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ANTIBACTERIAL AND ANTIFOULING POTENCY OF *Xestospongia testudinaria* EXTRACTS FROM BADI ISLAND, SPERMONDE ARCHIPELAGO

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ABSTRACT

The Sponge *Xestospongia testudinaria* belongs to the genus Xestospongia and produces various potential secondary metabolites. These secondary metabolites have been found to have potential antibacterial and antifouling compounds. The study aims to determine the antibacterial and antifouling potency of sponge *X. testudinaria*, using phytochemical and FTIR data as supporting data. Thick green extracts were used for screening antibacterial and antifouling tests. Samples with ration 1:4 w/v were macerated using *n*-hexane, ethyl acetate, and acetone for 3 x 24 h. The diffusion agar method was used to test antibacterial activities against *Vibrio* sp. and marine fouling bacteria. This study demonstrated the moderate potency of *X. testudinaria* extracts as antibacterial agents against *Vibrio* sp. The ethyl acetate crude extract of sponge *X. testudinaria* provided the highest inhibition zone against *Vibrio harveyi* (12.19 mm) at a concentration of 2 µg/25 µL. Moreover, an ethyl acetate extract with an average inhibition zone of 13.60 mm indicated the highest antifouling assay using agar diffusion. Meanwhile, phytochemical screening and FTIR were performed in the extract's presence of steroids, terpenoids, and alkaloids. In conclusion, the ethyl acetate extract of *X. testudinaria* can be one of the candidates for antibacterial drugs against *Vibrio* sp. and makes them very promising candidates for eco-friendly antifouling with further and comprehensive tests.

Keywords: Sponge**,** *Xestospongia Testudinaria*, Spermonde Archipelago, Antibacterial, *Vibrio* Sp., Antifouling.
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INTRODUCTION

There are approximately 9,000 known species of sponge in the world. According to the World Porifera Database, The Sulawesi Sea or Makassar Strait was reported which has 128 species of sponges (17 species of Calcarea, 97 species of Demospongiae, 13 species of Hexactinellida, and a species of Homoscleromorpha).[1] The order Poecilosclerida, which consists of 31 species, is the primary representative of the class Demospongiae. In addition, according to the most recent ecological study, the Spermonde Archipelago in Southwest Sulawesi has a larger beta diversity of sponge species.[2,3] Marine organisms, sponges, are potential sources of novel therapeutic medicines since they generate various chemicals with different bioactivities.[4] The Demospongiae class is the most abundant, approximately 90% of sponge species. One of the most researched genera within the Demospongiae class is Xestospongia. The genus Xestospongia is widely distributed worldwide, from the Indo-Pacific to the Caribbean, especially in Southeast Asia. In the Southeast Asian Sea, *Xestospongia* spp. has been reported to produce potential secondary metabolites, including alkaloids, steroids, fatty acids, quinone, etc. These compounds have been found to have antimicrobial agents.[5–7] The antibacterial activities of sponges have been studied in the world. Previous studies on antibacterial activities have been conducted only against human pathogens. Sponge *X. testudinaria* has potential antibacterial activity against various human bacterial pathogens.[8–10] Aaptamine, polyacetylenes, and sterols support antibacterial activities in the genus Xestospongia. These compounds have been isolated from Xestospongia and are active antibacterial agents against human pathogens.[11,12] However, all previous studies focused on biological activities against human pathogens. To our knowledge, studies concerning antibacterial activities against *Vibrio* sp. have rarely been conducted. Vibriosis is a bacterial disease that can cause death in shrimp and/or fish farming. This disease is a major problem in

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aquaculture, where cases are still quite high in South Sulawesi Province, Indonesia. Gram-negative bacteria from the Vibrionaceae family and the Vibrio genus cause various agents that cause vibriosis in shrimp or fish.[13,14] *Vibrio* sp. growth can be controlled with the quorum, and farmers often use antibiotics. However, antibiotics are no longer recommended because they have side effects on fish and consumers. This study initially sought alternative antibiotics from natural sources, such as sponges, regarding Vibriosis. Besides vibriosis, biofouling—the undesirable accumulation of organisms on submerged surfaces—is a major problem for several industries, including water monitoring, aquaculture, and marine transportation.[15] Along with negatively affecting infrastructure and machinery functioning, this problem has negative economic and environmental effects.[16] The attachment of fouling organisms has caused significant losses and shortened the service life of various marine structures, such as ships, docks, pilings, and offshore drilling support structures. The antifouling paints that developed in the mid-nineteenth century contained arsenic and mercury oxide as biocides. Another antifouling with paints containing tributyltin (TBT) was treated in the marine environment and human health.[17–20] With an increasing emphasis on investigating natural products as alternatives to conventional chemical-based approaches, the scientific community has been actively searching for efficient and environmentally acceptable antifouling solutions to address this urgent issue.[21] The special qualities of marine sponges have gained more attention recently as strong antifouling agents.[22,23] Sponges are a significant source of biomaterials and bioactive natural products that can find applications as antifouling products. They have evolved unique antifouling strategies that may contain potential solutions to this problem. Moreover, the study of sponges, especially species of *X. testudinaria*, as antifouling is still relatively limited.[18,19] Measuring the inhibition zone for antibacterial and antifouling tests is a preliminary procedure for finding drugs. Therefore, the research aimed to determine crude extracts of *X. testudinaria* from Badi Island, Spermonde Archipelago, Indonesia, against *Vibrio* sp. and marine fouling bacteria with phytochemical screening and FTIR. The results of this study are expected to be used as supporting data for further isolation of chemicals and the discovery of new and promising antibacterial and antifouling candidates.

EXPERIMENTAL

Sampling

Sponge *X. testudinaria* was collected by hand with scuba diving on the inner reef at 10-18 m depth on Badi Island, Makassar City, South Sulawesi Province, Indonesia. The samples were washed with seawater, freshwater, and distilled water to remove salt, epiphytes, and other impurities. After cleaning, the samples were weighed in wet and dried. The sample processing was conducted at the Faculty of Marine Science and Fisheries, Hasanuddin University, South Sulawesi Province, Indonesia.

Preparation of Extractions

Sample (sponges) (100 g) were cut into small pieces and made into powder form using the grinder. Then, samples with ration 1:4 w/v were macerated using *n*-hexane, ethyl acetate, and acetone for 3 x 24 h at room and stirred every 24 h. After that, the macerate from each solvent was filtered using Whatman filter paper (Cytiva, Sweden) and concentrated using a rotary vacuum evaporator (Buchi®, Switzerland) at 50°C until crude extracts were obtained.

Isolation of Marine Fouling Bacteria

Biofilm bacteria were isolated using a modified method of [17]. At the lowest low tide, a 15 x 10 cm wood panel is attached to the water substrate 50 cm below sea level. After 2 weeks of immersion, the panel is put into the cool box and brought to the laboratory to isolate biofilm bacteria. The panel is sprayed with sterile seawater, and then the panel surface is swabbed with a sterile cotton swab to Tryptic Soy Broth (TSB, Merck, Germany).

Antibacterial Test

Bacterial species (*Vibrio harveyi* (M-120), *V. alginolyticus* (B-425), and *V. parahaemolyticus* (T-170)) were gained from The Research Institute of Center for Brackish Water Aquaculture, Takalar, and Maros, South Sulawesi Province, Indonesia. The suspension's turbidity was standardized against 0.5 McFarland using a spectrophotometer at 600 nm. The bacterial inoculum was 10^8 cfu/ml. The sterilized Tryptic Soy Agar (TSA, Merck, Germany) media is cooled to 40°C. Then, about 5% bacterial culture of the agar volume

is added and poured into a petri dish (Pyrex, Japan).[24] Positive, and negative controls and crude extracts were used for antibacterial tests. The positive control test was performed using ciprofloxacin antibiotics, and dimethyl sulfoxide (DMSO, Merck, 802912) was used as the negative control. Meanwhile, about 4% of antifouling paint is used as positive control in the antifouling test. The agar diffusion method was applied in this test with slight modifications.[25,26] The blank discs (Oxoid, UK) with a diameter of 6 mm were placed on the bacterial medium's surface after soaking in the extracts at a concentration of 200 µg /disc. The samples were incubated for 24 h at 30°C. Distinct zones around the paper disc are signs of bacterial activities. All experiments were conducted in triplicates. Inhibition zone classifications are >15 mm as strong, 8–15 mm as moderate, and 1–8 mm as weak activities.[27]

Phytochemical Screening

Phytochemical screening was performed using Wagner, Meyer, and Dragendorf reagents to determine the alkaloid, Liebermann-Buchard reagent for steroids/terpenoids, and $FeCl_3$ flavonoid reagent.[28]

FTIR

Infrared resonance analysis characterization of functional groups in the sample used an FTIR spectrophotometer (FTIR 8501 Shimadzu).

Analysis Data

All experiments were performed with three replicates and presented as means ± standard deviation (S.D.). Statistical differences were tested by one-way analysis of variance (ANOVA) ($p<0.05$).

RESULTS AND DISCUSSION

Identification of Sponge

Sponge *X. testudinaria* was collected from Badi Island, Pangkep District, Indonesia, at a latitude of 4°58'21.976224 and a longitude of 119°17'4.923946. Badi Island is part of the Spermonde Islands group, which stretches along the southern Makassar Strait. Administratively, this oval-shaped island is in Mattiro Deceng Village, Liukang Tupabbiring District, Pangkajene Islands Regency, South Sulawesi Province, Indonesia. Genus Xestospongia thrives under various substrates like sand, rock, and dead coral rubble. This type is mainly found in depths over 10 m from the sea surface. It also has light blue at the external part and greyish yellow at the internal part as long as it is still alive.[8,29] The species *X. testudinaria* is a sponge from the Petrosiidae family. The color of this sponge is pink, with a massive growth form (large and compact structure) and rough, brittle surface consistency. The spicules are mega sclera oxea and strongyle. This species has a scent pungent smell. The distribution of this type can be found in all coral reef zones (lower fore reef, upper fore reef, and reef crest). *X. testudinaria* is a member of the demosponge class, which dominates more than 90% of the sponge types in the Spermonde Islands.[30] The secondary metabolites produced by sponges, especially *X. testudinaria*, have the potential antibacterial and antifouling to be used as raw materials for medicine and have toxicity that does not interfere with the presence of surrounding biota.

Antibacterial Test

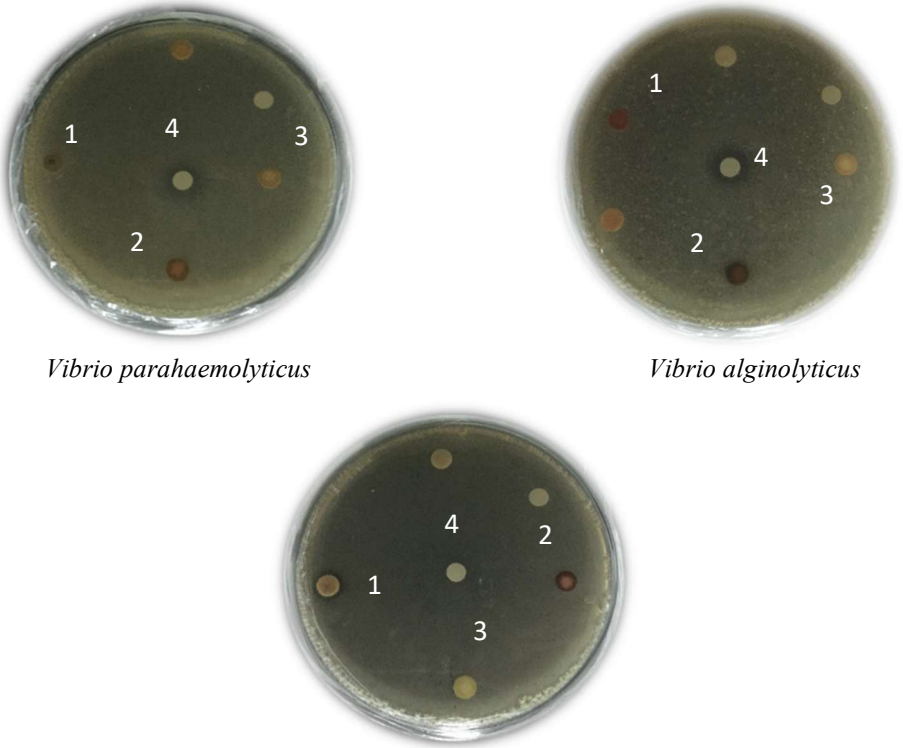
The antibacterial assay demonstrated that the crude *n*-hexane and ethyl acetate extracts inhibited the growth of three Gram-negative bacteria (Table-1 and Fig.-1). Compared to other extracts, the highest inhibition value was 12.19 mm in the ethyl acetate extract against *Vibrio harveyi*.

Table-1: Average Zone of Inhibition for Crude Extracts

Test samples	Inhibitory Zone (mm)		
	Va	Vp	Vh
N-hexane	8.91 ± 0.7^{b}	7.40 ± 1.03^{ab}	8.29 ± 1.23^{b}
Ethyl acetate	9.77 ± 0.28^{b}	8.01 ± 0.14^{b}	12.19 ± 0.55^{c}
Acetone	6.00 ± 0^{a}	6.00 ± 0^{a}	6.00 ± 0^{a}
Ciprofloxacin (Positive Control)	9.87 ± 1.88	11.53 ± 0.15	16.90 ± 1.02

Values are mean ± S.D.; Va (*Vibrio alginolyticus)*, Vp (*V. parahaemolyticus*), Vh (*V. harveyi*)

While the highest inhibition value of *V. alginolyticus* was observed with the *n*-hexane extract, the value was 8.91 mm. The crude acetone extract did not show antibacterial activity against any of the three Gram-negative bacteria. The value of the inhibition zone belongs to the moderate category.[27] This result was supported by a study that used microsymbionts derived from some sponges from Bali and Jepara, Indonesia, which showed potential antibacterial against *Vibrio* sp.[31]



Vibrio parahaemolyticus *Vibrio alginolyticus*

Vibrio harveyi

Fig.-1: The Result of The Antibacterial Test: *N*-Hexane (1), Ethyl Acetate (2), Acetone (3), and Positive Control (4) Against *Vibrio* sp.

The antibacterial activities of the ethyl acetate extract of sponge *X. testudinaria*, Badi Island, are like those of *X. testudinaria* from the Vietnamese Sea, which contains steroid compounds. These compounds are a form of sterols: a new sterol, langkosterol A, and two known sterols, xestosterol, and 24-hidroperoksi-24-vinil cholesterol. These compounds are active as antibacterial agents.[32] Besides, previous studies related to antibacterial showed that alkaloid compounds, amphetamine, isoaptamine, dimethyl (oxy) amphetamine, and dimethyl ketal isolated from the *X. testudinaria* have antibacterial activity against Gram-positive and *Staphylococcus aureus*. Meanwhile, *Escherichia coli* (Gram-negative) with MICs of 25, 6, 25, and <100 µg/mL, respectively.[11] The xestoquinol sulfate compound isolated from one of the Xestospongia genera also has antibacterial activity, with an IC50 of 125 µM against *S. aureus*.[9]

Antifouling Test

The antifouling test results of crude extracts against marine fouling bacteria can be seen in Fig.-2 and Fig.-3. The figures showed the formation of a zone inhibiting bacterial growth, which indicated antifouling activities. Ethyl acetate extracts with an average inhibition zone of 13.60 mm indicated the highest antifouling assay using agar diffusion. Hence, crude acetone extracts did not show antifouling activities (6.00 mm). Ethyl acetate extract has a broad spectrum as an antimicrofouling agent, which can inhibit various types of biofilm bacteria. It has the potential as an antifouling agent because it can inhibit various biofilm bacteria that cause microfouling.

Sponge *X. testudinaria* also exhibited potential as an antifouling agent, especially ethyl acetate and *n*-hexane extract. The results of this work are not only antibacterial but also contribute to increasing knowledge of natural sources for antifouling products.

Fig.-2: The Result of The Antifouling: *N*-Hexane (1), Ethyl Acetate (2), Acetone (3) Extracts, and Positive Control (4)

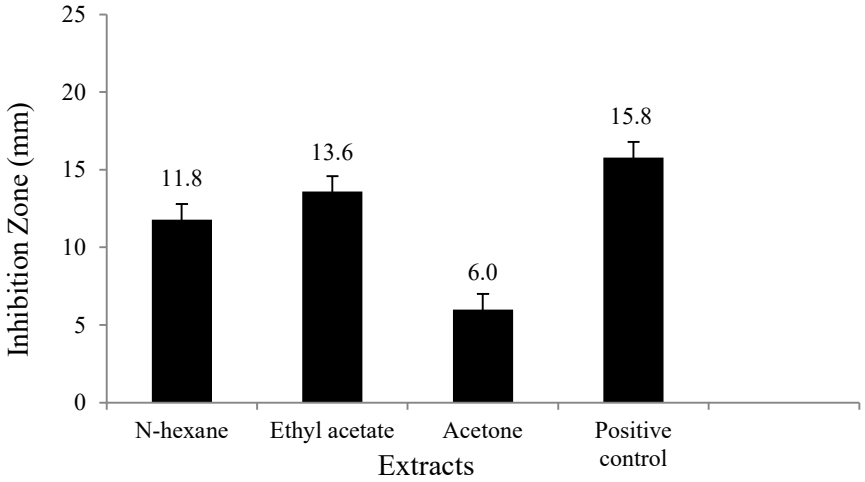


Fig.-3: The Average of Inhibition Zone Measurement in The Antifouling Test

Several previous studies have reported that sponges have inhibitory effects against marine fouling organisms for space during their life cycle. Moreover, the Mediterranean Sea on sponges of the family Irciniidae showed inhibition activities of mussel settlement (68%), and algal growth (>60%) caused by *Ircinia oros* extract at 30 µg/mL. Meanwhile, methanol extracts of *Haliclona* (*Soestella*) *caerulea* and *Ircinia* sp. (200 µg/mL) exhibited mussel settlement (35–45%).[18,33]

The other studies focusing on barnacles have investigated the antifouling activity of different Mediterranean sponge extracts (*I. oros*, *Sarcotragus spinosulus*, *Dysidea* sp., *Scalarispongia scalaris*, *Hippospongia communis*, with about 60% of barnacle settlement inhibition at a concentration of 100 µg/mL.[34] Following the results of the present study, the extract of *X. testudinaria* contains metabolites with antifouling activity that affect organisms. Metabolites proved effective in preventing mussel settling and inhibiting bacteria growth, and they were responsible for forming a film that facilitates further substrate colonization.[35]

Phytochemical Screening and FTIR

The results obtained were a green color change for Lieberman-Burchard (it means the presence of steroids) and a brown precipitate for Wagner, as in Table-2. The formation of a light brown precipitate indicates positive results for alkaloids in the Wagner test. The precipitate is potassium alkaloid, which makes Wagner's reagent, iodine, react with I^- from potassium iodide to produce I^{3-} ions that are brown. In the Wagner test, the K^+ metal ion will form a coordinate covalent bond with the nitrogen in the alkaloid to form a potassium-alkaloid complex that precipitates. The phytochemical test was intended to determine the class of secondary metabolite compounds in the extract.

Based on the IR spectrum data of ethyl acetate extract (Fig.-4) showed the presence of alkaloid groups, namely NH, indicated by the presence of N-H (str) bond absorption (3514 cm^{-1}), which is supported by the presence of C-N (str) bond absorption (1261 cm^{-1}). Absorption in the area of 2956 cm^{-1} and 2924 cm^{-1} shows the presence of CH sp^2 (str) and CH sp^3 (str) bonds supported by the absorption of CH_2 (bend) groups (1462 cm^{-1}) and CH_3 groups (1379 cm^{-1}). The absorption of the carbonyl ketone group C = O is also present

in the area of 1716 cm^{-1}. A phytochemical test and FTIR exhibited the compound class of the extracts. Most of the compounds are alkaloids, terpenoids, and steroids. Some alkaloids have been isolated from Xestospongia, including 3-alkyl pyridine alkaloid, captain, and manzamin C.[11,36,37]

Table-2: Results of Phytochemical Screening

No	Phytochemical	Extracts		
		N-hexane	Ethyl acetate	Acetone
1	Wegner	+	+	+
2	Meyer	-	-	-
3	Dragendorf	-	-	-
4	$FeCl_3$	-	-	-
5	Lieberman-Buchard	+	+	+

(-) Negative, (+) Weak positive, (++) Strong positive

Meanwhile, the results of steroid/terpenoids showed red and blue colors that were added to Lieberman-Buchard, which means positive. Some sterols have been isolated from Xestospongia.[32,38,39] In addition, a previous study in which four novel alkaloids of the amphetamine class were isolated supports the presence of antibacterial activity. These include amphetamine, isoaptamine, dimethyl (oxy) amphetamine, and its dimethyl ketal from an unidentified species of the Indonesian marine sponge of the genus Xestospongia. These novel compounds displayed antibacterial activity.[11] Marine metabolites exhibiting antifouling activities have also been reported: terpenoids, steroids, saponins, fatty acid-related compounds, bromotyrosine derivatives, and heterocyclic compounds. Agelasidine A was also the most promising compound identified as a potential antifouling agent.[19,34]

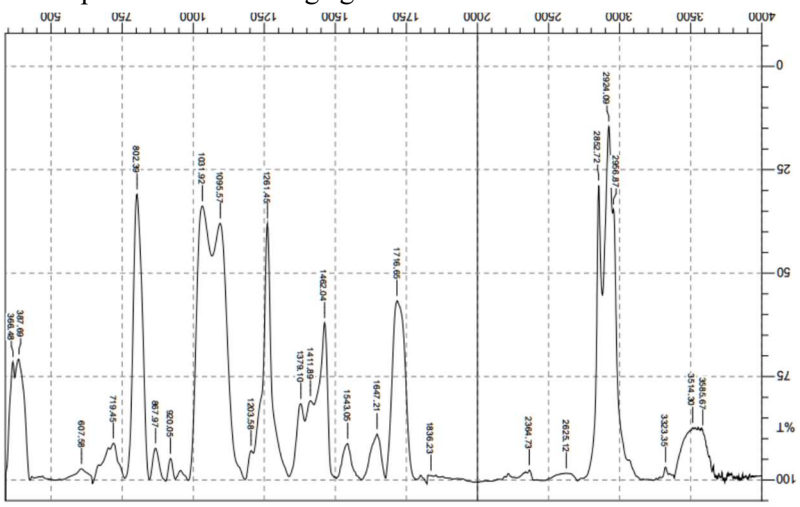


Fig.-4: FTIR Spectrum of Ethyl Acetate Extract of *Xestospongia testudinaria*

Controlling biofouling involves inhibiting the attachment process of larvae from invertebrates or spores from algae (microfouling). If the formation of biofilm bacteria (microfouling) can be inhibited in the early stages, it might also inhibit the attachment of macrofouling, and then biofouling can be controlled. More specifically, separation and structural elucidation are needed to reveal which compounds are bioactive against *Vibrio* sp. and marine fouling bacteria. Pure compounds are expected to exhibit potential biological activities and chemical diversity. Information regarding secondary metabolites potency of *X. testudinaria* can be used as a source of nutritious chemicals, particularly for treating diseases caused by pathogenic bacteria against *Vibrio* sp., and potentially be an environmentally friendly antifouling agent.

CONCLUSION

N-hexane and ethyl acetate crude extracts of *Xestospongia testudinaria* from Badi Island, South Sulawesi Province, Indonesia, are effective antibacterial agents against *Vibrio* sp. and antifouling against marine fouling bacteria. The ethyl acetate crude extract showed the highest inhibition zone among the extracts. In

addition, steroids, terpenoids, and alkaloid compounds were presented in FTIR and phytochemical tests. Moreover, further isolated compounds are required to identify these compounds specifically.

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CONFLICT OF INTERESTS

All authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing it, and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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