove plant *Acanthus ilicifolius*, often referred to as Jeruju, and that were acquired from the Taman Hutan Raya (TAHURA) Ngurah Rai, Bali, Indonesia.

METHODS AND MATERIALS

Study area

Endophytic fungal isolates were isolated from the stems, leaves, and root tissues of healthy, infection-free *A. ilicifolius* (also known as Jeruju) taken from the Mangrove Ecotourism Area of Segara Guna Batu Lumbang in Bali, Indonesia. The sampling site was located in Pemogan District, Denpasar City, at the geographical coordinates 8°44′02.9″S, 115°11′14.8″E (Figure 1).

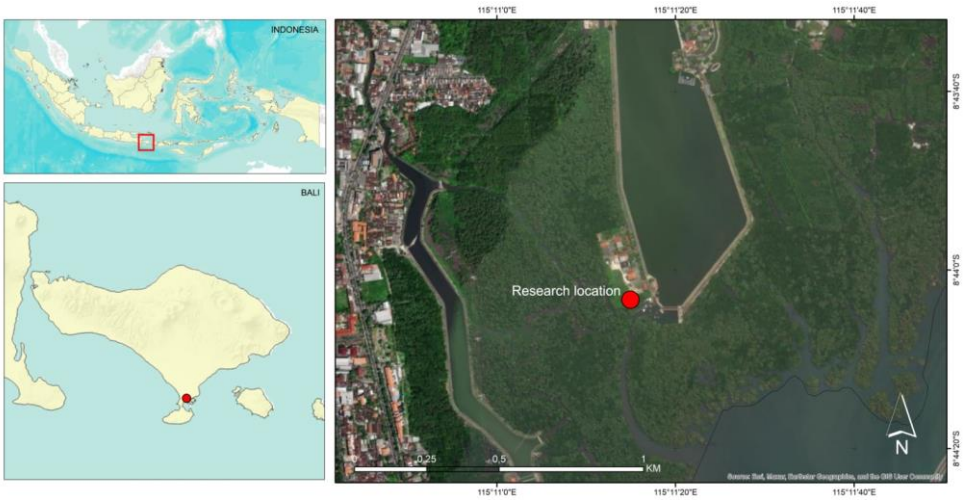


Figure 1. Area of study

Materials

The instruments employed in this study included a laminar airflow cabinet (BioBase, China) incubator (Memmert, China), hotplate (BioBase, China), drying oven (BioBase, China), inoculating loop (Sigma-Aldrich, Germany) 10 mL test tubes (Iwaki, Indonesia) Erlenmeyer flasks (250 mL and 500 mL) (Iwaki, Indonesia), glass stirring rods (Sigma-Aldrich, Germany), Petri dishes (Duran, Germany), aluminum foil (Klinpak, Indonesia), sterile cotton (OneMed, Indonesia), tissue paper (OneMed, Indonesia), *vacuum rotary evaporator* (Sigma Scientific Glass, Germany), orbital shaker (BioBase, China), analytical balance (OHAUS, China), separating funnel (Pyrex, Indonesia), autoclave (Hirayama, Japan), forceps

(IndoSurgicals, Indonesia), scissors (IndoSurgicals, Indonesia), compound microscope (Leica, Singapore), micropipettes (Eppendorf, US), microcentrifuge tubes (Thermo Fisher Scientific, UK), filter paper (Sigma-Aldrich, Germany), UV-Visible spectrophotometer (Thermo Fisher Scientific, UK), vortex mixer (BioBase, China), glass electrophoresis chamber (BioBase, China), sealing film (Sigma-Aldrich, Germany), thermal cycler (Labcycler 48) (SensoQuest GmbH, Germany), and a horizontal electrophoresis system (Mu-Pid EXu Submarine type) (BioRad, USA).

The ingredients employed were Potato Dextrose Agar (PDA) (HiMedia, India), Potato Dextrose Broth (PDB) (HiMedia, India), Nutrient Agar (NA) (Merck, USA), Mueller Hinton Agar (MHA) (Merck, USA), ethyl acetate (Merck, USA), *Methicillin-resistant Staphylococcus aureus* (MRSA) Strain 4430 stock culture, fresh plant tissues of *A. ilicifolius*, chloramphenicol 30 µg (Oxoid, UK), Quick-DNA™ Fungal Miniprep Kit (Zymo Research, USA), DNA polymerase (ThermoFisher, UK), deoxyribonucleotide triphosphates (dNTPs) (ThermoFisher, UK), 5× PCR buffer (Bioline) (ThermoFisher, UK), ITS1 and ITS4 primers (IDT) (Sigma-Aldrich, Germany), dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Germany), sterile distilled water (Sigma-Aldrich, Germany), agarose gel (Sigma-Aldrich, Germany), and Tris-acetate-EDTA (TAE) buffer (Sigma-Aldrich, Germany).

Procedures

A. ilicifolius's tissue sampling and sample surface sterilization

Root, stem, and leaf samples were collected from three healthy A. ilicifolius plants (nine total samples). Tissues were placed in sterile bags, transported on ice (~4°C), and stored at the Laboratory of Health and Science, Universitas Dhyana Pura for further analysis (Khwaja & Arunagirinathan, 2021). To eliminate epiphytic bacteria, all of *A. ilicifolius* tissues (roots, stems, leaves) underwent surface sterilization (Mulyani et al., 2024). The samples were sequentially immersed in 70% ethanol for 1 minute, 5.25% sodium hypochlorite for 5 minutes, and 70% ethanol for 30 seconds, before a final triple rinse with sterile distilled water and drying on sterile paper (Masumoto & Degawa, 2019; Sahu et al., 2022)

Endophytic fungi isolation

In order to prevent bacterial development, each surface-sterilized plant sample was aseptically divided into 1x1 cm segments and carefully put into Petri plates with PDA enriched with 500 ppm of chloramphenicol. For each organ (root, stem, and leaf), five segments were plated per dish, totaling nine Petri dishes: three for roots, three for stems, and three for leaves, each representing different *A. ilicifolius* individuals. This design allowed for broader endophytic fungal diversity while minimizing external microbial contamination. For seven days, every plate was incubated at room temperature (20°C) (Khalil et al., 2021; Toppo et al., 2024; Wiradana et al., 2024). The morphological characteristics of the resulting fungal colonies, such as color, texture, elevation, margin structure, and reverse pigmentation, were used to purify them. To obtain pure cultures, hyphal ends from various colonies were aseptically transferred to new PDA-chloramphenicol plates and cultured for a further seven days at 20°C.

Screening of antibacterial activity of endophytic fungi

An MRSA strain from the Udayana University culture collection was used to screen endophytic fungi for antibacterial activity via a dual-culture method (Wiradana et al., 2024). Fungal discs (1 cm) from 7-day-old cultures were co-cultured with MRSA on Nutrient Agar. Following a 24-hour incubation, the isolate showing the largest inhibition zone (with chloramphenicol and sterile water as controls) was chosen for molecular identification and a 30-day growth analysis in liquid PDB under static and shaker conditions to monitor biomass and pH (Septiana et al., 2017; Zhang et al., 2009).

Endophytic fungal cultivation

The cultivation phase was performed on the selected endophytic fungal isolate that exhibited the highest inhibitory activity in the preliminary screening. This assay aimed to determine the optimal fermentation period for the isolate under liquid culture

conditions. The rejuvenated fungal isolate, initially grown on PDA, was transferred into 1 L of PDB as a pre-culture medium and incubated for 7 days at 29 °C. Subsequently, 10 mL of the fungal filtrate from the pre-culture was inoculated into 250 mL of fresh PDB (main culture). The cultivation was carried out under both static and shaker conditions at ambient temperature for a period of 30 days. Sampling was conducted at 3-day intervals to assess the biomass yield (wet mycelial weight) and pH variations of the fungal broth throughout the fermentation period (Wiradana et al., 2024).

Molecular dentification

Molecular identification began with DNA extraction using a Quick-DNA Miniprep Kit. The ITS region of the rDNA was amplified via PCR using primers ITS1 and ITS4. The 25 µL PCR mixture included 7.5 µL of fungal DNA template, dNTPs, DNA polymerase, DMSO, and reaction buffer. Amplification was performed in a Labcycler 48 thermal cycler under the following conditions: initial denaturation at 95°C for 90 s; 35 cycles of denaturation (95°C, 60 s), annealing (56°C, 90 s), and extension (72°C, 120 s); followed by a final extension at 72°C for 15 min **(**Ariantari *et al*. 2023). PCR amplicons were visualized on a 1% agarose gel. Positive products were purified and sequenced by Macrogen Indonesia and First BASE Laboratories (Malaysia) using the Sanger method. The resulting sequences were identified via BLASTn analysis against GenBank, and phylogenetic relationships were inferred using the neighbor-joining method in MEGA 11 with 1,000 bootstrap replicates.

Screening Process of antibacterial activity using disk diffusion assay

Following optimal cultivation, the fungal culture was filtered to separate the filtrate from the mycelia. The filtrate was sequentially macerated with ethyl acetate, methanol, and n-hexane for three days each. The resulting extracts were concentrated via rotary evaporation at 40°C to yield crude extracts (‘Aini et al., 2022). Antibacterial activity against MRSA was evaluated using an agar well diffusion assay. Each extract was tested at 1000 µg/mL, with ciprofloxacin (10 µg/mL) as a positive control. After incubating inoculated plates at 37°C for 24 hours, the inhibition zones were measured. All tests were performed in triplicate (Rajreepa et al., 2020).

Phytochemical screening

The three crude fungal extracts were screened for major phytochemical classes using standard methods (Chua et al., 2022; Hagag et al., 2024). Alkaloids were detected by orange-red precipitates with Dragendorff's reagent and brown deposits with Wagner's reagent. The Liebermann-Burchard test indicated terpenoids (bluish-green) and steroids (reddish-purple). Saponins were identified by persistent frothing, phenolics by a blue-black color with $FeCl_3$, and flavonoids by a yellow color under UV light (366 nm) following the Wilson-Taubock test.

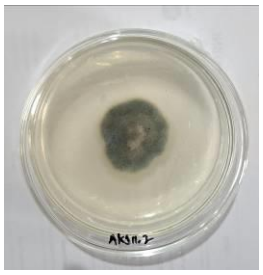
Data analysis

The antibacterial activity during the screening stage was evaluated based on standardized inhibition zone categories. Descriptive morphological characterisation of the endophytic fungus was carried out by examining both macroscopic and microscopic characteristics, and PCR was used for confirming identification. Evaluations of the fungal extract and cultivation period were conducted in triplicate. The data were statistically analyzed using IBM SPSS software (version 26.0). Significant differences were determined through one-way ANOVA, and means were compared using Duncan’s multiple range test at a significance level of $p < 0.05$.

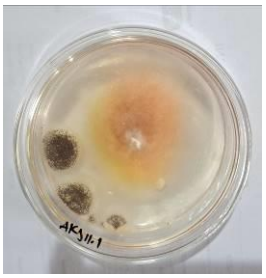
RESULTS AND DISCUSSION

A total of 31 endophytic fungal isolates were successfully obtained and subcultured on PDA medium for 7 days (Figure 2). These isolates consisted of 9 from the roots, 15 from the stems, and 7 from the leaves of *A. ilicifolius*. The macroscopic variation observed among the isolates revealed species diversity. The high number of isolates obtained in this study may be attributed to the extensive sampling across multiple plant parts, which increased the likelihood of recovering diverse endophytic fungi.

Roots



AK1



AK2



AK3



AK4



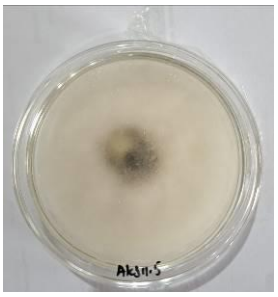
AK5



AK6



AK7

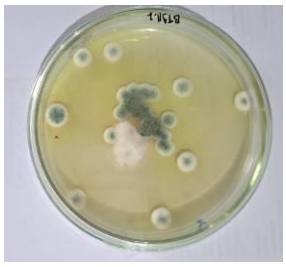


AK8

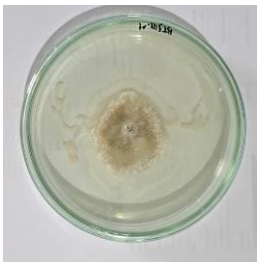


AK9

Stems



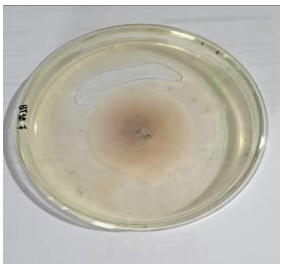
BA1



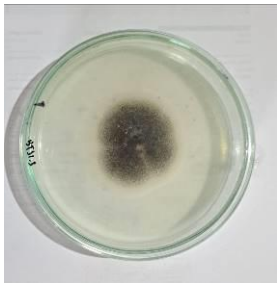
BA2



BA3



BA4









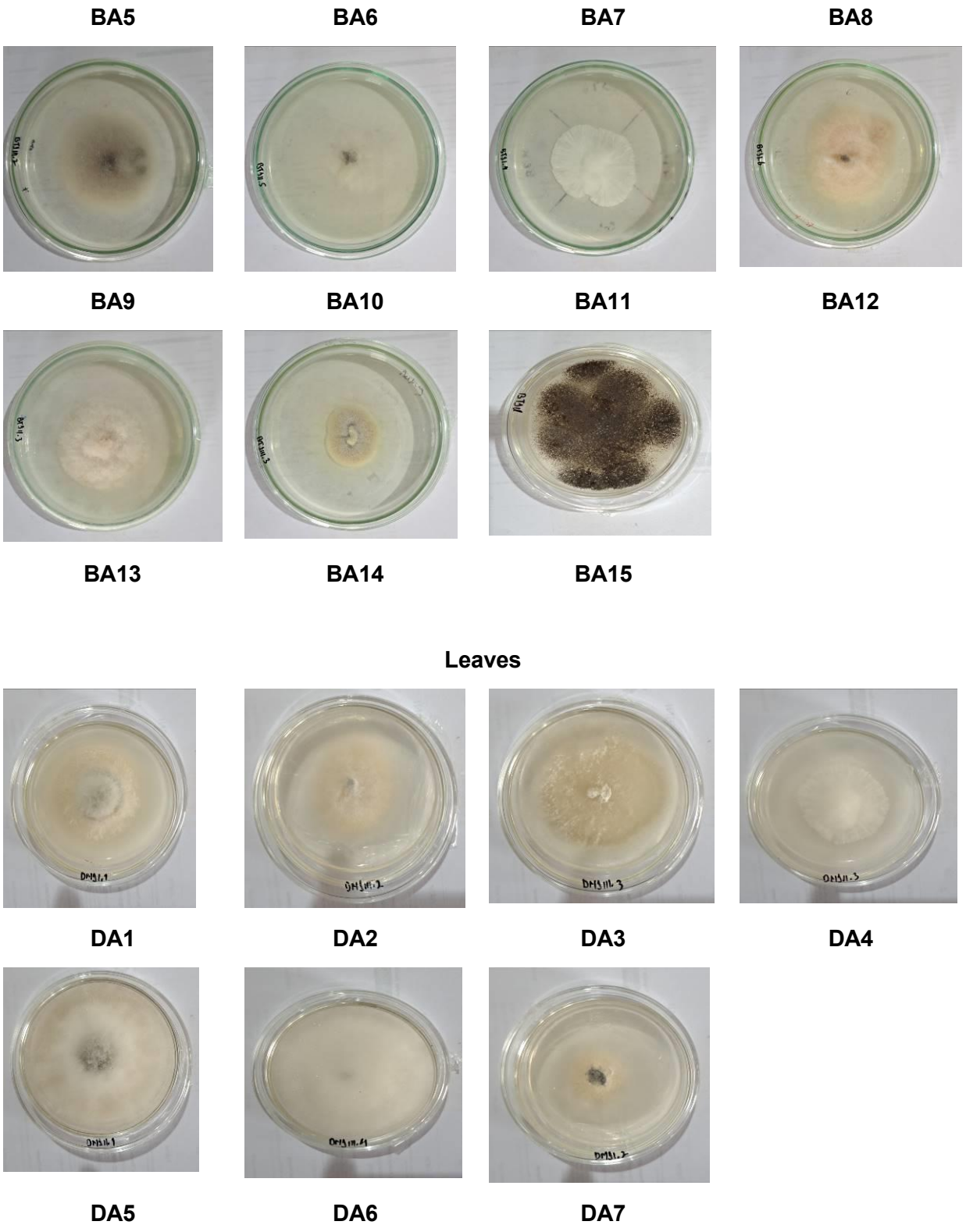


Figure 2. Endophytic fungal isolates from various tissue sections of *A. ilicifolius*.. Isolate codes: AK = root-derived isolates; BA = stem-derived isolates; DA = leaf-derived isolates.

Similar findings have been reported in previous studies, where surface disinfection techniques enabled the successful isolation of up to thirty endophytic fungal isolates from the stem and root tissues of *A. sparsifolia* Shap (Tuerdibieke et al., 2024). A total of 34 endophytic fungal isolates were recovered from the mangrove ecosystem in Dar es Salaam, Tanzania, with 13 of them exhibiting antimicrobial activity (Myovela et al., 2024). The leaves of the mangrove species *Ceriops tagal* have also yielded endophytic

fungus, with an isolation frequency of 11.66%; 96% of these were categorized within the phylum Ascomycota (Revathy et al., 2024).

The preliminary screening revealed that several endophytic fungi displayed moderate to no inhibitory activity against MRSA, with no isolates exhibiting strong antibacterial effects at this stage (Table 1). Notably, moderate inhibition was only observed in isolates symbiotically associated with root tissues, specifically isolates AK3 and AK5, with inhibition zones measuring 13.3 ± 1.5 mm and 14.3 ± 1.5 mm, respectively. For comparison, the positive control used in this study, chloramphenicol, produced an inhibition zone of 15.7 ± 0.6 mm, which also falls within the moderate category.

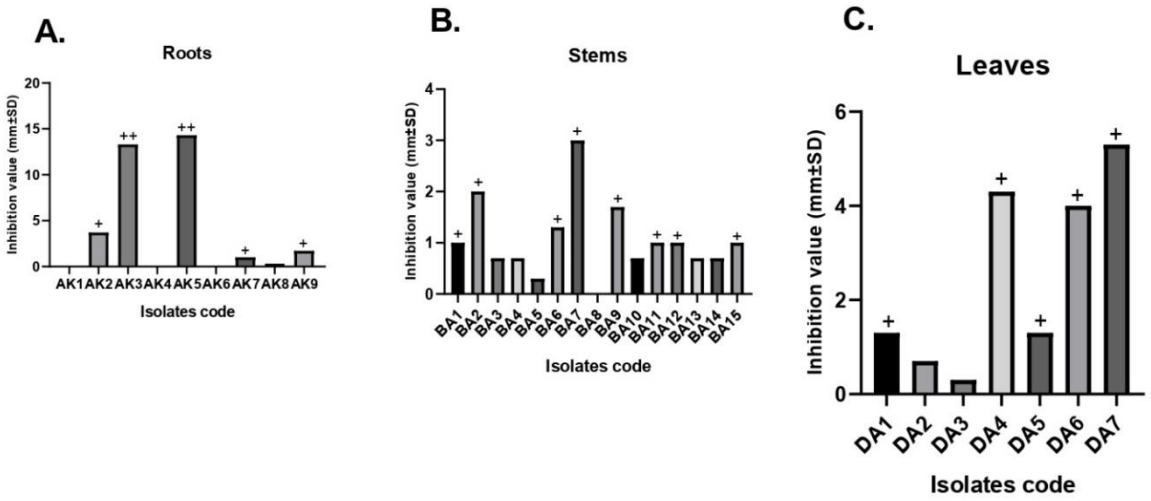


Figure 3. Endophytic fungi isolated from *A. ilicifolius* were tentatively screened for antibacterial activity against MRSA. Note: AK = endophytic fungal isolate from root tissues, BA = endophytic fungal isolate from stem tissues, DA = endophytic fungal isolate from leaf tissues. +++ = strong inhibitory activity (≥20 mm), ++ = moderate inhibitory activity (10–20 mm), + = weak inhibitory activity (1–9 mm), and - = no inhibitory activity or zone of inhibition ≤1 mm (Wiradana et al., 2024).

The results of the initial screening demonstrated that the inhibitory activity was predominantly exhibited by endophytic fungi symbiotically associated with the root tissues of *A. ilicifolius* against MRSA. This result is consistent with earlier research on endophytic fungi from *Syzygium aqueum*, in which two of ten isolates demonstrated potent antibacterial activity against *E. coli, S. aureus,* and *S. typhi* (Hiras Habisukan *et al*., 2021). A similar study on *Avicennia marina* mangrove endophytes revealed that two fungal isolates from root and bark tissues exhibited strong inhibition against *S. aureus* and *V. harveyi*. The root-derived isolate produced inhibition zones of 21.25 ± 0.56 mm and 21.03 ± 0.18 mm, while the bark isolate showed zones of 23.60 ± 0.77 mm and 21.80 ± 0.26 mm (Mulyani et al., 2024). Given their ability to synthesize novel antibiotics and bioactive metabolites, endophytic fungi have been extensively studied for their antimicrobial properties (De Silva et al., 2019; Sharma et al., 2016; Singh & Dubey, 2018). An important evolutionary adaptation to endophytes' internal existence, where they must contend with other microbes for scarce resources and space within the host plant, is their capacity to synthesis antibacterial chemicals (Anam et al., 2024; Drożdżyński et al., 2024). Research indicates that the majority of endophytic fungi can produce beneficial secondary metabolites, including steroids, lactones, phenols, and isocoumarin derivatives (Manganyi & Ateba, 2020). Among these, the AK5 isolate from *A. ilicifolius* roots showed superior antibacterial activity compared to other endophytic fungi from the same plant, specifically in its ability to synthesize anti-MRSA compounds. In a parallel finding, the HP-L1 isolate from Vietnamese nutsedge also

showed promising antimicrobial activity against critical nosocomial bacteria, including MRSA and *P. aeruginosa* (Nguyen et al., 2025).

Based on colony morphology, isolate AK5 exhibited a white-to-brown gradient, with peripheral white zones transitioning to a darker brown center. The colony was cottony, filamentous, and did not produce exudates. Microscopic observations further revealed that AK5 possessed septate hyphae and large, dark brown to black perithecia with hairy setae and visible ascospores (Figure 3).

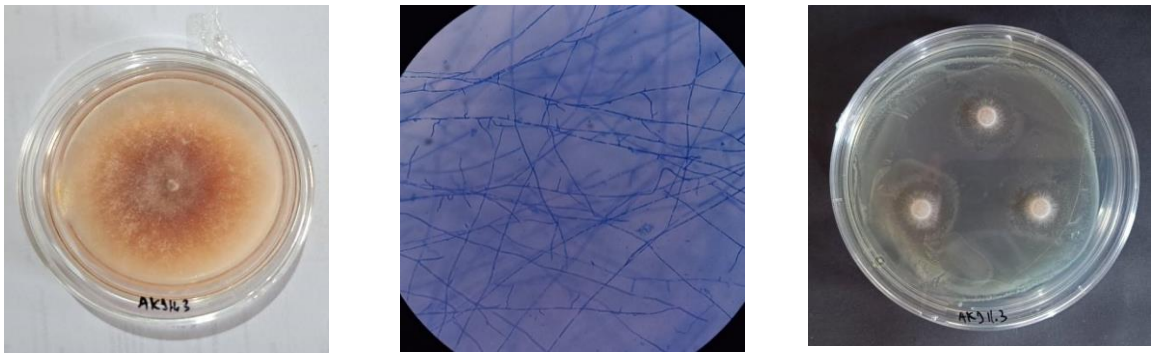


Figure 3. Characterization of AK5 isolate obtained from root tissues of *A. ilicifolius*. Note. **A.** Macroscopic appearance of the AK5 colony; **B.** Microscopic features of the fungal structures; and **C.** Antibacterial activity of AK5 against MRSA, as demonstrated by the inhibition zone formed on agar medium.

Cultivation of Endophytic Fungi

After three days of fermentation, the pH of the fungal cultures under both static and shaker conditions was recorded at 8. A gradual decrease in pH was observed under static conditions, reaching 5.53 by day 6 of fermentation. In contrast, the pH under shaker conditions remained relatively stable at 8.33 until day 6. A marked reduction in pH under shaker conditions began on day 9, dropping to 5.12, and fluctuated between pH 5 and 6 until day 30. A similar trend was observed under static conditions, where the pH consistently ranged between 5 and 6 throughout the remaining cultivation period (Figure 4).

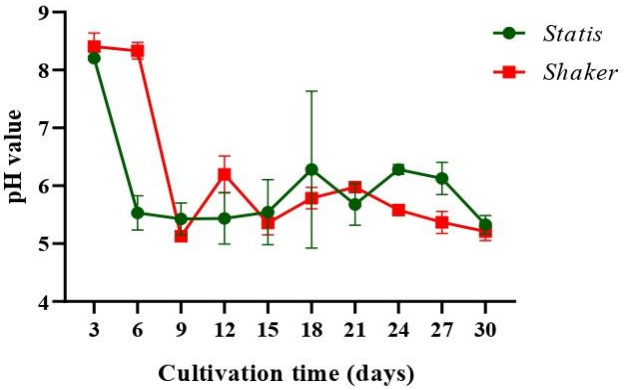


Figure 4. pH profile of the endophytic fungal culture medium during a 30-day fermentation under static and shaker conditions.

In contrast to studies where highly acidic conditions (pH 2–3) suppress fungal metabolism and enzyme production, the isolates in this study thrived in a moderately acidic range (pH 4–6). The observed vigorous growth and sporulation at a stable pH of 5–6 suggest these fungi are well-adapted to these conditions, avoiding the metabolic disruption seen in more extreme acidity (Yap et al., 2023; Pallem, 2019).

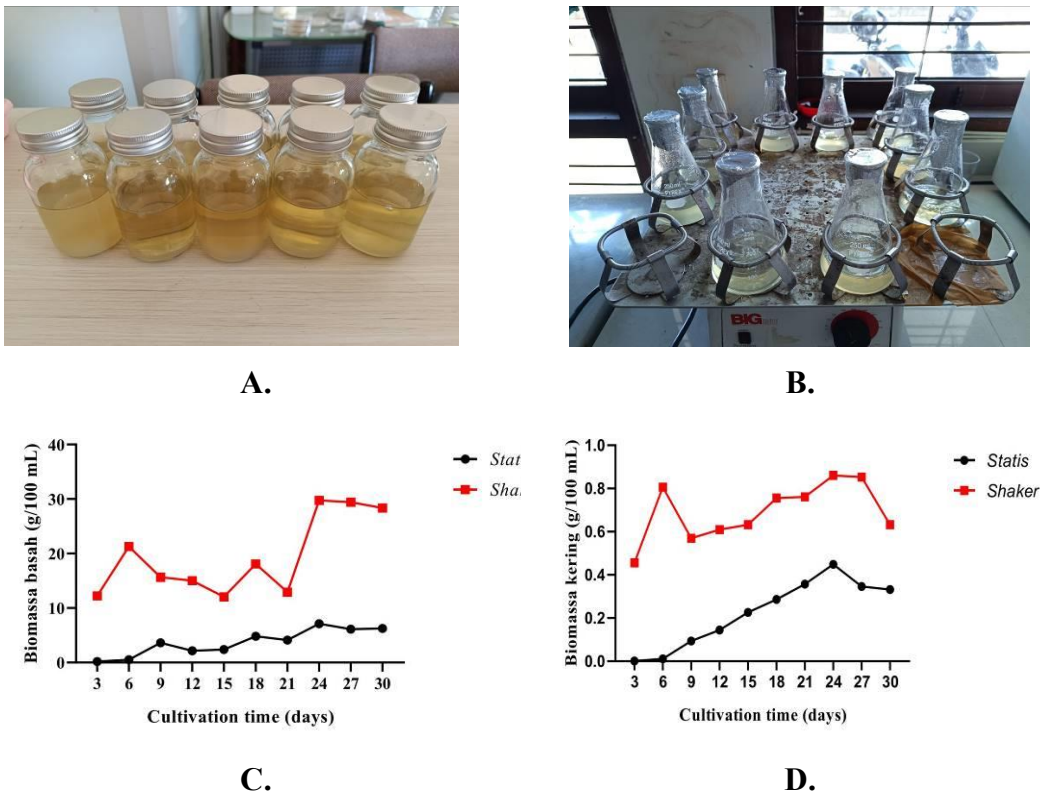


Figure 5. Cultivation of Endophytic Fungal Isolate Over a 30-Day Period in PDB Medium. **A.** Static culture condition, **B.** Shaker culture condition, **C.** Wet biomass of endophytic fungal mycelium, and **D.** Dry biomass of endophytic fungal mycelium.

The endophytic fungus reached its peak biomass on day 24 of the fermentation process, with shaker conditions yielding higher wet (29.73 g) and dry (0.86 g) weights than static conditions (7.12 g and 0.44 g, respectively; Figure 5C). This biomass is a potential source of lipids and other organic compounds, consistent with other endophytic fungi known to accumulate over 20% of their dry weight as lipids (Elfita et al., 2020). Electrophoretic separation verified effective amplification of the rDNA's ITS region in isolate AK5 with universal primers ITS1/ITS4, producing a distinct 500 bp amplicon (Figure 6). The presence of a distinct band in the sample (S) lane confirmed both the successful extraction of fungal genomic DNA and the effective amplification of the ITS region, which is widely used for species-level identification of fungi (Sharma et al., 2016).

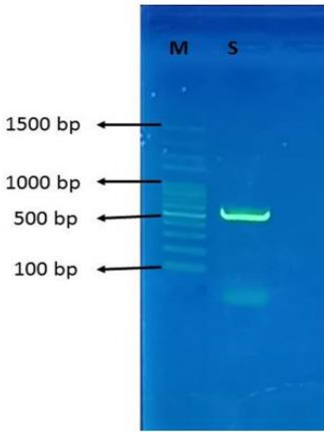


Figure 6. Electrophoresis results of the amplified DNA product from endophytic fungal isolate AK5 using ITS1 and ITS4 primers. Description: M = DNA ladder; S = endophytic fungal isolate AK5, showing a band at approximately 500 bp.

Sequencing analysis revealed that isolate AK5 in this study shared high similarity with *Chaetomium globosum* NW 24 (Accession No. MN326469.1). The nucleotide sequence obtained from the endophytic fungal isolate was aligned with closely related species to determine its phylogenetic relationship, showing 97–100% identity and query coverage with relevant species listed in the NCBI database. The resulting phylogenetic tree constructed from this analysis is presented in Figure 7.

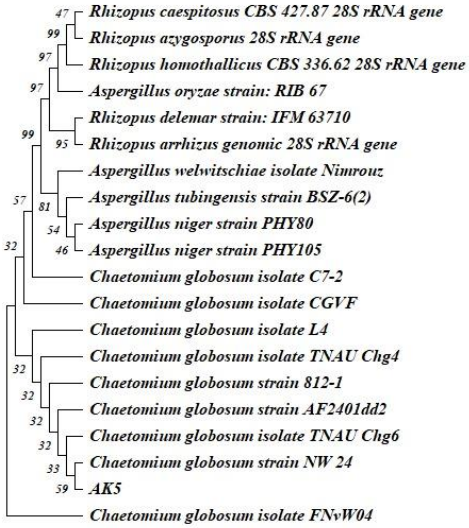


Figure 7. Phylogenetic tree of the endophytic fungal isolate AK5 obtained from the root tissue of *A. ilicifolius*, constructed based on ITS region sequence analysis.

This study provides the first report of *C. globosum* isolated from the root tissue of the mangrove *A. ilicifolius* (isolate AK5, Figure 7). This species is renowned for its bioactive potential; recent studies have documented strains producing anticancer polysaccharides, the membrane-disrupting antifungal agent chaeglobol A (Wang et al., 2024) and metabolites with broad-spectrum antimicrobial activity (Liu et al., 2024). Similarly, bioactive metabolites isolated from deep-sea sediment-derived *C. globosum* SD-347 demonstrated broad-spectrum antimicrobial efficacy against aquatic, plant, and human pathogens (Li et al., 2024). Additionally, *C. globosum* CGMCC 6882, obtained from *Gynostemma pentaphyllum*, displayed concentration-dependent growth inhibition against *S. aureus* and *E. coli* (Wang et al., 2023).

Antimicrobial efficacy of metabolites derived from endophytic fungi

The methanol extract of *C. globosum* NW24 demonstrated the strongest dose-dependent antibacterial activity against MRSA, with an inhibition zone of 17.00 ± 0.264 mm at 1 mg/mL. The ethyl acetate extract showed moderate activity (9.33 ± 0.057 mm at 1 mg/mL), while the n-hexane extract was inactive. As expected, ciprofloxacin (positive control) was highly effective (29.67 ± 0.057 mm), and the DMSO control showed no activity (Table 2).

Tabel 2. Inhibitory activity of *C. globosum* NW24 crude extracts derived from three solvents against MRSA

Extract Consentration (mg/ml)	Solvent		
	Ethyl acetate	n-hexan	Methanol
	Inhibition zone (mm)		
0.125	1.33±0.057^{b}	0.000 ± 0.000^{a}	0.000 ± 0.000^{a}
0.25	3.67±0.115^{c}	0.000 ± 0.000^{a}	0.67±0.057^{a}
0.5	6.00±0.000^{d}	0.000±0.000^{a}	5.00±0.100^{b}
1	9.33±0.057^{e}	0.000±0.000^{a}	17.00±0.264^{c}

Extract Consentration (mg/ml)	Solvent		
	Ethyl acetate	n-hexan	Methanol
Positive Control		29.67±0.057[f]	
Negative Control		0.000±0.000[a]	

Note: All extract concentrations were evaluated in triplicate for each solvent. Statistical significance ($p < 0.05$) was determined using Duncan's multiple range test, with differing superscript letters within columns denoting significant differences

Coastal populations traditionally use *A. ilicifolius* leaf preparations as therapeutic beverages for their antioxidant, antimicrobial, and anti-inflammatory properties (Natarajan et al., 2024). Consequently, these traditional uses have spurred research into the plant's pharmacology and its endophytic microbiome (Bai *et al*., 2014; Cai et al., 2017; Shah et al., 2024). The potent anti-MRSA activity of *C. globosum* NW24's ethyl acetate and methanol extracts, identified from 31 screened isolates, aligns with prior findings of antimicrobial endophytes from mangroves like *O. latifolia* (Hussein et al., 2024). Similarly, the *A. ilicifolius* endophyte *Talaromyces stipitatus* SK-4 yields potent depsidones (talaromyones A/B) active against *B. subtilis* (MIC = 12.5 µg/mL) (Cai et al., 2017).

While endophytes from mangroves like *A. ilicifolius* are known producers of antimicrobial compounds such as *Aspergillus flavipes* with its potent flavipesins research has largely focused on crude extracts. This highlights the need to explore the full bioactivity profile of our identified isolate, *C. globosum* NW24, beyond its initial antimicrobial potential (Bai et al., 2014). *Penicillium* sp. extracts from *Rhizophora apiculata* and *Bruguiera gymnorhiza* against *Salmonella typhi* (Ni et al., 2018). *Aspergillus sydowii* from *Sonneratia alba* showing broad-spectrum activity against *S. aureus, E. coli*, and *C. albicans* (Handayani et al., 2019).

Qualitative Phytochemical Evaluation of Endophytic Fungal Metabolites

The ethyl acetate and methanol extracts of *C. globosum* NW24, which demonstrated anti-MRSA activity, were selected for phytochemical screening and revealed the presence of multiple bioactive compounds (Table 3). The n-hexane fraction was inactive.

Table 3. Results of qualitative phytochemical analysis of the mushroom extract fraction, *C. globosum* NW24

Fungal extract	Phytochemical constituents						
	Phenolic	Tannin	Flavonoid	Saponin	Alkaloid	Steroid	Terpenoid
Ethyl acetate extract	+	+	+	-	+	-	-
Methanol extract	+	+	+	+	+	-	+
N-hexane extract	-	-	-	-	-	-	-

Note: +: positive and -: negative.

Phytochemical screening revealed the richest profile in the methanol extract of *C. globosum* NW24, containing phenolics, tannins, flavonoids, saponins, alkaloids, and terpenoids. The ethyl acetate extract contained fewer classes, while n-hexane showed none. This phytochemical diversity correlates with the methanol extract's superior anti-MRSA activity, though compound purification is needed to confirm the active metabolites' true efficacy (Bonev et al., 2008). This study is the first to document six major phytochemical classes in an endophytic fungus from *A. ilicifolius*. While other endophytes like *A. flavus* from *Dillenia indica* are known to produce similar antimicrobial compounds, our finding underscores the sophisticated metabolic networks of mangrove-associated fungi. These networks enable niche specialization through the production of diverse bioactive molecules like phenolics, alkaloids, and terpenoids (Gupta et al., 2025). While the *A. ilicifolius*-derived endophytic fungi in this study demonstrated only moderate anti-MRSA activity compared to controls, these results

remain scientifically valuable. Significant potential exists in the numerous uncharacterized pure compounds from mangrove endophytes. This underscores the need for advanced genomic and metabolomic investigations to fully elucidate these fungi's potential for pharmaceutical, agricultural, and industrial applications.

CONCLUSION

This study successfully isolated diverse endophytic fungi from *A. ilicifolius*, with root-associated isolates showing the most potent anti-MRSA activity. The identified isolate, *C. globosum* NW24, thrived under shaker fermentation, producing substantial biomass and maintaining a stable pH. The potent antibacterial activity of its methanol extract was linked to a diverse secondary metabolite profile. Further studies should focus on the purification of these active compounds and subsequent *in vivo* testing to assess their therapeutic potential.

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