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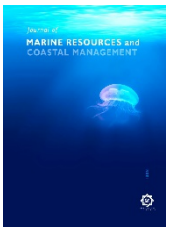

Antibacterial activities of the extracts of sponge *Agelas cervicornis* agains bacteria *Staphylococcus aureus*

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ABSTRACT

Infection is a health problem that can cause death in the world. An antibiotics is a treatment used to treat infectious diseases. If antibiotics are used continuously, they can cause resistance. One of the bacteria that causes skin infections and has been reported to be resistant to several antibacterials is Staphylococcus aureus. So we need an alternative to exploring antibacterial compounds derived from the ocean. Sponge extract from the genus *Agelas* has been reported to contain secondary metabolites that are antibacterial. This research aims to determine the antibacterial potential of sponge extract and the secondary metabolite of sponge extract, *Agelas cervicornis*. The extraction process uses maceration with methanol as a solvent. The tests carried out in this study included a phytochemical screening test and an antibacterial activity test carried out using the disc diffusion method to determine the inhibition zone produced against the pathogenic bacteria *Staphylococcus aureus*. The results of phytochemical screening showed that the sponge extract of *Agelas cervicornis* contained flavonoids, alkaloids, and terpenoids. The results of the antibacterial activity test of the sponge extract of *Agelas cervicornis* showed that all concentrations of 0.5 mg/ml, 1 mg/ml, and 1.5 mg/ml had weak inhibitory properties.

Keywords: *Agelas cervicornis*, antibacterial, sponge, *Staphylococcus aureus*

INTRODUCTION

Infections are an important health problem and the biggest cause of death in the world. One of the causes of infection is microorganisms. The most effective treatment in the case of infection is the use of antibiotics [1]. However, the use of antibacterials such as antibiotics derived from chemical compounds can cause bacterial resistance. *Staphylococcus aureus* has been reported to be resistant to oxacillin, penicillin, and other beta-lactam antibiotics [2]. Therefore, it is necessary to explore antibacterial compounds that come from natural ingredients and contain bioactive substances that can work as antibiotics [3].

Exploration for the use of natural materials is generally taken from land areas, while natural materials located in ocean areas have not been explored optimally. Some marine biota based on previous research that produces bioactive compounds are soft corals, mollusks, tunicates, bryozoans, and sponges [4].

In general, sponges appear to have no protection because they lack locomotion due to their sedentary life. Its survival relies on a constant flow of water to obtain food and oxygen. In addition, seawater has a fairly high potential for pollution. This makes sponges have an extra defense for their survival, so they are known to contain a variety of bioactive compounds [5]. The content of bioactive compounds that are rich in secondary metabolites in sponges is known to be able to ward off and inhibit pathogenic bacteria [6].

The sponge metabolite extract contains active compounds including peptides, terpenoids, steroids, acetogenins, alkaloids, cyclic halides, acetylenic phenolics, and other nitrogen compounds [7]. Several sponges belonging to the genus *Agelas* have been reported to have antibacterial activity. One example is in research, *Agelas cavernosa* sponge can inhibit the growth of pathogenic bacteria like *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. This allows other species in this genus to have secondary metabolites capable of inhibiting pathogenic bacteria [3].

Sponge sampling was carried out based on a preliminary survey and local stakeholders. The *Agelas cervicornis* type of sponge is known to be abundant in the waters of Kampung Kerapu, Situbondo Regency. Therefore, based on the abundant potential of sponges in the waters of Kampung Kerapu, this research aims to develop marine sponges of Kampung Kerapu that have high bioactive compounds as antibacterials.

METHODS

Research Location

The research was conducted from August 2021 to February 2022. The preliminary survey was conducted from August 24th-26th, 2021. Sampling was carried out on

September 16-18th, 2021 at the waters of Kampung Kerapu, Situbondo (Figure-1). Preparation of *Agelas cervicornis* sponge extract, antibacterial activity test, and phytochemical test were carried out at the Integration Laboratory of Sunan Ampel Islamic University, Surabaya.

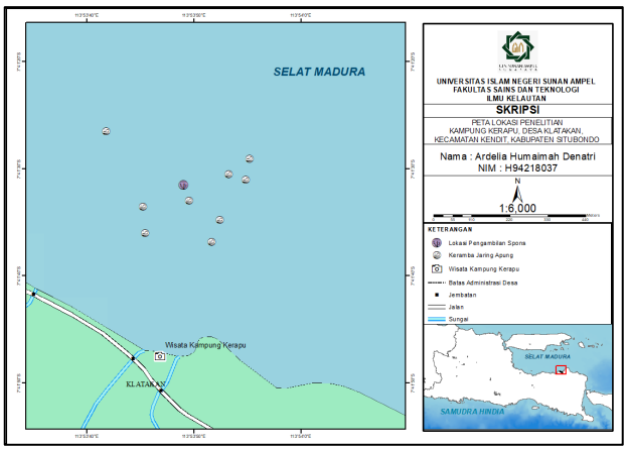


Figure-1. Sampling location maps

Sampling

A sampling of sponges was carried out using masks, snorkels, fins, zip locks, scissors, and cameras. After the sponges sample was successfully removed from the water, it was sprayed using sterile seawater and then stored in a callbox containing ice cubes and not exposed to direct sunlight.

Tools and Materials

The equipment used in this study was autoclave, bottles, vials, bunsen, petri dishes, coolbox, cotton swabs, funnels, erlenmeyer, beakers, measuring cups, scissors, hotplate, incubator, caliper, ossicle needle, camera, cotton, filter paper, laminar airflow, micropipette, mortar and pestle, tweezers, dropper pipette, plastic wrap, ziplock plastic, test tube rack, rotary evaporator, spreader, test tube, scale, and vortex mixer.

The basic ingredients used were sterile seawater, alcohol 70%, distilled water, Dragendorff, ice cubes, Ferric Chloride (FeCl) 1%, paper disc, Liebermann Burcand, methanol 96%, Nutrient Broth, *Agelas cervicornis*, *Staphylococcus aureus*, and Zobell Marine Agar.

Sterilization of Tools and Materials

Sterilization of tools is carried out before to avoid contamination when grown. The wet sterilization process was carried out with the help of a steam autoclave at 121°C for 15 minutes. The use of this temperature is because the pressure of 1 atm is equal to sea level [8].

Extraction of Sponge *Agelas cervicornis*

Extraction was carried out using the maceration method with methanol 96% as the solvent. The sample was weighed ±500gr, cut into small pieces, and then soaked in solvent for 1×24 hours with three repetitions at room temperature. After 1×24 hours, the solution was filtered using *Whatman* paper and then collected in bottles. Then the sample was soaked again with solvent and the process was repeated for up to 3×24 hours. The filtrate obtained was then evaporated using a rotary vacuum evaporator at a temperature of 40°C until the methanol separated and the thick extract remaining from the *Agelas cervicornis* sponge was then weighed.

Phytochemical Screening Test

Agelas cervicornis extract was subjected to phytochemical screening to ascertain the presence of secondary metabolites like flavonoids, alkaloids, terpenoids, steroids, saponins, and polyphenols.

1. Flavonoid test
   After wheighing 20 mg of *Agelas cervicornis* sponge extract, a few drops of a 10 percent NaOH solution were then added. If the sample turns a dark shade of black, brown, red, green, blue, or purple, flavonoids are present [9]
2. Alkaloid test
   Agelas cervicornis sponge extract in the amount of 20 mg was added to a test tube along with a few drops of HCl solution and thoroughly mixed. A few drops of Dragendorff's solution are also added; if a brick-red precipitate forms, the sample is positive for alkaloid chemicals [10]
3. Terpenoid and steroid test
   20mg of the extract *Agelas cervicornis* was placed in a test tube, and then Liebermann Burcand's solution was added in a few drops. If the color of the extracted sample changes to purple or red, this means terpenoid metabolites are present. However, if the hue changes to blue or green, it means steroid chemicals are present [11].
4. Saponin test
   Agelas cervicornis extract 20 mg was weighed into a test tube, 2 ml of hot water was added, and the test tube was shaken vertically. If samples produce lasting foam or bubbles after being shaken, they are considered to contain saponins [12].
5. Polifenol test
   Agelas cervicornis sponge extract, in the amount of 20 mg, was added to a test tube along with 10 drops of a 1 percent solution of $FeCl_3$. A color shift in the sample to purple, blue, or black will indicate the presence of phenol [13].

Antibacterial Activity Test

Paper disc with a 6 mm diameter that was subjected to three doses of 0.5 mg/ml, 1 mg/ml, and 1.5 mg/ml three times each. Chloramphenicol was employed as the positive control, and methanol was used as the negative control. After that, it was kept at 37°C for 24 and 48 hours for observation of the inhibitory zone.

Data Analysis

Following evaporation, the extract collected during the maceration process is determined as the yield value using Equation (1) [14].

$$Rendemen = \frac{Extract\ weight}{Material\ weight}\ x\ 100\% \qquad (1)$$

Antibacterial potential can be said to be high if at low concentrations it has large inhibitory power. The criteria for classifying antibacterial strength are as follows [15]: (a) Diameter >20 mm is *very strong*, (b) Diameter 10 – 20 mm is *strong*, (c) Diameter 5 – 10 mm is *medium*, and (d)

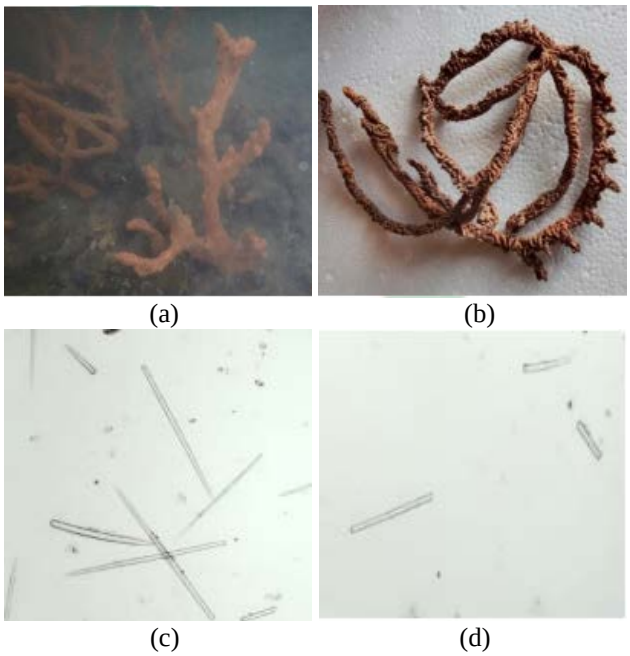
Diameter 0 – 5 mm is *weak*. After passing the homogeneity and normality tests, the data were then used to test statistical analysis for the diameter zone using the One Way-ANOVA test.

RESULTS AND DISCUSSIONS

Identification of sponge types is done by observing the morphology and shape of the spicules with the help of a microscope. Verification of the morphological form is carried out by comparing the documentation results with the sponge identification portal on the pages www.spongeguide.uncw.edu and www.marinespesies.org.

The sponge sample when in water is orange (Figure-2a) and dark brown when on land (Figure-2b). The consistency of the sample body is stiff, hard and difficult to tear. The sample has a long, erect, branched and cylindrical body. The spicules of the sponge samples observed were in the form of monaxon styloid (Figure-2c) and monaxon strongyle (Figure-2d).

The *Agelas cervicornis* species usually has a long, erect, branched and cylindrical body. It has a diameter of around 3 – 6 cm with a length of 50 – 100 cm. In general, it has a dark brown outer color and an orange inner color with a stiff body consistency, difficult to tear and hard. The spicules of this species are monaxon styloid and strongyle with acanthostyle fibers [16].



(a) (b) (c) (d)

Figure-2. (a) *Agelas cervicornis* sponge samples in water, (b) samples after being taken from the sea (c) monaxon styloid spicules, and (d) monaxon strongyle spicules

Rendemen Extract *Agelas cervicornis*

In this research, extract *Agelas cervicornis* produced a brown-green with 8.2 grams of weight. *Agelas cervicornis* sponge extraction findings revealed a low percentage of 1.64% (Table-1). The high yield number indicates that more extract was produced. Additionally, the yield is influenced by the active substances that are present. Therefore, if the yield is low, less extract is produced and there are fewer active chemicals in the extract as well [17].

Table-1. Rendemen extract of *Agelas cervicornis*

Extract	Sample Weight (gram)	Extract Weight (gram)	Rendemen (%)
Agelas cervicornis	500	8.2	1.64

The length of the maceration process and the temperature have an impact on the extraction yield size. More yield will result from maceration that lasts longer. This is due to the length of time that passes between the sample and the solvent's interactions, allowing for the breakdown of more secondary metabolites [18]. Temperature also has an impact on the amount of extract yield produced in addition to time. The sample's condition must be considered while choosing the temperature to prevent volatile chemicals from evaporating at high temperatures. Room temperature is the ideal setting for the maceration process [19].

Phytochemical Test

Agelas cervicornis sponge extract underwent a phytochemical analysis to determine the kinds of secondary metabolites it contains. In the science of pharmacology, secondary metabolites are frequently utilized as antibacterials. The change in hue of the sample solution was used to qualitatively conduct phytochemical testing. The usage of specific reagent types will result in color variations. The resulting modifications are compared to the predetermined standards [20].

Table-2 shows the examination of the Agelas cervicornis sponge extract's phytochemical test findings based on the color modifications that resulted from the addition of reagents for the flavonoid, alkaloid, terpenoid/steroid, saponin, and polyphenol chemicals groups.

The amount of chemicals found in the *Agelas cervicornis* sponge extract was significantly affected by the solvent employed in the extraction method. Methanol was employed as a solvent in this study. The ability of a substance to dissolve in the extraction solvent has a significant impact on the substance's efficacy [21] Methanol is a polar compound with a CH_3OH molecular structure with a hydroxyl group (-OH) and a metal group ($-CH_3$) that can attract polar, semi-polar, and non-polar compounds [22].

Antibacterial Activity Test

If the *Agelas cervicornis* sponge extract exhibits an inhibition against *Staphylococcus aureus* bacteria, which is demonstrated by the existence of a clear zone surrounding the paper disc, the antibacterial activity tests can be stated to be potentially effective. The effect of the extract on the microorganisms under test is evident in the clear zone.

Table-3 shows the findings of the evaluation of the *Agelas cervicornis* sponge extract's antibacterial inhibition zone against *Staphylococcus aureus* after 24 hours of observation. Table-4 shows the findings from the evaluation of the *Agelas cervicornis* sponge extract's antibacterial inhibition zone against *Staphylococcus aureus* after 48 hours of observation.

Table-2. Phytochemical test results of Agelas cervicornis extract

Compound	Reactor	Color Change Referances	Color Change of Sample	Results
Flavonoid	NaOH	Black, brown	Brownish red	+
Alkaloid	HCl + Dragendorff	Red prccipitate	Red prccipitate	+
Terpenoid/Steroid	Liebermann Burcand	Orange/blue	Brownish orange	+
Saponin	Hot Water	Foam	Doesn't foam	-
Polifenol	$FeCl_3$ 1%	Purple, Blue	Brownish	-

Table-3. The results of the 24-hour observation inhibition test

Concentration	Average diameter of the inhibition zone ± SD (mm)	Antibacterial strength
Chloramphenicol (positive control)	11.03 ± 0.47	Strong
Methanol (negative control)	0.00 ± 0.00	None
Aquades	0.00 ± 0.00	None
0.5 mg/ml	0.07 ± 0.05	Weak
1 mg/ml	1.10 ± 0.10	Weak
1.5 mg/ml	2.05 ± 0.05	Weak

Table-4. The results of the 48-hour observation inhibition test

Concentration	Average diameter of the inhibition zone ± SD (mm)	Antibacterial strength
Chloramphenicol (positive control)	11.75 ± 0.47	Strong
Methanol (negative control)	0.00 ± 0.00	None
Aquades	0.00 ± 0.00	None
0.5 mg/ml	0.13 ± 0.05	Weak
1 mg/ml	1.32 ± 0.10	Weak
1.5 mg/ml	2.22 ± 0.10	Weak

Agelas cervicornis sponge extract was tested for its antibacterial activity at three different doses, and the results indicate that it contains antibacterial chemicals with modest inhibitory power. The choice of the test concentration for the extract utilized is one of many variables that can affect this. Because the active chemicals or secondary metabolites in an extract with a high concentration will be more important than those in extracts with a low concentration, this will have an impact on the inhibitory power of antibacterial agents [23].

The amount of extract present also influences how quickly bacteria die. If the extract is administered at a high concentration and contains secondary metabolites, it will block the synthesis of bacterial cell walls, cell membranes, proteins, and nucleic acids, hastening the death of the bacteria [24].

Bacteriostatic and bactericidal types of antibiotics are separated based on how they work. The 24- and 48-hour observations were used to classify the antibacterial characteristics. Samples are considered bacteriostatic if they briefly bind to microbial cells to prevent their metabolism from developing, because when the concentration or stability drops, bacteria can repopulate. On the other hand, if the sample can stop growth and even result in microbial cell death, it can be classified as bactericidal [25].

In the positive control treatment, it was seen that there was a wider zone of inhibition compared to the *Agelas cervicornis* sponge extract used in the test. Chloramphenicol was chosen as a positive control because its activity can inhibit protein synthesis that takes place in ribosomes [26]. So in testing it was proven that the diameter of the inhibition zone was larger and inhibited the growth of *Staphylococcus aureus* bacteria.

The *Agelas cervicornis* sponge extract, which was extracted using methanol as a solvent and had concentrations of 0.5 mg/ml, 1 mg/ml, and 1.5 mg/ml, as well as a positive control in the form of chloramphenicol, had bactericidal activity, according to the study's findings based on the observation of the inhibition zones for 24 hours and 48 hours.

Statistical Test

Following the normality and homogeneity tests, which revealed that the data had a normal and homogeneous distribution, it was determined that H_0 is accepted and that the average inhibition zone of the *Agelas cervicornis* sponge extract at various concentrations did not differ. This was confirmed by the Anova test, which had a significance value of 0.941 and indicated that the value was >0.05. As a result, there is no real difference in the average inhibition zone. The ANOVA test findings reveal a value of >0.05 as a result of the selection of concentrations being substantially near to one another.

CONCLUSIONS

The presence of an inhibitory zone surrounding the paper disc suggests that the *Agelas cervicornis* sponge extract has antibacterial ability against the pathogenic bacteria *Staphylococcus aureus* in the waters of Kampung Kerapu. Concentrations of 0.5 mg/ml, 1 mg/ml, and 1.5 mg/ml displayed modest inhibition-level antibacterial action. A group of flavonoids, alkaloids, and terpenoids make up the secondary metabolites in the *Agelas cervicornis* sponge extract from the waters of Kampung Kerapu.

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