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Isolation and Identification of Secondary Metabolite from Marine Sponge *Callyspongia* sp. and its Antibacterial Potency

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Abstract: Marine sponge *Callyspongia* sp. is one full of potency as a source for discovering and developing novel antibacterial. This study aims to isolate the *Callypsongia* sp. and assay their antibacterial activity. *Callyspongia* sp. were macerated with ethyl acetate (3x24 hrs), isolated with vacuum liquid chromatography (VLC) and RC (radial chromatography), and determined their structure with ^{1}H and ^{13}C-NMR. The antibacterial activity was assayed with the microdilution method. From ethyl acetate extract of *Callyspongia* sp. was successfully 2 isolated compounds, namely, isolate C1 (cholesterol) and isolate C2 (Unknown alkaloid with carbonyl from aldehyde group). The extract has MIC>512 µg/mL against *Bacillus subtilis*, *Escherichia coli*, *Streptococcus mutans*, and *Salmonella enterica*. While in both isolates provided MIC value >256 µg/mL against *B. subtilis*, *E. coli*, and *S. mutans*, yet in *S. enterica* provided 128 µg/mL for isolate C1 and 256 µg/mL for isolate C2. In conclusion, ethyl acetate extract of *Callyspongia* sp. contains cholesterol and Unknown alkaloid with carbonyl from the aldehyde group, and they both exhibited low antibacterial susceptibility.

Keywords: *Callyspongia* sp.; isolation; VLC; RC; antibacterial; microdilution.

1. Introduction

Sponges are one of the biota components that make up coral reefs that are quite widely distributed. There are 15,000 species of sponges worldwide, and about 45% of bioactive compounds are found in sponges [1]. Sponges are marine natural products that potential as a source for novel drug discoveries. Many pharmacological activities are reported in sponges, including cytotoxic, kinase inhibitor, antibacterial, antiviral, antihyperlipidemic, antiproliferative, immunomodulatory, and anti-inflammatory [2-4]. The discovery of novel drugs from marine sources began in the mid-1970 and reported about 2500 new metabolites are found [5].

Callyspongia sp. is a sponge found in almost all Indonesian sea so that it is easily obtained [6]. It contains various secondary metabolites that can be used as a source of drugs

[7]. It reported having anticancer, antioxidant, immunomodulator, anti-inflammatory, antimicrobial and antiparasitic activity [8-10].

Infectious disease is a significant problem globally; one of them is a bacterial infection. To treat a bacterial infection, it requires antibiotics. However, the resistance of antibiotics is another major problem that occurs. Thus, it urgent to discover and develop novel antibiotics or antibacterial agents from natural products. Marine sponges provide many biological compounds that act as antibacterial [11,12].

One of the steps in discovering the novel drug is by isolating secondary metabolites in marine natural products. The isolation method is a technique of separating a component from a more complex mixture. The basis of this separation technique is the comparison of the properties of the component partition against the adsorbent [13]. The isolation process that must be carried out to obtain pure compounds includes extraction, fractionation, and purification [14]. According to the explanation above, we aim to isolate the secondary metabolite from marine sponge *Callyspongia* sp. and assay the extract and isolates' antibacterial activity.

2. Materials and Methods

2.1. Materials.

The material used was marine sponge *Callyspongia* sp., *Bacillus subtilis, Escherichia coli, Streptococcus mutans, Salmonella enterica.* The chemical was methanol (Merck®), ethyl acetate (Merck®), n-hexane (Merck®), chloroform (Merck®), distilled water, silica gel 60 GF_{254} (Merck®), silica 60 G (Merck®), cerium sulfate/ $CuSO_4$ (Merck®), Nutrient Agar/NA (Merck®), and Nutrient Broth/NB (Merck®). The apparatus used were a set of vacuum liquid chromatography (VLC), a set of radial chromatography (RC), a vacuum rotary evaporator (Buchi®), micropipette (Eppendorf®), Microwell plate (Greiner Bio-One®), spectrophotometer (Jenway), and NMR ^{1}H and ^{13}C spectra (JEOL).

2.2. Extraction and isolation.

A total of 259.7 g of dried extract of *Callyspongia* sp. was macerated with ethyl acetate (EtOAC) (1:10 w/v) for 3 x 24 hrs. The extract was concentrated under vacuum condition with a rotary evaporator (55°C) and yielded a total of 37.17 g concentrated extract (14.31%).

The extract was fractioned by vacuum liquid chromatography (VLC) and a mixture of n-hexane: ethyl acetate (7:3 v/v). The fraction was performed till obtained 7 fractions, which were Fraction A (1.371 g); fraction B (4.9 g); fraction C (4.7 g); fraction D (94 mg); fraction E (94 mg); fraction F (77 mg); and fraction G (5.8 g). Fraction A was continued fractioned using VLC with eluent n-hexane: ethyl acetate (7: 3 v/v) and obtained 20 fractions. The fraction 11 was a pure compound, coded as isolate C1. Other fractions were combined and obtained 8 main fractions, namely fractions 1-8. Fraction 6 (1.4 mg) was separated with radial chromatography (RC) with eluent dichloromethane: methanol (9:1 v/v) and obtained 14 fractions. Fraction 11 (10.2 mg) was a pure compound, coded as isolate C2.

2.3. Structure determination.

The structure of isolates was determined with ^{1}H NMR and ^{13}C NMR. The data obtained were compared to existed references.

2.4. Antibacterial activity.

Antibacterial activity was conducted according to the microdilution method. The bacteria used were *Bacillus subtilis, Escherichia coli, Streptococcus mutans,* and *Salmonella enterica* and incubated in nutrient agar (NA) at 37°C for 24 hrs. After that, the inocula were suspended in 0.9% NaCl to obtain the turbidity equivalent to 0.5 McFarland. A total of 100 µl media was put in each well of a microplate, continued by pipetting 100 µl of the sample into first wells and pipetting 100 µl the mixture to second wells, and so on up to the eighth wells to obtain concentrations 512 – 0.5 µg/mL. It was also conducted to chloramphenicol and DMSO for control. Following that, 100 µl of bacterias were added to wells. They were incubated for 16-20 hrs at 37°C and measured under spectrophotometer.

3. Results and Discussion

The spectra ^{13}C-NMR of isolate C1 exhibited 27 of carbons with 2 signals. The carbon is a typical methine carbon group of olefins that appeared at δC 121.8 ppm and δC 140.8 ppm. Other methine carbon derived from alkane chains at δC 56.8 ppm and 50.2 ppm, methylene carbon at δC 21.1 - 42.3 ppm, and methyl carbon at δC 11.94 - 19.4 ppm (Figure 1).

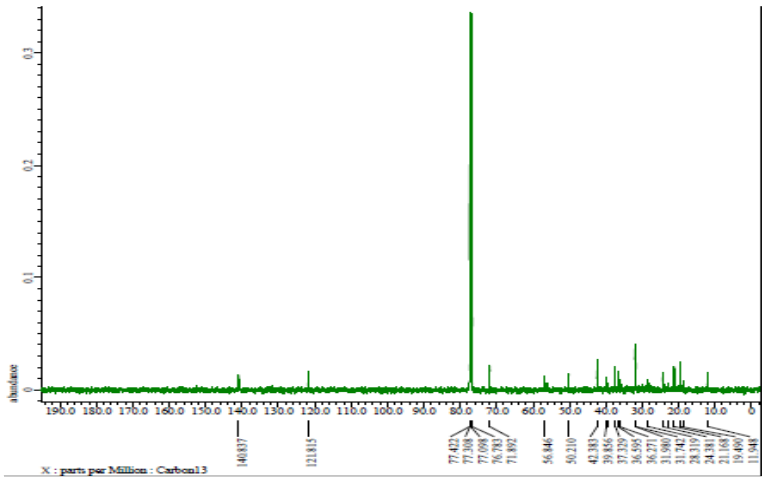

Figure 1. The Spectra ^{13}C-NMR of Isolate C1.

The ^{13}C-NMR analysis was also supported by the presence of a ^{1}H-NMR signal for the isolate C1 (Figure 2). ^{1}H-NMR analysis on C1 isolates exhibited 46 protons, 4 of which had a fairly large chemical shifting at 5.3; 5.1; 4.7; and 3.5 ppm. This magnitude indicates that the proton has a minimal electron density. Besides, the ^{1}H-NMR spectrum exhibited the buildup of protons with extensive integration. From the ^{1}H and ^{13}C-NMR data, it can be estimated that the molecular formula for isolate C1 is $C_{27}H_{46}O$. It is similar to cholesterol (Kalinowski et al., 1984). The data was: Cholesterol (1), ^{1}H NMR (500 MHz, $CDCl_3$) δH (ppm): 1.82 (*m*, H-4); 1.49 (*m*, H-12); 1.33 (1H, *m*, H-16); 1.26 (*m*, H-23); 1.12 (*m*, H-1); 0.82 (*m*, H-11); and 0.67 (*m*, H-24). ^{13}C NMR (125 MHz, $CDCl_3$) δC (ppm): 77.0 (C-25); 76.8 (C-17); 76.6 (C-14); 71.8 (C-3); 56.8 (C-13); 50.2 (C-9); 42.3 (C-4); 39.8 (C-12); 39.7 (C-22); 39.5 (C-20); 37.3 (C-10); 36.2 (C-16); 31.9 (C-8); 31.7 (C-25); 31.7 (C-2); 3.65 (C-1); 28.3 (C-7); 28.3 (C-23); 24.3 (C-15); 22.9 (C-26); 21.1 (C-11); 19.4 (C-19); 18.8 (C-21); 140.8 (C-5); 121.8 (C-6); 12.2 (C-27); and 11.9 (C-18).

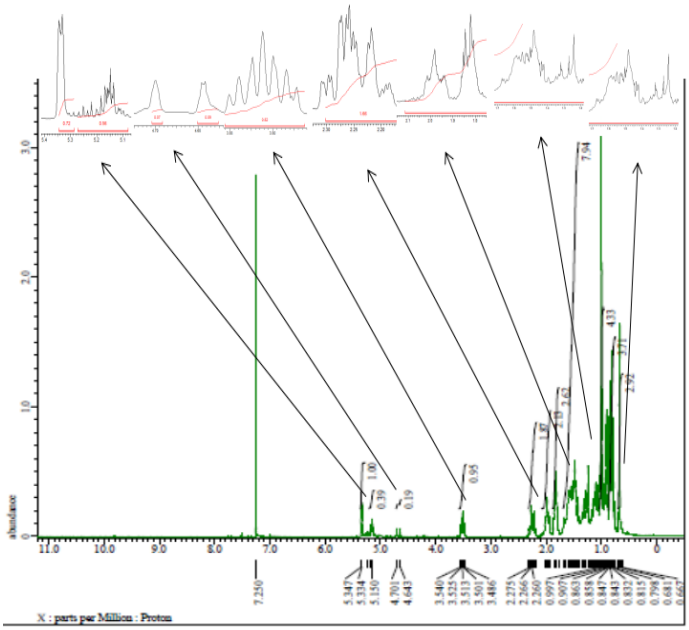

Figure 2. The Spectra ^{1}H-NMR of Isolate C1.

The C2 compound is obtained as a yellow solid. This compound exhibited dark spots on UV rays 254, spots are not visible at 366 nm light and after derivatization using, cerium sulfate followed by heating. According to $^{\text{the}}$ ^{13}C-NMR analysis of isolate C2 there was 10 carbon on its structure. Carbon at chemical shifting above 90 ppm, which were 125.8; 132.3; 176.6, and 178.0 ppm were olefin or Csp2 carbon. These four olefinic carbons allow the formation of two double bonds. The ^{13}C-NMR spectrum exhibited 12 types of carbon with 4 carbon peaks in this compound: a specific group, namely the quaternary carbon of the alkene group, which appeared at 178.06 and 176.63 ppm, and the methine carbon of the alkene group appeared at 132.37 and 125.85 ppm. Another methylene carbon derived from the alkane chain also appeared at 47.07-49.33 ppm (Figure 3).

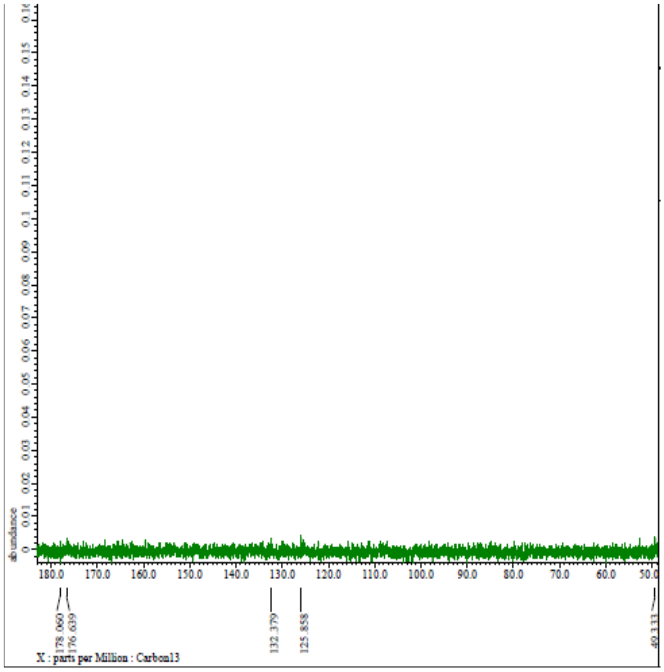

Figure 3. The Spectra ^{13}C-NMR of Isolate C2.

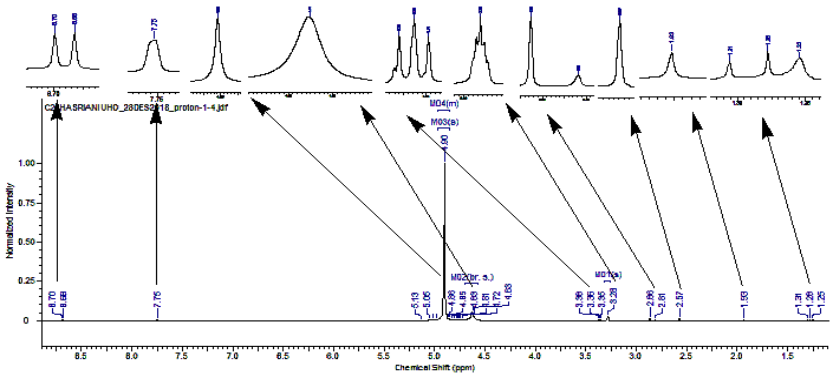

Figure 4. The Spectra ^{1}H-NMR of Isolate C2.

The 1H-NMR data exhibited 10 protons, 4 of which have a fairly large chemical shear, namely, 8.70; 7,75; 4.90, and 4.63 ppm. This magnitude indicates that the proton has a minimal electron density. The 1H-NMR spectrum of compound C2 shows 10 protons consisting of methylene protons (1.28; 1.93; 2.58, and 2.86 ppm) and methyne protons (7.75 ppm). From the ^{1}H and ^{13}C-NMR data, it can be estimated that the molecular formula for the isolate C2 is $C_{12}H_{10}$ (Figure 4). The data was: Unknown alkaloid with carbonyl from aldehyde group (2); yellow solid. Spectra of ^{1}H NMR (500 MHz, $CDCl_3$) δH (ppm): 8.7 (*d*, H-1); 7.75 (*s*, H-2); 4.9 (*s*, H-3); 4.63 (*s*, H-4); 3.38 (*t*, H-5); 3.28 (*m*, H-6); 2.86 (*d*, H-7); 2.58 (*s*, H-8); 1.93 (*s*, H-9); 1.28 (*m*, H-10). Spectra of ^{13}C NMR (125 MHz, $CDCl_3$) δC (ppm): 178.06 (C-1); 176.63 (C-2); 132.37 (C-3); 125.85 (C-4); 49.33 (C-5); 48.33 (C-6); 48.12 (C-7); 47.9 (C-8); 47.69 (C-9); 47.47 (C-10); 47.2 (C-11); and 47.05 (C-12).

According to the isolation and purification process, followed by structure determination, it was obtained 2 isolates, which were cholesterol (1) and unknown alkaloid with carbonyl from the aldehyde group (2). The structure of cholesterol is presented in Figure 5. The isolate 2 structure is undetermined with ^{1}H-NMR and ^{13}C-NMR.

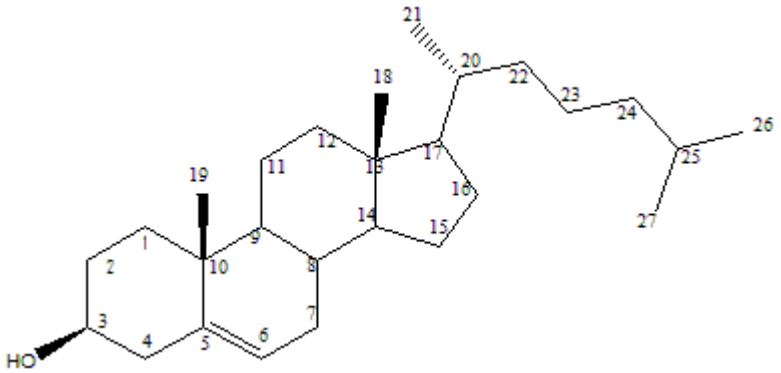

Figure 5. Cholesterol.

According to the antibacterial activity assay, the ethyl acetate extract of *Callyspongia* sp. was not demonstrating antibacterial activity with MIC value more than 512 µg/mL against all bacteria used. Simultaneously, the isolate C1 and isolate C2 provided MIC value more than 256 µg/mL, except for *S. enterica* with 128 dan 256 µg/mL, respectively (Table 1). Based on other studies showed that ethyl acetate extract was providing the lowest antibacterial activity against bacteria, it could be the reason the ethyl acetate extract of *Callyspongia* sp. was not providing antibacterial potency. The flavonoid, tannin, and phenolics contain in ethyl acetate extract was the lowest compared to methanolic or ethanolic extract [15]. Flavonoids, tannins,

and phenolic compounds are bioactive agents with many potencies, one of them as antibacterial by various mechanisms [16-18].

Table 1. MIC of Extract and Isolates from *Callyspongia* sp.

Sample	MIC Value (µg/mL)			
	B. subtilis	*S. mutans*	*E. coli*	*S. enterica*
EtoAC of *Callyspongia* sp.	>512	>512	>512	>512
Isolate C1	>256	>256	>256	128
Isolate C2	>256	>256	>256	256
Chloramphenicol	8	8	8	8

The control used was chloramphenicol. Chloramphenicol is an antibiotic that is used to treated infectious diseases. The chloramphenicol acts by binding to ribosome 50S of bacteria, thereby inhibiting bacteria's protein synthesis [19,20]. The MIC of chloramphenicol were 8 µg/mL (Table 1).

4. Conclusions

2 isolates were successfully isolated from *Callyspongia* sp., which were cholesterol and un-identified alkaloid, coded as C1 and C2. The extract of *Callyspongia* sp. was not exhibiting antibacterial properties against *B. subtilis*, *S. mutans*, *E. coli*, and *S. enterica.* On the other hand, isolate C1 and C2 were only exhibiting antibacterial properties against *S. enterica* according to MIC test.

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Conflicts of Interest

The authors declare no conflict of interest.

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