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Research Article

ANTIBACTERIAL AND ANTICANCER ACTIVITY OF PROTEIN SPONGES COLLECTED FROM THE WATERS OF KAPOPOSANG ISLAND OF SOUTH SULAWESI, INDONESIA

Andis Sugrani [1], Hasnah Natsir [2], M. Natsir Djide [3], and Ahyar Ahmad [2]*
[1]Health Analyst Program, Institute of Health Sciences Mega Rezky Makassar, Antang Raya Street No. 43, Makassar 90245, Indonesia
[2]Chemistry Department, Mathematic and Natural Science Faculty, Hasanuddin University, Perintis Kemerdekaan Street, Km 10 Tamalanrea, Makassar 90245, Indonesia
[3]Faculty of Pharmacy, Hasanuddin University, Perintis Kemerdekaan street, Km10 Tamalanrea, Makassar 90245, Indonesia
*Corresponding Author Email: ahyarahmad@gmail.com

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ABSTRACT

This study reported the protein bioactivity of two sponges *Haliclona fascigera* and *Clathria reinwardti,* collected from the waters of Kapoposang island of South Sulawesi in the search for potential new drugs. Protein extracts were subjected to antibacterial activity test and anticancer activity screening. Isolation of the protein fraction was carried out using a polar solvent (Tris-HCl buffer 0.1 M). Purification of protein was done by fractionation method using ammonium sulphate followed by dialysis and finally determination of protein content was carried out by Lowry method. An Antibacterial activity tests includes diffusion method using paper disc and anticancer activity screening with Brine Shrimp Lethality Test (BSLT). The results showed that protein from sponge *Clathria reinwardtii* (0-20% fraction) and sponge *Haliclona fascigera* (20-40% fraction) were effective in inhibiting the growth of *Staphylococcus aureus* and *Escherichia coli* bacteria, but not effectively as new antibacterial agents. The most toxic anticancer activity screening for shrimp larvae *A. salina* Leach was seen in the protein fraction of sponge *Haliclona fascigera* (20-40% fraction) with LC_{50} value = 0.013 µg/mL.

Keywords: *Haliclona fascigera, Clathria reinwardtii*, Protein, Toxicity, Antibacterial, Anticancer

INTRODUCTION

Indonesia is the archipelago country with the richest marine biodiversity in the world[1], and it is rich of marine biodiversity resources[2]. Its biodiversity includes plants, corals, sponges, mollusks, crustaceans, echinoderms, fish, reptiles, birds, and mammals[3].

Marine biodiversity Indonesia is use only as a food ingredient, but it did not use optimally in the medical and medicine fields[4]. Sponge is one of the marine organisms is contain many active compounds[1].

Sponges are one source of new prospective compounds. Many sponges' studies have produced novel compounds with unique structures and interest pharmacological activities[5] such as anticancer, antimicrobial, anti-inflammatory[6], antibiotics, antibacterial[7] and antimalarial[8].

Previous research, secondary metabolites compound found in several species of sponges, this compound is active as an antimicrobial, it have extracted with a non-polar solvent (chloroform), but from some other sponge species did not show antimicrobial activity[9]. These results have suggested some other compounds that actively like proteins, it produced in the body of the sponge as an antibacterial and so the sponge can still grow and develop well in the sea despite disruption of bacteria.

Research showed the presence of bioactive protein activity from sponges such as sponge protein as antibacterial[10] and as an antiviral[11,12]. Protein as an antimicrobial drug and anticancer have advantages such as good acceptability without side effect[13]. Besides that, proteins genes can be cloned, that are massively produced on an industrial scale through genetic engineering techniques.

In this research, isolated fraction of sponge protein from Kapoposang Island South Sulawesi is tested for antibacterial and anticancer activity. From this research, it is expected to generate new knowledge about the components of sponge bioactive protein, which can be used as a new drug ingredient.

MATERIALS AND METHODS

The materials used are sponge *Haliclona fascigera* and *Clathria reinwardtii*, pure culture of *Staphylococcus aureus* and *Escherichia coli*, *Artemia salina* Leach, buffer A (Tris-HCl 0.1 M pH 8.3; NaCl 2M; $CaCl_2$ 0.01M; β- mercaptoetanol 1% Triton X-100 0.5%) buffer B (Tris-HCl 0.1M pH 8.3; NaCl 2M; $CaCl_2$ 0.01M), buffer C (Tris-HCl 0.01M pH 8.3, NaCl 0.2M, $CaCl_2$ 0.01M), Ammonium sulphate (Merck), Lowry A, Lowry B, Albumine Bovine Serum (BSA) 4 mg/mL, chloramphenicol, marine sand, seawater, aluminum foil, 70% alcohol, tissue roll, paper disc and distillated water.

Sampling and Identification

Sponge samples collected manually around Kapoposang island of South Sulawesi. It was washed with sea water until clean, entered into plastic samples, labeled place and date, and then taken to laboratory[14,15]. Determination of Taxonomy of sponge was done in central research laboratory of UNHAS Seaweed and sponge.

Protein Isolation

Isolation of proteins using the procedure Schroder et al. has been modified. The collected sponges are cut into small pieces, homogenized by blender with buffer A and then it was filtering. Filtrate was freeze-liquid 2-3 times and then centrifuged at 4500 rpm 4°C for 20 minutes. The supernatant is stored in the refrigerator and then proceeds at the next stage[16].

Fractionation

Crude extract protein is fractionated using ammonium sulfate at saturation: 0-20%, 20-40%, 40-60% and 60-80% and then centrifuged at 13000 rpm at 4 °C for 10 minutes[17].

Dialysis

Protein fractions were included in the cellophane pouch, included into the buffer B solution then they are stirrer with a magnetic stirrer. The dialysis process has continued with buffer C and stopped until the buffer solution did not color.

Determination of Protein rate

The rate bioactive protein was determinate by the Lowry method Using Bovine Serum Albumine (BSA) as the standard[17].

Antibacterial Activity Test

The bioactive protein inhibitory used agar diffusion method. This Inhibitory test for the growth of *Staphylococcus aureus* and *Escherichia coli* bacteria. Medium Muller Hinton Agars (MHA) have sterilized by autoclaves 121 °C, than cooled into a temperature of 40°C - 45°C. After that 15 mL medium have poured aseptically into a petri dish and incorporated 0.2 mL suspension bacteria. Seven pieces paper discs have been mixed test compound. The paper discs have placed on the surface it. Petri dish have incubated for 24-48 hours at 37°C and then observed the zone of resistance.

Brine Shrimp Lethality Test (BSLT)

The test compounds were prepared in concentrations of 1, 10, 100 ppm in seawater. Shrimp larvae *Artemia salina* Leach were inserted into test compounds and added sea water to 5 mL. After 24 hours the live larvae were calculated with the help of a magnifying glass[18].

Data analysis

Data tabulated and calculated using probit analysis to obtain Lethal Concentration 50 (LC_{50}).

Table 1: The Pattern of Protein Distribution at Fractionation of Various Levels of Saturation $(NH_4)_2SO_4$ Sponge *Haliclona Fascigera* and *Clathria Reinwardtii.*

No.	Sponge Species	Protein Fraction	Protein concentration (mg/ml)	Volume of each Fraction (ml)	Total protein (mg)
1.	*Haliclona fascigera*	Crude extract	17.23	574	9892.32
		0-20 %	13.67	4.4	60.18
		20-40 %	31.16	9.6	299.17
		40-60 %	15.02	9.2	138.19
		60-80 %	22.74	3.6	81.87
2.	*Clathria reinwardtii*	Crude extract	15.67	550	8617.56
		0-20 %	31.22	32	998.97
		20-40 %	14.97	5.3	79.32
		40-60 %	6.49	6.3	40.89
		60-80 %	16.10	20	322.00

Table 2: Bioactivity of Crude Extract and Protein Fraction of Sponge *Haliclona fascigera* against *Staphylococcus aureus* and *Escherichia coli* for 24 Hours and 48 Hours

Bacterial	Protein Fraction	Average resistance zone diameter (mm)	
		24 hour	48 hour
Staphylococcus aureus	Crude extract	12.34	12.11
	0 – 20 %	13.90	13.01
	20 – 40 %	14.56	14.20
	40 – 60 %	13.23	12.95
	60 – 80 %	13.85	12.97
	Control (-)	6.00	6.00
	Control (+)	18.78	18.59
Escherichia coli	Crude extract	12.05	11.78
	0 – 20 %	13.56	13.05
	20 – 40 %	14.25	13.79
	40 – 60 %	12.15	12.07
	60 – 80 %	12.67	12.13
	Control (-)	6.00	6.00
	Control (+)	17.45	16.51

Table 3: Bioactivity of Crude Extract and Protein Fraction of Sponge *Clathria reindwardtii* against *Staphylococcus aureus* and *Escherichia coli* for 24 Hours and 48 Hours

Bacterial	Protein Fraction	Average resistance zone diameter (mm)	
		24 hour	48 hour
Staphylococcus aureus	Crude extract	12.89	12.28
	0 – 20 %	14.94	13.38
	20 – 40 %	12.45	12.00
	40 – 60 %	12.15	11.87
	60 – 80 %	12.70	12.39
	Control (-)	6.00	6.00
	Control (+)	19.13	18.91
Escherichia coli	Crude extract	12.34	12.47
	0 – 20 %	14.35	14.14
	20 – 40 %	12.55	12.53
	40 – 60 %	12.15	12.02
	60 – 80 %	12.45	12.51
	Control (-)	6.00	6.00
	Control (+)	17.96	17.75

Table 4: Calculation Result LC_{50} Shrimp Larvae *Artemia salina* Leach that died against some Protein Fractions

Sponge	Protein Fraction	LC_{50} (µg/mL)	Toxicity
Haliclona fascigera	Crude extract	4.645	Toxic
	Fraction 0 – 20 %	48.084	Toxic
	Fraction 20 – 40 %	0.013	Toxic
	Fraction 40 – 60 %	0.481	Toxic
	Fraction 60 – 80 %	0.016	Toxic
Clathria reinwardtii	Crude extract	67.764	Toxic
	Fraction 0 – 20 %	>1000	Non toxic
	Fraction 20 – 40 %	327.341	Toxic
	Fraction 40 – 60 %	97.724	Toxic
	Fraction 60 – 80 %	78.524	Toxic

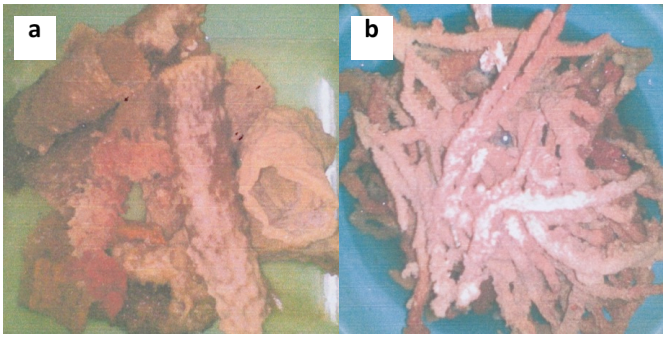

Figure 1: Species of sponge *Haliclona fascigera* (a) and *Clathria reinwardtii* (b).

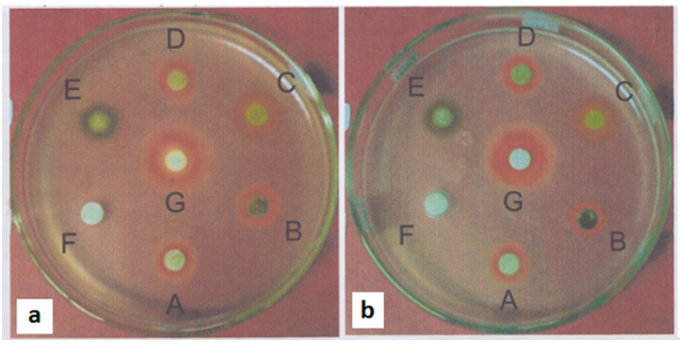

Figure 2:Antibacterial Activity of Crude Extract and Protein Fraction of Sponges *Haliclona fascigera* against bacteria (a) *Escherichia coli* and (b) *Staphylococcus aureus* (Exp. Crude extract, A; Protein Fraction 0 – 20 %, B; Protein Fraction 20 – 40 %, C; Protein Fraction 40 – 60 %, D; Protein Fraction 60 – 80 %, E; negative control (BSA), E; and positive control (Chloramphenicol)

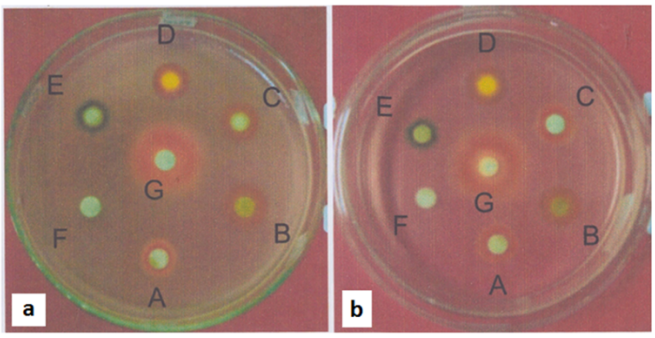

Figure 3: Antibacterial Activity of Crude Extract and Protein Fraction of Sponges *Clathria reinwardtii* against bacteria (a) *Escherichia coli* and (b) *Staphylococcus aureus* (Exp. Crude extract, A; Protein Fraction 0 – 20 %, B; Protein Fraction 20 – 40 %, C; Protein Fraction 40 – 60 %, D; Protein Fraction 60 – 80 %, E; negative control (BSA), E; and positive control (Chloramphenicol)

RESULTS AND DISCUSSION

Sampling and Identification

In this study, there are two species sponge collected from the waters of Kapoposang island of South Sulawesi. They identified in the laboratory of UNHAS Seaweed and sponge research center and they species knew *Haliclona fascigera* (Figure 1a) and *Clathria reinwardtii* (Figure 1b).

Protein rate Sponge *Haliclona fascigera* and *Clathria reinwardtii*

Protein rate sponges *Clathria reinwardtii* and *Haliclona fascigera* are determinate by Lowry method, this method used Bovine Serum Albumine (BSA) as standard [17]. The result of protein content show the crude extract of *Haliclona fascigera* is 17.23 mg/mL with total protein 9892.32 mg and *Clathria reinwardtii* is 15.67 mg/mL with total protein 8617.56 mg. The pattern of protein distribution in each fraction can be seen in Table 1.

Based on Table 1, the protein concentrations of the two species of sponges differed from each fraction. It may be due to differences in protein solubility in water, less solubility proteins were precipitation first than higher solubility proteins. The difference in solubility caused competition between proteins with ammonium sulfate salts in binding of protein solvents. The less solvent-binding proteins in the fractionation process, the protein then settles, so that the fraction with the highest protein concentration is a protein whose solubility is low in water. The highest protein concentration of *Haliclona fascigera* sponge found in the fraction of 20-40% of 31.16 mg/mL and in *Clathria reinwardtii* sponge found in the fraction of 0-20% of 31.22 mg/mL. These data have known, the protein *Haliclona fascigera* sponge is high solubility in water than protein from *Clathria reinwardtii* sponge.

Two bacteria were used to antibacterial test, that is gram-negative bacteria *Escherichia coli* and gram-positive bacterium *Staphylococcus aureus*, which are pathogenic in human. The bioactive protein inhibitory test for bacterial growth was performed using the agar diffusion method using a paper disc.

Bioactivity Antibacterial Protein Fraction of *Haliclona fascigera*

The results of the zone of inhibition of protein fraction of the *Haliclona fascigera* sponge on two bacteria, after 24 and 48 hours of incubation period are listed in Table 2. This Table shows each protein fraction having an antibacterial bioactivity against *Staphylococcus aureus* and *Escherichia coli*, as shown in (Figures 2a, b). The image shows a clear zone in each media. The clear zone shows all the studied sponge samples containing protein compounds capable of inhibiting the growth of pathogenic bacteria.

The strongest antibacterial activity during the 24-hour incubation period was found at a fraction of 20-40% with an area of 14.56 mm inhibition zone for *Staphylococcus aureus* and 14.25 mm bacteria for *Escherichia coli* bacteria. After 48 hours incubation there was no increase in inhibition zone to *E. coli* or *S. aureus* however inhibition in zone was decreased. This decrease indicates the bioactive protein contained in the *Haliclona fascigera* sponge is bacteriostatic. According to Wattimmena, an antibacterial is said bacteriostatic if the antibacterial is efficacious to inhibit bacterial growth but does not kill bacteria until within 48 hours[19].

Bioactivity Antibacterial Protein Fraction of *Clathria reinwardtii*

The inhibitory zone diameter of the protein fraction *Clathria reinwardtii* sponge on bacteria *Staphylococcus aureus* and *Escherichia coli* for the 24-hour incubation period is shown in Table 3. Table 3 shows all protein fractions having antibacterial bioactivity for *Staphylococcus aureus* and *Escherichia coli* as cited in Figures 3a, b. In, the picture is shows any clear zone in each medium. that all samples of sponges contain protein compounds that can inhibit the growth of pathogenic bacteria.

The strongest antibacterial activity for the 24-hour incubation period was at a fraction of 0-20% with inhibition zone of 14.94 mm for *Staphylococcus aureus* and 14.35 mm for *Escherichia coli* bacteria. After 48 hours incubation period there was no increase in inhibition zone for *Staphylococcus aureus* bacteria this means that the fraction of the protein is bacteriostatic. Inhibition zone for *Escherichia coli* bacteria on crude extract and fraction was found to be 60-80%. This shows the bioactive protein compound fraction 60-80% and the crude extract is bactericidal, the term bactericide is defined as a material killer bacterium[20]. Bactericides generally inhibit bacterial growth by irritating cell walls, agglomerating bacterial proteins due to acidity differences, as well as the hydrolysis and diffusion of cell caries that cause osmotic pressure differences[21].

Inhibition zone of the protein fraction and crude extract of two sponge samples tested with *Staphylococcus aureus* and *Escherichia coli*, none of the fractions exceeded the inhibition zone of the positive control (chloramphenicol). The activity of inhibition of protein fractions against pathogenic bacteria is small, it probably caused by the very low protein fraction concentration or because of the resistance of pathogenic bacteria to the bioactive protein.

An Inhibitory zone of the test sample of *Staphylococcus aureus* (gram-positive bacteria) is wider (more sensitive) than the inhibitory zone of the test sample against *Escherichia coli* (gram-negative bacteria). This is caused by the structure of gram-positive bacteria cell wall is simpler to facilitate the antibacterial compound to enter into the cell and find the target to work, while the gram-negative cell wall structure is more complex and layered three, the outer layer of lipoprotein, the middle layer of peptidoglycan and coating in lipopolysaccharides[20].

Activity of gram-positive test samples larger than gram-negative can also explain proteins present in *Clathria reinwardtii* and *Haliclona fascigera* sponges may be enzyme proteins, Ahmad's research reported enzyme proteins have high antibacterial bioactivity against gram-positive bacteria especially against *Salmonella typhi* and *Staphylococcus aureus* bacteria[22].

Fraction protein of sponge *Clathria reinwardtii* (0-20% fraction) and *Haliclona fascigera* (20-40% fraction) are effective in inhibiting the growth of *Staphylococcus aureus* and *Escherichia coli* bacteria. According to Cappucino et al*.,* an antibiotic ingredient is considered effective in inhibiting the growth of test microbes when it has a barrier diameter of ≥ 14 mm [23], but is not effectively used as a novel antibacterial agent because the zone of resistance does not exceed the positive control.

In general, the fraction proteins 0-20% from *Clathria reinwardtii* sponge had the strongest activity to both bacteria (*Staphylococcus aureus* and *Escherichia coli* bacteria). This is the earliest precipitating fraction, so this shows that the active protein is a protein that has a small solubility in water.

Screening for anticancer activity

In this study bioactivity test was performed to assess the anticancer activity. It is to determine whether the isolated protein fraction has anticancer activity or not. Prescreening of compounds suspected to be efficacious as anticancer was tested by Brine Shrimp Lethality Test (BSLT) method. It is widely used because it is cheaper, easier to develop, and there is no ethical rule in the use of test materials [24]. The test animal was used *Artemia salina* Leach. Based on some previous research results of this BSLT method indicate a specific correlation with anticancer test when having LC_{50}<1000 ppm.

According to Meyer et al., The death of *Artemia salina* Leach becomes a parameter to indicate the presence of active substances that are toxic. The LC_{50} value determined the level of toxicity of the test compound. The test substance said to be toxic if the value of LC_{50}<1000mL and did not say toxic if the value of LC_{50}> 1000mL. The smallest LC_{50} value is the more toxic compound [18]. In this study, toxicity tests for protein sponge *Clathria reinwardtii* and *Haliclona fascigera* used shrimp larvae *Artemia salina* Leach. The complete calculation of LC_{50} values has shown in Table 4.

All of the protein fractions sponge *Haliclona fascigera* and *Clathria reinwardtii* (Table 4) are toxic interval level, but the fraction 0-20% sponge *Clathria reinwardtii* are not toxic interval level. The protein fraction of the *Haliclona fascigera* sponge (20-40% fraction) gave the most toxic response to the shrimp larvae of *Artemia salina* Leach with LC_{50}= 0.013 µg/mL, it was suspected that this fraction was active as an anticancer agent but to ensure its validity, more tests are needed.

CONCLUSION

Based on the results, it can be concluded that the sponge protein *Clathria reinwardtii* (0-20% fraction) and *Haliclona fascigera* (20-40% fraction) effectively inhibits the growth of *Staphylococcus aureus* and *Escherichia coli* bacteria, but not effectively used as an antibacterial drug. In anticancer activity screening fraction of sponge protein, *Haliclona fascigera* (20-40% fraction) is most toxic to shrimp larvae *Artemia salina* Leach with LC_{50} value = 0,013 µg/mL.

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