Screening Activities of Crude Extracts Produced *Halodule* sp. Seagrass-Associated Fungus

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Abstract

Secondary metabolites from marine microorganisms, including marine-derived fungi, have consented to developing guide bioactive compounds. Marine-derived fungi were reported to be associated with various habitats, including seagrasses. The seagrass-associated fungus from the Indonesian marine area is still poorly unexplored. This study was presented to screen for the antimicrobial and antioxidant activity of crude extracts produced by fungi associated with Seagrass (Halodule sp.*) collected from Indonesia. Fresh samples were collected and kept fresh until they arrived in the Laboratory and immediately planted on Potato Dextrose Agar (PDA) Sea Salt media. Ten fungi were isolated and purified using a Malt Extract Agar (MEA) medium and subjected to fermentation treatment for 30 days using solid rice media. The compounds produced were collected by soaking directly using ethyl acetate (EA) for one hour while stirring mechanically. Evaporation of EA was carried out to obtain crude extracts. Each crude extract was subjected to antibacterial and antioxidant activities using the agar diffusion and DPPH methods, respectively. Reverse-phase Thin-Layer Chromatography (TLC) was used to observe the compound profile. The results of the activity test showed that two crude extracts from fungi with the code A.10.4 and B.5.1 had both activities, antibacterial (potent inhibition at 50% concentration and antioxidant (IC_{50} of 90.23 and 88.29 ppm, respectively). Another crude extract with the fungi code B.1.1 showed strong antioxidant activity (IC_{50} of 81.31 ppm) without antimicrobial activity. TLC results show different compound profiles from each crude extract and quite a good separation.*

Keywords: *fungi, antimicrobial, antioxidant, marine, seagrass, Halodule sp.*

Introduction

In the last few decades, marine micro-organisms, especially fungi, have been approved as a prominent resource for developing guide bioactive or new bioactive compounds (Ameen *et al.*, 2021). Given the fact that marine representation is more than 70% of the earth's surface and has an unexplored area for the discovery of bioactive compounds (Penesyan *et al.*, 2010). Indonesian marine is one of the areas still largely unexplored for the discovery of bioactive compounds from marine microorganisms, especially fungi (Thomas *et al.*, 2010). In the last five years, several studies have reported that crude extracts from the fermented product of fungi isolated from Indonesian marine areas have antimicrobial, cytotoxic, tyrosinase inhibitory, antiglycation, and antioxidant activities (Nursid *et al.*, 2019; Bakhtra *et al.*, 2022). Furthermore, several new compounds were reported to have been isolated from fungi isolated from Indonesian marine, including helicascolide C isolated from *Daldinia eschscholzii* (Tarman *et al.*, 2012), tetrahydrobostrycin, and 1-deoxytetrahydrobostrycin isolated from *Aspergillus* sp. 05F16 (Xu *et al.*, 2008), aspergione A–F and aspergillitine isolated from *Aspergillus versicolor* (Lin *et al.*, 2003).

The seagrasses are found throughout Indonesia, and they play a profound role in the nutrient cycling of the marine ecosystem (Abrams *et al.*, 2016). Seagrasses are groups of marine flowering plants that have adapted to the underwater lifestyle of coastal ecosystems (Wissler *et al.*, 2011). They reflect key habitat-forming species and constructors of coastal ecosystems (Wissler *et al.*, 2011). The seagrass-associated microbes, including fungi, have discrete environments within (Hurtado-McCormick *et*

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al., 2019). The content of photosynthesis, O_2, sanitation, and organic substrates in seagrass areas differs significantly from terrestrial regions (Hurtado-McCormick *et al.*, 2019). Hence, seagrass-associated fungi promote heterogeneity, activity, and community composition (Liu *et al.*, 2022). The heterogeneity of seagrass-associated fungi was reported, as 13 fungal strains were isolated and identified, including strains of *Cladosporium*, *Trichoderma*, and *Penicillium*, which were assigned to three different species: *Penicillium antarcticum*, *Penicillium atramentosum*, and *Penicillium atrovenetum* (Petersen *et al.*, 2019). Furthermore, phthalide derivatives including 3-[2′(R)-hydroxybutyl]-7-hydroxyphthalide, cyclopean, and cyclopeptine were isolated from fungi *Penicillium claviforme* seagrass-associated (Afiyatullov *et al.*, 2015). This study aimed to identify the antimicrobial and antioxidant activity of metabolic seconder produced by seagrass-associated fungi. The seagrass (*Halodule* sp.) was collected from the seagrass of Nipah Beach, Lombok, Indonesia (8°26'05.6"S 116°02'35.6"E). Solid-state fermentation was used to produce a crude extract from the fungus, a strain of fungus with antimicrobial and antioxidant activity.

Materials and Methods

Seagrass were collected in the morning when they were uninundated at Nipah Beach, Lombok, Indonesia (8°26'05.6"S 116°02'35.6"E). The samples were stored in sample plastic with seawater to maintain freshness and immediately transported to the laboratory for treatment. Furthermore, fungal growth induction was carried out using media containing Malt Extract (Sigma-Aldrich Inc., St. Louis, MO, United States) (15 g), Agar (Sigma-Aldrich Inc., St. Louis, MO, United States) (15 g) (MEA), sea salt (25 g), and chloramphenicol (0.2 g) in diH_2O (1 L), carried out after the samples were cut into small pieces and washed for 30 sec with 75% ethanol, 1 min with sterile NaCl, and 1 min with sterile water and incubated for 4–7 d at room temperature.

Pure fungus materials

Purifying fungi from the *Halodule* sp. was performed after incubation for 4-7 or faster when the fungus was observed growing on a PDA medium. The fungus was transferred to a petri dish containing MAE medium and sea salt (25 g) in diH_2O (1 L) and then incubated for 4–7 d at room temperature. The purification was repeated when it was observed that more than one fungus grew on a petri dish (Safwan *et al.*, 2022).

Solid-state fermentation

After the pure fungi had grown to fill the media in the petri dish, the fungi were fermented with sterile solid media. Each agar medium containing fungi was cut into small pieces and transferred to a 300 ml Erlenmeyer flask containing 25 g of rice, 3.5 g of malt extract, 3.5 g of sea salt, and 50 ml of water, then closed and kept to prevent contamination. Fermentation is carried out at room temperature with natural lighting and is not induced by light for 30 days. Control is carried out during fermentation by observing the presence of contaminants. Bacterial contamination is indicated by changes in the density of the media, which becomes porridge and is easy to pour. If contaminants occur during fermentation, re-fermentation will occur (Safwan *et al.*, 2022).

Crude extract materials and thin-layer chromatography

After fermentation process for 30 d, the secondary metabolites produced by each fungus were extracted using ethyl acetate solvent. A total of 50 ml of dH_2O was poured into an Erlenmeyer flask containing fermentation broth and stirred until it formed a slurry, then was added 100 ml of EA stirred, then sonicated for 30 min, and the EA layer was separated. The addition of EA to the slurry period was repeated 3 times, and the EA layer was collected. The filtrate layer of ethyl acetate was collected and then evaporated to obtain the crude extract. The crude extract obtained was collected and stored at cool temperatures before being used for the next stage. A gram of extract was dissolved in 1 ml methanol, and then chromatography was performed by C18 reversed-phase thin-layer plates with dichloromethane: methanol (10:1 v/v). The profile of TLC results was visualized on ultraviolet light at 254 nm and 366 nm, sprayed using 10% H_2SO_4, and then observed on heated (Safwan *et al.*, 2022).

Antimicrobial

Antibacterial activity was performed using the agar well diffusion method. Amoxicillin was used as a positive control. The crude extracts were dissolved in DMSO at a concentration of 1,000 ppm. The agar plate containing the Mueller Hinton Agar (MHA) medium and target pathogens bacteria (*Staphylococcus aureus* ATCC 25923) were prepared, and the paper discs were placed for the carrier. 50 ul of sample solution or positive control were added and incubated overnight at 37°C, and then the clear zone around the paper discs was observed to inhibit bacteria.

Antioxidant

The antioxidant performed used a free radical scavenging assay of the 2,2-diphenyl-1-picrylhydrazyl (DPPH). Vitamin C was used as a positive control. The crude extracts were dissolved in methanol at various concentrations, and a positive control was prepared. Then, add the DPPH solution (0,1 mM, 1 ml), mix by

vortex, and incubate for 30 mins at 27ºC. The absorbance was observed at λ 515 nm UV-vis spectrophotometer.

Results and Discussion

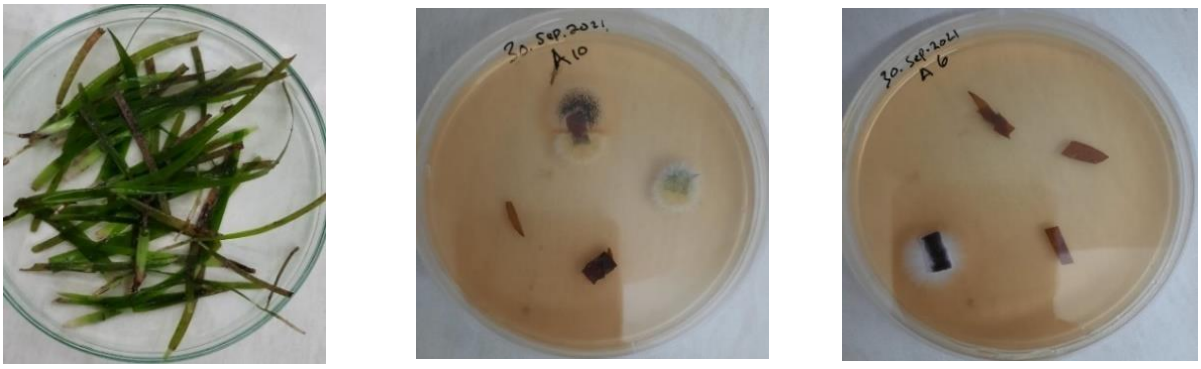
Isolation of fungus using PDA medium from *Halodule* sp. (Figure 1A) collected from the seagrass of Nipah Beach, Lombok, Indonesia (8°26'05.6"S 116°02'35.6"E), managed to grow some fungi from these plants. Figure 1B observed (morphological) several fungi growing from the same part of the plant, and in Figure 1C, it was observed that only one fungus grew from the sample. Figures 1 B and C observed some parts of the *Halodule* sp. There was unobserved fungus growth, indicating the controlling contamination of other microorganisms was carried out well in the isolation process.

Pure cultured fungus

A total of 10 fungi were isolated and purified (Figure 2) from Seagrass. All fungi were successfully isolated in a single strain and grown on an MEA medium. Some fungi were shown to grow fast after 4 days incubated, and some fungi grew slowly. Fungi A.10.4, A.10.1, and A.6.1 showed the fastest mycelium growth compared to fungi A.10.2, A.7.1, A.4.1, and A.10.3, incubated for 3 days on MEA medium. Fungi B.5.1, B.10.1, and A.B.1.1 showed the slowest mycelium growth incubated for 7 days on medium. The different mediums, including MEA, PDA, and Sabouraud Dextrose Agar (SDA), showed variations in mycelium and colony growth in fungi (Black, 2020). MEA is a shared medium used for over 100 years, probably due to its availability, low cost, and sufficient maltose content for fungal growth and fermentation (Jansen *et al.*, 2004). Based on previous research (Venkatachalam *et al.*, 2015), the results of observations of the diversity of seagrasses-endophytes collected from along the coast of Tamilnadu state, India, showed that *Aspergillus* species were the dominant fungi from *Halodule* sp. samples. In another study, the diversity of endophytic fungi isolated from *Halodule wrightii* leaves collected from the north-central Gulf of Mexico showed that the fungus *Trichocladium allopallonellum* dominated (Mata and Cebrián, 2013). Based on the growth pattern on agar media, the fungus isolated is probably *Colletotrichum salsolae* (A.10.4), *Aspergillus* sp., (A.10.1, B.5.1, B.10.1, dan B.1.1), *Arthroderma benhamiae* (A.10.2) (Hiruma *et al.*, 2015), *Pythium aphanidermatum* (A.7.1) (Ao *et al.*, 2024), *Aspergillus flavus* 5 (A.4.1), *Aspergillus fumigatus* (A.10.3), and *Sporangia Zygomycota* (A.6.1). Fungi *Aspergillus* is one of the fungi that has been found abundantly isolated from marine habitats (Safwan *et al.*, 2023). In our review, *Aspergillus* isolated from coral and algae was identified to produce secondary metabolite compounds with various activities such as antibacterial, anticancer, antioxidant, antifungal, and anti-inflammatory. In addition to being found in abundance from corals and algae, *Aspergillus* sp. is also abundant from deep-sea sediments and produces compounds such as terpenes, lactones, and polyketides (Safwan *et al.*, 2024).

Fermentation produced by the fungus

Fermentation of fungi isolated from *Halodule* sp. was carried out using a solid medium for 30 days in the flask (Figure 3A). Fermentation using the solid medium is a common and inexpensive method for fungi culture to produce the secondary metabolism (Carboué *et al.*, 2018). The composition of the medium has been described in the One Strain Many Compounds (OSMAC) method, and they produce different secondary compounds, increase the target product, or reduce the desired secondary metabolites (Bader *et al.*, 2020).



a b c

Figure 1. Leaves of the Seagrass (a), smaller segments of Seagrass, and fungus growing on MEA medium in 90 mm Petri dishes for 3 days incubated b and c.

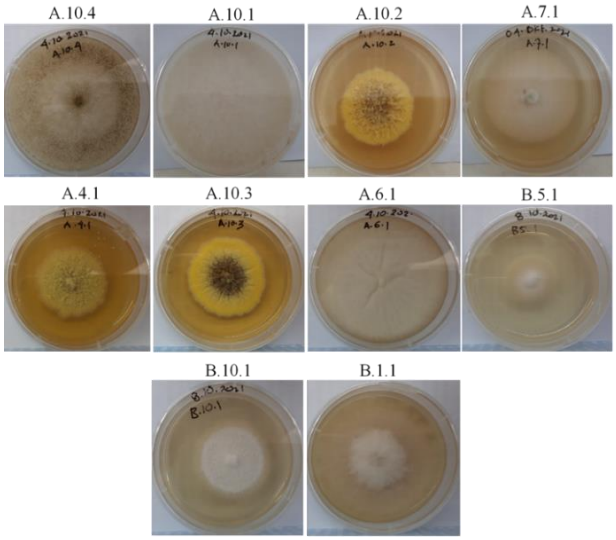


Figure 2. Colonies of pure culture fungi isolated from *Halodule* sp. on MEA medium 60 mm Petri dishes. A.10.4, A.10.1, A.10.2, A.7.1, A.4.1, A.10.3, and A.6.1 incubated for 3 days. B.5.1, B.10.1, and B.1.1 were incubated for 7 days.

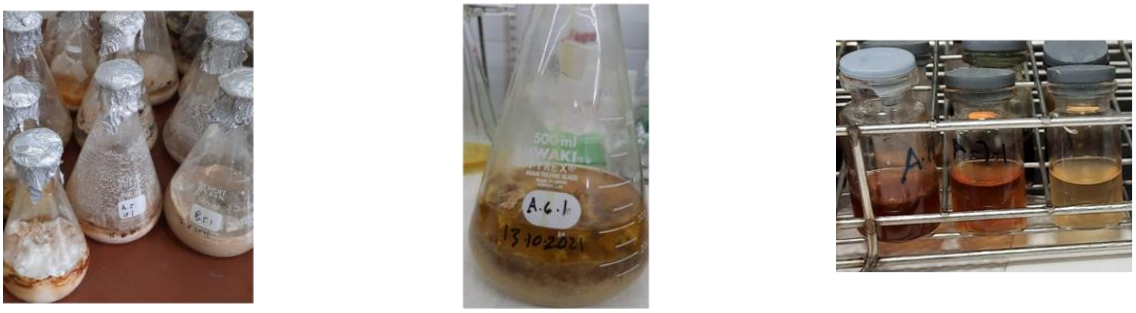


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Figure 3. The solid-state fermentation produced fungi for 30 days: A. Soaked of the fermented product with equal volumes of ethyl acetate, B. Dissolved of the crude extract with equal volumes of MeOH, C.

The extracted process of secondary metabolites produced by fungi isolated from *Halodule* sp. with equal volumes of ethyl acetate is shown in Figure 2B. Several studies have extracted secondary metabolites from the fungi-fermented product by directly soaking them in an organic solvent without lyophilizing and grounding them (Liu *et al.*, 2017). This study used powdered rice as a medium, which is an ungrounded fermented product. The crude extract was re-dissolved with MeOH (Figure 3C) for the reversed-phase thin-layer chromatography performed.

Profile of Thin-Layer Chromatography

Figure 4 shows the results of the TLC performed. The results show that each crude extract produced by fungi isolated from *Halodule* sp. has different TLC profiles, which indicates that the compounds produced by fungi are different for each strain. As seen in Figure 4, some fungi produced many compounds, as shown in TLC profile numbers 4, 7, and 8. These fungi have considerable potential to create new compounds.

Secondary metabolite compounds produced by fungi have diverse structures and groups. Our previous review of the diversity of compounds isolated from marine fungi in symbiosis with algae and corals showed various compounds such as terpenes, chromones, polyketides, alkaloids, and lactones. The results of this study, the TLC profile of the compounds shown in Figure 4, have explained the considerable variation of compounds produced by each fungus. Figures 4A, B, and C are the TLC results observed under UV366, UV254, and heating with $CeSO_4$, respectively. From observations under UV366, it was reported that it might be flavonoid and anthraquinone groups, such as 1'-Hydroxy-4',8,8'-trimethoxy-[2,2'-binaphthalene]-1,4-dione and (+)1-O-demethylvariecolorquinone A, were seen in fungi numbers 3, 4, 6 to 5 with blue, yellow, and orange fluorescence. Meanwhile, the observation results on

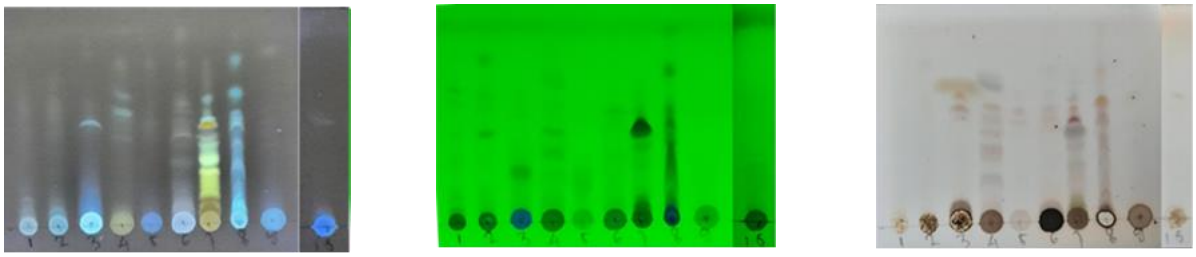
UV254 were thick black, which was observed on numbers 7 and 8, while on other numbers, it was not too thick. The compounds observed on UV254 have low fluorescence or chromo form groups of no more than 4, such as I-L and retinoid A-F compounds (Safwan *et al.*, 2024). Furthermore, the compounds observed on the plate when heating with $CeSO_4$ represent non-fluorescent compounds such as acrepeptin A-D (Safwan *et al.*, 2023), observed in fungus number 3.

Antimicrobial

Antimicrobials are among the most commonly used drugs. Recently, the occurrence of pathogenic bacteria resistance has become more serious, so the demand for antibacterial compounds is increasing. Natural products from fungal fermentation are considered important sources of antibacterial compounds due to the abundant diversity of fungal species and the diversity of secondary metabolites (Dos Reis *et al.*, 2019). In the past decade, many new bioactive natural compounds from marine fungi have been discovered with various activities such as cytotoxic, anticancer, antiviral, antibacterial, or antifungal (Safwan *et al.*, 2023). Antibacterial compounds from marine fungi have increased rapidly since 2010, and marine fungi have become an essential source of antibacterial compounds. In this study, all the crude extracts produced by fungus associated with *Halodule* sp. seagrass were performed for antimicrobial activity determined by the Kirby-Bauer disk diffusion method using *S. aureus* for indicator microbe. The microbial inhibitory activity is a quick and inexpensive assay for screening the activity of secondary metabolites. Meanwhile, secondary metabolites produced by microorganisms, including fungi, were reported to have good antimicrobial activity from various strains. Several fungal strains isolated from the marine were reported to produce secondary metabolites that have antimicrobial activity, such as *Pleosporales* sp., Westerdykella dispersal, and *Penicillium janthinellum* (Chen *et al.*, 2017; Xu *et al.*, 2019; Han *et al.*, 2021). Several secondary metabolites from these strains have been isolated, and the structurally were identified, such as peniciphenalenin G, peninaphone A, and penicilone B (Chen *et al.*, 2017; Xu *et al.*, 2019; Han *et al.*, 2021).

Antioxidant

In this study, all the crude extracts produced by fungus associated with *Halodule* sp. seagrass were performed for antioxidant activity determined by free radical scavenging using DPPH. The results of the antioxidant activities showed that three crude extracts with code A.10.4, B.5.1, and B.1.1 exhibited



a b c

Figure 4. C18 reversed-phase TLC profile with dichloromethane: methanol (10:1 v/v) of crude fungi extracts isolated from *Halodule* sp. by solid-state fermentation. Visualized ultraviolet light at 254 nm (A) and 366 nm (B) was sprayed using 10% H_2SO_4 (C). 1=A.10.4, 2=A.10.1, 3=A.10.2, 4= A.7.1, 5=A.4.1, 6=A.10.3, and 7=A.6.1, 8=B.5.1, 9=B.10.1, and 10=B.1.1.

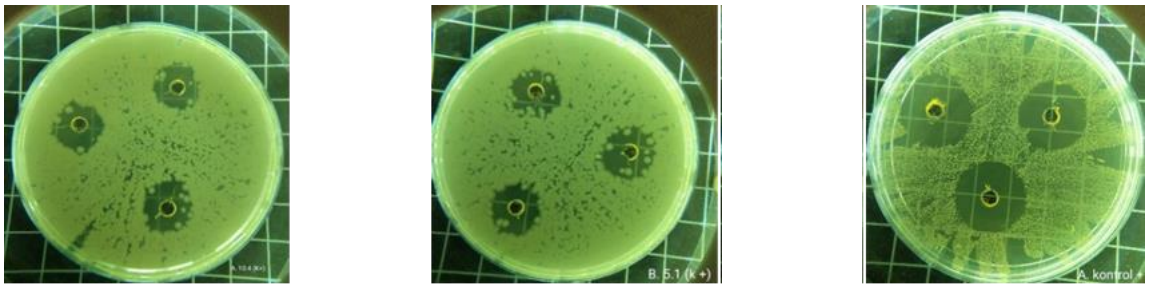


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Figure 5. Antimicrobial activity of crude extracts produced by fungi isolated from *Halodule* sp. by solid-state fermentation inhibited *S. aureus* growth by crude extract of A.10.4 (A), B.5.1 (B), and amoxicillin for control + (C).

potent DPPH radical scavenging with IC_{50} of 90.23 ppm, 88.29 ppm, and 81.31 ppm, respectively. Meanwhile, the antioxidant is a method of testing the activity of a secondary metabolite that is quite familiar, especially in the development of cosmetics. Several secondary metabolites produced by fungi isolated from marine were reported to have antioxidant activity, such as *Aspergillus flavus* isolated from sea sediments (Xiang *et al.*, 2021) and *Aspergillus unguis* isolated from seawater (Anh *et al.*, 2021).

Antioxidants have been used in dietary supplements to maintain health and prevent disease. Recently, synthetic antioxidants have shown carcinogenic and toxic properties, so researchers are faced with the search for natural antioxidants, which have led to identifying vitamins A, C, and E as antioxidants (Didier *et al.*, 2023). Fresh vegetables, fruits, tea, and wine are good sources of natural antioxidants. It is strongly associated with a lower risk of heart disease, so interest in natural antioxidants is increasing. Natural antioxidants from plants and fungi have been studied and play an essential role in improving health in the treatment of various diseases, and their safety has been documented (Młynarska *et al.*, 2024). Various compounds have been isolated, and many are phenolic, which are effective hydrogen donors and can inactivate lipid peroxidation, inhibit hydroperoxide decomposition into free radicals, and chelate metal ions (Jomova *et al.*, 2023). Phenolics from natural sources such as fermented fungi are better antioxidants than synthetic chemicals. Several strains have been shown to produce organic acid compounds that have two to four reactive hydroxyl groups, which can be associated with their antioxidant potential, such as gallic acid, ferulic acid, and ellagic acid (Chandra *et al.*, 2020). These organic acid compounds are produced under solid media fermentation conditions. Other compounds produced and isolated from various microbial sources that can scavenge free radicals are zeaxanthin and astaxanthin. These compounds have a long chain structure containing reactive hydroxyl groups, showing significant antioxidant effects (Chandra *et al.*, 2020). Based on the TLC results, the blue results in UV 366 are fluorescence from phenolic compounds. Almost all the fungi produced phenolic compounds.

Conclusion

Based on isolated and purified fungi from *Halodule* sp. seagrass, 10 pure fungi were obtained and grew on an MEA medium. Crude extracts numbers A.10.4 and B.5.1 showed inhibited *S. aureus,* and crude extracts numbers A.10.4, B.5.1, and B.1.1 exhibited DPPH radical scavenging. Based on the morphology of the isolated fungus, *Aspergillus* sp. is the fungus that is suspected to contain a compound that has antibacterial and antioxidant activity.

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