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Fermentation, bioactivity and molecular identification of endophytic fungi isolated from mangrove *Ceriops tagal*

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Abstract. *Putra IPYA, Utami KS, Hardini J, Wirasuta IMAG, Ujam NT, Ariantari NP. 2023. Fermentation, bioactivity and molecular identification of endophytic fungi isolated from mangrove* Ceriops tagal. *Biodiversitas 24: 3091-3098.* Mangrove-associated-endophytic fungi are recognized as a considerable source of bioactive compounds in drug discovery. This study evaluated the antimicrobial activity and toxicity of endophytic fungi isolated from *Ceriops tagal*. Four endophytic fungal isolates, documented as SU-1-4, were obtained. In addition, isolate SU-3 was further identified as *Hypoxylon mangrovei* according to the comparison of the internal transcribed spacer region. Each fungal isolate was fermented on a solid rice medium, then extracted with ethyl acetate at the end of fermentation. After that, the crude ethyl acetate extracts were partitioned between aqueous methanol and n-hexane, and the methanolic phases were subjected to phytochemical screening and antimicrobial and toxicity assays. Preliminary phytochemical identification suggested the presence of alkaloids, triterpenoids, and steroids in all fungal methanolic extracts. Among them, *H. mangrovei* SU-3 extract showed the most pronounced antimicrobial effects with minimum inhibitory concentration (MIC) values of 125 and 250 µg.mL^{-1} towards *Staphylococcus aureus* ATCC 6538 and *Staphylococcus epidermidis* ATCC 12228. Meanwhile, the toxicity assay employing the brine shrimp lethality test revealed that all fungal methanolic extracts were toxic, with LC_{50} values ranging from 173.09 to 227.76 µg.mL^{-1}. Isolation of antibacterial compounds and evaluation of their cytotoxicity on human cancer cells will be promising for further studies.

Keywords: Antibacterial, *Ceriops tagal*, endophytic fungi, *Hypoxylon mangrovei*, toxicity

INTRODUCTION

The emergence of antimicrobial and anticancer drug resistance has become a significant global health concern. In response to this issue, finding new drug candidates from natural products has been an ultimate strategy in pharmaceutical research for many years. For example, from 1981 to 2019, 63.2% of approved anticancer agents and 55.6% of approved antibacterial drugs were discovered using natural products as their starting point (Newman and Cragg 2020). Among the plethora of natural product sources, endophytic fungi represent a prospective production platform for novel drugs, as they are a relatively untapped source of secondary metabolites, which could potentially provide new leads for drug discovery and development (Tiwari and Bae 2022).

Endophytic fungi belong to a group of microbes that colonize the healthy tissue of plants without harming their host. Over the last two decades, there has been gaining interest in studying bioactive compounds produced by endophytic fungi, especially as antibacterial and cytotoxic agents (Tiwari and Bae 2022); for instance, a macrocyclic alkaloid produced by *Didymella* sp. IEA-3B.1 associated with *Terminalia catappa*, namely ascomylactam C, showed strong antibacterial properties towards several Gram-positive bacteria strains with MIC values of 3.1-6.3 µM (Ariantari et al. 2020). Additionally, a butyrolactone bulgariline B, isolated from *Bulgaria inquinans* residing in the mistletoe, possessed remarkable activity with an IC_{50} value of 1.8 µM against murine lymphoma cell line L5178Y (Ariantari et al. 2019).

As part of our attempt to unearth fungal endophytes' antibacterial and cytotoxic potential (Ariantari et al. 2021; Handayani et al. 2021), we are intrigued to explore the endophytic fungi associated with mangrove trees. Mangroves are recognized as a wealthy reservoir of endophytic fungi for drug discovery (Bibi et al. 2020) due to their capability to thrive in environmental adversities, including tidal transition, high salinity, and low oxygen levels (Feller et al. 2010). Furthermore, these stressors shape the adaptive metabolic pathway in the microbiomes of mangroves to produce unique functional metabolites, making them attractive sources of unusual bioactive compounds waiting to be discovered (Xu 2015; Liao et al. 2020).

Many studies have reported the isolation of endophytic fungi from various mangrove species (Bibi et al. 2020), including the *Ceriops tagal*. *Ceriops tagal* (Perr.) C.B. Rob. (Rhizophoraceae) is a mangrove plant widely nourishes in the coastal intertidal area of tropical and

subtropical regions, particularly in Africa, South Asia, South Pacific islands, and Southern China. This bark species has been used in traditional medicine to treat hemorrhage (Bandaranayake 1998). Phytochemical analysis revealed that *C. tagal* is rich in gallic acid, quercetin (Sachithanandam et al. 2022), terpenoids (Biswas et al. 2021; Zhang et al. 2021; Ahmad et al. 2022), and steroids (Egra et al. 2023). These compounds have been shown to have various potential therapeutic applications, including antitumor (Ni et al. 2018; Sachithanandam et al. 2022), antibacterial (Chacha et al. 2008; Egra et al. 2023), antioxidant (Sachithanandam et al. 2022; Egra et al. 2023), antihyperglycemic, analgesic, anti-inflammatory (Biswas et al. 2023), diuretic (Biswas et al. 2021), immunosuppressant (Zhang et al. 2021), and nematocidal (Ahmad et al. 2022) activities. For instance, vomifoliol, a sesquiterpene isolated from the hypocotyls extracts of *C. tagal*, displayed an immunosuppressant effect by inhibiting the dephosphorylation of NFAT1 and the proliferation of Jurkat cells (Zhang et al. 2021). Moreover, a dolabrane type of diterpene elucidated as ent-5*α*,3,15-dioxodolabr-1,4(18)-diene-2,16-diol from *C. tagal* showed moderate cytotoxicity towards various cancer cell lines with IC_{50} values ranging from 17.6 to 27.7 µM (Ni et al. 2018).

Furthermore, endophytic fungi isolated from *C. tagal* also have been found to produce diverse bioactive compounds as antimicrobial (Deng et al. 2018; Cai et al. 2020) and cytotoxic (Luo et al. 2020; Li et al. 2021). Antibacterial assay of talarocyclopenta B, a phenolicether derivative produced by *Talaromyces assiutensis* JTY2 associated with the leaves of *C. tagal*, exerted remarkable activity against a panel of pathogenic bacteria with MIC values from 1.25 to 5.00 µg.mL^{-1} (Cai et al. 2020). Seiricardine D, a new sesquiterpene afforded upon fermentation of endophytic *Cytospora* sp. from the hypocotyls of *C. tagal*, possessed antifungal activity towards *Magnaporthe oryzae* (Deng et al. 2018). Moreover, cytochalasin analogues, phychaetoglobin B, chaetoglobosins C, E, G, and V, isolated from the fungus *Chaetomium globosum* kz-19 inhabiting the twigs of *C. tagal* displayed strong cytotoxic effects towards HeLa and A549 cells with IC_{50} values ranging from 3.70 to 13.70 µM (Li et al. 2021). Another *C. tagal* endophyte, *Colletotrichum gloeosporioides*, was revealed to naturally synthesize (5R,7S)-5,7-dihydroxy-2-propyl-5,6,7,8-tetrahydro-4H-chromen-4-one with a cytotoxic effect against A549 cells with an IC_{50} value of 94 µM (Luo et al. 2020). Herein, we isolated four endophytic fungi from stems and leaves of *C. tagal*, collected from mangrove conservation in the south of Bali, and evaluated their antimicrobial and cytotoxic potential.

MATERIALS AND METHODS

Sample collection

Endophytic fungi were isolated from the fresh and infection-free stems and leaves of *C. tagal*, collected from Taman Hutan Raya Ngurah Rai, Bali, Indonesia, in August 2021. The collected plant parts were transported immediately to the laboratory for further processing, and the specimen of the plant parts were deposited at the Herbarium Biologi Udayana, Laboratory of Plant Taxonomy, Department of Biology, Faculty of Mathematics and Natural Sciences, Udayana University under specimen number of PY-CT01 (HBU).

Figure 1. Maps of Taman Hutan Raya Ngurah Rai, Bali, Indonesia (*left* and *middle*) as the site of sampling of the leaves and stems of *Ceriops tagal* (*right*) as the host plant of the isolated endophytic fungi

Materials

The agar media for fungal culture were prepared in sterile Petri dishes 90 mm (Esterile®) supplemented with malt extract (Merck®), Bacto agar (Difco BD®), yeast extract (Oxoid®), glycerol (Vivantis®), or chloramphenicol (Nalgene®). Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research) was used to extract the fungal genomic DNA. PCR mixture consisted of DNA polymerase, deoxyribonucleotide triphosphate (dNTP), buffer 5X (Bioline®), ITS1 and ITS4 primers (IDT®), dimethyl-sulfoxide (DMSO) (Sigma Aldrich®), sterilized water (Otsu-WI®), and extracted fungal DNA. Thermocycler Labcycler 48 was used to perform the PCR amplification. Gel agarose for electrophoresis was prepared from Agarose (1st BASE®) and tris-acetate-EDTA (TAE) buffer (Thermo Scientific®) and ran using Mu-pid EXu Submarine (Horizontal) type electrophoresis. Rice (Putri Sejati) was used for fungal fermentation. Methanol and n-hexane were redistilled before use. Antimicrobial activity was assayed against *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, Methicillin-resistant *Staphylococcus aureus* ATCC 3351, *Pseudomonas aeruginosa* ATCC 9027, *Propionibacterium acnes* ATCC 1223, and *Candida albicans* ATCC 10231. Using 96 well micro-plates (IWAKI), the tested bacterial strains were cultured in Mueller Hinton agar (MHA) and Mueller Hinton broth (MHB) (Himedia®), while *C. albicans* was cultured on Sabouraud dextrose agar (SDA) (Oxoid®) and Sabouraud dextrose broth (SDB) (Himedia®). For the toxicity test, *Artemisia salina* eggs (Supreme Plus®) were hatched in artificial seawater salt broth (Himedia®). This study does not involve experiments on animal and human subjects.

Procedures

Isolation of endophytic fungi

Endophytic fungi were isolated under the formerly described procedure (Ariantari et al. 2023). First, the plant samples were briefly washed thoroughly under running tap water and surface sterilized using 70% ethanol for 120 seconds. The samples were then rinsed with sterile distilled water to remove residual sterilizing agents and dried. Furthermore, a negative control was prepared to ensure the surface sterilization's sterility and the isolated fungi's endophytic nature. Next, the surface-sterilized samples were streaked onto an isolation agar plate supplemented with malt extract, Bacto agar, and chloramphenicol in demineralized water. Afterward, the same samples were aseptically dissected into 1×1 cm pieces of blocks and inoculated onto a separate isolation agar plate. Following this procedure, the agar plates were incubated at ambient conditions until fungal colonies were observed. If fungal growth is observed on the negative control dishes, the plates intended for isolating endophytic fungi are excluded from the subsequent purification steps. The process is repeated until no fungal growth is detected in the negative control, ensuring the reliability of the isolation procedure. Finally, the hyphal tip from a visually distinct colony was meticulously picked and transferred to a new agar plate to obtain pure fungal isolate. All isolated endophytic fungal strains were kept in the Laboratory of Molecular Forensic, Udayana University (NPA).

Identification of endophytic fungi

Based on the prominent activity in bioassay screening in this study, endophytic fungal isolate SU-3 was further subjected to molecular identification. Genomic DNA was extracted from the fungal colonies using Quick-DNA Fungal/Bacterial Miniprep Kit following the manufacturer's instructions. First, the internal transcribed spacer (ITS) region of the fungal rDNA was amplified using forward ITS1 (5′-TCCGTAGGTGAACCTGCGG-3') and reverse ITS4 (5′-TCCTCCGCTTATTGATATGC-3') primers. Then, in a microcentrifuge tube, a PCR mixture was prepared to comprise 0.5 µL of ITS1 primer, 0.5 µL of ITS4 primer, 0.5 µL of DNA polymerase, 0.5 µL of dNTP, 0.5 µL of DMSO, 5 µL of 5x buffer, 7.5 µL of fungal DNA, and sterile demineralized water up to 25 µL. Afterward, amplification was performed in Thermocycler Labcycler 48 initiated with pre-denaturation at 95°C for 90 sec, followed by 35 cycles of denaturation at 95°C for 60 sec, annealing at 56°C for 90 sec, and extension at 72°C for 120 sec, as described earlier (Ariantari et al. 2023). The amplification procedure was then terminated by a final extension at 72°C for 15 minutes. Subsequently, the PCR product was visualized through electrophoresis using 1% gel agarose at 70 volts for 45 minutes. The product was then submitted for sequencing to 1st BASE employing Sanger dideoxy sequencing. Using the BLASTN program, the obtained sequences were compared with the deposited sequences available in GenBank, and the phylogenetic relationships were determined using a neighbor-joining tree algorithm with 1,000 bootstraps.

Fermentation and extraction

Each fungal isolate was subjected to fermentation performed in two of 1,000 mL conical flasks, each containing 100 g rice in 110 mL distilled water. Aseptically, each agar plate containing pure fungal isolate was excised into agar blocks and spread onto the surface of the prepared rice media. They were incubated at room temperature in daylight conditions until the fungal mycelia thoroughly covered the rice media. After that, each rice culture was poured with 500 mL ethyl acetate, then agitated on an orbital shaker at 150 rpm for 6-8 hours. The liquid portion was collected through vacuum filtration, then concentrated using a rotary evaporator to yield crude ethyl acetate extract. Subsequently, this extract was subjected to liquid-liquid extraction between n-hexane and aqueous methanol containing 10% water. Each phase was concentrated into dryness under reduced pressure, and the methanol extract was subjected to further analysis and bioactivity tests.

Phytochemical screening

Phytochemical screening was conducted to qualitatively identify the presence of alkaloids, steroids/triterpenoids, polyphenols, saponins (Jones and Kinghorn 2012), and flavonoids (Ghannam et al. 2020) in each fungal extract. The alkaloids test was performed using Dragendorff's and

Wagner's reagents. The appearance of orange to orange-red precipitate after adding Dragendorff's reagent and brownish-to-yellowish precipitate following the reaction with Wagner's reagent suggests the presence of alkaloids in the test solution. Moreover, Liebermann-Burchard's test was performed for steroid/triterpenoid detection in fungal extracts. A bluish-green layer or the brownish/violet ring formation between the two immiscible solvents indicates the presence of steroids or triterpenoids in the tested solution. For qualitative analysis of saponins, Forth's test was done by vigorously shaking the test solution and observing the bubble formation. If there is no bubble formation indicates the absence of saponins, and vice versa. Polyphenols were detected by adding $FeCl_3$ to the test solution. The development of blue, blackish, or greenish-black coloration after the addition of $FeCl_3$ suggests the presence of polyphenols. Particularly, flavonoid screening was done by Wilson-Taubock's test by adding acetone, boric acid, oxalic acid, and ether to the test solution. The appearance of intensive yellow color under UV light 366 nm indicates flavonoids.

Antimicrobial test

The fungal extracts' antimicrobial properties were assessed using the broth microdilution technique following the protocol published by the Clinical and Laboratory Standards Institute (CLSI 2022). Before the test, each fungal extract was dissolved in DMSO. Then, on a 96-microwell plate, the prepared extract was diluted 2-fold in broth media to achieve a series of concentrations starting from 1.95 to 1,000 µg.mL^{-1}. The fungal extracts were evaluated towards several pathogenic microbial strains, including *S. aureus* ATCC 6538, *S. epidermidis* ATCC 12228, Methicillin-resistant *S. aureus* ATCC 3351, *P. aeruginosa* ATCC 9027, *P. acnes* ATCC 1223, and *C. albicans* ATCC 10231. Chloramphenicol and ketoconazole were included as positive controls, while broth medium containing 0.5% DMSO was used as the negative control. Each sample was tested in triplicates. The lowest concentration of extract that completely inhibits the visible growth of the microorganism is determined as the MIC value.

Brine Shrimp Lethality Test (BSLT)

BSLT was employed to examine the toxicity of the fungal extract according to the described procedure (Niksic et al. 2021) with slight modification. First, test solutions were prepared by dissolving the extract using 0.5% DMSO in artificial seawater. Next, to the solution, a 2-fold serial dilution was performed in artificial seawater to obtain a concentration range from 62.5 to 1,000 µg.mL^{-1}. Next, to carry out the BSLT, nauplii were prepared by incubating 20 g of *Artemisia salina* eggs in 300 mL artificial seawater inside a brine shrimp incubator equipped with continuous aeration and illumination at room temperature for 24 hours until they hatched. Using a disposable Pasteur pipette, 10 mature and healthy nauplii free from eggshell were transferred into vials containing test solution with the above concentration. The final concentration of 0.5% DMSO in artificial seawater was added as the negative control. The tests were conducted in triplicates. Each assay was incubated under constant lighting at room temperature for 24 hours. Afterward, the number of dead nauplii in each vial was enumerated, and each concentration's mean mortality percentage was calculated. Using SPSS version 26, the LC_{50} value of each extract was determined through Probit analysis.

RESULTS AND DISCUSSION

Isolation and identification of endophytic fungi

Four endophytic fungal strains were successfully isolated from mangrove *C. tagal* and coded as SU-1, SU-2, SU-3, and SU-4 isolates. Fungal isolates SU-1 and SU-2 were obtained from the leaves, while isolates SU-3 and SU-4 were afforded from the stems. The morphological appearance of these fungal isolates is shown in Figure 2.

Among the isolated fungal endophytes from *C. tagal*, isolate SU-3 was selected for further molecular identification due to its most pronounced activity on antimicrobial and toxicity assays. Amplification of its ITS region yielded a single DNA band of 500-750 base pair as displayed on gel agarose of electrophoresis (Figure 3).

Comparison of ITS region sequences through BLASTN search revealed that the isolate SU-3 belonged to the species *Hypoxylon mangrovei* (GenBank accession number: OQ726608) with a similarity percentage of 99.26%, query cover of 100%, and a maximum score of 977. This result was in line with phylogenetic analysis (Figure 4), which showed that isolate SU-3 was grouped into *Hypoxylon mangrovei* with bootstrap support of 90%.

Extraction

Methanolic and n-hexane extracts were obtained from the liquid-liquid partition of each fungal isolate's crude ethyl acetate extract (Table 1). Isolate SU-4 was found to produce the highest amount of extract, while the lowest one was yielded from isolate SU-1.

Phytochemical screening

Preliminary qualitative phytochemical analysis indicated that alkaloids, triterpenoids, and steroids were detected in each methanolic extract (Table 2). In addition, polyphenols were detected in the methanolic extract of isolates SU-3 and SU-4 and were absent in the extracts of isolates SU-1 and SU-2. No flavonoids and saponins were observed in all fungal extracts.

Antimicrobial assay

The antimicrobial assay showed that methanolic extract from isolate SU-3 possessed the most notable antibacterial activity against *S. aureus* and *S. epidermidis* with MIC values of 125 and 250 µg.mL^{-1}, respectively. The weaker activity was displayed by the extract of isolate SU-1 with MIC values of 250 and 500 µg.mL^{-1} towards *S. aureus* and *S. epidermidis*, respectively. The extract produced by isolate SU-4 also showed weak potency against these bacteria with MIC values of 500 and 250 µg.mL^{-1}, respectively. All extracts were inactive against MRSA, *P. aeruginosa*, *P. acnes*, and *C. albicans* up to 1,000 µg.mL^{-1} of the tested concentration (Table 3).

Brine Shrimp Lethality Test

The BSLT result (Figure 5) showed that extracts of SU-1-4 had LC_{50} values of 227.76, 188.91, 173.09, and 191.99 $\mu g.mL^{-1}$, respectively. All tested extracts were categorized as toxic because these LC_{50} values are less than 1,000 $\mu g.mL^{-1}$ (Meyer et al. 1982). The correlation between the BSLT outcome and the cytotoxicity of natural products on human solid tumor cell lines is noteworthy, indicating that this could be an initial method to assess the potential of an extract as an antitumor agent (Anderson et al. 1991).

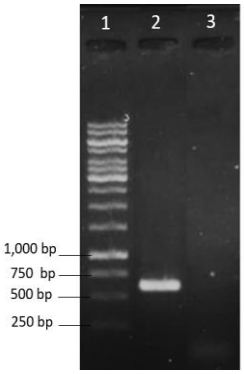

Figure 3. Electrophoregram of PCR product of amplified ITS region from SU-3. 1: 1 kb DNA marker; 2: SU-3; 3: negative control

Table 1. The amount of extract afforded following fermentation of endophytic fungal isolates from *C. tagal*

Code of fungal isolate	Amount of extract (mg)		
	Ethyl acetate extract	Methanol extract	N-hexane extract
SU-1	470.0	134.2	335.8
SU-2	776.6	384.1	392.5
SU-3	871.6	306.9	564.7
SU-4	1,339.0	565.8	773.5

Table 2. Result of phytochemical screening of fungal methanolic extract of endophytic fungal isolates from *C. tagal*

Phytoconstituents	Methanolic extracts from isolated endophytic fungi			
	SU-1	SU-2	SU-3	SU-4
Alkaloid	+	+	+	+
Triterpenoids	+	+	+	+
Steroids	+	+	+	+
Polyphenols	-	-	+	+
Flavonoids	-	-	-	-
Saponins	-	-	-	-

Note: + /- = indicated the presence/absence of the corresponding phytoconstituent

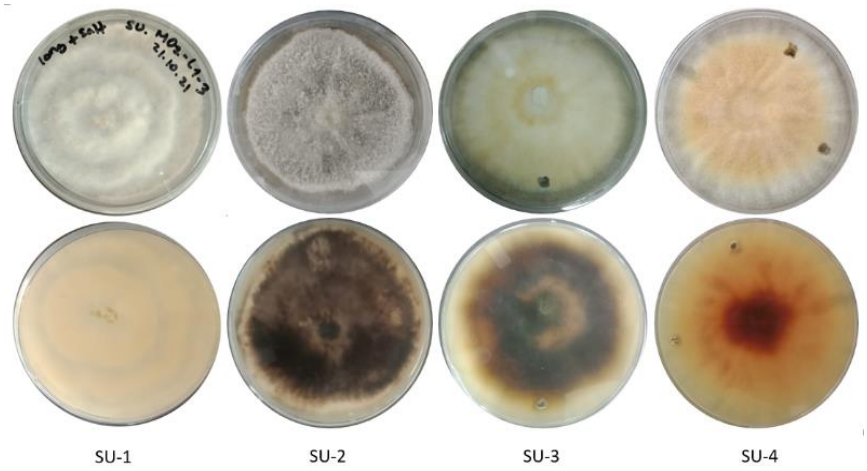

Figure 2. Morphological appearance of each endophytic fungi isolated from leaves and stems of *Ceriops tagal* cultured on agar media supplemented with malt extract, yeast extract, glycerol, and Bacto agar in demineralized water. The top and bottom pictures represent the respective fungal isolate's front and reverse views

Table 3. Antimicrobial properties of each fungal methanolic extract against several microbial strains

Code of methanolic extract	Minimum inhibitory concentration ($\mu g.mL^{-1}$) against several microbial strains					
	S. aureus ATCC 6538	*S. epidermidis* ATCC 12228	MRSA ATCC 3351	*P. aeruginosa* ATCC 9027	*P. acnes* ATCC 1223	*C. albicans* ATCC 10231
SU-1	250	500	>1,000	>1,000	1,000	>1,000
SU-2	1,000	1,000	>1,000	>1,000	1,000	>1,000
SU-3	125	250	>1,000	>1,000	1,000	>1,000
SU-4	500	250	>1,000	>1,000	1,000	>1,000

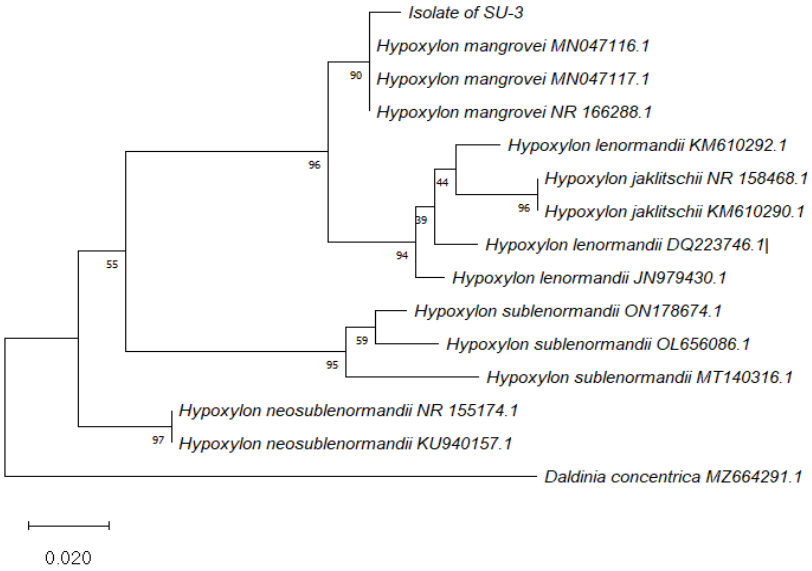

Figure 4. Phylogenetic relationship of isolate SU-3 based on comparing internal transcribed spacer (ITS) region using the neighbor-joining algorithm with 1,000 bootstraps

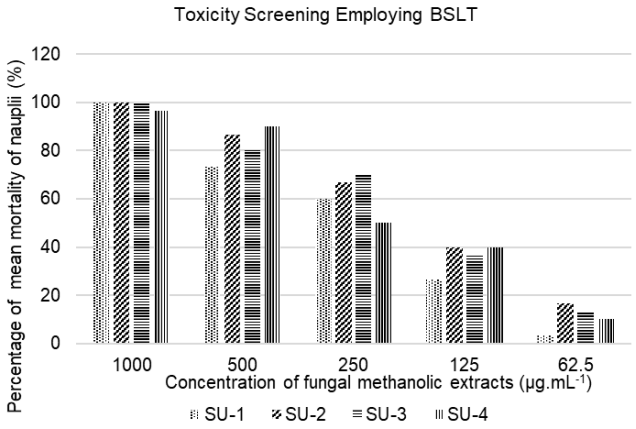

Figure 5. Percentage of mean (n=3) mortality of nauplii in each tested concentration of methanolic extract of isolates SU-1-4 from *C. tagal*

Discussion

Four endophytic fungi were isolated from mangrove *C. tagal* in this study. Among them, isolate SU-3 was determined as *Hypoxylon mangrovei* according to the ITS region identification. Several studies have revealed the diversity of endophytic fungi associated with *C. tagal*. For example, genera *Xylaria* and *Nodulisporium* were found previously to dominate the leaves of *C. tagal* in Andaman Island, India (Rajamani et al. 2018), while *Phomopsis* and *Pestalotiopsis* were frequently recovered from the stems and roots of *C. tagal* collected in the south coast of China (Xing and Guo 2011). However, in the present study, *H. mangrovei* was isolated from the stems of *C. tagal*. Previously, *H. mangrovei* was only reported as a saprobe on decomposing wood of *Rhizophora* sp. (Rhizophoraceae) mangrove collected in Prachaub Kiri Khan Province, Thailand (Dayarathne 2020). The distinct diversity of fungal endophytes among the same species of plant host is strongly influenced by the difference in the environmental condition of the host habitat (Lee et al. 2019; Wainwright et al. 2019). Other species of *Hypoxylon* were reported previously from various marine sources, including sponges (Leman-Loubière et al. 2017), algae (Medina et al. 2016; Vicente et al. 2021), and mangroves (Hou et al. 2021), as well as from the Chinese medicinal plant *Artemisia annua* (Gu et al. 2007; Liang et al. 2018).

Furthermore, we screened the antimicrobial activities and toxicities of the fungal methanolic extracts. Some of these extracts showed moderate antibacterial activity, while BSLT confirmed that these extracts were classified into the toxic category. The BSLT result of natural substances significantly correlates with their cytotoxicity in human solid tumor cells. Therefore BSLT is regarded as a pre-screening method for cytotoxicity against human cancer cells (Anderson et al. 1991). Among the tested extracts, the extract produced by *H. mangrovei* SU-3 showed the most pronounced potentials as antibacterial and cytotoxicity. The pharmacological activity of the tested fungal extract was influenced by its phytochemical constituents. Phytochemical screening indicated the presence of alkaloids, terpenoids, steroids, and polyphenols in *H. mangrovei* SU-3 extract. Previous studies have reported several compounds isolated from the extract of the genus *Hypoxylon*. For instance, terpenoids fendlerals A and B, along with fendlerin A, afforded from *Hypoxylon fendleri* of unidentified wood samples collected in Thailand, exhibited significant antimicrobial activities against *Plasmodium falciparum*, *Colletotrichum capsici*, and *Bacillus cereus* (Intaraudom et al. 2019). In addition, a multitude of terpenoids isolated from *Hypoxylon rickii*, such as botryanes, hypoxylan A, and rickitin A were found

to show cytotoxicity with IC_{50} values of 8.5 to 36.0 µg.mL^{-1} against murine cells (Kuhnert et al. 2015). Moreover, a group of α-pyrone alkaloids, namely hypotien, was successfully isolated from *Hypoxylon investiens* residing in *Blumea balsamifera* despite no antibacterial and cytotoxicity was shown (Yuan et al. 2019).

Similarly, several bioactive compounds with antimicrobial and cytotoxic activities also have been reported from endophytic fungi isolated from *C. tagal* in previous studies. For instance, alkaloids pyranonigrins A and S produced by *Talaromyces assiutensis* JTY2 were found to possess a broad spectrum of antifungal potential against an array of phytopathogenic fungi (Li et al. 2022). Other alkaloids, cladosporitin B and talaroconvolutin A, isolated from the extract of *Cladosporium* sp. HNWSW-1 showed cytotoxic potential towards several cancer cell lines with IC_{50} values ranging from 14.9 to 41.7 µM (Wang et al. 2018). Moreover, various bioactive terpenoids were also repeatedly isolated from endophytic fungi associated with *C. tagal*, including seiricardine (Deng et al. 2018), 2α-hydroxyxylaranol B, 4β-hydroxyxylaranol B (Zeng et al. 2015), among others. Interestingly, a terpenoid, 3,4-seco-sonderianol isolated from an unidentified endophytic fungus of the leaves of *C. tagal*, showed cytotoxicity against several cancerous cell lines with IC_{50} values starting from 9.2 to 25.4 µg.mL^{-1} (Zeng et al. 2015). Fungal endophytes of *C. tagal* were also reported before being capable of producing steroids. In a study by Deng et al. (2018), ergosterols, sitosterol, and stigmasterone were isolated and characterized from *Cytospora* sp. cultures. Among them, (22*E*,24*R*) 5,8-epidioxy-5α, 8α-ergosta-6,22*E*-dien-3ß-ol was found to be the most active against *Pseudomonas aeruginosa* and *Bacillus subtilis* with MIC values of 58.3 µM (Deng et al. 2018).

Overall, this study demonstrates the prospective antimicrobial properties and toxicity of the methanolic extracts from endophytic fungi associated with *C. tagal*. However, while the BSLT becomes a useful tool for cytotoxic screening, this assay only shows significant activity if the LD_{50} value is less than 30 µg.mL^{-1} (Meyer et al. 1982). Moreover, it is not tumor-specific (Meyer et al. 1982) and not applicable to compounds that need activation (Anderson et al. 1991). Further studies, therefore, should be performed to confirm the toxicity of the fungal methanolic extracts in the present study on human cancer cell lines.

In conclusion, four endophytic fungi were successfully isolated from *C. tagal*, one of which was identified as *H. mangrovei* SU-3. By far, this is the first report on the isolation of this fungus from *C. tagal* accompanied by its antibacterial assay with MIC values of 125 and 250 µg.mL^{-1} against *S. aureus* and *S. epidermidis*, along with its toxicity screening with LC_{50} value of 173.09 µg.mL^{-1}. Furthermore, apart from the biological diversity of endophytic fungi inhabiting *C. tagal* reported so far, these microorganisms have also reported capable of producing various bioactive compounds possessing antimicrobial and cytotoxic effects. Due to its potency, the isolation of antibacterial compounds from *H. mangrovei* SU-3 and the investigation of their prospective cytotoxicity on human cancer cells will be of scientific interest for future research.

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