JURNAL ILMIAH MEDICAMENTO

Vol.12 No.1 (March 2026)
page 115-130
https://doi.org/10.36733/medicamento.v12i1.13356
e-ISSN: 2356-4814

Original Article

Antimicrobial and Antioxidant Properties of Marine Fungi Associated with the Sponge *Pseudoceratina* sp. from Amed Coastal Waters, Bali, Indonesia

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Submitted : 31-12-2025
Revised : 06-02-2026
Accepted : 04-03-2026

Keywords: antimicrobial, antioxidant, marine fungi, marine sponges, *Pseudoceratina sp.*, toxicity.

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Abstract

Background: Marine fungi are organisms capable of growth and spore production in the marine environment and establishing symbiotic relationships with other marine organisms. Because of their high biochemical diversity, marine fungi are considered a potential source for the discovery of new natural compounds with various biological activities.
Objective: This study aims to conduct molecular identification and assess the bioactivity potential of the methanol extract obtained from endophytic fungi associated with the marine sponge *Pseudoceratina* sp. from the Amed Coastal Waters, Bali.
Methods: Eight marine fungi were isolated in this study and identified through molecular biology protocol as *Aspergillus tamarii* SP-3-1-4, *Aspergillus nomiae* SP-3-2 (A), *Penicillium citrinum* SP-3-2-1, *Aspergillus protuberus* SP-3-2-3, *Aspergillus sydowii* SP-3-2-4, *Aspergillus sydowii* RM NS SP-3B, *Aspergillus clavatonanicus* RM NS SP-3-2 A, and *Aspergillus nomiae* RM NS SP-3-2 B. The fungi were subsequently fermented on rice media containing salt and or without salt. In the final stage of fermentation, secondary metabolites were extracted using ethyl acetate, followed by liquid-liquid extraction using methanol containing 10% water and n-hexane. The resulting methanolic extracts were subjected to phytochemical analysis and bioassays.
Results: The phytochemical screening results of the methanol extracts showed that all extracts contained alkaloids. Among the tested extracts, the extract obtained from the fermentation of the fungus *A. protuberus* SP-3-2-3, on rice medium with the addition of salt, showed the highest activity against *Methicillin-resistant Staphylococcus aureus* ATCC 3351 with an inhibition zone diameter of 7.99±0.20 mm. The fungus *A. sydowii* SP-3-2-4 cultured with and without salt in rice media exhibited the most potent antioxidant capacity, with IC_{50} values of 37.02±1.12 and 32.48±0.81 µg/mL. Conversely, *A. nomiae* SP-3-2 (A), fermented without salt, displayed the highest toxicity with an LC_{50} value of 0.88±0.69 µg/mL.
Conclusion: Based on the pharmacological potential of extracts produced by the marine fungi associated with the sponge *Pseudoceratina* sp. found in the present study, identification of bioactive secondary metabolites and their mode of action is propitious for further investigation.

How to Cite: (*citation style* AMA 11th Ed.)
Adi IMAK, Witantri NKDE, Wulandari NMW, Leliqia NPE, Wirajana IN, Wibowo JT, Ware I, Ariantari NP. Antimicrobial and Antioxidant Properties of Marine Fungi Associated with the Sponge *Pseudoceratina* sp. from Amed Coastal Waters, Bali, Indonesia. *J. Ilm. Medicam.*, 2026;12(1): 115-130. https://doi.org/10.36733/medicamento.v12i1.13356

INTRODUCTION

Marine-derived fungi represent a prolific frontier in natural product discovery, characterized by exceptional metabolic plasticity and the ability to synthesize structurally unique secondary metabolites. Unlike their terrestrial counterparts, these microorganisms have evolved specialized biochemical pathways to thrive under extreme marine conditions, yielding a diverse array of scaffolds including terpenoids, steroids, and polyketides.[1] Sponges (Porifera), as ancient sessile invertebrates, serve as critical ecological niches for these fungal communities.[2] The symbiotic consortia within sponges are often driven by the host's adaptation to high-pressure, hypersaline, and hypoxic environments,

which triggers the production of potent defensive metabolites. Notable examples include drimane sesquiterpenoids, such as chartarolides A–C, and phenylspirodrimanes, which exhibited significant pharmacological activities.[3,4]

The genus *Pseudoceratina* is a sponge rich in secondary metabolites.[5] Among these metabolites are alkaloids, meroterpenoids, glycolipids, phenolics, sterols, and other nitrogenous compounds. These metabolites demonstrated a variety of pharmacological and biological activities, including antibacterial, antifungal, antimalarial, and cytotoxic activities.[6] A previous study on an endophytic fungus isolated from the marine sponge *Pseudoceratina purpurea*, which was isolated from the Sakuraguchi area, Ishigaki Island, Okinawa Prefecture, Japan, showed that the fungus was capable of producing xanthoquinodin. This compound possessed cytotoxic activity against various cancer cells.[7] To date, no studies have been reported concerning the activity of endophytic fungi isolated from *Pseudoceratina* within the Bali region.

The global surge in antimicrobial resistance necessitates the discovery of novel chemical scaffolds from untapped sources, such as sponge-associated marine fungi that possess unique biosynthetic pathways. In this study, we coupled precise molecular identification with antibacterial screening conducted under controlled salinity to mimic the marine environment. The comparison between these salinity levels is because marine fungi often require specific osmotic pressure to activate their secondary metabolism. Without the presence of salt, some bioactive compounds might remain unexpressed.[8] This integrated approach provides a reliable foundation for drug development by linking accurate fungal taxonomy with bioactivity produced under ecologically relevant conditions. This study aims to conduct molecular identification and assess the bioactivity potential of the methanol extract obtained from endophytic fungi associated with marine sponges from the Amed Coastal Waters, Bali. Herein, we report the results of the comprehensive phytochemical screening and biological assays performed on these fungal extracts.

METHODS

Instruments and Materials

Instruments

Bacterial absorbance was measured using a UV-Vis spectrophotometer (Thermo Scientific®). For molecular identification, technical procedures were conducted using a centrifuge (Hitachi®), a PCR thermal cycler (Sensoquest®), and a UV transilluminator (Enduro GDS®) for gel visualization. Antioxidant activity testing was performed using a microplate reader spectrophotometer (Thermo Scientific®, Lithuania).

Materials

In the fungal isolation process, agar media were used for fungal growth before the fungus was identified. The materials used in the preparation of agar media included a mixture of Bacto agar (Difco BD®, Maryland, USA), yeast extract (Oxoid®, France), malt extract (Himedia®, India), artificial sea salt (Himedia®, India), and glycerol (Vivantis®, Malaysia). For the isolation, purification, and fermentation of fungi, rice media (Putri Sejati®) (Indonesia) was used, with or without the addition of artificial sea salt. In the antimicrobial activity testing of the fungal extracts, media such as Sabouraud Dextrose Broth (SDB), Sabouraud Dextrose Agar (SDA), Mueller-Hinton Broth (MHB), and Mueller-Hinton Agar (MHA) from Oxoid® (France) and Himedia® (India), were used. In this test, antibiotics were also used as positive controls, namely ketoconazole (Zoralin®, Indonesia) and chloramphenicol (Nalgane®, USA). Another activity tested was antioxidant activity using DPPH (Smart-Lab®, Indonesia) as the primary reagent, and ascorbic acid (Supelco®, Germany) as the standard solution in the test. Fungal secondary metabolites were extracted using the maceration method with ethyl acetate, followed by liquid-liquid extraction with *n*-hexane and aqueous methanol. Ultimately, for molecular identification, ITS1 and ITS4 primers, DNA polymerase, nuclease-free water (Thermo Scientific, Lithuania), agarose (1st BASE®, Singapore), and TAE solution (1st BASE®, Singapore) were used.

Research Procedure

Sample Collection

The sponge *Pseudoceratina sp.* was obtained from the Amed Coastal Waters, located in Abang District, Karangasem Regency, Bali Province, Indonesia. The sponge samples were collected in June 2024. The sponge samples were placed in sterile 50 mL Eppendorf tubes and then placed in special storage containers to prevent damage. The prepared samples were then taken to the Integrated Laboratory at Udayana University for further experimental procedures.

Isolation of Marine Sponge-Derived Fungi

The agar medium used to isolate the marine sponge *Pseudoceratina* sp.-derived fungi was prepared using Bacto agar, malt extract, artificial sea salt water, chloramphenicol, and demineralized water. In addition to agar medium, rice media with or without artificial seawater were also used in the isolation process. The isolation stage was performed by inoculating the sponge sample into the agar medium. This procedure was conducted carefully to avoid damaging the medium, as damaged agar is more susceptible to contamination.[9] Isolation on rice medium was carried out similarly, by inoculating approximately 1 to 2 g of the sponge sample onto the rice medium. After that, the media containing the sponge sample were incubated at room temperature (25-27°C) for 7 days.[10] The fungal colonies that grew on the isolation medium and rice media were then transferred to a new agar medium to continue to the purification stage.

Fermentation and Extraction

The rice medium used in the fermentation process of the fungal isolate consists of two types, with and without the addition of artificial seawater. The rice medium was prepared by mixing 110 mL of water, 100 g of rice, and 3.8 g of artificial sea salt.[11] The medium was then sterilized using an autoclave. Small pieces of agar containing the fungal isolate were then inoculated onto the surface of the rice medium and spread evenly. The cultures were incubated for 7-14 days. Once the fungus has grown to cover the entire rice medium to the bottom surface, the extraction stage was carried out. Extraction was carried out by soaking the rice medium covered with fungus with 500 mL of ethyl acetate. The soaked medium was then cut into small pieces to facilitate the extraction process. After being cut into small pieces, the medium was agitated on an orbital shaker for 8 h to extract the compounds contained therein.[12] The resulting extract was filtered and evaporated to remove the solvent. In the final process, the extract was subjected to the separation stage using the liquid-liquid extraction method. Liquid-liquid extraction used a mixture of methanol containing 10% water and n-hexane. This methanol extract was then utilized for further analysis and bioactivity testing. The final weight of the methanol extract obtained after evaporation was recorded as the yield of extract.

Molecular Identification

Molecular identification was performed by comparing the deoxyribonucleic acid (DNA) sequence from the Internal Transcribed Spacer (ITS) region. This process used two primers, the ITS1 forward primer (5'-TCCGTAGGTGAACCTGCGG-3') and the ITS4 reverse primer (5'-TCCTCCGCTTATTGATATGC-3').[13] Fungal DNA was first extracted using the Quick-DNA Fungal/Bacterial Miniprep kit, following the protocol. The ITS region of the extracted DNA was then amplified using a Labcycler 48 Thermocycler. The reaction mixture contained 1 µL of ITS1 primer, 1 µL of ITS4 primer, 20 µL of DNA polymerase, 17 µL of nuclease-free water, and 1 µL of fungal genomic DNA as template. DNA amplification occurs in several stages. Pre-denaturation was performed at 95°C for 1.5 minutes, followed by 35 cycles of denaturation, annealing, and extension. Each cycle consisted of denaturation at 95°C, annealing at 56°C, and extension at 72°C, each lasting 1 minute. The process ended with a final extension at 72°C for 15 minutes. After amplification, the PCR product was examined by electrophoresis using a 1% agarose gel in a 10× TAE buffer solution. The gel was run for 45 minutes at 75 volts. The PCR product was then submitted for sequencing analysis. The purified PCR products were sequenced using the Sanger dideoxy termination method on an ABI 3730xl Genetic Analyzer (Applied Biosystems®). The obtained DNA sequence was compared with nucleotide sequence data in the NCBI GenBank database using the Basic Local Alignment Search Tool (BLAST) for nucleotide program. Species identification was determined based on the BLASTn search results by evaluating several key indicators such as Query Coverage, Percent Identity and E-value. A sequence was considered a high match species if it exhibited a Query Coverage and Percent Identity of ≥ 99%, with an E-value close to 0.0. These parameters ensure that the alignment is statistically significant and covers most of the analyzed sequence length. Subsequently, a phylogenetic tree was reconstructed using MEGA 11 software to determine evolutionary relationships among fungal isolates. The phylogenetic tree was built using the Neighbor-Joining method with 1,000 bootstrap replications to ensure the reliability of the branching.[14]

Phytochemical Screening

Phytochemical screening is a qualitative analysis technique used to identify the presence of particular compound groups, such as alkaloids, flavonoids, terpenoids, triterpenoids, steroids, saponins, and polyphenols. The extract solution was prepared before testing. Alkaloid testing was carried out using Bouchardat, Wagner, and Dragendorff reagents. For the alkaloids test, 100 µL of the dissolved extract solution was taken, and three drops of each of the alkaloid testing reagents were added. Positive results for alkaloid testing were indicated by the appearance of a brown to black precipitate after the addition of Bouchardat reagent, an orange precipitate after the addition of Dragendorff reagent, and a yellowish-white precipitate after the addition of Wagner reagent.[15] The positive results for alkaloids were determined based on a consistent reaction across at least two different reagents. This approach was

taken to avoid false-positive interpretations and ensure the reliability of the phytochemical screening.[16] Steroid and triterpenoid testing was performed using Liebermann-Burchard (LB) reagent. One to two drops of LB reagent were added to the prepared extract solution. The appearance of a bluish-green color indicated a positive result for steroid testing. In contrast, a positive result for triterpenoid testing was indicated by the appearance of a purple to brownish ring at the boundary between the two solvents.[17] Saponin testing was carried out by preparing 200 µL of extract solution, which was then shaken vertically for 10 seconds, followed by the addition of 2N HCl solution. A positive saponin test result was indicated by the formation of foam that does not disappear after the addition of HCl. Flavonoid testing was carried out by preparing 200 µL of extract. The following reagents: oxalic acid, ether, acetone, and boric acid, were added to the extract solution and observed under ultraviolet (UV) light at 366 nm. The appearance of yellow fluorescence indicates a positive result for flavonoids.[18] The polyphenol test was carried out by preparing 200 µL of extract solution, to which one to three drops of 10% $FeCl_3$ solution were added. A positive result for polyphenols was indicated by the appearance of a dark blue to black or greenish-black color. Terpenoid testing was performed by reacting 200 µL of extract with 100 µL of chloroform and 100 µL of H_2SO_4. A positive result for terpenoid testing was indicated by the appearance of a reddish-brown layer at the top of the solution.[19]

Antimicrobial Test

Antimicrobial testing was conducted using the disc diffusion method. The tested bacteria included *Methicillin-resistant Staphylococcus aureus* (MRSA) ATCC 3351, *Streptococcus mutans* ATCC 35668, *Streptococcus epidermidis* ATCC 12228, *Bacillus cereus* ATCC 11778, *Candida albicans* ATCC 10231, *Escherichia coli* ATCC 25922, and *Staphylococcus aureus* ATCC 25923.[20,21] The tested extract solution was prepared by dissolving 50 mg of extract into 1 mL of solvent (distilled water: methanol) at a ratio of 1:9 to produce a final concentration of 5%. Next, suspension of bacterial and fungal cultures was prepared, followed by incubation using an incubator at 37°C for 18-24 h for bacterial and 48-52 h for fungal.[22] Afterward, 600 µL of bacterial suspension equivalent to a turbidity standard of 0.5 McFarland was prepared, then spread on agar media for antibacterial testing. For the antifungal assay, 550 µL of fungal suspension was spread on agar media. Next, sterile discs were placed on the agar medium, and 10 µL of the test solution was added to each sterile disc. As a negative control, 10 µL of methanol was used. Chloramphenicol and ketoconazole were used as reference drugs. The assay was done in triplicate.

Antioxidant Test

The antioxidant activity of the tested extracts was determined using the DPPH (2,2-diphenyl-1-picrylhydrazide) method. For comparison, ascorbic acid was used as a standard solution prepared in a concentration range of 4 to 12 µg/mL. For the samples, a concentration range of 20 to 500 µg/mL was used. The initial stage of the antioxidant test was carried out by mixing 40 µL of 0.5 mM DPPH solution with 160 µL of sample or standard solution in methanol in a 96-well microplate. Additionally, a blank was prepared by mixing 40 µL of DPPH solution with 160 µL of pure methanol to determine the maximum radical absorbance. The mixture was then incubated in a dark room for 30 minutes to trigger the reaction. After the incubation process was completed, the absorbance value of the tested solution was measured using a spectrophotometer at a wavelength of 517 nm.[23] This test was performed three times (triplicate). Free radicals from DPPH will react with antioxidant compounds that can donate protons, thereby neutralizing the radicals.[24]

Brine Shrimp Lethality Test

The Brine Shrimp Lethality Test (BSLT) is a method used to test the toxicity of extracts. *Artemia salina* larvae were used for this assay. Artificial seawater was prepared by mixing 9.5 g of artificial sea salt broth with 300 mL of distilled water, before the shrimp egg hatching process.[25] To hatch *A. salina* eggs, eggs were incubated for 48 hours in artificial seawater under continuous light at room temperature.[26] For the assay, a 24-well microplate was used. The tested extract solution was prepared in artificial seawater and serially diluted to achieve the final concentration ranging from 1 to 1000 µg/mL. Next, ten *A. salina* larvae were added to each well. As a negative control, only artificial seawater and larvae were added to the wells. The experiment was done in triplicate. The microplate was incubated at room temperature for 24 hours under continuous light. The number of dead larvae was observed and % mortality was calculated by comparing the number of dead larvae to the larvae used in each well. Next, the lethal concentration 50% (LC_{50}) was calculated using probit analysis of extract concentration vs the number of dead larvae.

Data Analysis

Antioxidant activity was presented as an IC_{50} value. Based on IC_{50} values, the antioxidant capacity of each extract was categorized into five groups: very strong, strong, moderate, weak, and inactive. Antioxidant capacity is considered very strong if the IC_{50} falls below 50 µg/mL, strong in the range of 50-100 µg/mL, moderate in the range of

100-250 µg/mL, weak in the range of 250-500 µg/mL, and inactive if the IC_{50} is more than 500 µg/mL.[27] The IC_{50} value was calculated by analyzing the linear relationship between the sample concentration and the percentage of inhibition. The percentage of inhibition of the extract against free radicals was calculated using the following equation (1).

$$\%\text{Inhibition} = \frac{A-B}{A}\ x\ 100 \quad \ldots\ldots (1)$$

A is blank absorbance, and B is sample absorbance.

Antibacterial activity was measured by determining the diameter of the clear zone, known as the inhibition zone, which forms around the sterile disc in tests using the disc diffusion method. Based on the clear zone, the diameter of the inhibition zone can be divided into four categories: weak if the diameter is less than 5 mm, moderate if it is in the range of 5-10 mm, strong if it is in the range of 10-20 mm, and very strong if the diameter of the inhibition zone is more than 20 mm.[28] The diameter of the inhibition zone was calculated using the following equation (2).

$$\text{Inhibition zone} = \frac{(A-C)+(B-C)}{2}\ x\ 100 \quad \ldots\ldots (2)$$

A: vertical diameters of the clear zone; B: horizontal diameters of the clear zone; and C: diameter of the disc.

Toxicity testing on the extract was conducted by calculating the mortality of brine shrimp larvae (*A. salina*) at all tested concentrations during the incubation period (24 hours). The toxicity test observed the number of dead larvae, which was then analyzed using probit values. The mortality percentage was calculated using the following equation (3).

$$\%\ \text{Mortality} = \frac{Number\ of\ dead\ larvae}{Total\ number\ of\ larvae}\ x\ 100\% \quad \ldots\ldots (3)$$

The LC_{50} of the tested extract was calculated using probit analysis of extract concentration vs the number of dead larvae, using Microsoft Excel software. LC_{50} values can be categorized into three groups: high, medium, and low. The toxicity is considered high if the LC_{50} value is below 100 µg/mL, medium if it is in the range of 100-1000 µg/mL, and low if it is above 1000 µg/mL.

RESULT AND DISCUSSION

Fungal Isolation and Molecular Identification

The isolation procedure of endophytic fungi from *Pseudoceratina* sp. yielded eight fungal isolates, encoded as SP-3-1-4, SP-3-2 (A), SP-3-2-1, SP-3-2-3, SP-3-2-4, RM NS SP-3 B, RM NS SP-3-2 A, and RM NS SP-3-2 B. The isolation process of endophytic fungi from the *Pseudoceratina* sp. was carried out using two treatments, with and without the addition of salt. The addition of salt to the rice medium was done with the aim of mimicking the living environment of endophytic fungi with their natural environment. This condition is necessary so that the fungi can adapt, develop, and prevent death due to osmotic shock. The macroscopic appearances of the fungal isolates are shown in **Figure 1.**

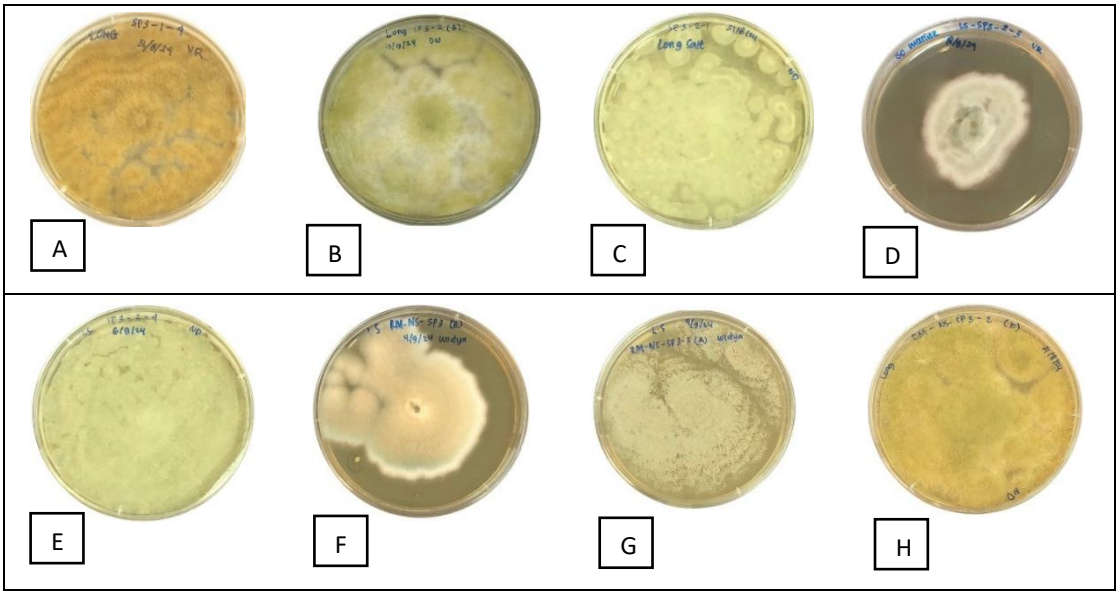

Figure 1. Morphological appearance of fungal isolates: SP-3-1-4 (A), SP-3-2 (A) (B), SP-3-2-1 (C), SP-3-2-3 (D), SP-3-2-4 (E), RM NS SP-3 B (F), RM NS SP-3-2 A (G), RM NS SP-3-2 B (H), view from the top of the agar media.

Molecular identification method by amplification of Internal Transcribed Spacers (ITS), particularly the ITS 1+4 region, has been widely used. The high speed and genetic variability between species make the ITS region an accurate genetic marker for species taxonomy. In this study, amplification of this region of eight fungal isolates encoded as SP-3-1-4, SP-3-2 (A), SP-3-2-1, SP-3-2-3, SP-3-2-4, RM NS SP-3 B, RM NS SP-3-2 A, and RM NS SP-3-2 B produced DNA fragments of 500-700 base pairs (bp), as shown in **Figure 2.** This result is in accordance with established literature, which states that the ITS region in fungi typically ranges from 500 to 700 bp**.**[29] Based on the results of identification using the Basic Local Alignment Search Tools (BLAST) method in **Table 1**, isolate SP-3-1-4 was identified as *Aspergillus tamarii*, based on its similarity (100%) to *A. tamarii* (MT340979.1). Next, isolate SP-3-2 (A) showed 100% similarity to *Aspergillus nomiae* (PX362631.1). The fungal isolate SP-3-2-1 was identified as *Penicillium citrinum*, as it exhibited high similarity to *P. citrinum* (PX094005.1). Meanwhile, isolate SP-3-2-3 had 100% similarity with *Aspergillus protuberus* (PQ932326.1). Fungal isolates SP-3-2-4 and RM NS SP-3 B showed the closest similarity to *Aspergillus sydowii* (MT079126.1 and PV687296.1). The fungal isolate RM NS SP-3-2 A was identified as *Aspergillus clavatonanicus* based on its closest similarity to the GenBank sequence MN735923.1, while isolate RM NS SP-3-2 B revealed the highest similarity to *Aspergillus nomiae* (MN871698.1). This result was also supported by the phylogenetic analysis as depicted in **Figure 3**.

Table 1. Result of molecular identification of the isolated fungi from the marine sponge *Pseudoceratina* sp. collected from Amed Coastal Waters using the Basic Local Alignment Search Tools (BLAST) method.

Isolate code	Taxa with the closest relationship (accession number)	Number of base pairs	Percent similarity	Query cover	E-value
SP-3-1-4	*Aspergillus tamarii* (MT340979.1)	574	100%	100%	0.0
SP-3-2 (A)	*Aspergillus nomiae* (PX362631.1)	559	100%	100%	0.0
SP-3-2-1	*Penicillium citrinum* (PX094005.1)	530	100%	100%	0.0
SP-3-2-3	*Aspergillus protuberus* (PQ932326.1)	537	100%	100%	0.0
SP-3-2-4	*Aspergillus sydowii* (MT079126.1)	542	100%	100%	0.0
RM NS SP-3 B	*Aspergillus sydowii* (PV687296.1)	529	100%	100%	0.0
RM NS SP-3-2 A	*Aspergillus clavatonanicus* (MN735923.1)	563	100%	100%	0.0
RM NS SP-3-2 B	*Aspergillus nomiae* (MN871698.1)	559	100%	100%	0.0

All isolates had 100% similarity with a query cover value of 100% and an E-value of 0.0, as shown in **Table 1**. Among the isolated fungi, only isolate SP-3-2-1 belongs to *Penicillium*, the remaining fungal isolates belong to the genus *Aspergillus*, although both genera have been repeatedly reported as symbionts of marine sponges. Previous studies reported that the species *A. sydowii* and *A. nomiae* were obtained from sponges collected in the Mandeh Sea, South Sumatra, Indonesia. Isolate *A. sydowii* initially appeared as a dense white cottony colony. While literature often describes mature *A. sydowii* as having blue-green hues, the observed white morphology is consistent with the early growth stage or predominantly vegetative mycelia of the species when cultured in specific media. Isolate *A. nomiae* colony showed a granular to velvety texture with a coloration that transitioned from yellowish-green to dark olive green as it matured. The reverse side of the colony was typically pale to light brown. A notable characteristic observed was the abundant production of dark brown to blackish sclerotia. [30,31] The species *P. citrinum* was previously also obtained from Wakatobi Waters, Southeast Sulawesi. Macroscopically, *P. citrinum* was characterized by a velvety white-green colony with a distinct yellowish exudate on the surface. [32]

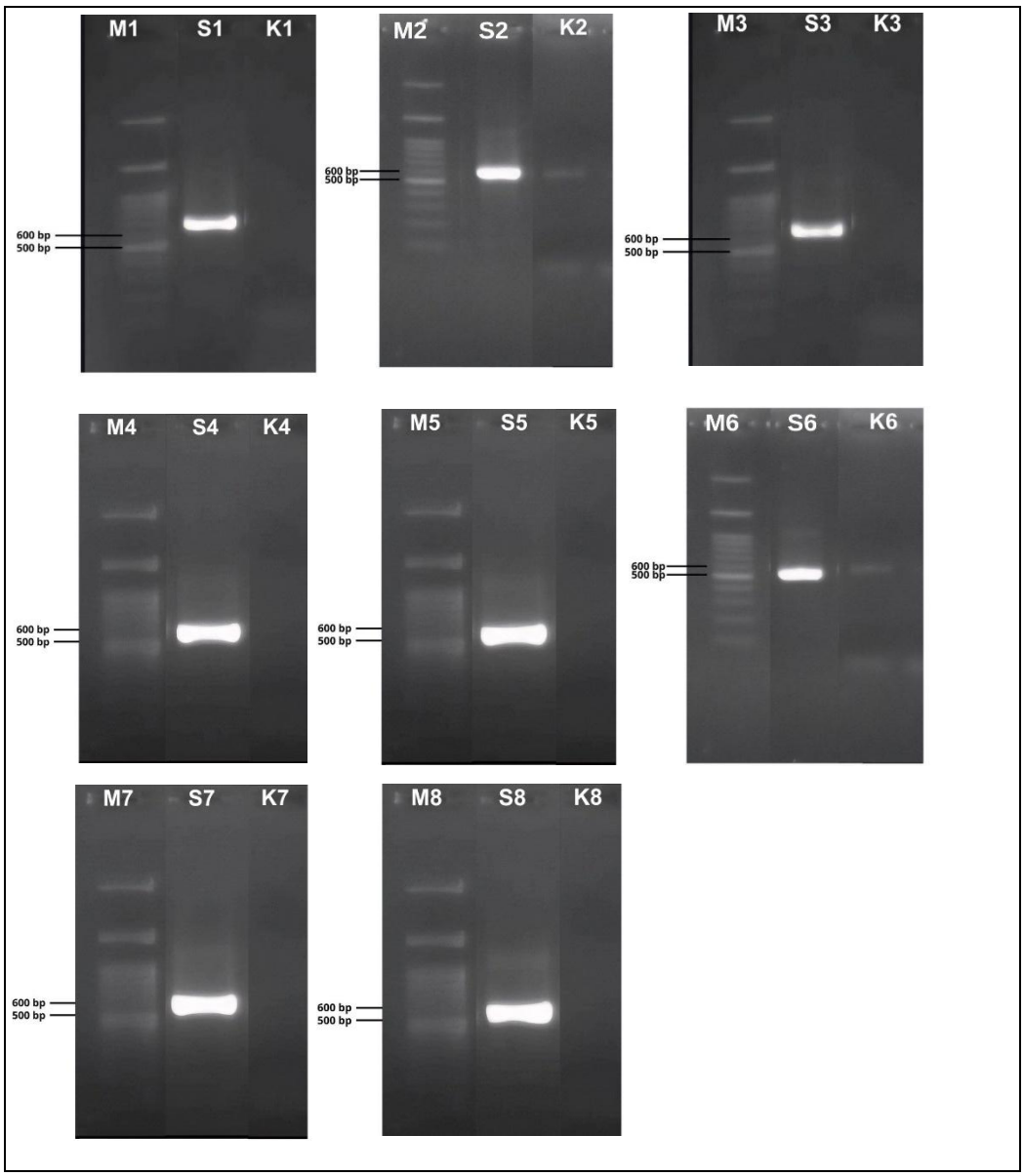

Figure 2. DNA bands after PCR amplification of the ITS1+4 region, visualized under ultraviolet light following agarose gel electrophoresis. M1-8: DNA ladders, S1-8: DNA samples SP-3-1-4 (S1), SP-3-2 (S2) (B), SP-3-2-1 (S3), SP-3-2-3 (S4), SP-3-2-4 (S5), RM NS SP-3 B (S6), RM NS SP-3-2 A (S7), RM NS SP-3-2 B (S8). K1-8: negative control.

Figure 3. Phylogenetic tree of the fungal isolates from the marine sponge *Pseudoceratina* sp. collected from Amed Coastal Waters, Bali, Indonesia, based on comparison of the Internal Transcribed Spacer (ITS) regions using neighbor-joining algorithm with 1,000 bootstraps.

Fermentation and extraction

In this study, the ethyl acetate (EtOAc) extract afforded after fungal fermentation was further processed through liquid-liquid extraction, resulting in the methanol (MeOH) and *n*-hexane extracts, with the extract yields presented in **Table 2**. The methanol extract was selected for subsequent bioassay testing. The n-hexane extract was primarily obtained as a pre-extraction or defatting step to remove non-polar lipids and fatty acids from the fungal biomass.[33] Because this fraction consists mostly of fatty acids, it was deemed unsuitable for downstream bioactive molecules development and excluded from further bioactivity testing.[34] The presence of these thick oils and fats can physically block the penetration of antimicrobial active compounds. Consequently, compounds intended to act against target cells may not penetrate effectively, potentially leading to inaccurate or inhibited bioassay results.

Table 2. The amount of extract from the fermentation of fungal strains derived from the marine sponge *Pseudoceratina* sp.

Fungal isolate	Salt in media*	Extract's amount (g)		
		EtOAc	MeOH	*n*-hexane
Aspergillus tamarii	With	0.34	0.23	0.11
SP 3-1-4	Without	0.44	0.30	0.14
Aspergillus nomiae	With	1.01	0.54	0.47
SP-3-2 (A)	Without	0.86	0.41	0.44
Penicillium citrinum	With	0.77	0.29	0.47
SP-3-2-1	Without	2.15	0.35	1.80
Aspergillus protuberus	With	1.93	1.89	0.03
SP-3-2-3	Without	0.95	0.32	0.62
Aspergillus sydowii	With	1.53	0.32	1.21
SP-3-2-4	Without	0.98	0.51	0.47
Aspergillus sydowii **RM NS SP-3 B (S)**	With	1.20	0.22	0.97
Aspergillus clavatonanicus	With	0.85	0.41	0.43
RM NS SP-3-2 A	Without	0.80	0.20	0.60
Aspergillus nomiae	With	0.78	0.36	0.41
RM NS SP-3-2 B	Without	0.92	0.21	0.70

*Fermentation was done on 2 flasks (1L) of rice media for each condition

Phytochemical Screening

The results of phytochemical screening of extracts of fungal isolates cultivated in the rice media with the presence or without the addition of salt (**Table 3**) showed that alkaloids were detected in all fungal extracts. Flavonoids were detected in several fungal extracts, including *A. nomiae* RM NS SP-3-2 B extract, obtained from fermentation with and without salt addition. None of the fungal extracts contained saponins and steroids. Terpenoids were detected in the fungal extracts of *A. nomiae* RM NS SP-3-2 B and *A. protuberus* SP-3-2-3 afforded after fermentation with the addition of salt, as well as from *A. clavatonanicus* RM NS SP-3-2 A extract from salt-free fermentation. Meanwhile, triterpenoids were found in numerous fungal extracts, either from fermentation with the addition of salt or without salt. Polyphenols were detected in fewer fungal extracts, including *P. citrinum* SP-3-2-1 extracts. Our results in **Table 3** suggested that more secondary metabolite groups were detected in fungal extracts obtained from salt addition fermentation, indicating that salinity is a vital environmental trigger. In marine-derived fungi, the presence of salts introduces osmotic pressure that activates secondary metabolic pathways that typically remain silent in salt-free conditions. This physiological response stimulates the biosynthesis of specialized bioactive compounds as an adaptation strategy to survive in their natural marine habitat.[35]

Table 3. Phytochemical screening of methanolic extracts produced from the rice fermentation of marine fungi associated with the sponge *Pseudoceratina* sp.

Extract of fungal isolate	Salt in media	Class of secondary metabolites						
		Alkaloids*	Flavonoids	Polyphenols	Saponins	Terpenoids	Steroids	Triterpenoids
***Aspergillus tamarii* SP 3-1-4**	With	(+)	(-)	(-)	(-)	(-)	(-)	(+)
	Without	(+)	(+)	(-)	(-)	(-)	(-)	(+)
***Aspergillus nomiae* SP-3-2 (A)**	With	(+)	(-)	(+)	(-)	(-)	(-)	(+)
	Without	(+)	(-)	(-)	(-)	(-)	(-)	(+)
***Penicillium citrinum* SP-3-2-1**	With	(+)	(+)	(+)	(-)	(-)	(-)	(+)
	Without	(+)	(-)	(+)	(-)	(-)	(-)	(+)
***Aspergillus protuberus* SP-3-2-3**	With	(+)	(+)	(-)	(-)	(+)	(-)	(+)
	Without	(+)	(-)	(-)	(-)	(-)	(-)	(-)
***Aspergillus sydowii* SP-3-2-4**	With	(+)	(-)	(-)	(-)	(-)	(-)	(+)
	Without	(+)	(-)	(+)	(-)	(-)	(-)	(+)
***Aspergillus sydowii* RM NS SP-3 (B)**	With	(+)	(-)	(-)	(-)	(-)	(-)	(+)
***Aspergillus clavatonanicus* RM NS SP-3-2 A**	With	(+)	(-)	(+)	(-)	(-)	(-)	(+)
	Without	(+)	(-)	(-)	(-)	(+)	(-)	(-)
***Aspergillus nomiae* RM NS SP-3-2 B**	With	(+)	(+)	(+)	(-)	(+)	(-)	(-)
	Without	(+)	(+)	(-)	(-)	(-)	(-)	(+)

The symbol (+/-) indicates the presence or absence of secondary metabolites in the extract.
*The presence of alkaloids was identified using Bouchardat, Dragendorff's and Wagner's reagents

Several previous studies have reported bioactive secondary metabolites from the species of *Aspergillus* and *Penicillium,* as found in this study. However, none of those studies ever reported metabolites of *Aspergillus* and *Penicillium* fungi associated with *Psceudoceratina* sp. collected from Bali Waters. Fungi isolated from different environments seemed to have the capability to produce different secondary metabolites. For example, *A. sydowii* SCSIO00305 isolated from the marine sponge, *Stelletta* sp., collected from the South China Sea, was reported capable of producing alkaloids cyclotryprostatins B, E and fumitremorgin B. Meanwhile, the fungus *A. sydowii* CPCC 401353 associated with the marine sponge, *Spongia obscura*, collected from Florida, United States, was reported to produce the polyphenol, 3-hydroxybenzoic acid.[36] Another study revealed that *A. tamarii* associated with the marine sponge, *Fanthalia* sp., yielded a new alkaloid, speradine A. The fungus *P. citrinum* 2015PF07 isolated from the marine sponge, *Phyllospongia foliascens*, collected from the South China Sea, was reported to produce alkaloids, scalusamide A and pyrrolidine.[37] To date, no studies have revealed the secondary metabolites of *A. clavatonanicus* and *A. protuberus* isolated from marine sponges.

Antimicrobial Test

The fungal extract of *A. tamarii* SP 3-1-4 exhibited inhibitory activity against *S. epidermidis* and *C. albicans* (**Table 4**). Extract of *A. nomiae* SP-3-2 (A) showed weak inhibition only against *S. epidermidis*, while *A. nomiae* RM NS SP-3-2 B showed strong to mild inhibition against several tested bacteria, including Gram-negative *E. coli*. However, only the extract of *A. nomiae* RM NS SP-3-2 B obtained from cultivation with the presence of salt in rice media showed pronounced antimicrobial activity. In contrast, *P. citrinum* SP-3-2-1 did not exhibit inhibitory activity against the tested microbial strains. The species *A. protuberus* SP-3-2-3 predominantly demonstrated antimicrobial activity from the fermentation in medium with salt addition, whereas *A. sydowii* SP-3-2-4 extract showed most of its inhibitory activity after cultivation in medium without salt addition. However, although belonging to the same species, *A. sydowii* RM NS SP-3 (B) tended to exhibit antimicrobial activity when the extract was obtained from salt addition fermentation. Weak inhibitory activity against *S. epidermidis* and *S. mutans* was also observed for the *A. clavatonanicus* RM NS SP-3-2 A extract. The distinct metabolite profiles observed in the presence of salt indicate a significant metabolic switching triggered by osmotic pressure.[38] In marine-derived fungi, salinity serves as a crucial chemical cue that simulates their natural ecological niche, thereby inducing the expression of cryptic or silent gene clusters (BGCs).[39] This phenomenon not only alters the chemical diversity but also enhances the production of specialized metabolites as a form of chemical defense against high-salinity stress.

Fungal species found in our studies were also reported earlier as symbiont microorganisms from terrestrial ecosystems. For example, *A. tamarii* was isolated before from *Lycoperdon umbrinum*, and exhibited antimicrobial activity against *S. aureus* ATCC 6538, *B. subtilis* ATCC 663, and *E. coli* ATCC 25922.[40] Antimicrobial activity was also reported for *P. citrinum* isolated from *Jatropha heyni*, with inhibition against *S. aureus* ATCC 6538.[41]

Table 4. Diameter of the inhibition zone in the antimicrobial assay of the methanolic fungal extracts against the tested Gram-positive and Gram-negative bacteria, as well as *C. albicans*.

Extracts of fungal isolate	Salt in media	Diameter of inhibition growth zone (mm) against microbial strains (mean ± SD)						
		S. aureus ATCC 25923	*E. coli* ATCC 25922	MRSA ATCC 3351	*B. cereus ATCC 11778*	*S. mutans ATCC 35668*	*S. epidermidis ATCC 12228*	*C. albicans ATCC 10231*
***Aspergillus tamarii* SP 3-1-4**	With	(-)	(-)	(-)	(-)	(-)	1.08±0.04	(-)
	Without	(-)	(-)	(-)	(-)	(-)	(-)	3.40±0.02
***Aspergillus nomiae* SP-3-2 (A)**	With	(-)	(-)	(-)	(-)	(-)	0.66±0.02	(-)
	Without	(-)	0.60±0.26	(-)	(-)	(-)	1.47±0.03	(-)
***Penicillium citrinum* SP-3-2-1**	With	(-)	(-)	(-)	(-)	(-)	(-)	(-)
	Without	(-)	(-)	(-)	(-)	(-)	(-)	(-)
***Aspergillus protuberus* SP-3-2-3**	With	2.27±0.04	1.13±0.03	7.99±0.20	4.16±0.12	(-)	2.48±0.06	(-)
	Without	(-)	(-)	(-)	(-)	(-)	1.35±0.02	(-)
***Aspergillus sydowii* SP-3-2-4**	With	6.51±0.02	(-)	(-)	(-)	2.21±0.02	4.72±0.39	(-)
	Without	3.74±0.018	1.21±0.01	2.91±0.13	1.84±0.03	1.73±0.00	2.98±0.11	(-)
***Aspergillus sydowii* RM NS SP-3 (B)**	With	(-)	1.31±0.04	(-)	(-)	13.57±0.07	1.41±0.02	(-)
***Aspergillus clavatonanicus* RM NS SP-3-2 A**	With	(-)	(-)	(-)	(-)	(-)	0.89±0.01	(-)
	Without	(-)	(-)	(-)	(-)	0.10±0.00	0.95±0.04	(-)
***Aspergillus nomiae* RM NS SP-3-2 B**	With	(-)	1.66±0.06	(-)	5.35±0.15	6.31±0.06	2.31±0.05	(-)
	Without	(-)	(-)	(-)	(-)	(-)	(-)	(-)
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

Remarks: The symbol (+/-) indicates the presence or absence of an inhibition zone in the extract.
*Ketoconazole is used as positive control in the antifungal test, while chloramphenicol is used as a positive control in the antibacterial tests.

Antioxidant Test

Antioxidant testing was performed using the DPPH method. In this method, ascorbic acid was used as a reference. Based on **Table 5**, four extracts did not have antioxidant activity, namely *A. sydowii* RM NS SP-3 (B), *A. clavatonanicus* RM NS SP-3-2 A, *A. nomiae* RM NS SP-3-2 B, and *A. nomiae* SP-3-2 (A) fermented without the addition of salt to the medium. This can be seen from the IC_{50} value, which is above 500 µg/mL. The *A. sydowii* SP-3-2-4 extract showed very strong antioxidant activity in both media with and without salt addition, with IC_{50} values of 37.02±1.12 and 3.48±0.81 µg/mL, respectively. The extract of *A. tamarii* SP 3-1-4 tended to show moderate and weak antioxidant activity with IC_{50} values of 125.66±6.74 and 323.43±11.75 µg/mL, respectively. Meanwhile, the extract from *P. citrinum* SP-3-2-1 showed strong activity with IC_{50} values of 69.56±1.72 and 100.72±7.57 µg/mL, respectively. Strong and moderate antioxidant activity was also demonstrated by the extract of *A. protuberus* SP-3-2-3 with IC_{50} values of 76.17±3.45 and 170.11±0.90 µg/mL, respectively. There is no difference in the antioxidant activity of fungal extracts obtained from rice fermentation, with or without the addition of salt. This may indicate that the biosynthetic pathways for antioxidant compounds, such as phenolics and flavonoids, are likely constitutively expressed in these strains, meaning they are produced consistently regardless of external salinity triggers. Furthermore, the nutrient-rich composition of the rice medium may have already provided optimal conditions for secondary metabolite production, whereby the addition of salt did not impose further osmotic stress necessary to significantly alter the antioxidant profile.[42]

Previous studies reported that the species *A. sydowii* isolated from marine algae exhibited very strong antioxidant activity with an IC_{50} value of 30.8 µg/mL. [43] Another study revealed that the species *P. citrinum*, isolated from Parmotrema in Sri Lanka, showed strong antioxidant activity with an IC_{50} value of 68.6±4.03 µg/mL.[44]

Table 5. Antioxidant test result of the methanolic fungal extracts from marine sponge *Pseudoceratina* sp. using the DPPH method.

Extract of fungal isolate	Salt in media	IC_{50}±SD(µg/mL)	Category*
Aspergillus tamarii	With	125.66±6.74	Moderate
SP 3-1-4	Without	323.43±11.75	Weak
Aspergillus nomiae	With	305.52±9.69	Weak
SP-3-2 (A)	Without	>500	In active
Penicillium citrinum	With	69.56±1.72	Strong
SP-3-2-1	Without	100.72±7.57	Strong
Aspergillus protuberus	With	76.17±3.45	Strong
SP-3-2-3	Without	170.11±0.90	Moderate
Aspergillus sydowii	With	37.02±1.12	Very Strong
SP-3-2-4	Without	32.48±0.81	Very Strong
Aspergillus sydowii **RM NS SP-3 (B)**	With	>500	In active
Aspergillus clavatonanicus	With	>500	In active
RM NS SP-3-2 A	Without	>500	In active
Aspergillus nomiae	With	>500	In active
RM NS SP-3-2 B	Without	>500	In active
Ascorbic Acid**		6.79±0.10	Very Strong

*Category refers to the study by Endra *et al*. (2021)[45]

**In the antioxidant assay, we used ascorbic acid as the reference standard.

Brine Shrimp Lethality Test Method (BSLT)

Table 6 presents toxicity test results for extracts using the BSLT method, expressed as LC_{50} ± standard deviation values. This assay is commonly used as an initial screening to identify extracts that potentially possess cytotoxic properties.[46] The BSLT serves as a preliminary indicator of bioactivity, as toxicity levels toward brine shrimp larvae have been shown to correlate positively with potential cytotoxicity against cancer cells.[47] Most of the extracts exhibited high toxicity, except for the *A. sydowii* SP-3-2-4 extract fermented on rice medium with added salt, which demonstrated medium toxicity. A previous study reported that marine sponge-derived *P. citrinum* isolates exhibited high toxicity, with an LC_{50} value of less than 30 µg/mL. [48] High toxicity is often due to bioactive secondary metabolites such as alkaloids, terpenoids, steroids, and flavonoids. These metabolites play an important role in the defense mechanism against harsh environments, and their presence contributes to high toxicity values. [49]

Table 6. Toxicity test result of the methanolic fungal extracts isolated from the marine sponge *Pseudoceratina* sp. using the BSLT method.

Extract of fungal isolate	Salt in media	LC_{50}±SD (µg/mL)	Category*
Aspergillus tamarii	With	2.73±1.06	High Toxic
SP 3-1-4	Without	1.08±0.44	High Toxic
Aspergillus nomiae	With	2.01±1.97	High Toxic
SP-3-2 (A)	Without	0.88±0.69	High Toxic
Aspergillus nomiae	With	0.94±0.90	High Toxic
SP-3-2-1	Without	37.39±9.33	High Toxic
Aspergillus protuberus	With	13.46±8.65	High Toxic
SP-3-2-3	Without	37.51±3.99	High Toxic
Aspergillus sydowii	With	101.1±8.88	Medium Toxic
SP-3-2-4	Without	13.0±1.84	High Toxic
Aspergillus sydowii **RM NS SP-3 (B)**	With	45.94±5.05	High Toxic
Aspergillus clavatonanicus	With	34.88±5.78	High Toxic
RM-NS SP-3-2 A	Without	36.39±7.18	High Toxic
Aspergillus nomiae	With	64.14±4.90	High Toxic
RM-NS-SP-3-2 B	Without	39.61±11.11	High Toxic

*: LC_{50} >1000 µg/mL: Non-toxic, LC_{50} 500-1000 µg/mL: Low toxic, LC_{50} 100-500 µg/mL: Medium toxic, LC_{50} 0-100 µg/mL: High Toxic[50]

Overall, this study demonstrated that fungi isolated from the sponges *Pseudoceratina* sp. were fermented on rice medium, both with and without salt supplementation. The fungi isolated from *Pseudoceratina* sp. were predominantly species of the genus *Aspergillus*. The results of secondary metabolite analysis via phytochemical screening revealed a significant difference between extracts afforded from fermentation with and without salt supplementation. Most extracts with added salt tended to yield more varied secondary metabolite groups compared to those without salt. Similar results were obtained in the antimicrobial and antioxidant assays, in which most of the extracts with salt supplementation demonstrated better activity. These findings are consistent with previous studies. For example, the fungus *Spicaria elegans* was isolated from marine sponges collected in Jiaozhou Bay, China. This fungus was cultivated on salt media at concentrations of 3% and 10%. The extract from the fungal fermentation in 10% salt medium showed antimicrobial activity against *S. aureus* with a zone of inhibition value of 1.53±3.00 mm.[51] In the toxicity test, no significant difference in activity was observed between the extracts from fungal cultivation with and without salt addition. Future studies should focus on the bioassay-guided isolation of specific bioactive fractions, involving several chromatographic techniques to further characterize the secondary metabolites detected in this secondary metabolite screening, specifically targeting *A. sydowii* SP-3-2-4 for its antimicrobial and antioxidant activity, as well as *A. nomiae* SP-3-2 (A), and *A. tamarii* SP 3-1-4 for its toxicity potential in the BSLT observed in the present study.

Limitation of the study

While providing valuable insights and a foundation for further research, this study is subject to certain limitations. As the sponge-associated fungi in the present study was isolated only from *Pseudoceratina* sp. collected from Amed Coastal Waters, Bali, the fungal diversity found may not fully represent the fungal symbiotic population in different locations. Furthermore, the phytochemical analysis remained at the preliminary screening, where the specific active compounds responsible for the observed bioactivities were not yet isolated, nor identified. Moreover, the cytotoxicity potency of the tested extracts was restricted to the BSLT data as an initial screening. Based on these few shortcomings, future research should focus on the isolation of bioactive fungal secondary metabolites employing a bioassay-guided isolation procedure, followed by metabolite identification through LC-HRMS/MS and extensive NMR analysis. Moreover, advanced cytotoxicity assay of the fungal extracts obtained from this study towards various human cancer cell lines and non-cancerous cells is strongly warranted to validate their selective anticancer potential against cancer cells and to rule out general toxicity. Additionally, as we observed different secondary metabolite profile between extracts afforded from the fermentation with the addition of salt and salt-free conditions on phytochemical screening, application of the "One Strain MAny Compounds" (OSMAC) approach, by varying a wider range of fermentation parameters, such as nutrient sources, type of media (solid and broth), addition of different kind of salts (NaBr, $NaNO_3$, NaCl), pH and temperature, could further explore the fungi's capability in producing novel bioactive secondary metabolites. Eventually, expanding the sampling site would provide a more comprehensive understanding of the biological diversity of *Pseudoceratina* sp.-associated fungi.

CONCLUSION

In this study, we isolated and identified eight fungal strains associated with the marine sponge, *Pseudoceratina* sp., collected from the Amed Coastal Waters, Bali, Indonesia. The isolated fungal species predominantly belong to the genus *Aspergillus*. Phytochemical analysis revealed the presence of secondary metabolites in the fungal extracts, and more varied secondary metabolite classes were detected in the extracts obtained from the fungal fermentation with the addition of salt in rice media. In the antimicrobial assay, some fungal extracts, such as extracts of *A. protuberus* SP-3-2-3 and *A. nomiae* RM NS SP-3-2 B from salt-added fermentation, showed better inhibition against the tested microbes than those from the salt-free fermentation. Moreover, the extract of *A. sydowii* SP-3-2-4 demonstrated potent activity in the antioxidant assay, with an IC_{50} value of 32.48±0.81 µg/mL, positioning it as a promising source of anti-radical agents. Most of the fungal extracts also showed medium to high toxicity in BSLT, which indicates their potential for further investigation of cytotoxic compounds. Owing to the bioactivities observed in this study, phytochemical characterization of secondary metabolites and their mechanism of action, particularly as antimicrobial, antioxidant and cytotoxicity, are promising to be investigated further.

FUNDING

Research funding from the Institute for Research and Community Service (LPPM) of Universitas Udayana, through "Kampus Merdeka" research grant (no.: B/266.46/UN14.4.A/PT.01.03/2024 and B/230.58/UN14.4.A/PT.01.03/2025) is gratefully acknowledged.

ACKNOWLEDGMENTS

The authors would like to thank the Institute for Research and Community Service (LPPM) of Udayana University for the research funding and the Karangasem Regency Government for facilitating sample collections. We also thank the National Research and Innovation Agency of Indonesia (BRIN) for providing the research facilities.

GENERATIVE AI DISCLOSURE STATEMENT

Portions of this manuscript were assisted by Grammarly for correcting grammar and language improvement. The authors have verified the accuracy and originality of all content. No images were manipulated using AI.

AUTHOR CONTRIBUTION STATEMENT

I Made Agus Kusuma Adi: Investigation, Data curation, Data analysis, Writing original draft. **Ni Komang Diah Eka Witantri**: Investigation, Data curation. **Ni Made Widya Wulandari**: Investigation, Data curation. **Ni Putu Eka Leliqia**: Methodology, Supervision, Revising the manuscript. **I Nengah Wirajana**: Methodology, Resources, Revising the manuscript. **Joko Tri Wibowo**: Methodology, Resources, Supervision, Review and revising the manuscript. **Ismail Ware**: Methodology, Review and revising the manuscript. **Ni Putu Ariantari**: Conceptualization, Methodology, Resources, Supervision, Review and editing of the manuscript.

CONFLICT OF INTEREST DECLARATION

The authors declare no conflict of interest.

REFERENCES

1. Lopez-Moya F, Barretta R, Gonçalves MFM, Esteves AC, Alves A. Marine Fungi: Opportunities and challenges. *Encyclopedia*. 2022;2(1):559-577. doi:10.3390/encyclopedia2010037
2. Wang Z, Qader M, Wang Y, Kong F, Wang Q, Wang C. Progress in the discovery of new bioactive substances from deep-sea associated fungi during 2020-2022. *Front Mar Sci*. 2023;10:1232891. doi:10.3389/fmars.2023.1232891
3. Barzkar N, Sukhikh S, Babich O. A comprehensive review of marine sponge metabolites, with emphasis on *Neopetrosia* sp. *Int J Biol Macromol*. 2024;280(Pt 2). doi:10.1016/j.ijbiomac.2024.135823
4. Yuan Y, Lei Y, Xu M, Zhao B, Xu S. Bioactive terpenes from marine sponges and their associated organisms. *Mar Drugs*. 2025;23(3):96. doi:10.3390/md23030096
5. Kurimoto SI, Inoue K, Ohno K, Kubota T. Ceratinadin G, A new psammaplysin derivative possessing a cyano group from a sponge of the genus *Pseudoceratina*. *J Org Chem*. 2024; 20(1): 3215-3220.doi:10.3762/bjoc.20.267

6. Mohamed HM, Murshid SSA, Alreemi RM, Alyahyawy OY, Mohamed GA, Ibrahim SRM. The genus *Pseudoceratina*: phytochemistry, biosynthesis, and pharmacology. *Results Chem*. 2025;18(7):102714. doi:10.1016/j.rechem.2025.102714
7. Ueda JY, Takagi M, Shin-Ya K. New xanthoquinodin-like compounds, JBIR-97, -98 and-99, obtained from marine sponge-derived fungus *Tritirachium* sp. SpB081112MEf2. *J Antibiot*. 2010;63(10):615-618. doi:10.1038/ja.2010.92
8. Gonçalves MFM, Paço A, Escada LF, Albuquerque MSF, Pinto CA, Saraiva J, Duarte AS, Rocha-Santos TAP, Esteves AC, Alves A. Unveiling biological activities of marine fungi: The effect of sea salt. *Appl Sci*. 2021;11(13). doi:10.3390/app11136008
9. Esmeralda M, Ngedo E, Tria K, Dewi N, Putu N, Leliqia E, Ariantari P. Uji toksisitas ekstrak metanol jamur yang diisolasi dari spons *Phyllospongia foliascens* menggunakan metode Brine Shrimp Lethality Test (BSLT). *J PharmUdayana*. 2025;14(1):41-50. doi:10.24843/JFU.2025.v14.i01.p08
10. Wulandari NMW, Dwijayanti NK, Putri NPAE, Putra IPYA, Leliqia NPE, Wibowo JT, Dwija IBNP, Ariantari NP. Bioactivities and molecular identification from marine sediment-derived fungi isolated from Amed and Tulamben Beaches. *J Ilm Medicam*. 2025;11(1):74-85. doi:10.36733/medicamento.v11i1.10745
11. Ariantari NP, Putra IPYA, Leliqia NPE, Yustiantara PS, Proborini MW, Nugraheni N, Zulfin UM, Jenie RI, Meiyanto E. Antibacterial and cytotoxic secondary metabolites from endophytic fungi associated with *Antidesma bunius* leaves. *J Appl Pharm Sci*. 2023;13(7):132-143. doi:10.7324/JAPS.2023.101347
12. Putra IPYA, Ariantari NP, Wibowo JT, Leliqia NPE, Dwija IBNP, Wulandari NMW, Dwijayanti NK, Putri NPAE, Dewi KTN. *Phyllospongia foliascens*-derived fungi: molecular identification, antimicrobial activity and secondary metabolite profile of their methanolic extracts. In: *BIO Web of Conferences*. Vol 176. EDP Sciences; 2025. doi:10.1051/bioconf/202517602003
13. Putra IPYA, Utami KS, Hardini J, Wirasuta IMAG, Ujam NT, Ariantari NP. Fermentation, bioactivity and molecular identification of endophytic fungi isolated from mangrove *Ceriops tagal*. *Biodiversitas*. 2023;24(5):3091-3098. doi:10.13057/biodiv/d240565
14. Ariantari NP, Leliqia NPE, Putra IPYA, Nugraheni N, Jenie RI, Meiyanto E. Endophytic fungi from red ginger (*Zingiber officinale* var. rubrum) as promising source of antimicrobial and cytotoxic secondary metabolites. *J Appl Pharm Sci*. 2024;14(11):100-110. doi:10.7324/JAPS.2024.178823
15. Dubale S, Kebebe D, Zeynudin A, Abdissa N, Suleman S. Phytochemical screening and antimicrobial activity evaluation of selected medicinal plants in Ethiopia. *J Exp Pharmacol*. 2023;15(1):51-62. doi:10.2147/JEP.S379805
16. Maheshwaran L, Nadarajah L, Senadeera SPNN, Ranaweera CB, Chandana AK, Pathirana RN. Phytochemical testing methodologies and principles for preliminary screening/qualitative testing. *J Asian Plant*. 2024;12(5):11-38. doi:10.9734/aprj/2024/v12i5267
17. Iqbal M, Jariah AA. Phytochemical screening of secondary metabolite compounds in tammate leaf extract (*Lannea coromandelica* (HOUTT.) MERR.) from Pangkep Regency using various extraction methods. *J Health Science*. 2025;5(1):59-66. doi:10.47679/jchs.2025107
18. Godlewska K, Pacyga P, Najda A, Michalak I. Investigation of chemical constituents and antioxidant activity of biologically active plant-derived natural products. *Molecules*. 2023;28(14):1-77. doi:10.3390/molecules28145572
19. Hoang TNN, Phan TT, Phan TKL, Nguyen NHV, Dong TAD, Le THA. Phytochemical screening, extraction, and determination of the bioactivities of the extract-enriched polyphenols and saponins from *Musa balbisiana* Fruit. *J Food Process Preserv*. 2023;2023. doi:10.1155/2023/2581641
20. Walker RD. *Standards for Antimicrobial Susceptibility Testing.* Vol 60. 1999.
21. Ariantari NP, Putra IPYA, Dwiyani NMW, Leliqia NPE, Wirajana IN, Yustiantara PS, Febriansah R. Antimicrobial, cytotoxicity, and LC-MS/MS analysis of methanolic extract of *Fusarium proliferatum* ZO-L2-4, an endophytic fungus isolated from *Zingiber officinale* Roscoe. *J Pharm Pharmacogn Res*. 2025;13(1):152-162. doi:10.56499/jppres23.1899_13.1.152
22. Gajic I, Kabic J, Kekic D, Jovicevic M, Milenkovic M, Mitic Culafic D, Trudic A, Ranin L, Opavski N. Antimicrobial susceptibility testing: a comprehensive review of currently used methods. *Antibiotics*. 2022;11(4):1-26. doi:10.3390/antibiotics11040427
23. Baliyan S, Mukherjee R, Priyadarshini A, Vibhuti A, Gupta A, Pandey RP, Chang CM. Determination of antioxidants by DPPH radical scavenging activity and quantitative phytochemical analysis of *Ficus religiosa*. *Molecules*. 2022;27(4). doi:10.3390/molecules27041326

24. Nugraheni TS, Setiawan I, Putri AA, Sukmawati AW, Khasanah LN, Nisa LK, Putri LNH, Wulandari SK, Riswana SA. Tinjauan artikel: macam-macam metode pengujian aktivitas antioksidan. *J Pharm*. 2023;13(1):2656-8950. doi:10.51601/ijhp.v5i1.407
25. Ariantari NP, Putra IPYA, Wibowo JT, Putri SAMDA, Nabila CIB, Kristiani NKA, Wirasuta IMAG. Evaluation of antibacterial and toxicity properties of marine endophytic fungi from red algae *Gracilaria salicornia* and *Chondrus* sp. *BIO Web Conf*. 2024;92:1-12. doi:10.1051/bioconf/20249202019
26. Nerdy N, Lestari P, Sinaga JP, Ginting S, Zebua NF, Mierza V, Bakri TK. Brine shrimp (*Artemia salina* leach.) lethality test of ethanolic extract from green betel (*Piper betle* linn.) and red betel (*Piper crocatum* ruiz and pav.) through the soxhletation method for cytotoxicity test. *Open Access Maced J Med Sci*. 2021;9:407-412. doi:10.3889/oamjms.2021.6171
27. Fithrotunnisa Q, Arsianti A, Kurniawan G, Qorina F, Tejaputri NA, Azizah NN. In vitro cytotoxicity of *Hibiscus sabdariffa* Linn extracts on A549 lung cancer cell line. *Phcog J*. 2020;12(1):14-19. doi:10.5530/pj.2020.12.3
28. Hasbi N, Rosyunita R, Rahim AR, Wardoyo EH, Arnawati IA, Haza SS, Hulfifa LN, Alamsyah ADCF, Validika IKS. Phytochemical screening and antibacterial activity test of sumbawa white honey against *Bacillus megaterium*. *J Scientis*. 2023;9(10):8707-8712. doi:10.29303/jppipa.v9i10.4913
29. Viotti C, Chalot M, Kennedy PG, Maillard F, Santoni S, Blaudez D, Bertheau C. Primer pairs, PCR conditions, and peptide nucleic acid clamps affect fungal diversity assessment from plant root tissues. *Mycology*. 2024;15(2):255-271. doi:10.1080/21501203.2023.2301003
30. Handayani D, Dwinatrana K, Rustini R. Antibacterial compound from marine spone derived fungus *Aspergillus sydowii* DC08. *Rasayan J Chem*. 2022;15(4):2485-2492. doi:10.31788/RJC.2022.1546971
31. Artasasta MA, Yanwirasti Y, Taher M, Djamaan A, Ariantari NP, Edrada-Ebel RA, Handayani D. Apoptotic activity of new oxisterigmatocystin derivatives from the marine-derived fungus *Aspergillus nomius* NC06. *Mar Drugs*. 2021;19(11):1-10. doi:10.3390/md19110631
32. Sabdaningsih A, Liu Y, Mettal U, Heep J, Riyanti, Wang L, Cristianawati O, Nuryadi H, Sibero MT, Marner M, Radjasa OK, Sabdono A, Trianto A. A new citrinin derivative from the Indonesian marine sponge-associated fungus *Penicillium citrinum*. *Mar Drugs*. 2020;18(227):1-12.
33. Grillo G, Capaldi G, Radošević K, Jakopović Ž, Markov K, Brncic M, Gallina L, Calcio Gaudino E, Cravotto G. Unlocking the bioactive potential of pomegranate peels: A green extraction approach. *Antioxidants*. 2023;12(10). doi:10.3390/antiox12101796
34. Sander A, Petračić A, Rogošić M, Župan M, Frljak L, Cvetnić M. Feasibility of different methods for separating n-hexane and ethanol. *Separations*. 2024;11(5). doi:10.3390/separations11050151
35. Said G, Ahmad F. Effects of salt concentration on the production of cytotoxic geodin from marine-derived fungus *Aspergillus* sp. *Turk J Biochem*. 2022;47(4):399-402. doi:10.1515/tjb-2022-0058
36. Ibrahim SRM, Mohamed SGA, Alsaadi BH, Althubyani MM, Awari ZI, Hussein HGA, Aljohani AA, Albasri JF, Faraj SA, Mohamed GA. Secondary metabolites, biological activities, and industrial and biotechnological importance of *Aspergillus sydowii*. *Mar Drugs*. 2023;21(8). doi:10.3390/md21080441
37. Gu Y, Ding P, Liang Z, Song Y, Liu Y, Chen G, Li JL. Activated production of silent metabolites from marine-derived fungus *Penicillium citrinum*. *Fitoterapia*. 2018;127(2):207-211. doi:10.1016/j.fitote.2018.02.012
38. Salazar-Alekseyeva K, Herndl GJ, Baltar F. Influence of salinity on the extracellular enzymatic activities of marine pelagic fungi. *J Fungi*. 2024;10(2). doi:10.3390/jof10020152
39. Behera AD, Das S. *Ecological Insights and Potential Application of Marine Filamentous Fungi in Environmental Restoration*. Vol 22. Springer Netherlands; 2023. doi:10.1007/s11157-023-09655-2
40. Ogbole OO, Adebayo-Tayo BC, Salawu KM, Okoli VC. Molecular Identification and antimicrobial activity of endophytic fungi *Aspergillus tamarii* (Trichomaceae). *Nig Journ Pharm Sci*. 2017;16(1):41-48. doi:10.24843/JNP.2017.v16.i01.p41
41. Ashoka GB, Shivanna MB. Antibacterial, antioxidant, and anticancer activities of *Penicillium citrinum* Thom. endophytic in *Jatropha heynei*. *J Appl Pharm Sci*. 2023;13(3):196-207. doi:10.7324/JAPS.2023.95958
42. Isah T, Umar S, Mujib A, Sharma MP, Rajasekharan PE, Zafar N, Frukh A. Secondary metabolism of pharmaceuticals in the plant in vitro cultures: strategies, approaches, and limitations to achieving higher yield. *Plant Cell Tissue Organ Cult*. 2018;132(2):239-265. doi:10.1007/s11240-017-1332-2
43. Hu X, Li X, Meng L, Wang B. Antioxidant bisabolane-type sesquiterpenoids from algal-derived fungus *Aspergillus sydowii* EN-434. *J Oceanol Limnol*. 2020;38(5):1532-1536. doi:10.1007/s00343-020-0049-y

44. Samanthi KAU, Wickramarachchi S, Wijeratne EMK, Paranagama PA. Two new bioactive polyketides from *Curvularia trifolii*, an endolichenic fungus isolated from *Usnea* sp., in Sri Lanka. *J Natl Sci Found*. 2015;43(3):217-224. doi:10.4038/jnsfsr.v43i3.7950

45. Endra P, Ricka I. Aktivitas antioksidan fraksi etil asetat dan air ranting buah parijoto (*Medinilla speciosa* Blume) dengan perendaman radikal bebas DPPH. *J Pharm Cend*. 2021;5(2):135-144. doi:doi.org/10.31596/cjp.v5i2.143

46. Wulandari S, Okta FN, Utami SP, Giovani SDC. Uji toksisitas ekstrak etil asetat biji kebiul (*Caesalpinia bonduc* L.) pada ekstraksi maserasi. *J I Phar*. 2024;11(2):32-46. doi:10.52161/jiphar.v11i2.590

47. Ismail MA, Vitayani S, Aulia N, Mangarengi Y, Karim M. Cytotoxicity test of Green betel leaf ethanol extract (*Piper betle* L .) using shrimp larvae (*Artemia salina* L .) using the Brine Shrimp Lethality Test (BSLT) method. *J Health Pharm*. 2025;5(1):138-149. doi:doi.org/10.51601/ijhp.v5i1.407

48. Handayani D, Artasasta MA, Mutia D, Atikah N, Rustini, Tallei TE. Antimicrobial and cytotoxic activities screening of fungal secondary metabolites isolated from marine sponge *Callyspongia* sp. *AACL Bioflux*. 2021;14(1):249-258. doi:doi.org/10.31596/cjp.v5i2.143

49. Khasanah NW, Karyadi B, Sundaryono A. Phytochemical test and toxicity of *Hydnophytum* sp. against *Artemia salina* Leach. *J Science Edu*. 2020;4(1):47-53. doi:10.33369/pendipa.4.1.47-53

50. Satya NA, Pradana DLC, Kolib A, Aprilia CA. Brine shrimp lethality test on aqueous extract of *Caesalpinia Sappan* L. *JFIOnline*. 2021;13(1):62-67. doi:10.35617/jfionline.v13i1.54

51. Wang Y, Lu Z, Sun K, Zhu W. Effects of high salt stress on secondary metabolite production in the marine-derived fungus *Spicaria elegans*. *Mar Drugs*. 2011;9(4):535-542. doi:10.3390/md9040535