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Original Article

Bioactivities and Molecular Identification from Marine Sediment-Derived Fungi Isolated from Amed and Tulamben Beaches

Bioaktivitas dan Identifikasi Molekuler Jamur yang Diisolasi dari Sedimen di Pantai Amed dan Tulamben

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Abstract

Marine fungi have demonstrated the ability to produce promising new bioactive compounds. This study aims to isolate fungi from the marine sediments collected from Amed and Tulamben Beaches, Bali, Indonesia and investigate the bioactivity of the fungal extracts. Following fungal isolation, pure isolates were obtained and fermented on rice media containing salt and without salt. Next, secondary metabolites were extracted with ethyl acetate, then separated by liquid-liquid extraction using methanol containing 10% water and *n*-hexane. The resulting MeOH extracts were analyzed for their phytochemical content and tested for their antimicrobial and antioxidant activities using the disc diffusion and 2,2-diphenyl-1-picrylhydrazyl (DPPH) method, respectively. In total, three fungal isolates were obtained. Molecular identification from the Internal Transcribed Spacer (ITS) region of the isolates showed the closest relationship to *Aspergillus* sp., *Aspergillus tamarii,* and *Trichoderma* sp. Phytochemical analysis showed all fungal extracts contained alkaloids and polyphenols. Moreover, extract from *Trichoderma* sp. SED-TU (1) (NS) inhibited *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Streptococcus mutans* with inhibition zone diameters of 4.69±0.16; 3.51±0.01, and 3.49±0.25 mm, respectively. Extract from *Aspergillus tamarii* RM-S-SED-AM (S) inhibited *Candida albicans* with an inhibition zone of 7.04±0.22 mm and also had strong antioxidant activity with an IC_{50} value of 62.00±0.35 µg/mL. This study indicates that fungal isolates from marine sediments have great potential as a source of bioactive natural products.

Abstrak

Jamur laut merupakan salah satu sumber senyawa bioaktif baru yang menjanjikan. Penelitian ini bertujuan untuk mengisolasi jamur dari sedimen laut yang dikumpulkan dari Pantai Amed dan Tulamben, Bali, Indonesia dan menguji bioaktivitas ekstrak jamur yang diperoleh. Setelah isolasi jamur, isolat murni diperoleh dan difermentasi pada media beras yang mengandung garam dan tanpa garam. Pada akhir fermentasi, metabolit sekunder diekstraksi dengan etil asetat, kemudian dipisahkan dengan ekstraksi cair-cair dengan metanol yang mengandung 10% air dan *n*-heksana. Ekstrak MeOH yang dihasilkan dianalisis kandungan fitokimianya dan diuji aktivitas antimikroba dan antioksidannya dengan menggunakan metode difusi cakram dan 2,2-difenil-1-pikrilhidrazil (DPPH). Pada penelitian ini diperoleh tiga isolat jamur. Identifikasi molekuler dari daerah *Internal Transcribed Spacer* (ITS) isolat menunjukkan hubungan terdekat dengan *Aspergillus* sp., *Aspergillus tamarii,* dan *Trichoderma* sp. Analisis fitokimia menunjukkan semua ekstrak jamur mengandung alkaloid dan polifenol. Selain itu, ekstrak dari *Trichoderma* sp. SED-TU (1) (NS) dapat menghambat *Staphylococcus aureus*, *Staphylococcus epidermidis*, dan *Streptococcus mutans* dengan diameter zona hambat berturut-turut sebesar 4,69±0,16; 3,51±0,01 dan 3,49±0,25 mm. Ekstrak dari *Aspergillus tamarii* RM-S-SED-AM (S) menghambat *Candida albicans* dengan zona hambat sebesar 7,04±0,22 mm dan juga memiliki aktivitas antioksidan yang kuat dengan nilai IC_{50} sebesar 62.00±0.35 µg/mL. Penelitian ini mengindikasikan bahwa isolat jamur yang diisolasi dari sedimen laut memiliki potensi yang besar sebagai sumber metabolit bioaktif.

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INTRODUCTION

Various health problems related to infectious diseases and cell damage due to oxidative stress are still an essential concern worldwide.[1,2] The widespread and incorrect use of antimicrobial drugs in the community can cause new problems, including the increasing incidence of drug resistance, which complicates the treatment of infectious diseases.[3] On the other hand, the decrease in the body's ability to counteract free radicals causes the onset of various degenerative diseases, such as cancer, diabetes, and cardiovascular disease.[4]

To date, the development of new drugs faces significant global challenges, primarily due to the increasing cases of antibiotic resistance and the prevalence of degenerative diseases. Natural products are known as potential sources for drug discovery, including antimicrobial agents and antioxidants. The utilization of marine fungi shows a promising source of bioactive compounds such as antimicrobial and antioxidant.[5]

Fungi isolated from marine environments, such as sediments, have attracted significant attention due to their potential to produce new bioactive compounds as antimicrobial and antioxidant.[5] A new compound, diorcinol K, and three previously known compounds, namely diorcinols D, F, and I, were obtained from marine sediment-derived *Aspergillus* sp. CUGB-F046. These compounds showed significant antibacterial activity against *S. aureus* and methicillin-resistant *S. aureus* (MRSA) with Minimum Inhibitory Concentration (MIC) values of 3.125, 6.25, and 6.25 µg/mL, respectively.[6] On the other hand, *Aspergillus* sp. SCSIO 41024 was isolated from deep-sea sediments in the South China Sea and yielded cyclopiazonic acid (CPA), speradine A, and asperorydine H. These compounds showed good antioxidant activity in an assay employing DPPH with IC_{50} values of 190.1, 31.9, and 228.4 µg/mL, respectively.[7]

In this study, we reported potential fungal isolates with antimicrobial and antioxidant activities. The isolates were obtained from marine sediments collected at Amed and Tulamben beaches in Bali, Indonesia. Following fungal cultivation on rice media with and without the addition of artificial sea salt, the resulting methanolic extracts were subjected to metabolite analysis and bioassays, including antimicrobials and antioxidants.

METHODS

Instrumental and Materials

Instrumental. A microplate reader spectrophotometer (Thermo Scientific®, Lithuania) was used for antioxidant testing. The DNA extraction kit from Zymo Research®, USA (Quick-DNA™ Fungal/Bacterial Miniprep Kit) was used for DNA extraction.

Materials. Agar media was prepared using malt extract (Himedia®, India), Bacto agar (Difco BD®, Maryland USA), yeast extract (Oxoid®, France), artificial seawater salts broth (Himedia®, India), and glycerol (Vivantis®, Malaysia). Rice media (Putri Sejati®, Indonesia) with or without the addition of artificial seawater salts broth (Himedia®, India) was used for fungal isolation, purification, and fermentation. Mueller-Hinton Agar (MHA) (Oxoid®, France), Mueller-Hinton Broth (MHB) (Himedia®, India), Sabouraud Dextrose Agar (SDA) (Oxoid®, France), and Sabouraud Dextrose Broth (SDB) (Himedia®, India) were used in the antimicrobial activity test. Chloramphenicol (Nalgane®, USA) and ketoconazole (Zoralin®, Indonesia) were used as a positive control for the antimicrobial assay. The antioxidant activity test used DPPH (Smart-Lab®, Indonesia). Ascorbic Acid (Supelco®, Germany) was used as a reference solution for the antioxidant assay. Ethyl acetate, methanol, and *n*-hexane were used in fungal maceration and liquid-liquid extraction. Primers (ITS1 and ITS4), DNA polymerase, nuclease-free water (Thermo Scientific, Lithuania), agarose (1st BASE®, Singapore), and tris-acetate-EDTA (TAE) solution (1st BASE®, Singapore) were used in the molecular identification process.

Sample Collection

Marine sediment was collected from Amed Beach, Abang District, Karangasem Regency (8°21'00.8 "S 115°40'58.4 "E) and Tulamben Beach, Kubu District, Karangasem Regency (8°16'32.5 "S 115°35'34.8 "E) in June

2024. Samples were placed in 50 mL sterile Eppendorf tubes and stored in the collection box. The sample was processed at the Integrated Laboratory of Universitas Udayana.

Isolation of marine-sediment-derived-fungi

Isolation of marine sediment-derived fungi was carried out following the procedure described in the protocol[8]. In brief, agar media for fungal isolation contains malt extract, Bacto agar, artificial seawater salts broth, and chloramphenicol in demineralized water. In addition, rice media with and without the addition of artificial seawater salts were also used for fungal isolation. Isolation was carried out by inoculating ±1–2 g of marine sediment onto agar and rice media and incubating them at room temperature (28 °C) for 7 days. Each fungal colony grown on agar and rice medium with different morphological appearances was transferred to a new agar medium for purification. The agar media for purification contains a mixture of malt extract, Bacto agar, and artificial seawater salts broth in demineralized water.

Molecular identification

Molecular identification was carried out through comparison of DNA sequences of the Internal Transcribed Spacer (ITS) region using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') as forward primer and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') as reverse primer. Prior to the polymerase chain reaction (PCR) process, the fungal DNA was extracted using the Quick-DNA Fungal/Bacterial Miniprep Kit according to the package protocol. The ITS region of the extracted DNA was then amplified using a Labcycler 48 Thermocycler in a mixture containing 1 µL ITS1 primer, 1 µL ITS4 primer, 20 µL DNA polymerase, 17 µL nuclease-free water, and 1 µL extracted fungal genomic DNA as template. For DNA amplification, the thermocycler was set under pre-denaturation conditions at 95°C for 1.5 minutes, followed by 35 cycles of denaturation at 95°C, annealing at 56°C, and extension at 72°C; each cycle was run for 1 minute and ended with a final extension at 72°C for 15 minutes. Next, the amplicons were analyzed by electrophoresis using 1% agarose gel in 10× TAE buffers (Tris-acetate-EDTA) at 75 volts for 45 minutes. PCR products were then submitted to Genetika Science Laboratory for sequencing analysis. The obtained sequences were then compared with nucleotide sequences using the Basic Local Alignment Search Tool (BLAST) program for nucleotides in the National Center for Biotechnology Information (NCBI) GenBank. The reference sequences of interest and the fungal sequences were aligned in pairs using the MUSCLE method. Phylogenetic trees were reconstructed using MEGA software version 11.0.11 by neighbor-joining method with 1,000 bootstrap replications.[9]

Fermentation and extraction

Each fungal isolate was fermented on rice media with and without the addition of artificial seawater salts. The rice media contained 100 g of rice, 110 mL of demineralized water, and an additional 3.8 g of artificial seawater salts for the rice media containing salt. Aseptically, each agar medium containing pure fungal isolates was cut into pieces and spread onto the surface of the prepared rice medium. The samples were incubated at room temperature until the fungal mycelia covered the rice media. The fermented media were macerated using 500 mL of ethyl acetate in an orbital shaker at 150 rpm for 8 hours. The resulting macerate was filtered and concentrated using a rotary evaporator to remove the solvent. The extract was partitioned using liquid-liquid extraction with a 2:1 ratio of methanol containing 10% water and *n*-hexane. The methanol extract was further analyzed regarding its bioactive content and bioassay.[10]

Phytochemicals Screening

Extracts with a concentration of 6000 µg/mL were prepared. Analysis to detect the presence of alkaloids, terpenoids, steroids, triterpenoids, saponins, polyphenols, and flavonoids was performed with phytochemical screening. For the alkaloid test, Dragendorff's, Wagner's, and Bouchardat's reagents were used. To the 100 µL extract solution, 100 µL of 2N HCl and three drops of each reagent were added. The presence of an orange precipitate after the addition of Dragendorff's reagent, a white to yellow precipitate after adding Wagner's reagent, and a brown to black precipitate after the addition of Bouchardat's reagent indicates the presence of alkaloids.[11] Terpenoid testing was conducted by reacting 200 µL of the extract solution with 100 µL of chloroform and 100 µL of H_2SO_4. The presence of a reddish-brown layer on top indicates a positive result

for terpenoids.[12] Steroid and triterpenoid tests were performed using the Liebermann-Burchard reaction. A bluish-green color suggests the presence of steroids, while a brown or purple ring at the boundary between the two solvents indicates the presence of triterpenoids.[13] For the saponin test, 200 µL of the extract solution was shaken vertically for 10 seconds, followed by adding one drop of 2N HCl. If the resulting foam does not disappear, it indicates the presence of saponins.[14] The polyphenol test was carried out by reacting 200 µL of the extract solution with a 10% $FeCl_3$ solution. A color change to dark blue, blackish-blue, or blackish-green indicates the presence of polyphenols.[13] The flavonoid test was conducted by evaporating 200 µL of the extract solution, followed by the addition of 200 µL each of acetone, boric acid, oxalic acid, and ether. The solution was then observed under UV_{366} light. Intense yellow fluorescence indicates the presence of flavonoids.[13]

Antimicrobial test

Antimicrobial assay was conducted using disc diffusion methods against the following bacterial strains: *Staphylococcus aureus* ATCC 25923, *Methicillin-resistant Staphylococcus aureus* (MRSA) ATCC 3351, *Streptococcus mutans* ATCC 35668, *Staphylococcus epidermidis* ATCC 12228, *Bacillus cereus* ATCC 11778, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 10231. The test solution was prepared by dissolving 50 mg of extract with 1 mL of solvents (methanol: distilled water=9:1) to produce a final concentration of 5%. Suspension of bacterial and/or fungal cultures was made at as much as 10 mL, and the incubation process was carried out for 18-24 hours for bacteria and 48-52 hours for fungi at 37°C. For the assay, 600 µL of the bacteria suspension, equivalent to 0.5 McFarland standard turbidity, was spread on agar media for antibacterial testing and 550 µL of the fungal suspension for antifungal testing. Next, sterile discs were placed on agar media, and 10 µL of test solution was added to each sterile disc.[15] For the negative control, 10 µL of methanol was added to the sterile disc, and antibiotic chloramphenicol with a concentration of 30 µg/mL or antifungal ketoconazole with a concentration of 15 µg/mL was used as a positive control, followed by incubation for 18-24 hours for the antibacterial test and 48-52 hours for the antifungal test. The experiment was performed in triplicate.

Antioxidant test

The antioxidant activity of the tested extracts was estimated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay.[16–18] Ascorbic acid as a reference solution was prepared with concentrations of 4, 6, 8, 10, and 12 µg/mL. Samples were prepared with a series of concentrations starting from 20 to 500 µg/mL. For the assay, 40 µL of 0.5 mM DPPH and 160 µL of sample dissolved in MeOH were added to a 96-well microplate, followed by incubation for 30 minutes at room temperature in a dark room. Next, the absorbance was measured using a microplate reader spectrophotometer at 517 nm wavelength. When DPPH as a free radical encounters a proton-donor substrate such as an antioxidant, the radicals are scavenged, reducing the absorbance. The experiment was performed in triplicate.

Data analysis

The antimicrobial activity was determined by measuring the diameter of the inhibition zone around the sterile discs using a caliper. Antimicrobial activity was analyzed based on its inhibition category. The antimicrobial activity is classified into four categories: weak inhibition (<5 mm), moderate (5–10 mm), strong (10–20 mm), and very strong (>20 mm).[19,20] The diameter of the inhibition was calculated as follows:

$$\text{Inhibition zone} = \frac{(A-C)+(B-C)}{2} \quad \text{..... (1)}$$

A: Vertical diameters of the clear zone
B: Horizontal diameters of the clear zone
C: Diameter of disc

The antioxidant activity was quantitatively analyzed and categorized according to its IC_{50}. Antioxidant activity is considered very strong if the IC_{50} value is below 50 µg/mL; strong: IC_{50}= 50-100 µg/mL, moderate: IC_{50}= 101-250 µg/mL, weak: IC_{50}= 250-500 µg/mL, and inactive when the IC_{50} is more than 500 µg/mL.[21] The IC_{50} value was calculated from the linear regression of sample concentration *versus* percent inhibition. The inhibition percentage of the extract against free radicals was calculated as follows:

$$\%\text{Inhibition} = \frac{A-B}{A} x100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

A: Blank absorbance
B: Sample absorbance

RESULT AND DISCUSSION

Fungal isolation and molecular identification

Fungal isolation from marine sediments resulted in three fungal isolates, encoded as RM-NS-SED-AM (1) and RM-S-SED-AM obtained from Amed Beach, and SED-TU (1) obtained from Tulamben Beach. Isolate RM-NS-SED-AM (1) was obtained from the fungal isolation procedure on rice media without the addition of salt, while RM-S-SED-AM was isolated from rice media supplemented with salt. Meanwhile, SED-TU (1) was isolated from the isolation procedure on agar media. The use of isolation media with and without salt addition aims to isolate a broader diversity of marine fungi. Facultative marine fungi are capable of growing in both saline and non-saline conditions, whereas obligate marine fungi require a saline environment for optimal growth. The macroscopic appearance of fungal isolates is shown in **Figure 1.**

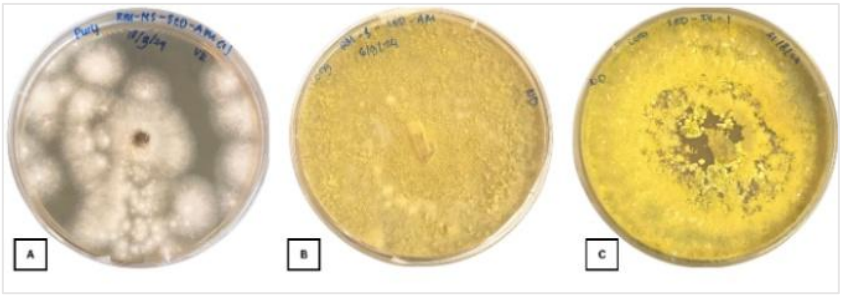

Figure 1. Morphological appearance view from the top of the agar media of fungal isolates: RM-NS-SED-AM (1) (A), RM-S-SED-AM (B), and SED-TU (1) (C).

Molecular identification of species based on sequence comparison using ITS1 and ITS4 evolves rapidly and exhibits higher genetic variability between species. Therefore, the ITS region can be utilized for species-level identification.[22] Amplification of the ITS region from isolates RM-NS-SED-AM (1), RM-S-SED-AM, and SED-TU (1) produced DNA bands of approximately 500–700 bp (**Figure 2**). The isolates were classified into three different species with high similarity percentages (>99%) and E-values of 0.0, as shown in **Table 1**. Isolate RM-NS-SED-AM (1) was identified as *Aspergillus* sp., showing 100% similarity to *Aspergillus* sp. PP070052.1. Isolate RM-S-SED-AM was identified as *Aspergillus tamarii*, with 100% similarity to *Aspergillus tamarii* JQ257030.1. Meanwhile, isolate SED-TU (1) was identified as *Trichoderma* sp., showing 100% similarity to *Trichoderma* sp. MK871051.1 and supported by bootstrap values greater than 86 in the phylogenetic analysis (**Figure 3**).

Previous research reported that the *Aspergillus* genus, namely *A. tubingensis* was found from the area with high salt concentrations (hypersaline) and higher ambient temperatures.[23] This species was also isolated from the bottom sediments of the eastern Gulf of Finland.[24] Moreover, previous studies also reported *A. tamarii* species afforded from marine sediments from the Mediterranean Sea, Alexandria,[25] as well as from the upper layer of sediments in the Velikaya Salma Strait located on the southwestern shore of Kandalaksha Bay in the White Sea.[26] Meanwhile, one of the species of *Trichoderma*, namely *T. lixii* was obtained before from coastal marine waters in Nha Trang Bay and Van Bay of Phong Bay, Vietnam.[27]

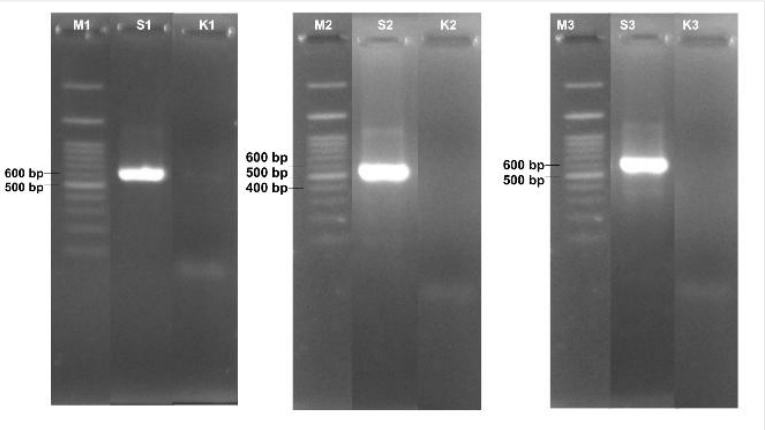

Figure 2. Electrophoregram of PCR products from amplification of ITS region; M1-3: 100 bp DNA ladders, S1: DNA sample with code *Aspergillus* sp. RM-NS-SED-AM (1), S2: DNA sample with code *A. tamarii* RM-S-SED-AM, S3: DNA sample with code *Trichoderma* sp. SED-TU (1). K1-3: negative controls.

Table 1. Molecular identification using GenBank's Basic Local Alignment Search Tool (BLAST) for nucleotides.

Isolate Code	Taxa with the Closest Relationship	Number of basepairs	Percent similarity	Query Cover	E. Value
RM-NS-SED-AM (1)	*Aspergillus* sp. (PP070052.1)	517	100%	100%	0.0
RM-S-SED-AM	*Aspergillus tamarii* (MH345887.1)	573	100%	99.82%	0.0
SED-TU (1)	*Trichoderma* sp. (MK871051.1)	610	100%	100%	0.0

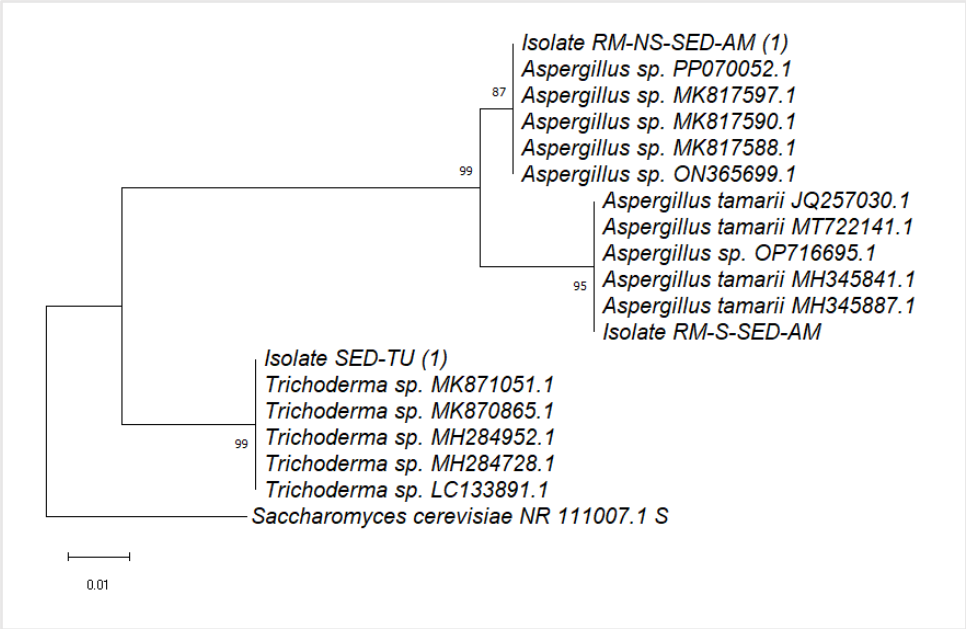

Figure 3. Phylogenetic tree of isolates *Aspergillus* sp. RM-NS-SED-AM (1), *Aspergillus tamarii* RM-S-SED-AM, and *Trichoderma* sp. SED-TU (1) based on comparison of Internal Transcribed Spacer (ITS) regions using neighbor-joining algorithm with 1,000 bootstraps.

Fermentation and extraction

The methanol and *n*-hexane extracts obtained from the liquid-liquid partitioning of the crude ethyl acetate extract of each fungal isolate are shown in **Table 2**. Only methanol extracts were further used for analysis and bioassays. The *n*-hexane solvent has non-polar characteristics which predominantly dissolves compounds such as oils and fats with relatively large molecular weights. The presence of these compounds can inhibit the diffusion process and block the penetration of antimicrobial active compounds into cells,[28]

therefore only methanolic extracts were used for further bioassays in this study. In addition, previous studies have also reported that extracts obtained from the methanolic phase showed the highest antioxidant activity.[29,30]

Table 2. The amount of extract afforded following fermentation of the marine sediment-derived fungi.

Fungal isolate	Salt in media	Extract's amount (g)		
		MeOH extract	n-hexane extract	EtOAc extract
Aspergillus sp. RM-NS-SED-AM (1)	With	1.14	0.73	1.87
	Without	0.79	0.27	1.06
Aspergillus tamarii RM-S-SED-AM	With	0.28	0.19	0.47
	Without	0.31	0.26	0.57
Trichoderma sp. SED-TU (1)	With	0.83	0.50	1.33
	Without	0.85	0.52	1.37

Phytochemicals Screening

Phytochemical screening of the obtained methanol extracts showed that all extracts contained alkaloids and polyphenols (**Table 3**). Flavonoids were detected in the extracts of *Aspergillus* sp. RM-NS-SED-AM (1) and *Trichoderma* sp. SED-TU (1) cultured on rice medium supplemented with salt. Triterpenes were identified in the extracts of *Aspergillus* sp. RM-NS-SED-AM (1) and *Aspergillus tamarii* RM-S-SED-AM. None of the extracts exhibited the presence of saponins, terpenes, or steroids. We observed no difference in the phytochemical profile of extracts afforded from fungal isolates *Aspergillus* sp. RM-NS-SED-AM (1) and *Aspergillus tamarii* RM-S-SED-AM regarding salt occurrence in the media. However, in the *Trichoderma* sp. SED-TU (1) extracts, flavonoids were only detected in the methanol extract when the fungus was fermented on rice medium with salt addition.

There are limited reports on secondary metabolites from *Aspergillus tamarii* from marine sediment by far. However, a previous study on *A. tamarii* M143 led to the isolation of a new pentacyclic oxindole alkaloid (Separadine A) from the cultured broth of the fungus. The fungus was obtained from driftwood at a seashore in Okinawa.[31] Alkaloids such as asperazine, asperazines B and C, and pestalamide D were also reported to be produced by *A. tubingensis* OUCMBIII 143291, which was isolated from tidal zone sediments in Dongying, China.[32] Research on one of the *Trichoderma* species, *Trichoderma* lixii, collected from Mentawai Island, Indonesia, led to the isolation of several secondary metabolites. Trichodermamides A and B, aspergillazine A, and DC1149B are classified as alkaloids. Ergosterol peroxide is identified as a steroidal compound, while 5-hydroxy-2,3-dimethyl-7-methoxy-chromone belongs to the polyphenol class. Cerebrosides C and D are categorized as glycosphingolipids, and nafuredin A is classified as polyketide. Lastly, harzianumols E and F are identified as sesquiterpenes, a subgroup of the terpene class.[33]

Table 3. Phytochemicals screening of methanol extracts from the marine sediment-derived fungi.

Fungal isolate	Salt in media	Class of secondary metabolites						
		Alkaloids	Flavonoids	Polyphenols	Saponins	Terpenes	Steroids	Triterpenes
Aspergillus sp. RM-NS-SED-AM (1)	With	(+)	(+)	(+)	(-)	(-)	(-)	(+)
	Without	(+)	(+)	(+)	(-)	(-)	(-)	(+)
Aspergillus tamarii RM-S-SED-AM	With	(+)	(-)	(+)	(-)	(-)	(-)	(+)
	Without	(+)	(-)	(+)	(-)	(-)	(-)	(+)
Trichoderma sp. SED-TU (1)	With	(+)	(+)	(+)	(-)	(-)	(-)	(-)
	Without	(+)	(-)	(+)	(-)	(-)	(-)	(-)

Note: (+/-) indicates the presence/absence of the corresponding secondary metabolite class.

Antimicrobial test

The antimicrobial activity of the tested extracts was indicated by the mean diameter of the inhibition zone±standard deviation (SD). Among them (**Table 4**), *Trichoderma* sp. SED-TU (1) extracts afforded from rice culture without the addition of salt (NS) showed potential inhibition against *S. aureus*, *S. epidermidis*, and *S. mutans* with zone inhibitions of 4.69±0.16, 3.51±0.01, and 3.49±0.25 mm, respectively. Meanwhile, *Aspergillus*

sp. RM-NS-SED-AM (1) extract, which was obtained from rice culture without the addition of salt (NS), showed potential inhibition against *B. cereus* growth with a zone inhibition of 3.19±0.85 mm. On the other hand, *Aspergillus tamarii* RM-S-SED-AM extract from rice culture containing salt (S) had the most potent inhibition against *C. albicans* with a zone inhibition of 7.04±0.23 mm. None of the tested extracts had antibacterial activity against the tested Gram-negative bacterium *E. coli* and MRSA. Although the disc diffusion results indicate generally weak inhibition categories, the presence of clear inhibition zones using extracts at a low tested concentration (5%) suggests that the tested samples possess potential antibacterial activity.

Many previous studies have shown that bioactive compounds with antimicrobial activity have been found in extracts produced by marine fungi from the genera *Aspergillus* and *Trichoderma*. Aside from marine ecosystems, the antibacterial activity of secondary metabolites isolated from these genera is also widely reported from fungal isolates derived from terrestrial environments, which indicates the broad distribution of these genera in nature and their potential as producers of bioactive compounds, including antibacterial. For example, *A. tamarii* extract, isolated from the inner living tissues of the fruiting bodies of *Lycoperdon umbrinum* mushroom in the botanical Garden, University of Ibadan, was shown to have antimicrobial activity against *S. aureus* ATCC 6538 and *E. coli* ATCC 25922, with zone inhibitions of 14.5±0.707 and 23±1.414 mm.[34] Extracts of *A. tubingensis* obtained from the lichen *Parmelia caperata* of Similipal Biosphere Reserve, India exerted antimicrobial activity against Gram-positive bacteria, *B. subtilis* and *S. aureus* with inhibition zone diameters of 20.30±0.57 and 16.50±0.50 mm. This extract also showed antifungal activity against *C. albicans*, with an inhibition zone reaching 25.30±1.73 mm.[35] *Trichoderma lixii* extract, the fungus isolated from mangrove *Sonneratia alba* grown in Bungus, West Sumatra, Indonesia, also showed inhibition against the growth of *S. aureus*, *E. coli* and *C. albicans*, with inhibition zone diameters of 14.37±0.01, 13.65±0.00 and 14.57±0.00 mm, respectively.[36]

Table 4. Diameter of inhibition zone (mm) of fungal extracts against the tested bacterial and fungal strains

Fungal isolate	Salt in media	Diameter of inhibition growth zone (mm) against microbial strains (mean±SD)						
		***S. aureus* ATCC 25923**	***E. coli* ATCC 25922**	***B. cereus* ATCC 11778**	***S. mutans* ATCC 35668**	***S. epidermidis* ATCC 12228**	**MRSA ATCC 3351**	***C. albicans* ATCC 10231**
Aspergillus sp. RM-NS-SED-AM (1)	With	(-)	(-)	(-)	(-)	(-)	(-)	(-)
	Without	(-)	(-)	3.19±0.85	(-)	(-)	(-)	(-)
Aspergillus tamarii RM-S-SED-AM	With	(-)	(-)	(-)	(-)	(-)	(-)	7.04±0.23
	Without	0.22±0.00	(-)	(-)	(-)	0.21±0.01	(-)	(-)
Trichoderma sp. SED-TU (1)	With	(-)	(-)	(-)	(-)	(-)	(-)	(-)
	Without	4.69±0.16	(-)	(-)	3.49±0.25	3.51±0.01	(-)	(-)
Positive control*		21.79±0.00	23.97±6.54	22.10±0.76	21.69±0.92	14.06±0.01	3.30±0.00	25.24±0.03

Note: (-) indicates that there is no antimicrobial activity. All tests were performed with three replicates.
*Chloramphenicol is used as a positive control in the antibacterial tests, while ketoconazole is used as a positive control in the antifungal test.

Antioxidant test

The antioxidant activity of the tested extracts was shown by the mean of IC_{50}±standard deviation (**Table 5**). Ascorbic acid was used as a reference and showed prominent antioxidant activity. Among the tested extracts, *Aspergillus tamarii* RM-S-SED-AM extract cultured on rice media containing salts had strong antioxidant activity with an IC_{50} of 62.00±0.35 µg/mL. In contrast, its extract from rice culture without salt was inactive. Extracts of *Aspergillus* sp. RM-NS-SED-AM (1) cultured on rice media containing salt, and *Trichoderma* sp. SED-TU (1) cultured on rice media with or without salt exhibited moderate antioxidant activity with IC_{50} values of 149.33±5.46, 106.02±2.51, and 163.85±3.56 µg/mL, respectively. Meanwhile, *Aspergillus* sp. RM-NS-SED-AM (1) (NS) extract from rice culture without adding salt only showed weak antioxidant activity with an IC_{50} value of 305.72±8.19 µg/mL.

Previous studies have shown that extracts and compounds from the *Aspergillus* and *Trichoderma* genera have antioxidant activity. For example, the extract of soil-derived *A. tubingensis*, which was isolated

from a soil sample close to the Sohag-Qena Road, Sohag Governorate, Egypt was shown to have very strong antioxidant activity with an IC_{50} value of 1.2±0.2 µg/mL.[37] A study on *Trichoderma* sp. Jing-8, isolated from the stem of *Panax notoginseng* collected in Wenshan, Yunnan Province, China, led to the isolation of alternariol and alternariol-1′-hydroxy-9-methyl ether, which demonstrated potent radical-scavenging activity with an IC_{50} value of 12 µg/mL.[38]

Table 5. Antioxidant activity of extracts from marine sediment-derived fungi using the DPPH method.

Fungal isolate	Salt in media	IC_{50}±SD (µg/mL)	Category*
Aspergillus sp. RM-NS-SED-AM (1)	With	149.33±5.46	Moderate
	Without	305.72±8.19	Weak
Aspergillus tamarii RM-S-SED-AM	With	62.00±0.35	Strong
	Without	>500	Inactive
Trichoderma sp. SED-TU (1)	With	106.02±2.51	Moderate
	Without	163.85±3.56	Moderate
Ascorbic Acid**		6.79±0.10	Very strong

*Category refer to the study by Endra *et al*. (2021)[21]
**Ascorbic acid was used as a reference compound in the antioxidant assay.

In the present study, each isolated marine sediment-derived fungus was cultured on rice media with or without salt. The secondary metabolite analysis showed no apparent differences among the secondary metabolites detected in extracts from both cultures, except for *Trichoderma* sp. SED-TU (1) extract. However, in the antimicrobial and antioxidant tests, the presence or absence of salt in rice media seemed to influence the activity of the extracts. This finding is in line with previous studies. For example, the extract of the fungus *Phialosimplex* isolated from seawater from Dona Paula beach grown on media containing 15% NaCl showed higher antioxidant activity,[39] suggesting that salt in media could affect the production of secondary metabolites, which in turn also influence their bioactivity. As the method for phytochemical analysis used in our study is only able to detect the presence of a particular class of secondary metabolites, analysis using HPLC-DAD or LC-MS would be more beneficial to detect particular secondary metabolites produced by fungi grown on different media compositions for further investigation.

CONCLUSIONS

Three fungal isolates obtained from the marine sediments of Amed and Tulamben beaches were identified as *Aspergillus* sp. RM-NS-SED-AM (1), *Aspergillus tamarii* RM-S-SED-AM, and *Trichoderma* sp. SED-TU (1). Phytochemical analysis revealed that all methanol extracts contained alkaloids and polyphenols. Antimicrobial tests showed that extracts from the culture of all three isolates without salt have superior antibacterial activity compared to the culture without salt. However, only the culture of *A. tamarii* RM-S-SED-AM with salt showed antifungal activity against *C. albicans*. Moreover, antioxidant test revealed that this extract possess strong antioxidant activity. Therefore, the findings from this study regarding the effect of salt on the culture can serve as the starting point of further investigations, particularly in the search for compounds responsible for the antibacterial, antifungal, and antioxidant activities of each fungal strain.

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CONFLICT OF INTERESTS

The authors declare no conflicts of interest.

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