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Phytochemical, antioxidant, and antimicrobial analysis of *Trichoderma asperellum* isolated from ascidian *Eudistoma* sp

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ABSTRACT

Ascidians and their associated fungi are prolific producers of bioactive natural products. This present study was aimed at examining the characteristics of phytochemicals, antioxidants, and antimicrobial activity of the culturable fungi associated with *Eudistoma* sp. that were collected from the waters of Bunaken Island, North Sulawesi, Indonesia. Endophytic fungi were isolated using the dilution method on sabouraud dextrose agar supplemented with chloramphenicol. The pure fungal isolates were grown in a rice medium for 15 days under static conditions and daylight at room temperatures. The fungus and the rice medium substrate were extracted with EtOAc, evaporated, and freeze-dried, yielding a dry extract. The antioxidant activities were assessed using the 2,2-diphenyl-1-picrylhydrazyl assay. The antibacterial activity of the ethyl acetate fungal symbiont extract was determined using the Kirby–Bauer disk diffusion method, employing *Candida albicans*, *Staphylococcus aureus*, *Escherichia coli*, *Aeromonas hydrophila*, and *Salmonella* sp. as indicator pathogenic microbes. The phytochemical screening revealed the presence of secondary metabolites such as alkaloids, triterpenoids, tannins (1.11 mg/ml), flavonoids (3.76 mg/ml), and phenolics (5.98 mg/ml) in the fungal extract. The EtOAc extract of the fungus had a moderate antimicrobial activity against *C. albicans*, *E. coli*, and *S. aureus* but a strong antibacterial activity against *A. hydrophila* and *Salmonella* sp. Molecular identification using the ITS1-4 region of DNA revealed that the fungal strain had 99.37% identity with *Trichoderma asperellum*. Hence, this fungus can be further investigated as a potential source of antioxidants as well as broad-spectrum antimicrobials.

INTRODUCTION

Marine-derived fungi have garnered considerable attention due to producing beneficial bioactive compounds (Cardoso *et al*., 2020; Jin *et al*., 2016). Recently, screening for bioactive compounds isolated from ascidians has gained popularity due to the fact that many of these compounds exhibit antioxidant and antibacterial activity (Casertano *et al*., 2020; Dou and Dong, 2019). Antimicrobial agents, which include antibiotics, antiviral agents, and antifungal agents, are critical components of the treatment of infectious diseases (Leekha *et al*., 2011). However, as a result of indiscriminate antimicrobial use, microorganisms have evolved resistance mechanisms (Wang *et al*., 2018). This has become a significant issue for public health in recent years. Hence, the research has been focused on identifying sources of natural antibiotics. In addition to antibiotics, antioxidants have garnered researchers' attention in medicine due to their numerous health benefits (Dhalaria *et al*., 2020). Antioxidant and antibacterial activity has been demonstrated in several fungi

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Figure 1. Ascidian *Eudistoma* sp. collected from Bunaken waters.

associated with marine organisms such as ascidians (Da Luz Calado *et al*., 2021; Ramesh *et al*., 2021). It has been established that approximately 8% of the bioactive molecules isolated from ascidians are the result of symbiotic microorganisms (Casertano *et al*., 2020). Microorganisms associated with ascidians are a relatively untapped resource, particularly in Indonesia, despite the fact that Indonesia has one of the world's largest seascapes. Certain ascidians found in North Sulawesi are unique to the region and are not necessarily found elsewhere, and their habitat contributes to the unique fungi associated with them. Certain compounds isolated from marine ascidians-derived fungi that are taxonomically related to or identical to terrestrial fungi have been identified, such as *Aspergillus*, *Penicillium*, *Talaromyces*, and *Trichoderma* species (Nicoletti and Vinale, 2018). *Eudistoma* sp. is one of the ascidian species which is known to be abundant and capable of producing bioactive compounds that have been widely used. The present study examined culturable fungi associated with *Eudistoma* sp. that were collected from the waters of Bunaken Island, North Sulawesi, Indonesia, for the presence of phytochemical, antioxidant, and antimicrobial activity.

MATERİALS AND METHODS

Sample material

Ascidian *Eudistoma* sp. (Fig.e 1) was obtained from the Bunaken waters of North Sulawesi, Indonesia, with coordinates 01°36′49.46″N, 124°46′03.17″S, at a depth of 7 m (Fig. 2). Following that, the sample was surface-sterilized three times with sterile seawater and stored in a plastic bag. Samples were immediately transported to the laboratory using a coolbox for the isolation of the symbiotic fungi.

Isolation, cultivation, and extraction of symbiotic fungi

Symbiotic fungi were isolated from the ascidian and cultivated using the method of Kjer *et al*. (2010) with slight modification by Sandrawati *et al*. (2020). The ascidian was cut into small fragments, and approximately 10 g was dissolved in sterile seawater to a volume of 100 ml in an Erlenmeyer. After gently shaking the Erlenmeyer flask, the solution was diluted to a concentration of 10^{-6}. One milliliter of the sample was aseptically poured onto an sabouraud dextrose agar medium containing chloramphenicol in Petri dishes. After that, the plates were incubated for 5–7 days at a temperature ranging from 25°C to 27°C. Colonies exhibiting distinct forms were classified as distinct isolates. After that, each distinct colony was purified using the streak method to obtain pure isolates. Pure fungal isolates were cut into 1 × 1 cm squares and grown in a rice medium at room temperature for 4–6 weeks. When the entire rice medium was completely covered with fungal mycelia, the fungus had reached its maximum potential and was ready to harvest. The pure fungus was grown in a 250 ml Erlenmeyer bottle in a rice medium, each containing 10 g of rice in 10 ml of marine and distilled water with a ratio of 1:1 for 15 days in static conditions and at room temperature under daylight conditions. Following incubation, the fungal and rice medium substrate were extracted three times with EtOAc (100 ml) at the ratio of 1:1 for 24 hours and filtered. A rotary evaporator was used to evaporate the filtrate, yielding a thick extract of the fungus. The sample was then freeze-dried for 2 × 24 for further analysis.

Table 1. The reagents used for the analysis of phytochemical compounds of the fungal extract.

Phytochemicals	Reagents
Phenolics	$FeCl_3$ 5%
Flavonoids	Concentrated HCl + Mg
Steroids Triterpenoids	Lieberman Burchard
Saponins	PHCl + Aquadest
Tannin	$FeCl_3$ 1%
Alkaloids	Mayer ($HgCl_2$ + KI) Dragendorff (KI + $B_5H_9N_4O_{22}$) I_2 + KI

Phytochemical analysis

Phytochemical analyses were carried out following the procedure of Damongilala *et al*. (2021). The fungal ethyl acetate extract stock was prepared by weighing 100 mg of dry extract and adding it to a 10 ml volumetric flask with methanol p.a to obtain an extract with a concentration of 10 mg/ml (10,000 ppm). A stock solution with a concentration of 1 mg/ml (1,000 ppm) was made by taking 1 ml of the 10,000 ppm extract and adding it to a 10 ml volumetric flask with methanol p.a. Phytochemical compounds analyzed from fungal extracts and their reagents can be seen in Table 1.

Antioxidant activity assay

The antioxidant activities of the isolated fungus were assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay, as described by Sanger *et al*. (2021) The samples and positive control DPPH were dissolved in methanol with final concentrations of 25, 50, 75, 100, and 125 ppm. DPPH was dissolved at concentrations of 0.05 mg/ml (0.5 µM) in anhydrous ethanol (EtOH). DPPH will bind the hydrogen donated by the antioxidants in the sample. A color shift from dark purple to light yellow indicated the binding reaction had taken place. After 100 minutes of reaction, the color change was observed using a UV-vis spectrophotometer (Shimadzu UV-Vis 1800, Japan) at an

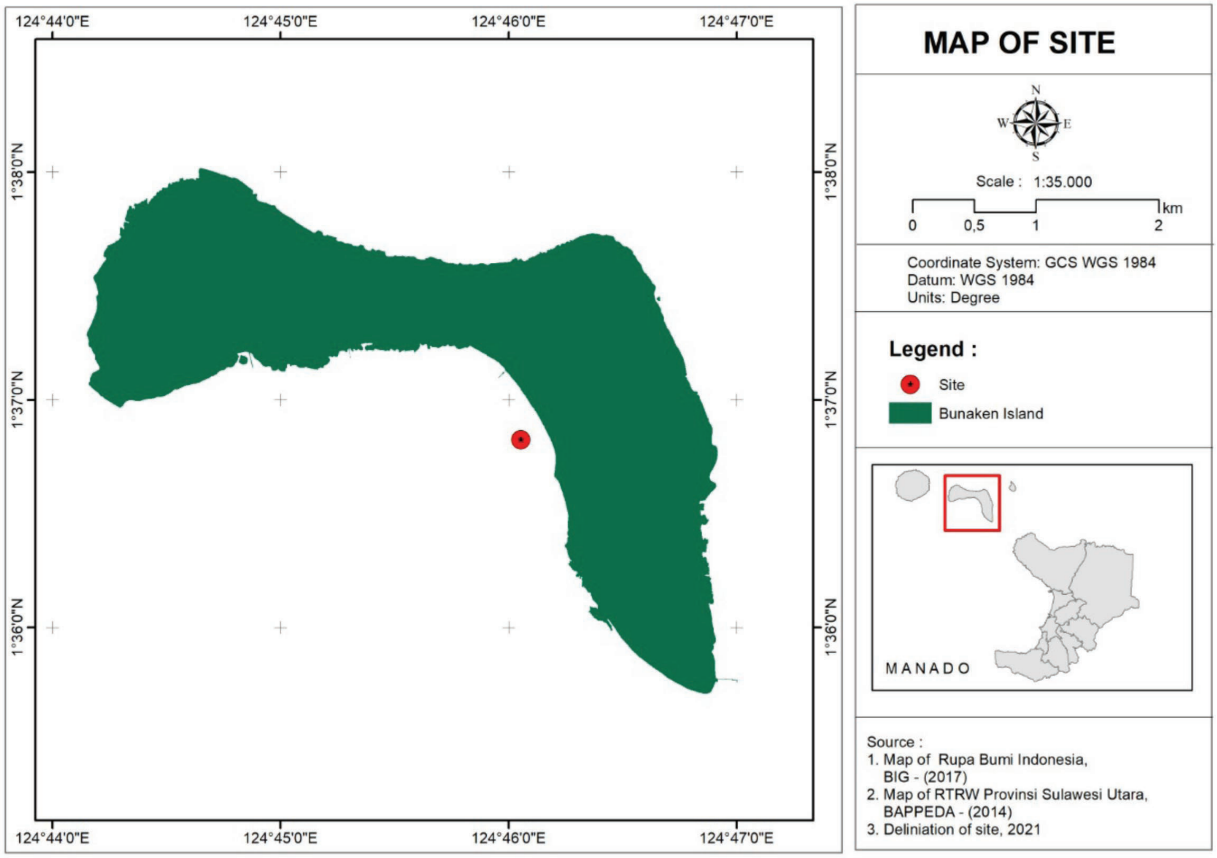

Figure 2. Ascidian sampling location.

absorbance of 517 nm. The strength of the antioxidant activity of a compound is classified based on Blois (1958), which is very strong (< 50 µg/ml), strong (50–100 µg/ml), moderate (100–150 µg/ml), and weak (150–200 µg/ml).

Antimicrobial activity assay

The antibacterial activity of the ethyl acetate fugal symbiont extract was determined in this study using the Kirby-Bauer disk diffusion method, which was slightly modified from Hudzicki (2009). The indicator microbes used in this study (*Candida albicans*, *Staphylococcus aureus, Escherichia coli, Aeromonas hydrophila*, and *Salmonella* sp.) were obtained from the Laboratory of Molecular Biology and Marine Pharmacy, Faculty of Fisheries and Marine Sciences, Sam Ratulangi University, Manado, Indonesia. These fungi and bacteria were cultured and maintained in liquid B1 media. Using B1 agar media, the antimicrobial activity of the ethyl acetate extract from fungal symbionts was tested. Each fungal extract with a concentration of 10 mg/ml (10,000 ppm), positive control (chloramphenicol 1,000 ppm), and negative control (methanol p.a) was dripped as much as 20 µl on each 6 mm diameter sterile paper disc and allowed to dry. This procedure was repeated three times for each indicator microbe. Disc papers were placed on previously prepared test media. Observations were made after incubation for 1 × 24 h and 2 × 24 hours. The resulting clear zone of inhibition was measured using a ruler, and the results were recorded in mm. The diameter of the inhibition zone is used to categorize the strength of antibacterial activity according to Davis and Stout (1971) as follows: very strong (≥20 mm), strong (10 to 20 mm), moderate (5 to <10 mm), and weak (≤5 mm).

Molecular identification of the fungus

Fungi isolated from the ascidian were identified molecularly using the ITS1-4 marker following the protocol of Handayani *et al*. (2019, 2021). DNA isolation was carried out using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, D6005). Polymerase chain reaction amplification was performed using MyTaq HS Red Mix (Bioline; BIO-25048ITS1-4 primers were used to perform bidirectional sequencing).

RESULTS AND DISCUSSIONS

Marine-derived fungi are an abundant source of structurally novel natural products, which include a wide range of chemical compounds and pharmacological applications. Marine-derived fungi are those that are isolated in a marine environment. This is in contrast to the traditional definition of marine fungi, which are both obligatory and facultative inhabitants of the marine environment (Pang *et al*., 2016). The current study focuses on the exploration of a marine-derived fungus, or more precisely an ascidian-derived fungus obtained from the Bunaken seawaters of North Sulawesi, Indonesia, as a source of antimicrobials. As shown in Figure 1, the ascidian under study was the *Eudistoma* sp.

Molecular identification of the fungus

The pure fungus was isolated from the ascidian *Eudistoma* sp., as shown in Figure 3. The fungal strain was identified as

Figure 3. Pure culture of *T. asperellum* AFBN 4 isolated from the ascidian *Eudistoma* sp.

Trichoderma asperellum based on DNA sequence amplification and sequencing, with sequence assembly 799 bp of the ITS1-4 region, and an identity percentage of 99.37%. Ascidian-derived fungi *Trichoderma* sp. is a potential natural resource containing a wide range of structurally novel natural products containing a wide range of abundant chemical substances with numerous applications in the field of pharmacology. So far, 78 different types of metabolites from this fungus have been identified, and the majority of these metabolites have therapeutic properties, allowing them to be used as a source of new drug discovery (Su *et al*., 2018). Up until now, there has been very little known about *T. asperellum*, which has been isolated from ascidians. However, a marine-derived fungus, *T. asperellum*, which produced six peptaibols known as asperelines A–F, was successfully isolated from the sediment of Antarctic Penguin Island (Ren *et al*., 2009). Additionally, *T. asperellum* cf44-2, an algal endophyte, also yielded seven previously unknown terpenes, including bisabolane, cyclonerane, and harziane derivatives (Song *et al*., 2018).

Phytochemical constituents analysis of fFungal EtOAc extract

The EtOAc extract of *T. asperellum* AFBN 4 was subjected to phytochemical analysis to determine the presence of alkaloids, triterpenoids, steroids, tannins, flavonoids, saponins, and phenolics. The phytochemical screening revealed the presence of secondary metabolites such as alkaloids, triterpenoids, tannins (1.11 mg/ml), flavonoids (3.76 mg/ml), and phenolics (5.98 mg/ml) in the fungal extract. The result is nearly identical to that discovered in previous studies, indicating that these compounds are present in the species *Trichoderma* sp (Ikram *et al*., 2019; Omomowo *et al*., 2020). The attractive chemical structures and notable biological activities of *Trichoderma* have attracted great attention (Zhang *et al*., 2021). As a result, numerous reviews have been published on various aspects of *Trichoderma*, not only for the chemical diversity of the metabolites, but also for the diverse bioactivities and possible applications (Zeilinger *et al*., 2016). Alkaloids trichoderamides A (136) and B (137) were produced by *T. gamsii*, an endophyte of *Panax notoginseng* (Ding *et al*., 2015). *Trichoderma* has been reported to produce the terpenoids trichothecenes (Shi *et al*., 2020; Yamazaki *et al*., 2020). In addition, *Trichoderma* has been reported to produce phenolic and flavonoid compounds as well (Yusnawan *et al*., 2020).

Table 2. Antioxidant activity of fungal extracts using the DPPH method.

Concentration of extract (ppm)	Absorbance (λ 517 nm)	Inhibition %	IC_{50} (µg/ml)
25	0.437	49.24	
50	0.383	55.51	
75	0.340	60.51	32.89
100	0.316	63.38	
125	0.161	80.30	
DPPH	0.861	—	

Antioxidant activity

The isolated fungal antioxidant activity was determined using the DPPH radical scavenging assay. This assay is frequently used to determine the presence of antioxidant activity within an organism. The IC_{50} value is used to determine the antioxidant activity of an extract using the DPPH method. This value represents the concentration of the extract that results in a 50% loss of DPPH activity. As presented in Table 2, the antioxidant activity of fungal extracts is 32.89 g/ml, which puts it in the category of a strong antioxidant. *Trichoderma* sp. has been known to produce antioxidant substances, as previously reported (Su *et al*., 2018; Zhang *et al*., 2017). The cosmetics and pharmaceutical industries can benefit from the radical scavenging properties of a variety of natural compounds derived from marine sources, according to numerous studies (Gogineni and Hamann, 2018; Wu *et al*., 2018).

Aeromonas hydrophila *Escherichia coli* *Salmonella sp.* *Staphylococcus aureus* *Candida albicans*

Figure 4. Antimicrobial activity of *T. asperellum* AFBN against pathogenic microbes.

Table 3. Diameter of inhibition zone of *T. asperellum* AFBN extract against several pathogenic microbes.

Pathogenic bacterial strains	Diameter of inhibition zone (mm ± SD)		
	After 24 hours	Positive control	Negative control
Candida albicans	8.5 ± 0.30	20 ± 0.27	0 ± 0.00
Aeromonas hydrophila	15 ± 0.17	20 ± 0.50	0 ± 0.00
Escherichia coli	9.5 ± 0.56	17 ± 0.20	0 ± 0.00
Salmonella sp.	10 ± 0.44	19 ± 0.20	0 ± 0.00
Staphylococcus aureus	8.5 ± 0.20	12 ± 0.17	0 ± 0.00

Trichoderma asperellum contains a variety of phytoconstituents, including alkaloids, triterpenoids, tannins, flavonoids, and phenolics. The antioxidant activity of phenolic compounds is significant (Shad *et al*., 2014). According to recent study, antioxidant properties are ascribed to the presence of polyphenols, flavonoids, alkaloids, terpenoids, and carotenoids (Farag *et al*., 2020; Larayetan *et al*., 2019). Antioxidants can neutralize reactive oxygen and nitrogen species, preventing the resurgence of a wide range of degenerative diseases such as cancer, autoimmune disorders, cardiovascular disease, and neurodegenerative disease (Vitale *et al*., 2020). As a result, antioxidants derived from *T. asperellum* can be studied further for potential use in medicinal applications, possibly in place of currently used synthetics.

Antimicrobial activity

A large number of marine-derived fungi have antimicrobial properties (Sumilat *et al*., 2020) (Handayani *et al*., 2021; Sandrawati *et al*., 2020). The results of the antimicrobial activity analysis of *T. asperellum* AFBN are shown in Table 3 and Figure 4. According to Davis and Stout (1971), the antimicrobial activity of the fungal extract against *C. albicans*, *E. coli*, and *S. aureus* was moderate, while its activity against *A. hydrophila* and *Salmonella* sp. was classified as strong. Several previous studies have also reported that *Trichoderma* extract has antibacterial activity, including against methicillin-resistant *S. aureus* (Sadykova *et al*., 2015), *S. aureus* (Haryani *et al*., 2019; Leylaie and Zafari, 2018), *C. albicans* (Sadykova *et al*., 2015), *E. coli* (Haryani *et al*., 2019; Khethr *et al*., 2008; Leylaie and Zafari, 2018), and *Salmonella typhi* (Reena and Nagar, 2014). The antibacterial activity of the *Trichoderma* extract against *A. hydrophila* has not been previously reported. Additionally, *Trichoderma* sp. isolated from mangrove *Ceriops tagal* was also effective against *Vibrio algionlyticus* (Haryani *et al*., 2019). Similarly, *Trichoderma reesei* (CGF-11) exhibited antibacterial activity against a variety of phytopathogenic bacteria (Ikram *et al*., 2019).

CONCLUSION

Trichoderma asperellum AFBN has been isolated from ascidian *Eudistoma* sp. from Bunaken waters. This fungal EtOAc extract contained phytoconstituents such as alkaloids, triterpenoids, tannins, flavonoids, and phenolic compounds. Additionally, it also possesses significant antioxidant activity. The EtOAc extract of the fungus has broad-spectrum antibacterial activities. As a result, *T. asperellum* may be investigated further for potential commercialization in the health and pharmacoindustries.

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CONFLICT OF INTERESTS

None.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included within this research article.

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