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Antimicrobial Activity Test of Methanol Extract of *Haliclona* sp. from Sibolga Waters

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Abstract: *Haliclona* sp. is a genus of demosponges in the family Chalinidae. Demospongiae are a type of sponge that lives in marine environments and is known for its unique, often branching skeletal structure. The use of antibiotics has led to the selection of pathogenic microorganisms in humans that are more resistant to many types of drugs, resulting in poorer treatment outcomes in cases of infection. Bacteria can become resistant to antibiotics due to improper dosing, inaccurate diagnosis, and inappropriate use of antibiotics against the causative bacteria. This study aims to determine the antimicrobial activity of the methanol extract of *Haliclona* sp. as an inhibitor of pathogenic microbes. This research employs a descriptive method with antagonistic testing. The results of the methanol extraction process of the symbiotic bacteria in *Haliclona* sp. produced a thick, dark brown extract. The results of the antagonistic test showed that the clear zones formed on the three test microbes were categorized as strong.

Keywords: Antimicrobial; *Haliclona* sp.; Methanol

Introduction

Haliclona sp. from marine sponge species has shown its potential as an antimicrobial agent. The extract was found to have immunomodulatory activity, effectively suppressing non-functional and functional immune responses in Wistar rats. This suppression includes a reduction in immune cell profiles (total WBC count, lymphocytes, platelets, spleen cells, and bone marrow cells) and the splenosomatic index, as well as the ex vivo proliferation of bone marrow and spleen cells. HSCE demonstrated strong immunosuppressive activity, particularly myelosuppression. This indicates that HSCE could be a potential candidate for immunosuppressive drugs, with a focus on its antimicrobial properties (Gunathilake et al., 2020).

The use of antibiotics has led to the selection of pathogenic microorganisms in humans that are more resistant to many types of drugs, resulting in poorer treatment outcomes in cases of infection (Arias & Murray, 2009). Pathogenic bacteria can develop resistance vertically (Lenski, 1998) or horizontally through gene transfer (Högberg et al., 2010). These resistance genes are acquired either from the local human microbiota or from environmental microorganisms exposed to individuals (e.g., through food, water, or soil).

The resistance of pathogenic bacteria to antimicrobials has become a major issue in global health (Negara, 2014). Bacteria can become resistant to antibiotics due to improper dosing, inaccurate diagnosis, and inappropriate use of antibiotics against the causative bacteria (Sugireng & Lio, 2020). Methicillin-resistant *Staphylococcus aureus* (MRSA) is a type of *Staphylococcus aureus* that is resistant to antibiotics. MRSA can develop resistance to antibiotics through genetic mutations caused by improper use of antibiotics (Sugireng & Lio, 2020).

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antibiotics. MRSA can develop antibiotic resistance through genetic mutations caused by improper use of antibiotics (Sugireng & Lio, 2020). Acute Hepatopancreatic Necrosis Disease (AHPND) is a bacterial disease that affects shrimp. This disease has caused significant economic losses for the shrimp industry, particularly in Asian countries such as Thailand, Vietnam, and Malaysia, as well as in South America and the United States (Kumar et al., 2020). AHPND is caused by a specific strain of the Gram-negative bacterium *Vibrio parahaemolyticus* that carries a conjugative plasmid (pVA1) of approximately 69 kbp, which contains the pirABvp genes (Lee et al., 2015).

The Centers for Disease Control and Prevention (CDC) in the United States currently classifies *Candida albicans* as the third most common bloodstream pathogen in patients, with a mortality rate of up to 50% (Wisplinghoff et al., 2004). In terms of treatment, conventional azoles and polyene antifungals are commonly used to control infections caused by ***C. albicans*** (Pereira et al., 2021). However, the adaptive behavior of ***C. albicans*** allows it to form biofilms, which pose a challenge in treatment.

Method

Location

Haliclona sp. was collected with the help of scuba diving by local residents at a depth of approximately 10 meters in the waters of Sibolga, North Sumatra. During sample collection, the sponges were gathered as needed, then placed in plastic bags containing seawater and oxygen, and subsequently stored in a cool box.

Research Procedure

Isolation of Sponge Symbiont Bacteria

Haliclona sp. was cut and weighed 1 gram, then aseptically ground using a mortar and pestle. The isolation method applied was the pour plate method. The next step was dilution, with dilution ranges from 10^{-1} to 10^{-3}. The purpose of dilution is to obtain isolates that are not too dense and can represent various types of bacteria present in the sample.

The resulting bacterial suspension was then aseptically inoculated into Marine agar media in Petri dishes, and placed in an incubator at 37°C for 24 hours. Subsequently, morphological characterization was conducted, focusing on size, shape, color, and elevation, with reference to guidelines from Cappucino & Sherman (1998).

Purification of Sponge Symbiont Bacteria Haliclona sp.

Purification of sponge symbiont bacteria involves picking a single colony, while paying attention to morphological characteristics such as size, shape, color, and elevation (Cappucino & Sherman, 1998). From colonies with different morphologies, a single colony is aseptically picked and inoculated by streaking onto the same media used during initial isolation. Subsequently, it is incubated at 37°C for 24 hours. The goal of purifying bacterial isolates is to separate the inoculation results comprising various colonies of different bacterial types, thus obtaining pure bacterial colonies in each bacterial culture. The bacterial colony chosen for purification is the dominant colony (Pastra & Surbakti, 2012). The bacterial isolation process is repeated until pure isolates are obtained (Marzuki, 2018).

Fermentation and Extraction of Potential Sponge Symbiont Bacterial Isolates

The most potential isolates of sponge symbiont bacteria were each inoculated using 200 ml of marine broth media, then shaken at room temperature for 72 hours (Murniasih et al., 2018). The cultures were harvested and centrifuged to separate the pellet and supernatant using centrifugation at 6000 rpm for 15 minutes (Sunny et al., 2015). The supernatant was extracted with methanol and ethyl acetate in a 1:1 ratio (Murniasih & Rasyid, 2010). The extracts were dried using a rotary evaporator and stored in a refrigerator before conducting antimicrobial tests.

Antimicrobial Activity Testing of Haliclona sp. Sponge Symbiont Bacterial Extracts

Microbial cultures for testing were inoculated by picking a single colony and suspending it in a tube containing 5 ml of 0.9% physiological NaCl solution (Rotty et al., 2015). A cotton swab containing the test bacteria was swabbed onto the surface of Mueller-Hinton Agar (MHA) using the swab method and left for a while to allow the test bacteria to absorb into the media. Oxoid paper discs were impregnated with 50 µL of sponge symbiont bacterial extract and placed on the surface of the agar plates previously inoculated with the test bacteria. A positive control using Ciprofloxacin at a concentration of 200 µg/ml was included, and the plates were then incubated for 24 hours at 37°C. The diameter of the inhibition zones was classified into strong (> 20.0 mm), moderate (> 2.5 ≤ 20.0 mm), and weak (1.0 mm ≤ 2.5 mm) categories (Gutiérrez et al., 2019).

Data Analysis

Data were analyzed descriptively to describe the antimicrobial activity of methanol extracts from Haliclona sp. sponge symbiont bacteria.

Results and Discussion

In the antimicrobial activity test of *Haliclona sp.* sponge symbiont bacteria extracts using the antagonism

test, only one isolate was selected based on the largest clear zone in the antimicrobial activity test of bacterial supernatant, namely isolate Sp510-1(4). The criteria for antibacterial strength used in the study categorize clear zone diameters as follows: ≤ 5 mm is categorized as weak, 5-10 mm as moderate, 10-20 mm as strong, and ≥ 20 mm as very strong (Retnowati & Katili, 2023).

In Figure A, B, and C, the results of the antagonism test indicate that the clear zones formed around the three test microbes are categorized as strong (as seen in Table 1). This proves that the methanol extract from the marine sponge *Haliclona* sp. exhibits significant antibacterial activity due to containing various bioactive secondary metabolites. Previous studies have identified that this extract is effective against various Gram-positive and Gram-negative pathogenic bacteria. Secondary metabolites such as alkaloids, terpenoids, and polyphenols in this extract function by damaging the bacterial cell wall, disrupting protein synthesis, and interfering with bacterial cell membrane function (Hoppers et al., 2015).

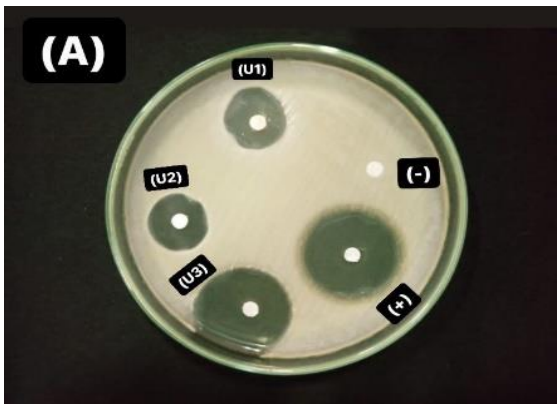

Figure 1. The results of the antagonism test of methanol extract from *Haliclona sp*. sponge symbiont bacteria against the pathogenic bacterium *S. aureus*

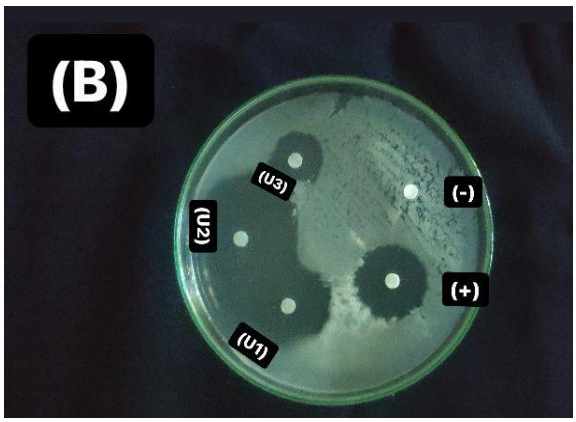

Figure 2. The results of the antagonism test of methanol extract from *Haliclona* sp. sponge symbiont bacteria against the pathogenic bacterium *V. parahalemotycus*

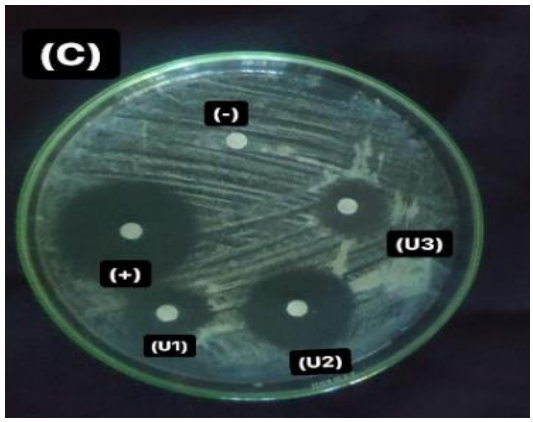

Figure 3. The results of the antagonism test of methanol extract from Haliclona sp. sponge symbiont bacteria against the pathogenic *C. albicans*

Table 1. Clear zones formed on Methanol extract of sample isolate Sp510-1(4)

Methanol Extract of Sample Isolate Sp510-1(4)	Clear Zone (mm)		
	Staphylococcus aureus	*Vibrio parahalemotycus*	*Candida albicans*
U1	25.1	20	22
U2	22.7	19.7	19.7
U3	20.4	21.6	19.3
Positive Control	40.9	40.8	31
Negative Control	0	0	0

The methanol extract of the selected isolate Sp510-1(4) has 3 replicates marked U1, U2, and U3. For *S. aureus*, U1 is 25.1 mm, U2 is 22.7 mm, and U3 is 20.4 mm, with an average of 22.7 mm. For *V. parahalemotycus*, U1 is 20 mm, U2 is 19.7 mm, and U3 is 21.6 mm, with an average of 20.4 mm. For *C. albicans*, U1 is 22 mm, U2 is 19.7 mm, and U3 is 19.3 mm, with an average of 20.3 mm.

Based on the criteria of Davis & Stout (1971), the crude extract methanol are effective extracts for inhibiting *Staphylococcus aureus* because these extracts and fractions fall into the strong category for inhibiting *Staphylococcus aureus*. Generally, Gram-positive bacteria are more sensitive to compounds with antimicrobial activity compared to Gram-negative bacteria. The difference in sensitivity between Gram-positive and Gram-negative bacteria can be attributed to differences in the cell wall structure of each type of bacteria.

The positive control showed a significant difference because it produced the highest antibacterial activity against the test bacteria compared to the negative control, extract, or test fractions. For this test, the antibiotic used was ciprofloxacin. According to Katzung (2004), ciprofloxacin is a broad-spectrum bacteriostatic antibiotic. The research results showed that the diameter of the inhibition zone formed by ciprofloxacin was larger

for the Gram-negative bacterium Escherichia coli (36.30 mm) compared to the Gram-positive bacterium Staphylococcus aureus (27.65 mm). This is consistent with Katzung (2004) statement that for most Gram-negative bacteria, ciprofloxacin only requires a concentration of 0.2-5 µg/mL, whereas for most Gram-positive bacteria, inhibition occurs at concentrations of 1-10 µg/mL. This indicates that Gram-negative bacteria are more sensitive to ciprofloxacin than Gram-positive bacteria. Ciprofloxacin works by inhibiting protein synthesis in bacterial cells. It binds reversibly to the 50S ribosomal subunit, preventing the binding of amino acids to the ribosome. Ciprofloxacin specifically binds to the acceptor site (the initial binding site of aminoacyl-tRNA) or to the peptidyl site, which is a critical binding site for peptide chain elongation (Katzung, 2004).

The negative control showed a significant difference compared to the positive control, extract, and test fractions. The negative control used, which was methanol, did not show any inhibition zones in tests against the Gram-positive bacterium *Staphylococcus aureus*, the Gram-negative bacterium *Vibrio parahaemolyticus*, and the yeast *Candida albicans*. This indicates that the control used did not affect the antimicrobial test, so the inhibition observed was not due to the solvent but rather due to the activity of compounds present in the *Haliclona* sp. sponge.

Conclusion

The methanol extract from the *Haliclona* sp. sponge symbiont bacteria, particularly isolate Sp510-$^{-1}$(4), shows strong antibacterial activity. Clear zones of 22.7 mm against *S. aureus*, 20.4 mm against *V. parahaemolyticus*, and 20.3 mm against *C. albicans* indicate the extract's effectiveness, categorized as strong.

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Author Contribution
Conceptualization, writing—original draft preparation, and visualization, U.M., D.S., and K.N.; methodology, formal analysis, and writing—review and editing U.M.; validation and investigation, D.S. and K.N.; resources, U.M. and D.S.; data curation, K.N. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest
The authors declare no conflict of interest.

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