Toxicity and Radical Scavenger Properties of Various Extracts of Sponge *Clionidae* sp. Kangean Islands

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**Abstract.** Sponge *Clionidae* sp. is a multicellular animal that has active secondary metabolites that are used for survival. Sponge has cytotoxic, anticancer and antitumor properties and can be used in the pharmaceutical field, but the toxicity and antioxidant levels must be done to determine the uses level. Toxicity levels of sponge *Clionidae* sp. against *Artemia salina* L. and the IC\(_{50}\) value of the antioxidant was tested against DPPH radicals. Extraction of sponge *Clionidae* sp. was carried out with sonication using methanol, n-butanol, ethyl acetate and n-hexane as solvents. Then, the crude extract was tested for toxicity levels by the BSLT method and the antioxidant test by the DPPH method. Sponge extracts with ethyl acetate has the highest LC\(_{50}\) at 62.50 ppm and n-butanol has the highest IC\(_{50}\) values of 77.34 ppm. Identification of extract sponge with ethyl acetate was carried out using a UV-Vis spectrophotometer, resulting a maximum wavelength at 407.5 nm, ethyl acetate sponge extract showed the presence of O-H stretch groups 3327 cm\(^{-1}\), C-H stretch 2922 cm\(^{-1}\), C=C aromatic 1462 cm\(^{-1}\), and C=O 1711 cm\(^{-1}\) by FT-IR. The dominant compound in ethyl acetate extract that identified in LC-HRMS are chlorhexidine, 1,3,7-octanetriol, tobramycin, stigmatellin Y and maraniol.

1. **Introduction**

As many as 71% of the earth's surface is covered by sea water that makes sea have a variety of natural resources. Marine organisms are very diverse both biological and animal including algae, corals, sponges and other intervertebrate animals [1]. Indonesia is a country that rich in natural products, one of that is a sea sponge. Sugumaran, et al [2] stated that marine organisms, especially sponges are the best source for antimicrobial and antitumor substances. However, until now the utilization of marine products has not been maximized especially in the pharmaceutical field [3]. Marine metabolites and other natural products work in competition with their environment. Therefore, many marine organisms have evolved into efficient metabolites and exhibit high toxicity or biological activity even at very low concentrations [2]. Bioactive metabolic substances are often found in certain concentrations produced by marine sponges [4].

Sponge can be the most promising organism for drug development because it has the ability to synthesize a variety of organic products and can be a major source of biologically active marine products [5]. The last few decades, sea sponge has been considered as a material to find bioactive chemicals related to the presence of primary and secondary metabolites in it. Sea sponges produce a high concentrations of cytotoxic substances by secrete a toxic mucus that can kill other marine species as a defense against themselves [6]. Compounds from marine sources such as sponges have bioactive properties such as antitumor, anticancer, antiproliferation, cytotoxic and antibiotics. These compounds have been isolated from marine sources having chemical compounds such as phenols, alkaloids,
terpenoids, polyester, and other secondary metabolites found in sea sponges, seaweed and dinoflagellates [7].

Figure 1. Sponge Clionidae sp.

Sponge from the Clionidae family is a type of digging organism that can dig calcareous substrates such as rocks, coral reefs or oyster shells. Previous research of sponge Clionidae sp. reported by Palermo, et al. in 1998 that the sponge Clionidae sp. from the genus Cliona was isolated and was identified had an aromatic hexacyclic alkaloid, Storniamides A-D. In addition, it was also reported that the isolate Cliona chilensis also contained a tripeptide alkaloid namely Celenamide E. [8].

Brine Shrimp Lethality Test (BSLT) is a method for screening the active compounds from natural materials. BSLT method are used to determine the LC$_{50}$ value and detect the toxicity level of compounds [9]. The antioxidant activity was tested using the DPPH radical solutions 2,2-Diphenyl-1-pikrilhidrazil, in the presence of antioxidant compounds that able to donate the H atom or the electron, the absorbance of the solution will be decrease and form a stable molecule. The antioxidant test using DPPH method is used to determine the IC$_{50}$ value. IC$_{50}$ value is the concentration of the extract to scavenging 50 % of radical[10]

Antioxidant test on sponge extract needs to be done to determine the antioxidant activity of the dominant compound containing phenolic or heteroaromatic compounds on the dominant component of the extract [11]. A drug must be among the lowest drug concentrations in plasma to get the desired drug working. These compounds can be used as drugs if the using does not exceed the LC$_{50}$ value of the toxicity value. Therefore, a toxicity test is necessary to be evaluated [12].

Based on the background above, this study was conducted with the aim of identifying secondary metabolite compounds contained in the sponge extract of the family Clionidae sp. and conducting toxicity tests and their antioxidant tests.

2. Experimental Section

2.1. Chemicals and Instrumentation
The materials used in this study were sponge Clionidae sp. from Kangean Island, ethyl acetate (Smart Lab), n-hexane (Full time), n-butanol (Smart Lab), ethanol 96% (Merck) and methanol (Full time), Dimethyl Sulfoxide (DMSO) solution (Sigma Aldrich), sea water and Artemia salina L. and 2,2-Diphenyl-1-picyrylhidrazyl (Sigma-Aldrich). The instrument used in this research were sonicator, rotary evaporator (IKA RV 10 digital), UV Vis Spectrophotometer (Shimadzu 1600), FTIR spectrophotometer (Shimadzu 8400S, extract was analyzed using KBr pellet), LC HRMS (Thermo Fisher Scientific).

2.2. Sponge Collection
Sponges were collected from Kangean Island, Madura, Indonesia. Sponges were stored in a freezer and cleaned with ethanol 96 %. After that sponge cutted into small pieces and dried in the room temperature (not exposed to direct sunlight) for 2-3 days.

2.3. Sponge Extraction
The sponges were grounded to a fine powder. The powdered sponge was extracted with different solvents, n-hexane, ethyl acetate, n-butanol and methanol with sonicator for 1 h. The extracts were filtered through filter paper and concentrated with rotary evaporator. The crude extract was stored at 5 $^\circ$C and used in toxicity and antioxidant assay.
2.4. Toxicity Assay with BSLT (Brine Shrimp Lethality Test) Method

Subramani, et al. in 2013 [13], was used to study the toxicity test of sponge with different solvents using *Artemia salina L.* method. The toxicity properties known by the mortality number of *Artemia salina L.*, and the LC$_{50}$ (Lethal Concentration 50) can be determined.

2.4.1. The Hatching of Brine Shrimp Larvae. Brine shrimp were hatched in 150 mL of seawater under aeration condition for 48 h. After 48 h the eggs hatched and shrimp larvae were ready to be used for toxicity testing of sponge extract.

2.4.2. Preparation of Sample Solution. The crude extracts of methanol, n-butanol, ethyl acetate were made a concentration of 200 ppm as a stock solution by adding 1% Dimethyl Sulfoxide (DMSO) to the total solution. Then, a dilution was carried out of 5 ppm; 10 ppm; 25 ppm; 50 ppm; 100 ppm; 125 ppm; 150 ppm by using sea water.

2.4.3. Toxicity Test of Sponge Extract with Shrimp Larvae. Twenty larvae shrimp were putted into vial bottle and the 5 mL of test solution was added with respective concentrations; 5 ppm; 10 ppm; 25 ppm; 50 ppm; 75 ppm; 100 ppm; 125 ppm; 150 ppm, then observed after 24 h and observed the number of dead larvae. 1% DMSO and sea water were added as blank control and compared with the test solution with a concentration of 5 ppm; 10 ppm; 25 ppm; 50 ppm; 75 ppm; 100 ppm; 125 ppm; 150 ppm. LC$_{50}$ by making a curve between % mortality and concentration. % mortality was calculated by the equation:

\[
\text{\% mortality} = \frac{\text{Number of initial larvae} - \text{Number of dead larvae}}{\text{Number of initial larvae}} \times 100\%
\]

2.5. Antioxidant Test of Sponge Extract with DPPH Radical Methods

2.5.1. Preparation of Solution. Crude sponge extract was made a concentration of 250 ppm as a stock solution. Then, the solution is carried out to a concentration of 12.5 ppm; 37.5 ppm; 62.5 ppm; 87.5 ppm; 125 ppm; 150 ppm; 187.5 ppm using methanol.

2.5.2. Antioxidant Test of Sponge Extract. The antioxidant test with the DPPH method was adapted from Hanani, et al. in 2005 [3], it was carried out by adding 4 mL test solution with a concentration of 0 ppm; 12.5 ppm; 37.5 ppm; 62.5 ppm; 87.5 ppm; 125 ppm; 150 ppm; 187.5 ppm into a vial bottle and 1 mL DPPH 0.1 mM. The test control blank was used by adding 4 mL methanol and 1 mL DPPH 0.1 mM. Control blanks and test solutions were added with DPPH 0.1 mM and incubated at 37 °C for 30 minutes. Measurements were taken at 515 nm. IC$_{50}$ values were calculated by making a curve between % inhibition and concentration. % inhibition was calculated by the equation [14]:

\[
\text{\% inhibition} = \frac{\text{Absorbance blank} - \text{absorbance sample}}{\text{absorbance blank}} \times 100\%
\]

2.6. Characterization of Sponge Extract

2.6.1. Characterization of Sponge Extract Using UV-Vis Spectrophotometer. The method for characterizing sponge extract products with UV-Vis spectrophotometer was adapted from Pore, et al. in 2016 [15] crude sponge extract was dissolved in methanol and then measurements were taken at wavelengths of 200-800 nm. The maximum wavelength is obtained from the maximum absorbance value. The instrumentation that used was UV-Vis Spectrophotometer Shimadzu 1600.

2.6.2. Characterization of Sponge Extract Using FTIR Spectrophotometer. Sponge extract was mixed with KBr and made the pellets and inserted into the sample holder. Then, infrared light will pass through the sample so that the molecules in the sample will vibrate. The system on the computer will analyze the data in the form of spectrum in the form of %T and wavenumbers. The data was used to predict the functional groups which are constituent compounds of sponge extract.
2.6.3. Characterization of Sponge Extract Using LC-HRMS. Samples that were ready to be filtered with milipore and injected with syringes were 5 µL with a flow rate of 0.7 mL/min.

3. Result and Discussion

3.1. Crude Sponge Extract

From the extraction of sponges with various solvents, the results are described in the Table 1.

| No | Solvent     | Initial Mass (g) | Extract Mass (g) | % Yield |
|----|-------------|------------------|------------------|---------|
| 1  | Methanol    | 24               | 1.89             | 7.875   |
| 2  | n-Butanol   | 24               | 0.40             | 1.67    |
| 3  | Ethyl Acetate | 24           | 0.32             | 1.33    |
| 4  | n-Hexane    | 24               | 0.11             | 0.45    |

The polar extract which is methanol extract has the highest yield (7.875 %) and n-butanol extract (1.67 %), this means that many polar compounds are distributed in to the polar solvent. Meanwhile, ethyl acetate and n-hexane extract have 1.33 % and 0.45 % of yield, respectively; which means the distribution into non polar solvent.

3.2. Toxicity Test Value of Sponge Extract with Artemia salina L.

Based on the results of toxicity test, the linear equation is obtained to determine the LC$_{50}$ value of the toxicity test. The LC$_{50}$ value are described in the Table 2.

| No | Sponge Extract | LC$_{50}$ (ppm) |
|----|----------------|-----------------|
| 1  | n-Butanol      | 101.77          |
| 2  | Methanol       | 95.72           |
| 3  | n-Hexane       | 75.61           |
| 4  | Ethyl Acetate  | 62.50           |

The LC$_{50}$ values in the toxicity test with BSLT method for n-hexane, ethyl acetate, butanol and methanol sponge extracts. Each sponge extract obtained LC$_{50}$ values with concentrations <1000 ppm. In this study, it was known that each extract had toxic properties with an LC$_{50}$ value <1000 ppm. Ethyl acetate sponge extract has the lowest concentration LC$_{50}$ value among other sponge extracts of 62.50 ppm. The toxicity category can be seen from Table 3.

| Categories          | LC$_{50}$ values |
|---------------------|------------------|
| Supertoxic          | ≤ 5 mg/kg        |
| Very Toxic          | 5-50 mg/kg       |
| Toxic               | 50-500 mg/kg     |
| Toxic Medium        | 0.5-6 g/kg       |
| Mild Toxic          | 5-15 g/kg        |
| Practically non-toxic | >15 g/kg        |

From Table 3, ethyl acetate, n-hexane, methanol and n-butanol extracts have a toxic category.

3.3. Antioxidant Test Value of Sponge Extract with DPPH Radicals

Figure 2 was describing free radical DPPH scavenging activity of various extract sponge Clionidae sp.
Figure 2. DPPH Radical Scavenging of Various Extract Sponge in Difference Concentration.

Table 4. The IC\textsubscript{50} Value of Antioxidant Test.

| No | Sponge Extract | IC\textsubscript{50} (ppm) |
|----|----------------|--------------------------|
| 1  | Methanol       | 172.27                   |
| 2  | n-Hexane       | 161.17                   |
| 3  | Ethyl Acetate  | 98.96                    |
| 4  | n-Butanol      | 77.34                    |

The IC\textsubscript{50} value was described at Table 4. IC\textsubscript{50} was defined as the concentration of the extract sponge solution scavenging 50\% of radical. n-Butanol extract has the highest IC\textsubscript{50} value 77.34 ppm which means has the higher ability of free radical scavenging using the free radical DPPH method.

3.4. Characterization Result

Table 5. Characterization result with UV – Vis and FTIR Spectrophotometer.

| No | Sponge Extract | Maximum Wavelength (nm) | Wavenumber (cm\textsuperscript{-1}) |
|----|----------------|-------------------------|-------------------------------------|
| 1  | Ethyl Acetate  | 407.5                   | 3327 (Broad-Intense; O-H),          |
|    |                |                         | 2922 (Sharp-Intense; C-H sp\textsuperscript{3}). |
|    |                |                         | 1711 (Sharp-Intense; C=O)           |
| 2  | n-Hexane       | 405                     | 3414 (Broad-Intense; O-H),          |
|    |                |                         | 2924 (Sharp-Intense; C-H sp\textsuperscript{3}). |
|    |                |                         | 1740 (Sharp-Intense; C=O)           |
| 3  | Metanol        | 405                     | 3375 (Broad-Intense; O-H),          |
|    |                |                         | 2918 (Sharp-Intense; C-H sp\textsuperscript{3}). |
|    |                |                         | 1638 (Sharp-Intense; C=O)           |
| 4  | n-Butanol      | 401.5                   | 3377 (Broad-Intense; O-H),          |
|    |                |                         | 2922 (Sharp-Intense; C-H sp\textsuperscript{3}). |
|    |                |                         | 1705 (Sharp-Intense; C=O)           |
| RT (min) | Chemical Formula and Area | Compound Name and Relative Mass | Chemical Structure |
|---------|--------------------------|-------------------------------|--------------------|
| 26.392  | C_{22}H_{30}Cl_{2}N_{10}  | Chlorhexidine (505.4 g/mol)  | ![Chemical Structure](image1) |
| 0.898   | C_{8}H_{18}O_{3}         | 1,3,7-Octanetriol (162.23 g/mol) | ![Chemical Structure](image2) |
| 21.533  | C_{18}H_{37}N_{5}O_{9}  | Tobramycin (467.5 g/mol)    | ![Chemical Structure](image3) |
| 19.215  | C_{29}H_{40}O_{6}        | Stigmatellin Y (484.28 g/mol) | ![Chemical Structure](image4) |
| 12.99   | C_{12}H_{12}O_{3}        | Maraniol (204.07 g/mol)      | ![Chemical Structure](image5) |
Chlorhexidine is a biguanide compound used as an antiseptic agent with antibacterial activity. Chlorhexidine reacts with the negative charge of microbial cell surface, thereby destroying the integrity of the cell membrane [16]. 1,3,7-Octanetriol belongs to the organic compounds known as a fatty alcohol. 1,3,7-Octanetriol can be found at plant and animals [17].

Maraniol and stigmatellin Y are compounds that have chromone groups. Chromone group is a natural compound consisting of 4H-benzopyran-4-one. Chromone group can be found in several species of higher organism. Chromone group has antioxidant activity by reacting directly with free radicals and through the chelation process of Fe (II) and Cu (II). Chromone compounds or chromone compound derivatives are known with their anticancer properties. The treatment of cancer, especially breast cancer with chromone compounds, makes modulation of drug transportation more efficient to a higher level of chemotherapy [17].

Tobramycin is identified as part of a complex antibiotic. Tobramycin can inhibit protein synthesis by binding with 30S ribosomes from ribosomes of prokaryotic cells. Tobramycin has antibacterial properties against *Staphylococcus pneumoniae* and tobramycin are active against many aerobic and facultative gram-negative bacteria including *Enterobacteriaceae, Pseudomonas aeruginosa* and *Acinetobacter sp.*[18,19].

**Conclusions**

Sponge extract with ethyl acetate solvent has the highest LC50 value 62.50 ppm and the other sponge extracts obtained toxic properties with LC50 values concentrations <1000 ppm. Meanwhile, sponge extract with n-butanol solvent has the highest IC50 value 77.34 ppm which means has the higher ability of free radical scavenging. Furthermore, the dominant compound of ethyl acetate extracts are chlorhexidine, 1,3,7-octanetriol, tobramycin, stigmatellin Y and maraniol.

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