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Antibacterial activity of ethyl acetate extract of symbiont fungus *Aspergillus* sp. in the sponge *Rhabdastrella* sp. from Gili Layar Island, Lombok, Indonesia

Mahfur Mahfur[1*], Arina Kamila[2], Jihan Fatin Abidah[2], Putu Oka Samirana[3], Baso Didik Hikmawan[4], Diah Pratimasari[5]

[1]Department of Pharmacy, Faculty of Pharmacy, Pekalongan University, Pekalongan, Indonesia.
[2]Bachelor of Pharmacy Program, Faculty of Pharmacy, Pekalongan University, Pekalongan, Indonesia.
[3]Department of Pharmacy, Faculty of Mathematics and Natural Science, Udayana University, Bali, Indonesia.
[4]Pharmaceutical Research and Development Laboratory of Farmaka Tropis, Faculty of Pharmacy, Universitas Mulawarman, Samarinda, Indonesia.
[5]Nasional College of Health Science Sukoharjo, Sukoharjo, Indonesia.

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ABSTRACT

Sponges are animals that can live in symbiosis with microorganisms, one of which is a fungus. Fungi associated with this sponge can produce bioactive compounds that have pharmacological activity. One example is having antibacterial activity by inhibiting the growth of or killing pathogenic bacteria. The purpose of this study was to determine the type of fungus associated with the sponge *Rhabdastrella* sp., the secondary metabolites produced, and its antibacterial activity. The sponges were cultivated to grow symbiotic fungi on saline Sabouraud dextrose agar media. Fermentation was carried out in Sabouraud dextrose broth saline liquid medium to increase secondary metabolite productivity. The fungus symbiont will extract with ethyl acetate solvent. Ethyl acetate was chosen because it separated salt and water, so a group of pure organic compounds could be obtained. Identification of secondary metabolites was carried out by chromatography (thin layer chromatography and gas chromatography). The antibacterial activity of the ethyl acetate extract of the symbiont fungus *Aspergillus* sp. in the sponge *Rhabdastrella* sp. was determined using the well diffusion method. Molecular identification showed that *Aspergillus* sp. was the most dominant type of fungus associated with *Rhabdastrella* sp. The fungus extract of *Aspergillus* sp. showed the presence of alkaloids, flavonoids, terpenoids, and phenolics. The inhibitory effect of *Aspergillus* sp. extract against *Escherichia coli*, *Staphylococcus aureus*, and *Streptococcus pyogenes* was compared with the standard drug ciprofloxacin. The results of this study indicate that the ethyl acetate extract of *Aspergillus* sp. from *Rhabdastrella* sp. has antibacterial activity against some causative organisms.

INTRODUCTION

Sponges have recently attracted high interest from researchers because they contain microorganisms that have not been identified from the marine environment but which have the potential as new chemicals (Brinkmann *et al*., 2017; Calcinai *et al*., 2017; Mahfur *et al*., 2022b; Pallela dan Ehrlich, 2016). Sponges contain chemical compounds that have many pharmacological activities, such as antiviral, antifungal, antibacterial, and antimalarial, anti-inflammatory, and neurosuppressive effects (Carroll *et al*., 2019; Mahfur *et al*., 2022a; Novanna *et al*., 2018; Shady *et al*., 2019; Zhang *et al*., 2021). Although exploration of sponges as a source of active compounds can interfere with the survival of sponges due to their scarcity, this can be prevented by isolating microorganisms that have a symbiosis with sea sponges, including fungi (Brinkmann *et al*., 2017; Handayani dan Artasasta, 2017; Hikmawan *et al*., 2018).

Fungal symbionts in sponges can produce the same compounds as sponges because genetic transfer occurs from the sponge to the fungal body. Fungal symbiosis with sponges can occur in the sponge body cells' cytoplasm (Casertano *et al*., 2020; Pejin dan Karaman, 2017; Thacker dan Freeman, 2012). Previous research showed that

*Corresponding Author
Mahfur Mahfur, Department of Pharmacy, Faculty of Pharmacy, Pekalongan University, Pekalongan, Indonesia.
E-mail: mahfur.isfa @ gmail.com

symbiosis between fungi and sponges has the potential to be a source of compounds that produce antibacterial and cytotoxic effects (Brinkmann *et al*., 2017; Setyowati *et al*., 2017). Fungi found in symbiosis with sponges are generally *Aspergillus fumigatus, Trichoderma reesei, Aspergillus flavus, Aspergillus nomius,* and *Penicillium* sp. (Handayani and Aminah, 2017; Setyowati *et al*., 2018).

Rhabdastrella sp. is a sponge that belongs to the Ancorinidae family. It is native to the Indo-West Pacific and tropical oceanic waters of Asia (Manalundong and Uy, 2021; Mioso *et al*., 2017). Most of the compound groups isolated from *Rhabdastrella* sponges are triterpenoids which have been shown to have anticancer activity (Lai *et al*., 2021; Trang *et al*., 2022). To the best of our knowledge, fungal symbionts in *Rhabdastrella* sp. have never been studied. However, based on the activity of the sponge isolate, it is possible that the sponge symbiont also has anticancer and antibacterial activity. In this study, the symbiont sponge extract will be tested for antibacterial activity against *Escherichia coli, Staphylococcus aureus,* and *Streptococcus pyogenes.*

Recently, antimicrobial activities of medicinal sources have been investigated to ascertain claims of therapeutic properties (Sallau *et al*., 2016, 2022; Uttu *et al*., 2021, 2015). The bacteria *E. coli, S. aureus,* and *S. pyogene* are types of bacteria that are often found as sources of disease in humans (Affi Kakou *et al*., 2021; Rustamova *et al*., 2020; Valentin *et al*., 2011). They also belong to a type of bacteria that is already resistant to several existing antibacterials (Abdelfattah *et al*., 2016; Asif, 2016). Therefore, is necessary to look for alternative sources of new antibacterials, one of which can be from *Rhabdastrella* sp.

This study aims to determine the type of fungus symbiotic with the sponge *Rhabdastrella* sp., and the compounds and the antibacterial activity in the symbiont fungus.

MATERIALS AND METHODOLOGY

Sampling and cultivation of symbiont fungus

Rhabdastrella sp. samples were collected by scuba diving at a depth of around 12 meters on Gili Layar Island in Sekotong, West Lombok, Indonesia. Sponges were identified at the Marine Natural Product Laboratory at Diponegoro University in Semarang, Indonesia. The sponges *Rhabdastrella* sp. were sterilized with 70% ethanol for 30 seconds and were then cut into small pieces. Afterward, they were cultivated on Sabouraud's Dextrose Agar Saline with the addition of 1 ml ciprofloxacin and put in the incubator at a temperature of 25°C for 14 days (Setyowati *et al*., 2018).

Purification and identification of symbiont gungus

The purification process was carried out based on the morphological appearance of each different colony (Hidayat *et al*., 2021). This process was carried out by taking one more dominant isolate growing on the media and planting it in new media until a pure isolate was obtained (Setyowati *et al*., 2017). Identification was carried out by observing the symbiont fungus macroscopically, microscopically, and molecularly based on partial locus genetic analysis in the internal transcribed spacer (ITS). Molecular identification began with DNA extraction from a 7-day-old fungus with a Zymo Research extractor. DNA amplification was carried out using the polymerase chain reaction (PCR) which involved the thermal cycler method with ITS 1 and ITS4 as DNA regions (Setyowati *et al*., 2018). PCR product quality was seen using electrophoresis on 1% agarose. The results of the PCR visualization were analyzed using PT at 1st Base Laboratories Sdn Bhd, Malaysia. DNA sequences were analyzed for homology using the basic local alignment search tool (www.ncbi.nlm.nih.gov). Phylogenetic trees were analyzed using MEGA 7.0 software, while statistical analysis used the neighbor-joining method with 1,000 bootstrap replication fields (Larasati *et al*., 2021).

Fermentation and extraction of fungal secondary metabolites

Fermentation was carried out by inoculating pure fungal on an Sabouraud dextrose broth (SDB) medium and incubating it at 37°C for 10 days with a shaker. The fermented symbiont fungus was separated between the supernatant and mycelia. The supernatant obtained was 90 ml and extracted with a 1:1 ratio of ethyl acetate. The ethyl acetate extract obtained was concentrated with a rotary evaporator at 60°C (Setyowati *et al*., 2017).

Identification of metabolite compound profiles

Identification of secondary metabolites used thin layer chromatography (TLC) with a mobile phase of chloroform: methanol 9:1. The results of the TLC analysis were seen in visible light with UV light 254 and 366 nm and sprayed with a reagent. The identification of compounds used gas chromatography-mass spectra (GC-MS) (Shimadzu GCMS-QP2010SE). Injection in GC-MS with GC-MSQP-2010 ultra running conditions was carried out in a column oven with a temperature of 50°C and an injection with a temperature of 250°C. The carrier gas used was helium with a pressure of 100 kPa and a flow rate of 89.3 ml/minute (Kanjana *et al*., 2019). The grouping and writing of identification results followed previous research (Ibrahim *et al*., 2021; Risikat *et al*., 2022).

Antibacterial activity test

The antibacterial method used well diffusion on Muller Hinton agar media with three bacteria: *E. coli* ATCC25922*, S. aureus* ATCC25923, and *S. pyogenes* ATCC19615. Bacterial suspension used 0.5 McFarland standard whose concentration was 1.5 × 108 CFU/ml. The testing was performed with extract concentrations of 50; 25; 12.5; and 6.25 mg/ml, positive control (ciprofloxacin) and negative control (ethyl acetate). The petri dish was incubated at 37°C for 1 × 24 hours. After incubation, the clear zone was measured from each test (Handayani and Aminah, 2017).

RESULTS AND DISCUSSION

Symbiont fungus cultivation

The results of the cultivation showed the presence of fungal growth. The *Rhabdastrella* sp. sponges obtained two fungus isolates with white (MIC 6B1) and green (MIC 6B2) characteristics (Fig. 1). The growth of fungi was evident, with several microorganisms collaborated in the filter feeder animal

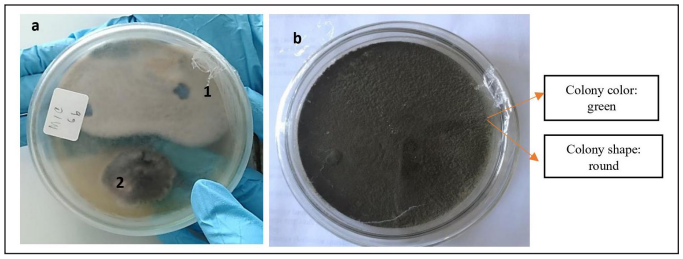


Figure 1. (a) Sponge symbiont fungus *Rhabdastrella* sp.: (1) dominant fungi MIC 6B1 and (2) fungi MIC 6BA. (b) Results of MIC 6B1 purification.

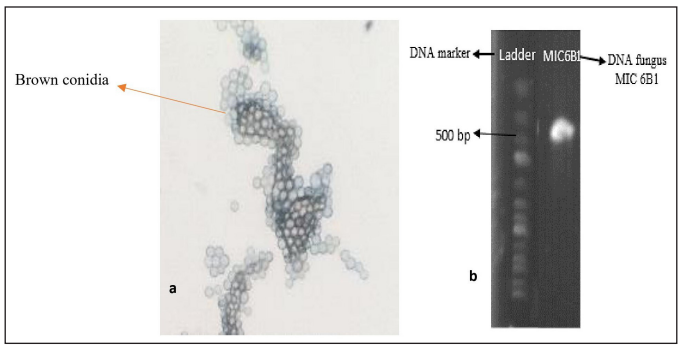


Figure 2. (a) Results of microscopic identification. (b) Results of visualization of molecular PCR identification.

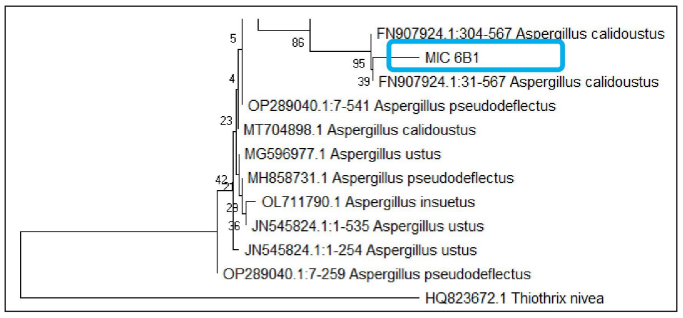


Figure 3. Phylogentic tree of fungus MIC 6B1 (*Aspergillus* sp.).

tissue, and in this case, the fungus throve. The symbiotic relationship between the fungi and sponges is mutualistic in which genetic transfer occurs from the body of the sponge to the body of the fungus (Chu *et al*., 2021; Gaino *et al*., 2014; Panggabean *et al*., 2022). Through this relationship, fungi can also produce secondary metabolites that are the same as or similar to the symbiotic sponge (Hong *et al*., 2022; Nguyen dan Thomas, 2018; Samirana *et al*., 2021).

Purification and identification of symbiont fungus

The purification process aims to obtain pure MIC 6B1 isolates based on their morphology (Setyowati *et al*., 2017). The symbiont fungus used for the purification is a fungus that is 14 days old because it is in the logarithmic/ exponential phase range where fungal cell division occurs very quickly and constantly (Kjer *et al*., 2010; Rendowaty *et al*., 2017). The purification was carried out for the dominant

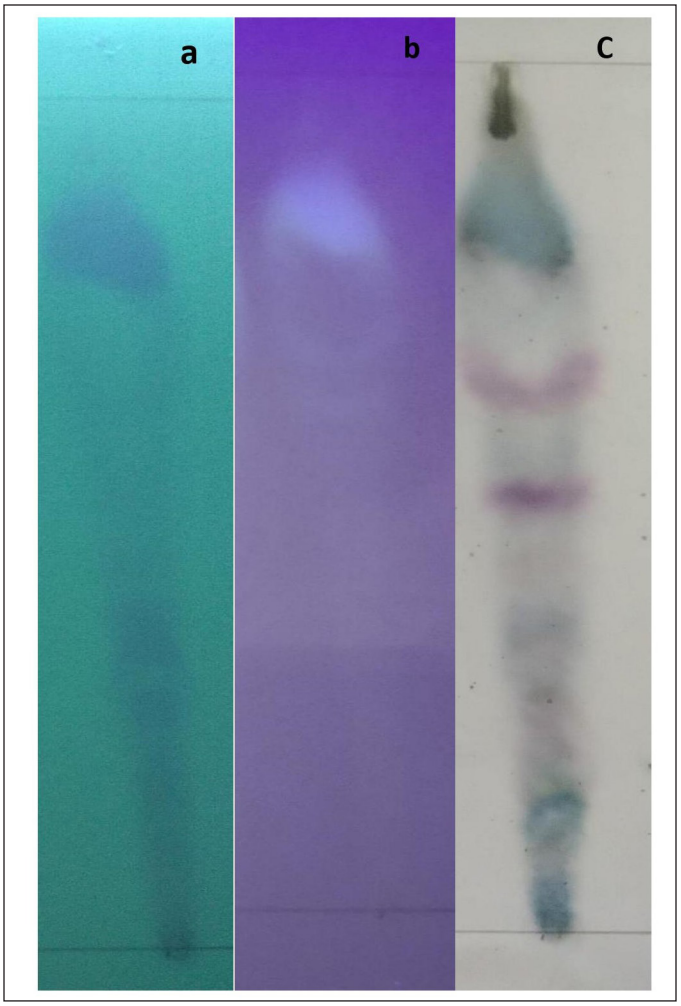


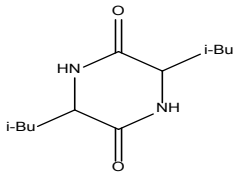
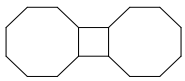
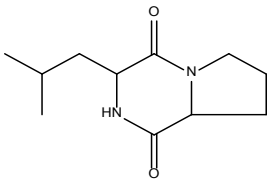
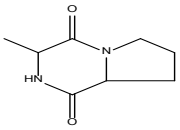
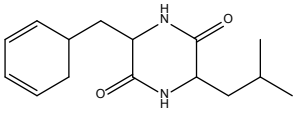
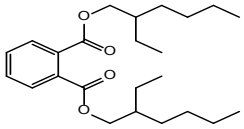
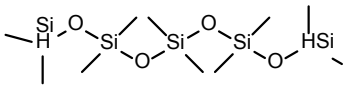
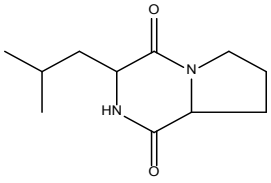
Figure 4. Profile of secondary metabolites from ethyl acetate extract fungus *Aspergillus* sp. with mobile phase chloroform: methanol (9:1) (a) 254 UV, (b) 366 UV, and (c) Vanilin. As. Sulfat.

Table 1. Results of classes of compounds identified in ethyl acetate extract of *Aspergillus* sp.

S/N	Class of compounds	Methods	Result	Remark
1	Alkaloids	Dragendorf Reagent	+	Brownish red spot under visible light
2	Flavonoids	Sitroboric Reagent	+	Light blue fluorescence spots under UV 366
3	Terpenoids	Vanillin Sulfuric acid	+	Dark purple spot under visible light
4	Phenolic	Fe-Cl_3 Reagent	+	Brown spots under visible light

fungus, which was MIC 6B1 with white mycelium (Fig. 1). The purification obtained green fungus with round colonies (Fig. 1). The dominant fungi which initially had white colonies turned green after 14 days of rearing. This can happen because of the process of adaptation to the environment in the growth medium (Höller *et al*., 2000). The results of the macroscopic

Table 2. Result of compounds suggested by GC-MS.

Peak	RT	Compound Name	Structure	Molecular Weight	Concentrations (%)
1	19.20	2,5-Piperazinedione, 3,6-bis(2-methylpropyl)	 $C_{12}H_{22}N_2O_2$	226	28.78
2	29.23	Cyclobuta[1,2:3,4]dicyclooctene,	 $C_{16}H_{28}$	220	10.70
3	18.28	1,4-diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0] nonane	 $C_{11}H_{18}N_2O_2$	210	10.72
4	17.34	1,4-diaza-2,5-dioxobicyclo[4.3.0]nonane	 $C_7H_{10}N_2O_2$	154	8.57
5	20.79	3-benzyl-6-isobutyl-2,5-dioxo-piperazin	 $C_{14}H_{18}N_2O_2$	260	8.55
6	20.89	1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester (CAS) Bis(2-ethylhexyl) phthalate	 $C_{24}H_{38}O_4$	390	8.38
7	28.84	1,1,3,3,5,5,7,7,9,9-Decamethyl-Pentasiloxane	 $C_{10}H_{32}O_4SI_5$	356	8.36
8	18.31	1,4-diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0] nonane	 $C_{11}H_{18}N_2O_2$	210	8.19
9	21.28	Silicone grease	-	-	7.70

identification of the colonies were round, green colonies with white on reverse and had a texture like cotton. The results of microscopic identification also showed the presence of round and pale brown conidia (Fig. 2). The MIC 6B1 fungal strains were identified by DNA sequencing (Fig. 2) and registered in GenBank (accession number OQ451587, www.ncbi.nlm.nih.gov). The isolates showed 93.90% homology and 74% query cover to *Aspergillus calidoustus* (Fig. 3). A maximum identity value of above 97% indicates that the isolate has a high degree of homology and is likely of the same species. Low percent homology makes the sample classified into *Aspergillus* sp. The fungus *Aspergillus* sp. has pale brown radiating conidia consisting of catenate conidia. Previous research conducted by Manuel *et al*. (2021) found that *Aspergillus* sp. isolates showed the growth of white cotton colonies which turned green over time.

Fermentation and extraction of fungal secondary metabolites

The fungus *Aspergillus* sp. from the sponge *Rhabdastrella* sp. was fermented in liquid media SDB saline to increase the production of secondary metabolites. Fungal growth during the fermentation period occurred from day 7 to day 14 in which there was an increase in secondary metabolites production. Meanwhile, starting on the 15th day, the growth of the fungus relatively decreased (Rendowaty *et al*., 2017; Zhang *et al*., 2019). In this study, harvesting was carried out on the 10th day of fermentation following research by Samirana *et al*. (2021) which revealed that fermentation on the 10th day produced more extracts and maximum secondary metabolites. At the extraction stage, the choice of solvent is an important factor in obtaining the optimal amount of extract. Ethyl acetate was chosen as a solvent because the sample used was a marine natural product containing salt. Therefore, ethyl acetate functions to separate salt and water, so the metabolites produced from the extraction process are a group of pure organic compounds without any other unwanted ingredients (Al-Saleem *et al*., 2022; Ibrahim *et al*., 2022; Kumar *et al*., 2018). The average yield produced in the extraction was 0.077% ± 001% w/v.

Identification of metabolite compound profiles

The TLC profile results with chloroform: methanol 9:1 mobile phase at 254 and 366 nm UV light and sprayed with reagents to determine the presence of secondary metabolites such as alkaloids, flavonoids, terpenoids, and phenols (Kaur *et al*., 2017) as seen in Figure 4 and Table 1. Research by Tanod *et al*. (2020) found that the fungus *Aspergillus* sp. as a sponge symbiont produced alkaloid, terpenoid, and phenolic compounds which produced antibacterial activity. The active compound of the ethyl acetate extract was identified quantitatively using the GC-MS instrument to determine the specific compound content in the ethyl acetate extract of the fungus *Aspergillus* sp. Table 2 shows the chemical compound of ethyl acetate extract of *Aspergillus* sp. Of all chemical components found in the ethyl acetat extract of *Aspergillus* sp., the biggest secondary metabolite compound was 2,5-piperazinedione, 3,6-bis(2-methylpropyl) with 28% composition. These compounds are likely to have contributed to the extract's antifungal effects (Raut *et al*., 2021).

Antibacterial activity test

An antibacterial activity test was carried out to determine the potential of the ethyl acetate extract of *Aspergillus* sp. in inhibiting bacterial growth. The test was carried out on the Gram-negative bacteria *E. coli* ATCC25922, the Gram-positive bacteria *S. aureus* ATCC25923, and *S. pyogenes* ATCC19615. The ethyl acetate extract of *Aspergillus* sp. could inhibit the growth of the bacteria which was characterized by the formation of clear zones around the wells after 24 hours of incubation (Table 3). The antibacterial activity of *Aspergillus* sp. extract against *E. coli, S. aureus*, and *S. pyogenes* at a concentration of 50 mg/ml showed inhibition zones on bacterial media of 16.77 ± 1.73, 7.00 ± 0.41, and 6.41 ± 0.84 mm. The results of the test showed that the inhibition of ethyl acetate extract on *E. coli bacteria* was greater compared to *S. aureus* and *S. pyogenes* (Fig. 5). The ethyl acetate extract of the fungus *Aspergillus* sp. is known to contain organic compounds based on the identification of secondary metabolite profiles. These compounds have chemical components that contain nitrogen atoms in their structure, so they are classified as alkaloid compounds that have antibacterial activity (Beesoo *et al*., 2017; Casertano *et al*., 2020). The natural alkaloid antibacterial mechanism shows that alkaloids can disrupt bacterial cell membranes, affect DNA function, and inhibit protein

Table 3. Results of the inhibition zone.

No.	Bacteria	Extract concentration (mg/ml)	$\vec{x}$ inhibition zone (mm)
1	*E. coli*	50	16.77 ± 1.73
		25	12.99 ± 3.35
		12.5	7.81 ± 3.36
		6.25	6.94 ± 1.27
		CP	19.71 ± 0.96
		CN	0.0
2	*S. aureus*	50	7.00 ± 0.41
		25	5.90 ± 0.55
		12.5	5.50 ± 1.07
		6.25	4.50 ± 0.88
		CP	14.89 ± 0.65
		CN	0.0
3	*S. pyogenes*	50	6.41 ± 0.84
		25	5.42 ± 0.76
		12.5	4.48 ± 0.67
		6.25	4.41 ± 0.78
		CP	11.41 ± 0.64
		CN	0.0

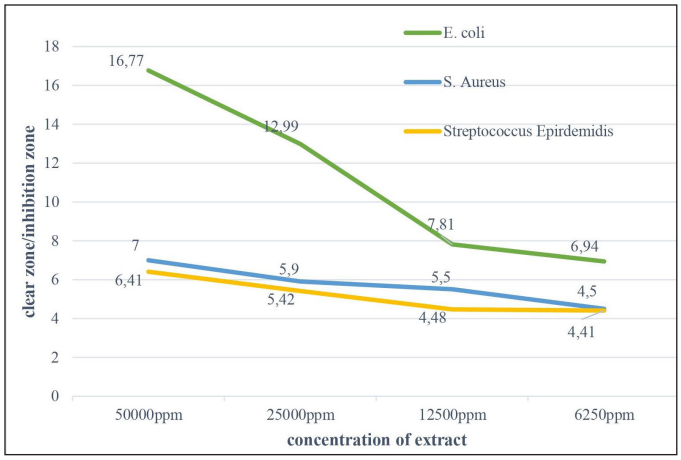


Figure 5. Antibacterial activity of extract ethyl acetate *Aspergillus* sp. against pathogenic bacteria.

synthesis. Thus, natural alkaloids are potentially active against various bacteria, including *E. coli*, *S. aureus*, and *S. pyogenes* (Gao *et al*., 2019; Liu *et al*., 2020; Yan *et al*., 2021). The other compounds in the extract, such as flavonoids, phenols, and terpenoids, also have antibacterial activity. Flavonoids have an antibacterial mechanism of inhibiting nucleic acid synthesis, inhibiting the function of the cytoplasmic membrane, and inhibiting the energy metabolism of bacterial cells (Beesoo *et al*., 2017; Xie *et al*., 2014). The antibacterial mechanism of terpenoid compounds is by destroying the bacterial cell membrane which will affect the basic properties of bacteria and as an inhibitor of protein synthesis which can achieve an antibacterial effect by blocking every process of the protein synthesis pathway (Huang *et al*., 2022). The antibacterial mechanism of phenolic compounds is by modifying the permeability of cell membranes, the changes in various intracellular functions induced by the binding of phenolic compounds to enzymes, and thus, phenolic compounds can increase their antimicrobial activity by supporting their interactions with cell membranes (Bouarab *et al*., 2019).

CONCLUSION

The fungus *Aspergillus* sp. (MIC 6B1) is the dominant type of fungal symbiont associated with the sponge *Rhabdastrella* sp., which has antibacterial activity and has the potential as a source of chemical compounds for isolation.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to the conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

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

DATA AVAILABILITY

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

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