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Research Reports

Isolation and Molecular Identification of Endophytic Fungi Associated with Brown Algae for Inhibiting *Escherichia coli* ESBL

Isolasi dan Identifikasi Molekuler Jamur Endofit Berasosiasi Dengan Alga Coklat untuk Menghambat *Escherichia coli* ESBL

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ABSTRACT

Background: The marine environment is the main source of research on natural products in the future. In addition, marine microorganisms have been identified as a natural source capable of developing new antibiotic compounds, including controlling urinary tract infections caused by *Escherichia coli* ESBL. **Purpose:** This study aims to isolate, select, and test the potential of brown macroalgae endophytic fungi (Phaeophyceae) collected from the coastal waters of Sanur, Bali Province. **Methods:** Three types of brown algae were collected from Sanur Beach and their endophytic fungi were isolated using PDA media. Antibacterial activity was determined by measuring the inhibition zone and determining the inhibition category. The selected isolates were cultured and the phytochemical profile was determined qualitatively. In addition, molecular identification using the Internal Transcribed Spacer (ITS) primer set and comparison with the GenBank (NCBI) database were carried out in this study. **Results:** The results showed that 10 isolates were successfully isolated from three types of brown macroalgae. It was found that isolates T1, S1, and P3 had the highest inhibition in the very strong category. There were variations in mycelial weight, pH value, and inhibition shown by the culture filtrate of the three endophytic fungal isolates against *Escherichia coli* ESBL. Isolate S1 had the highest phytochemical profile, namely alkaloids, triterpenoids, saponins, and phenolics. The three endophytic fungal isolates showed isolate T1 (*Phlebiopsis magnicystidiata*) (MT5617191), isolate P3 (*Neurospora crassa* strain RT3M) (MT1028551), and isolate S1 *(Peniophora sp.)* (MH2680421). **Conclusion:** The results of this study provide initial information regarding the potential of bioprospecting brown macroalgal endophytic fungi as a source of new antibiotics against *Escherichia coli* ESBL.

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ABSTRAK

Latar Belakang: Lingkungan laut adalah sumber utama penelitian tentang produk alami di masa depan. Selain itu, mikroorganisme laut telah diidentifikasi sebagai sumber alami yang mampu mengembangkan senyawa antibiotik baru, termasuk mengendalikan infeksi saluran kemih yang disebabkan oleh *Escherichia coli* ESBL. **Tujuan:** Penelitian ini bertujuan untuk mengisolasi, menyeleksi, dan menguji potensi jamur endofit makroalga coklat (Phaeophyceae) yang dikumpulkan dari perairan pantai Sanur, Provinsi Bali **Metode:** Tiga jenis alga coklat dikumpulkan dari Pantai Sanur dan jamur endofitnya diisolasi menggunakan media PDA. Aktivitas antibakteri ditentukan dengan mengukur zona inhibisi dan menentukan kategori inhibisi. Isolat yang dipilih dikultur dan profil fitokimia ditentukan secara kualitatif. Selain itu, identifikasi molekuler menggunakan primer set Internal Transcribed Spacer (ITS) dan perbandingan dengan database GenBank (NCBI) dilakukan dalam penelitian ini. **Hasil:** Hasil penelitian menunjukkan bahwa 10 isolat berhasil diisolasi dari tiga jenis makroalga coklat. Ditemukan bahwa isolat T1, S1, dan P3 memiliki inhibisi tertinggi dalam kategori sangat kuat. Terdapat variasi berat miselia, nilai pH, dan inhibisi yang ditunjukkan oleh filtrat kultur dari ketiga isolat jamur endofit terhadap *Escherichia coli* ESBL. Isolat S1 memiliki profil fitokimia tertinggi, yaitu alkaloid, triterpenoid, saponin, dan fenolik. Ketiga isolat jamur endofit menunjukkan isolat T1 (*Phlebiopsis magnicystidiata*) (MT5617191), isolat P3 (*Neurospora crassa* strain RT3M) (MT1028551), dan isolat S1 *(Peniophora sp.)* (MH2680421). **Kesimpulan:** Hasil penelitian ini memberikan informasi awal mengenai potensi bioprospeksi jamur endofit makroalga coklat sebagai sumber antibiotik baru terhadap *Escherichia coli* ESBL.

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Kata kunci: *Escherichia coli* ESBL; Jamur Endofit; Makroalga Coklat; Lingkungan Laut; Resistensi Antibiotik

INTRODUCTION

The occurrence of antimicrobial resistance (AMR) has preceded the discovery and commercialization of new antimicrobial agents for the control of infectious diseases in the community. This has resulted in increased detection of bacteria and antimicrobial resistance genes in natural environments, especially those heavily influenced by human activities, such as agriculture, aquaculture, and suboptimal waste processing or disposal (Larsson and Flach, 2022). On the other hand, anthropogenic and geogenic pollutants in the environment, along with antimicrobials, may indirectly induce or facilitate the process of AMR (Kraemer *et al.*, 2019; Rosiana *et al.*, 2022, Wasilah *et al.*, 2021). Heavy metals have been widely reported to be co-selective for AMR (Poole, 2017), including towards an environment that is unaffected by large-scale antimicrobial use (Imran *et al.*, 2019).

Various reports of increasing morbidity and mortality due to resistant infections have revealed an ongoing global increase in antimicrobial resistance (AMR), which is now widely recognized as a global public health threat. In summary, the main known causes of AMR include bacterial mutations (von Wintersdorff *et al.*, 2016). Infections caused by resistant microorganisms can result in more severe clinical infections, treatment failure, longer hospitalizations, and likely higher health care costs (Mancuso *et al.*, 2021). On the other hand, the reduced effictiveness of first-line antimicrobial therapy, which was previously effective against a wide range of bacteria, has resulted in the use of last-line antimicrobials, which are more expensive and have more side effects (Rawson *et al.*, 2021).

Extended-spectrum beta lactamase (ESBL) is an enzyme that can cause resistance to most beta-lactam antibiotics, including penicillin, cephalosporins, and monobactam aztreonam (Paterson and Bonomo, 2005). *Escherichia coli* is one of the microorganisms that can produce ESBL (Hayer *et al.*, 2023). The circulation of *Escherichia coli* ESBL is very widespread, including from domestic animals (Falodun *et al.*, 2022), wildlife (Dalazen *et al.*, 2023), agricultural-livestock environments (Al-Mustapha *et al.*, 2023), and healthcare settings (Azuma *et al.*, 2022), thus having the potential to spread resistance. Recent evidence shows that the large proportion of *Escherichia coli* ESBL in the aquatic environment is of great concern, and these bacteria are found in human fecal samples, which contributes to the presence of antibiotic resistance in the aquatic environment (Li *et al.*, 2023). The majority of plasmid-encoded ESBLs are of the TEM, SHV, or CTX-M types, with CTX-M being the most common ESBL type in *Escherichia coli* and *K. pneumonia* (Rossolini *et al.*, 2008). The spread of blaCTX-M-15 poses a major public health threat because isolates expressing this gene are generally resistant to various antibacterial treatments (Agyekum *et al.*, 2016).

Endophytes are endosymbiotic microorganisms that invade the intercellular spaces of plants in their natural habitat (Oukala *et al.*, 2021, White *et al.*, 2019). Plants provide habitat and food for endophytic microorganisms in this mutualistic relationship, while endophytic microbes protect against diseases and pest infections and induce tolerance to abiotic stresses, such as drought and salinity (Rana *et al.*, 2020). Bioactive compounds produced by *Pseudomonas syringae*, for example, can cause stomatal closure, allowing pathogens to enter the apoplast (Melotto *et al.*, 2006). In addition, endophytic microbes play an important role in the ecological carbon-nitrogen cycle (Peñuelas and Terradas, 2014). They have the ability to influence plant biogeography and ecosystem function by controlling host performance in a variety of environmental situations (Guerreiro *et al.*, 2018). At the metabolic level, metabolites indicate the physiological condition of plant organisms. These metabolites are the end products of gene transcription and protein expression, as well as the basic building blocks of the phenotype of an organism (Jin *et al.*, 2017). On the other hand, metabolites can also influence or control gene transcription and expression, as well as protein activity (Rinschen *et al.*, 2019). Internal and external influences, such as growth, plant type, and environmental conditions, can affect the concentration of metabolites or the type of active compound produced. Ultimately, these changes will affect the metabolic pathway (Trivedi *et al.*, 2020).

Endophytes and their secondary metabolites have received increasing attention in recent years, especially for medical and industrial applications, as well as for the development of new biological control agents in the sustainable agricultural sector (Deutsch *et al.*, 2021; Li *et al.*, 2022). The marine environment is currently considered as one of the most important sources in the field of natural products research, as marine organisms have demonstrated extraordinary biological, biochemical, and biosynthetic potential (Newman and Cragg, 2014). Research into marine natural products is often considered important for the identification of new chemical structures, especially those with unconventional mechanisms of action (Permatasari *et al.*, 2022; Widhiantara *et al.*, 2022).

The relationship between macroalgae and their endophytic fungus has been reported in the ecological field. However, research on endophytic fungus from tropical locations is still very limited. Several recent findings have detailed the isolation of endophytic fungi and bacteria and the discovery of their bioactive compounds as materials for producing new antibiotics from macroalgae (de Felício *et al.*, 2015; Kamat *et al.*, 2022; Mathew *et al.*, 2023). The need for the availability of new antimicrobial sources that can reduce the use of commercial antibiotics can be produced by endophytic fungi. Research shows that endophytic microorganisms have been shown to produce up to 166 molecules with new chemical structures and antibacterial activity. On the other hand, endophytic microorganism offer lower commercial production costs, easier to culture, and controlled fermentation conditions to support commercial-scale synthesis of antibiotic compounds (Silva *et al.*, 2022). The results of previous studies also summarize the compounds isolated from endophytic fungi that have bioactivities such as sources of antibiotic, antioxidant, cytotoxic, antitumor, antimalarial, anti filarial and enzyme inhibition (Nalini and Prakash 2017).

However, studies on the potential of endophytic fungi, especially those isolated from brown macroalgae (Phaeophyceae) in controlling infectious diseases such as *Escherichia coli* ESBL, are still limited. Secondary metabolites produced by endophytic fungi are very useful if developed as alternative agents to control infections from *Escherichia coli* ESBL and to produce new antibiotic compounds from the marine environment. Therefore, this study aims to isolate, identify, and test the inhibitory power of endophytic fungi that were isolated from brown macroalgae collected from the coastal waters of Sanur, Bali Province as a new source for producing secondary metabolites and biological control agents in controlling Escherichia coli ESBL. The findings of this study could potentially reduce dependence on pharmaceutical treatments by exploring natural substances as alternatives to antibiotics.

MATERIAL and METHOD

Brown Macroalgal Sampling

Brown macroalgae (*Sargassum aquifolium, Padina australis,* and *Turbinaria ornata*) were collected from the waters of Sanur, Bali Province at the lowest low tide conditions in the intertidal zone. The algae were collected randomly from three beaches in Sanur, namely Mertasari Beach, Semawang Beach, and Sindhu Beach. The collected macroalgae were placed in a sterile plastic clip measuring 20 x 30 cm, labeled, and taken to the laboratory for further analysis. During transportation, brown macroalgae samples were stored in a cooler box filled with ice gel. Upon arrival at the laboratory, brown macroalgae were washed with sterile distilled water to remove contaminats and stored in the refrigerator until used for isolation of endophytic fungi at a later stage.

Isolation of Endophytic Fungi from Brown Macroalgae

Endophytic fungi were isolated in laminar airflow under sterile conditions. The brown macroalgae were cleaned with fresh water and sterilized using 70% ethanol (Merck, UK) three times for three seconds, followed by three washes for one minute with distilled water. To ensure that optimal surface sanitation of macroalgae, the fungi were tested by inoculation on potato dextrose agar (PDA) (Merck, UK). The absence of fungal growth indicated that the macroalgae surface was sterile. Isolation was carried out in 90 mm Petri dishes (Iwaki Pyrex, Japan) with brown macroalgae first sliced into small pieces (0.5 x 0.5 cm) and placed in sterile PDA growth medium **(Figure 1)**.

Endophytic fungi were isolated using at least two Petri dishes, with five pieces each, for each brown macroalgae. The Petri dish was then incubated at 25ºC for seven days (Deutsch *et al.*, 2021). Fungus that successfully grew were collected and inoculated on new PDA media for purification and placed in a "Y" shape. Fungus that showed different colonies were collected and inoculated on sterile PDA media that had been added with chloramphenicol (Sigma-Aldrich, USA). The fungal isolates that grew were used for the selection stage.

Selectivity of Endophytic Fungi

The selection of endophytic fungi was performed directly on the test microbe, namely pure E. coli ESBL, which is a collection culture from the Clinical Microbiology Laboratory of Dr. Kariadi Central General Hospital, Semarang, Central Java, obtained from urine samples of patients positive for urinary tract infections (UTIs). This isolate was identified and its susceptibility pattern was obtained using the Vitek®MS (bioMerieux) test (Prastiyanto et al., 2022). The test bacteria werefirst rejuvenated in nutrient agar (NA) slant (Merck, UK) and incubated for 24 hours at 37ºC. Subsequently, 100 µL of bacterial culture which had been incubated at 37ºC for 24 hours was added to 20 mL of new NA media. The dish containing the mixture of NA and bacterial culture was then allowed to solidify. The fungal isolate on seven-day-old PDA

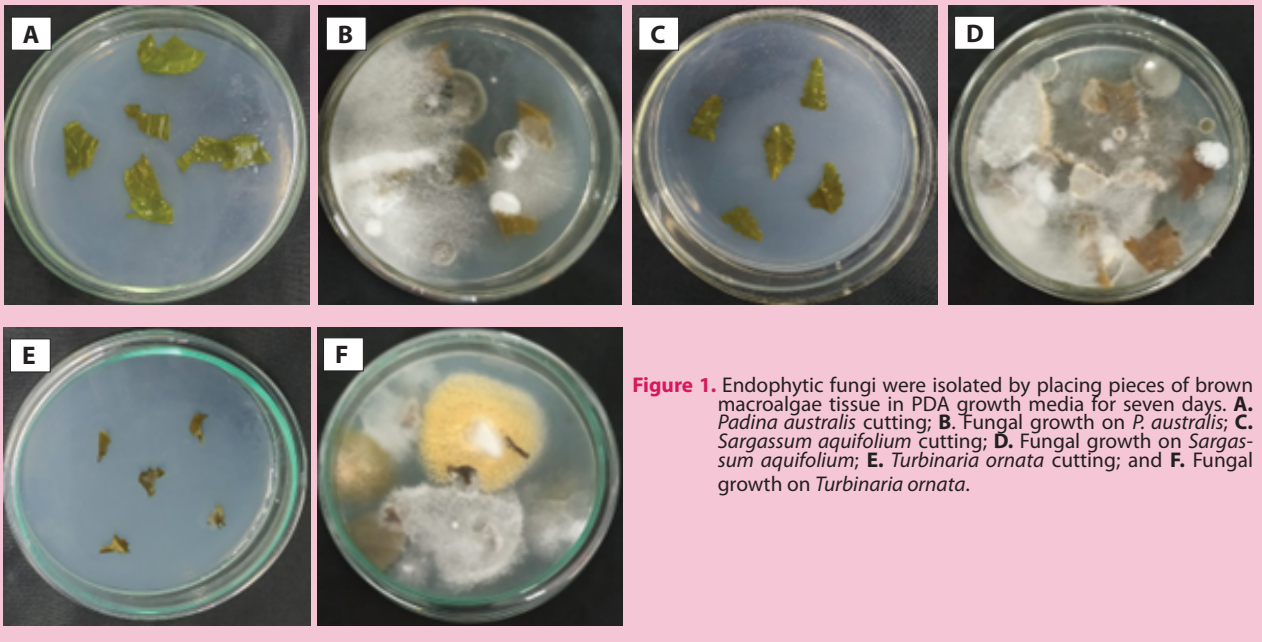


Figure 1. Endophytic fungi were isolated by placing pieces of brown macroalgae tissue in PDA growth media for seven days. **A.** *Padina australis* cutting; **B**. Fungal growth on *P. australis*; **C.** *Sargassum aquifolium* cutting; **D.** Fungal growth on *Sargassum aquifolium*; **E.** *Turbinaria ornata* cutting; and **F.** Fungal growth on *Turbinaria ornata.*

media was then cut into rounds (1 cm in diameter) and transferred to the plate., which was incubated at 37ºC for 24 to 48 hours.

The measurement of antimicrobial activity was adapted from Apsari *et al.*, (2019). Antimicrobial activity was indicated by the large diameter of endophytic fungal colonies. Antimicrobial activity was examined quantitatively (%) by comparing the inhibition zone with the growth of colonies added with the antibiotic ampicillin (Sigma-Aldrich, USA) as positive control. Meanwhile, the negative control was only cultured with *E. coli* ESBL. To obtain representative results, each test for endophytic fungi was repeated three times. In addition, the antibacterial activity of each endophytic fungi was categorized as follows: no antibacterial activity (-), 0-1 mm (+), 1-3 mm (++), 3-7 mm (+++), and 7-15 mm (++++). The zone of inhibition was measured to confirm the activity and antibacterial category.

Cultivation of Selected Endophytic Fungus

Endophytic fungi from each brown macroalgae with the highest resistance were subjected to the cultivation stage. The cultivation test aimed to obtain the best cultivation time for each endophytic fungus. Three selected endophytic fungi that had been purified on PDA media were cultured on potato dextrose broth (PDB) (Merck, UK) media (preculture) for seven days at room temperature with temperatures ranging from 27ºC to 29ºC. A 10 mL of fungi from the preculture results was taken and inoculated into 200 mL of PDB media in a sterile Erlenmeyer (Iwaki, Japan). The cultivation stage was carried out at room temperature for 30 days under static cultivation conditions. Fungi harvesting was carried out at three-day intervals to determine the growth rate of the isolate. The tests carried out for each harvest consisted of calculating the dry biomass of the fungi, the pH value, and the antibacterial activity of the culture filtrate of the fungi growth medium.

Dry fungal biomass was determined by filtering the growing mycelium using filter paper, then drying in an oven at a temperature of 40ºC. The dry weight of the mycelium was weighed to obtain the biomass of endophytic fungi. The growth curve was determined based on the biomass weight of the fungi. During the observations, the pH value and antibacterial activity against *E. coli* ESBL were tested at a single concentration (100 µL). Cultivation in larger amounts of media was carried out after identification of the optimal growth time for each endophytic fungus. The cultivation was carried out by inserting 15 mL of selected endophytic fungi preculture PDB media into six 500 mL Erlenmeyer flasks (Iwaki, Japan), each containing 350 ml of sterile PDB media. The fungi were cultured at room temperature until the optimum time was reached, which corresponds to the time at which the growth rate was determined.

Extraction

The extraction stage was carried out on culture media that had previously been separated from the successfully grown fungal mycelia. The extraction process was carried out using ethyl acetate (p.a.) (Merck, UK) solvent in the culture medium. The medium was shaken using a shaker for 24 hours. The extract obtained was separated from the medium using a separating funnel. This procedure was repeated three times. The extract was then concentrated using a vacuum rotary evaporator at a temperature of 40ºC to obtain a crude extract from the culture medium. The crude extract of this endophytic fungus was used for phytochemical profile screening tests.

Phytochemical Profile Screening

The phytochemical profile screening of each endophytic fungal extract was carried out qualitatively. The phytochemical profiles analyzed included alkaloids, flavonoids, terpenoids, and steroids. This test was based on color changes that occurred in each reagent according to the company's method.

Morphological Identification

Morphological identification of each potential endophytic fungus was carried out by paying attention to the characteristics of colony growth on PDA medium and additional characteristics identified microscopically (conidial shape, hyphae, and growing zone). Identification of the selected endophytic fungi was carried out descriptively in accordance with the findings of Hasyyati *et al.*, (2017).

DNA Extraction

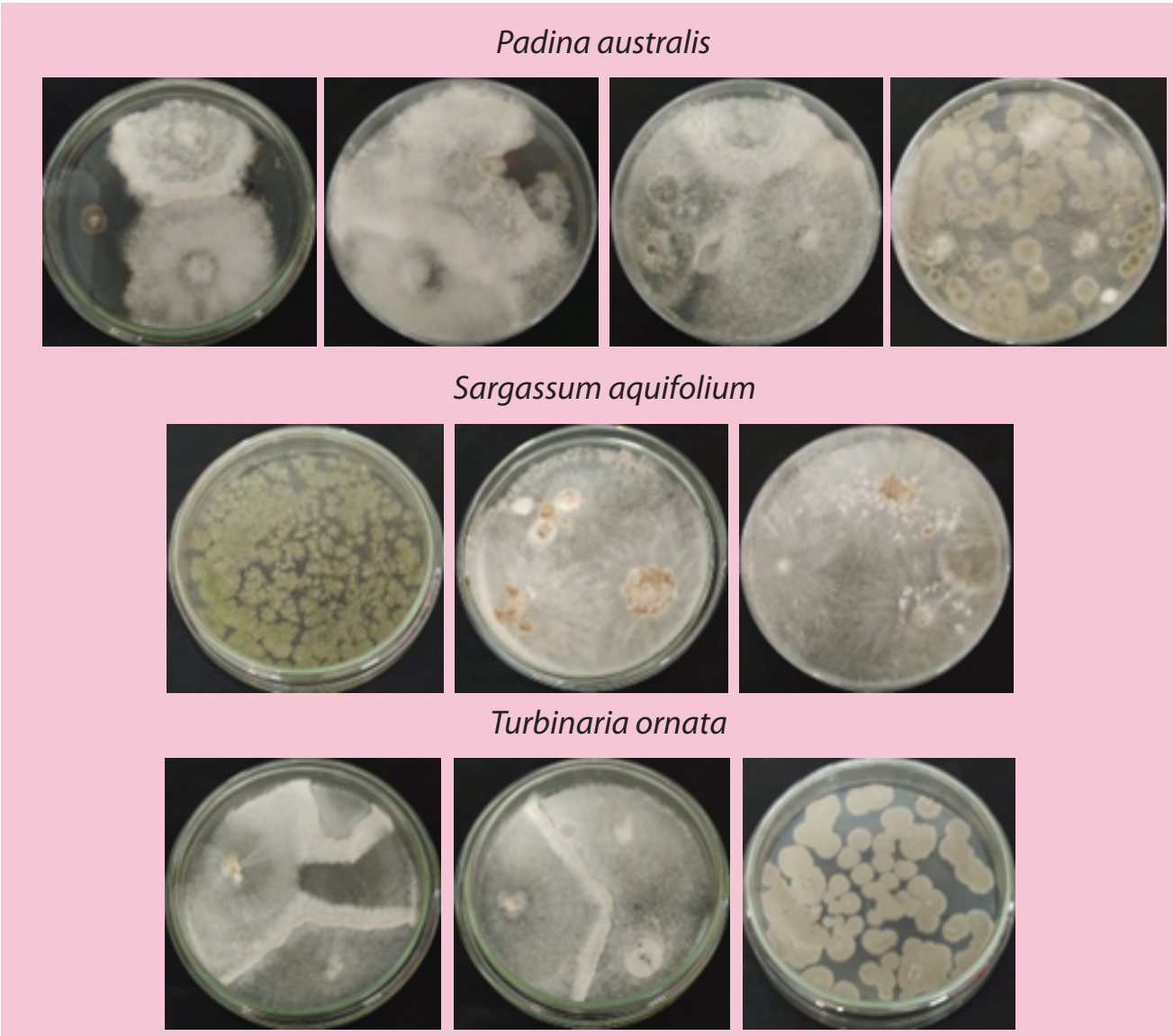
Single colony cultures of endophytic fungi were incubated for four days in PDB media at 25ºC and 150 rpm. After four days, the culture was centrifuged at 6,000 rpm/4.032 g for 10 minutes, and 100 mg pellets (wet weight) were used for the DNA extraction step using a fungal DNA extraction kit according to company's procedures (Zymo, USA) (Thi Minh Le *et al.*, 2019).

Amplification by PCR and Sequencing

The ITS region was amplified using ITS1 and ITS4 primers. The cycle program included 96ºC for five minutes, 39 cycles at 96ºC for 45 seconds, 55ºC for 45 seconds, and 72ºC for 60 seconds, with a final extension at 72ºC for five minutes. A total of 3 µl of PCR product was subjected to agarose gel electrophoresis (1.2%) and visualized under UV light, by immersing the gel in 0.5 µg/ml ethidium bromide solution for three minutes, followed by rinsing with water for five minutes. The PCR product of 22 µl was purified using a DNA fragment purification kit. The PCR products (30 µl of 20 ng/µl) were sent for sequencing (Macrogen, Singapore).

Sequencing and Phylogenetic Analysis

Sequencing results were compared with the NCBI GenBank database using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST) for fungal species identification. Clustal W software (BioTools Organization, http://www.clustal.org/) was used for nucleotide sequence alignment. A phylogenetic tree with 1,000 replications of bootstrap analysis was created using MEGA 11 software (The Pennsylvania State University, USA; https://www.megasoftware.net/).



Picture 2. Endophytic fungal isolates from each brown macroalgae species (Phaeophyceae) from Sanur Beach, Bali Province.

Data Analysis

Data on the inhibitory activity of fungal colonies at the selection stage, growth curves, and effectiveness of selected endophytic fungal extracts were analyzed using descriptive analysis, which is the average of three replications along with the standard deviation, and presented in the form of tables and graphs. SPSS software version 23.0 was used to determine the 95% confidence interval, which shows the significant difference between each treatment. If the results of the analysis of variance were significantly different, the Duncan test was performed. Phytochemical profile identification as well as morphological and molecular identification were analyzed descriptively and presented in tables and figures.

RESULTS

Isolation of Endophytic Fungi

A total of 10 endophytic fungi isolates were successfully isolated from three brown macroalgae collected from the waters of Sanur Beach. Among the selected brown macroalgae samples, Padina australis recorded the highest number of endophytic fungi, namely four isolates, and three isolates each for *S. aquifolium* and *T. ornata* (**Figure 2**). Comparison of the number of endophytic fungi that were isolated based on differences in colony shape in each brown macroalgae species revealed a variety of strains that differed in macroscopic appearance. However, further research is needed to confirm the number of endophytic fungal brown macroalgae in other coastal regions in Bali Province.

Selection of Brown Macroalgal Endophytic Fungus

The antagonist test of endophytic fungal isolates from brown macroalgae against *E. coli* ESBL was carried out directly, which was the initial stage to obtain three fungal isolates whose extracts were able to inhibit the growth of *E. coli* ESBL. The results of the analysis of variance (ANOVA) showed that there were differences in the inhibitory power of endophytic fungal colonies on the growth of *E. coli* ESBL (**Table 1**). The

Table 1. Mean Inhibitory ± Standard Deviation (SD) of The Diameter Of Endophytic Fungal Isolates of Brown Macroalgae on The Growth of *Escherichia coli* ESBL

No.	Endophytic Fungal Isolates	Mean (mm) ± SD	Inhibition Category
1.	T1	23.1 ± 0.70^{a}	++++
2.	T2	9.7 ± 0.90^{b}	+++
3.	T3	5.0 ± 0.88^{b}	+++
4.	S1	15.2 ± 0.35^{c}	++++
5.	S2	15.1 ± 0.22^{c}	++++
6.	S3	14.3 ± 0.24^{bc}	++++
7.	P1	15.9 ± 0.45^{bc}	++++
8.	P2	13.8 ± 0.62^{bc}	++++
9.	P3	17.5 ± 0.15^{bc}	++++
10.	P4	16.9 ± 0.30^{bc}	++++
11.	Control (+)	43.2 ± 0.27^{d}	++++
12.	Control (-)	0^{e}	-

Note: Different letter notations in the same column indicate significant differences between treatments with a p value <0.05 based on the DUNCAN test. Note: Activity without antibacterial (-), 0 – 1 mm (+), 1 – 3 mm (++), 3 – 7 mm (+++), and 7 – 15 mm (++++).

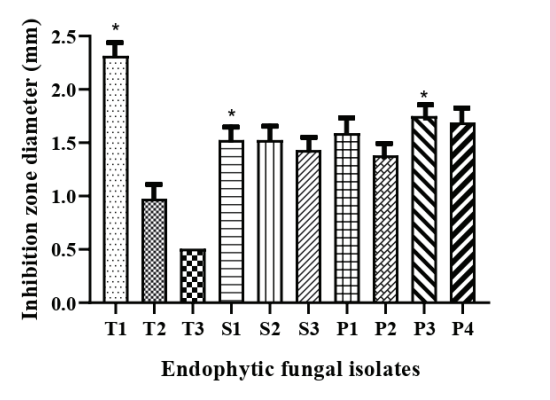
Table 2. Qualitative Phytochemical Profile of Ethyl Acetate Extract Of Brown Algae Endophytic Fungi

Compound	Reactor	P3 Extract	S1 Extract	T1 Extract
Alkaloids	Wagner	+	+	+
Flavonoids	HCl + Mg powder	-	-	-
Triterpenoids	Lieberman-Burchard	-	+	-
Saponin	Hot water + HCl	-	+	-
Phenolic	$FeCl_3$ 10%	+	+	+
Tannin	FeCl 1%	-	-	-
Steroids	Lieberman-Burchard	-	-	-

highest inhibitory effect was shown by isolate T1 at 2.316 ± 0.704 mm, followed by isolates S1 and P3 at 1.525 ± 0.350 mm and 1.750 ± 0.152 mm, respectively (**Figure 3**). The different species of fungal isolates of *E. coli* ESBL is potential as an antimicrobial. This is due to the fact that 10 isolates of endophytic fungi from brown macroalgae consisted of several different types of fungi. Therefore, they had different inhibitory activities. A total of three endophytic fungus, namely isolates T1 from *T. ornata*, S1 from *S. aquifolium*, and P3 from *P. australis*, were further identified and their growth rates were observed on PDA medium. The colonies of each selected fungal isolate are shown in **Figure 4**.

Cultivation of Endophytic Fungi Isolates

The growth of each selected endophytic fungal isolate was



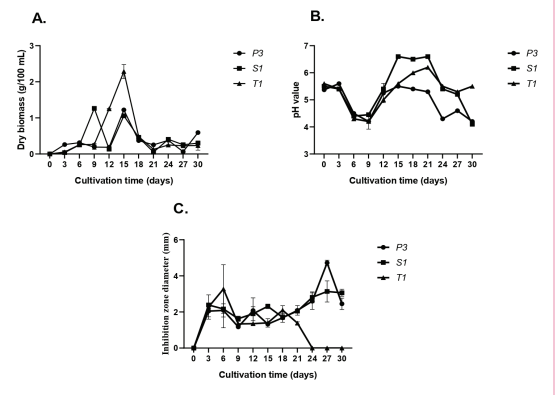
Picture 3. Diameter of the inhibition zone of endophytic fungal isolates of brown macroalgae on the growth of E. coli ESBL

determined based on the dry weight of the mycelium. The mycelium weight of each endophytic fungal isolate increased starting from day 3 of the exposure period. The highest mycelium weight of each endophytic fungal isolate varied depending on the heating time. The highest mycelium weight for isolate T1 was obtained on day 15, namely 2,285 g/100 mL. Furthermore, the highest mycelium weight for isolate S1 was on day 9, namely 1,264 g/100 mL, and isolate P3 on day 15, namely 1,221 g/100 mL (**Figure 5A**).

The pH value was measured to determine the pH conditions in the culture medium during the cultivation process. The results showed that the pH value of the endophytic fungal medium that was cultivated for 30 days was in the range of four to six (B). The highest pH value for each endophytic fungi varies, namely 6.2 (T1), 5.5 (P3), and 6.6 (S1). The pH value tended to decrease at the end of the cultivation period for all endophytic fungi isolates starting from day 24 to dat 30.

The inhibitory power of endophytic fungi was tested to determine the ability of each culture filtrate from fungal isolates based on the best time to inhibit the growth of *E. coli* ESBL. The highest inhibition of *E. coli* ESBL growth from each endophytic fungal culture filtrate was isolate T1 on day 6, namely 32.7 mm, followed by isolates S1 and P3 on day 27, which were 31.3 mm and 47.2 mm (**Figure 5C**). A decrease in

Picture 4. Three endophytic fungal isolates were selected from each brown macroalgae from Sanur Beach which had the highest inhibitory activity on the growth of E. coli ESBL. **A.** Isolate T1 from *T. ornata*; **B.** Isolate S1 from *S. aquifolium*; and **C.** Isolate P3 from *P. australis*.



Picture 5. Cultivation period of selected endophytic fungal isolates. A. endophytic fungal mycelium biomass weight; B. pH value; and C. diameter of the inhibition zone of endophytic fungal filtrate culture for 30 days. Isolate T1 of *T. ornata*; isolate S1 of *S. aquifolium*; and isolate P3 of *P. australis*.

inhibitory power occurred in isolate T1 on days 24 and 30, and on day 30 for isolates S1 and P3.

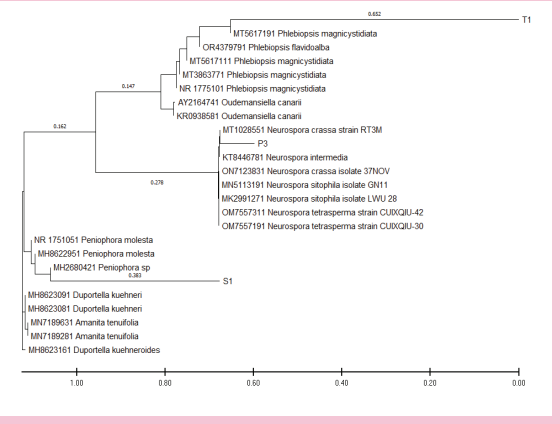
Phytochemical Profile

This qualitative test was carried out to determine the compounds contained in the ethyl acetate extract of endophytic fungi. S1 isolate extract showed a profile of alkaloid, triterpenoid, saponin and phenolic compounds. The number of phytochemical profiles in isolate S1 was greater than the other two isolates, namely P3 and T1, each of which was positive for alkaloid and phenolic compounds (Table 2). The endophytic fungal extract of isolate S1 has a profile of metabolites, such as alkaloids, triterpenoids, saponins and phenolics.

Identification of Potential Endophytic Fungi

Microscopic identification was performed on each endophytic fungal isolate using the Lactophenol Blue (LB) dye. The results of microscopic identification of endophytic fungi are shown in Figure 6. The microscopic morphology of each endophytic fungal isolate in this study had varying structures. Primarily, the three endophytic fungal isolates had a cell wall structure, but only isolates T1 and P3 showed a septum in their morphology. Similarly, the nuclei were seen in isolates S1 and P3. Therefore, it can be concluded that isolates T1 and P3 belong to septate hyphae based on cell division. No conidia were found in the three endophytic fungal isolates and no growing zones were formed. Similarly, the macroscopic characteristics of each endophytic fungal isolate grown on PDA medium showed a white surface, white-brown colony reserves, and a cottony texture.

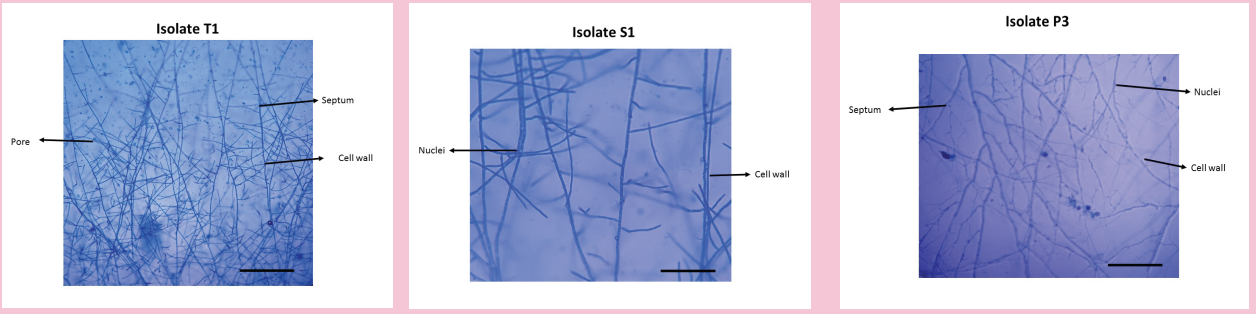
Sequencing results submitted to NCBI for the three endophytic fungal isolates showed that isolate T1 was similar to *Phlebiopsis magnicystidiata* (MT5617191), isolate P3 was similar to *Neurospora crassa* strain RT3M (MT1028551), and isolate S1 was similar to *Peniophora sp.* (MH2680421) (**Figure 7**). Interestingly, the three endophytic fungal isolates were in different clades, such as isolate T1 in clade 1, isolate P3 in clade 2, and isolate S1 in clade 3.



Picture 7. Phylogenetic tree analysis of endophytic fungal isolates from three brown macroalgae in Sanur Beach, Bali

DISCUSSION

A total of 10 endophytic fungal isolates were successfully isolated from three species of brown macroalgae. Three selected endophytic fungal isolates showed inhibitory activity against the growth of *Escherichia coli* ESBL. Collection sites and species of brown macroalgae were randomly selected in the coastal waters of Sanur, Bali. The presence of varying endophytic fungi in each brown macroalgae can most likely be attributed to abiotic factors, such as temperature, light, salinity, and nutrients. However, these factors were not inves-



Picture 6. Microscopic observation of the morphology of each endophytic fungal isolate from brown macroalgae. Isolate T1 of *T. ornata*; isolate S1 of *S. aquifolium*; and isolate P3 of *P. australis*.

tigated in this study. Several studies have also documented variations in endophytic fungal diversity based on the macroalgae genus. Endophytic fungi have been successfully isolated from red macroalgae (*Bostrychia tenella*), Ceramiales, and Rhodophyta from the Brazilian sea. This study found 45 strains of endophytic fungi and 10 selected strains were tested for cytotoxicity as well as antifungal and antibacterial activity (de Felício *et al.,* 2015). A smaller number of endophytic fungi isolates were found in the research of Harikrishnan *et al.,* (2021), who isolated endophytic fungi from macroalgae on the west coast of South India. This study obtained five endophytic fungal isolates which were tested for their anticancer, antioxidant, and antimicrobial activities. These findings suggested that macroalgae acting as a habitat for endophytic microorganisms serve not only as a suitability factor, but also environmental and biogeographic factors.

Antagonism assay of endophytic fungal isolates of brown macroalgae against *Escherichia coli* ESBL was carried out directly, which was the initial selection for screening the three selected isolates. Isolate T1 obtained from *T. ornata* had the highest inhibition rate at this initial selection stage, followed by isolate P3 from *P. australis* and isolate S1 from *S. aquifolium*. The difference in inhibitory activity of endophytic fungal isolates can be attributed to be the differences in endophytic fungal species. The composition of the endophytic fungi and the phytochemical profile produced by each isolate largely determines the antimicrobial potential produced. Certain types of endophytic fungi can produce metabolite profiles that are similar to their hosts, but there are also those that produce different compounds. The isolates selected in this study had phytochemical profile components such as alkaloids, triterpenoids, saponins, and phenolics for isolate S1, as well as alkaloids and phenolic compounds for isolates P3 and T1. The strategy developed by these microorganisms is in the form of allelochemicals that are able to limit the growth of competing organisms and reduce the negative impacts caused by competitors in the surrounding environment (Konarzewska *et al.,* 2020; Poveda, 2021). Secondary metabolites can support the development of organisms by inhibiting the growth of competing organisms. Interestingly, this secondary metabolite can also inhibit the growth of various types of endophytic fungi (Xie *et al.,* 2019).

The growth rate of endophytic fungal isolates was determined based on the weight of dry mycelium produced within 30 days of cultivation period. Fungi have four growth phases, namely lag/adaptation, log/growth, stationary, and death. The endophytic fungal isolates in this study had an adaptation phase from day 0 to day 12, experienced growth on day 15, and tended to decrease on day 18. Compared with previous research, the weight of mycelium produced in this study was higher on day 15, especially by isolates T1 and P3 and on day 9 by isolate S1. The weight of mycelium produced from previous research was only 0.6985 g/100 ml and 0.7180 g/ml on the days 24 and 27, respectively, by the RS1A endophytic fungi isolated from coastal plants of Sarang Semut (Nurzakiah *et al.,* 2020).

The pH value of the media produced by each endophytic fungal isolate ranged between four and five, especially at the end of the cultivation period. These results are supported by similar research which revealed that RS1A and RS3 endophytic fungal isolates from coastal plants of Sarang Semut that were cultivated for 27 days had pH values ranging from four to five (Nurzakiah *et al.,* 2020; Sahara, 2013). The pH value of the culture medium is very important to determine the optimization of abiotic parameters that support the growth of endophytic fungi to carry out the fermentation process. The fermentation process to support the growth and recovery of endophytic fungi must have at least a slightly acidic pH range of 5.8 to 6.0 (An *et al.,* 2020). Single culture media such as PDA can also be recommended in research focusing on the cultivation of endophytic fungi because they are easy to cultivate (Gong *et al.,* 2019; Ibrahim *et al.,* 2021).

The inhibition test of the culture filtrate of each endophytic fungal isolate in inhibiting the growth of Escherichia coli ESBL showed varying results at each cultivation time. Interestingly, isolates T1 and S1 had the highest inhibition on day 30 of cultivation, longer than isolate P3 which showed inhibition on day 6 of cultivation. This suggested that the antimicrobial compounds produced from each isolate accumulated in different phases. In contrast to the results of this study, the time required for endophytic fungi isolated from seagrass to produce the enzyme β-glucosidase was on day 15 (Oktavia *et al.,* 2014). Reduced nutrition allows endophytic fungi to experience stress. To overcome this, they produce more secondary metabolites in the medium environment (Chaudhary *et al.,* 2022; Verma *et al.,* 2021). The metabolic waste products in the final phase tend to accumulate and ultimately reduce the number of endophytic fungi, which is supported by a decrease in mycelial weight on the last day.

The results of molecular identification showed species differences for each selected endophytic fungal isolate. Morphologically, *Phlebiopsis spp.* is similar to *Scopuloides spp.* and several Phanerochaete species based on the characteristics of lamprocystidia and simple insulated generative hyphae (Zhao *et al.*,2021). There are still few studies mentioning the use of *P. magnicystidiata,* especially as a biological control agent. Neurospora crassa is an endophytic fungud that has antimicrobial activity and is reported to be able to control burn wound infection pathogens that are resistant to commercial drugs (El-Zawawy *et al.,* 2022). To the best of our knowledge, this study is likely the first to report *N. crassa* isolated from brown macroalgae. Previous research has successfully reported *N. crassa* isolated from *Lycium shawii* containing high levels of phenolic compounds (Ali *et al.,* 2020). The endophytic fungus Peniophora sp. isolated from the marine environment showed laccase production activity of 709.03 U/L when cultured at a temperature of 28ºC for 21 days in MA2ASW media with added artificial sea water (Bonugli-santos *et al.,* 2010). The laccase enzyme is an enzyme capable of degrading lignin, which is important in chemical, food, agricultural, and cosmetic applications. However, research showing the antibacterial activity of *Peniophora sp.* Is still very limited.

CONCLUSION

There were 10 isolates of endophytic fungi isolated from brown macroalgae in Sanur Beach, with three isolates selected from each brown macroalgae. Culture filtrates of endophytic fungi had a very high inhibition category on the growth of ESBL-producing *Escherichia coli*. The inhibitory ability exerted by the three extract isolates can be attributed to the content of the secondary metabolite profile produced. Molecular identification revealed that the three isolates were identified as isolate T1 (*Phlebiopsis magnicystidiata*) (MT5617191), isolate P3 (*Neurospora crassa* strain RT3M) (MT1028551), and isolate S1 (*Peniophora sp.*) (MH2680421). However, further research is needed to investigate the antioxidant compounds, reduced expression of genes related to virulence factors, and anti-biofilm activity of these isolates on the growth of pathogenic bacteria.

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CONFLICT of INTEREST

The authors have no conflicts of interest to declare.

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ETHICAL APPROVAL

This study did not involve experimental animals. Therefore, ethical approval was not required.

AUTHORS' CONTRIBUTIONS

Conceptualization, project acquisition, and implementation of the study: PAW, AAAPP, EW, JMF, and NSDP. Analysis and interpretation of data: NKYS, IGW, IMGSS, and FRP. Drafting the manuscript: PAW, NSDP, ANMA. Revision of the manuscript: PAW, AAAPP, NSDP, and ANMA.

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