INVESTIGATING THE BIOLOGICAL ACTIVITIES OF *CLATHRIA VULPINA* AND *STYLISSA CARTERI* THROUGH IN VITRO AND IN SILICO APPROACHES

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Abstract

Background: Myocardial infarction is one of the deadly diseases characterized by the presence of blood clot inhibiting the circulation of blood to the heart, caused by ecto-ATPase. Sponges *Clathria vulpina* and *Stylissa carteri* have bioactive compounds that can be an alternative in inhibiting ecto-ATPase. This research aimed to find the potential of bioactive compounds from Indonesian *Clathria vulpina* and *Stylissa carteri* in acting as inhibitory agents for ecto-ATPase. **Methods:** The bioactive compounds were extracted by maceration using methanol solvent. The bioactive compound from the sponge extracts were profiled using GC-MS. Hemolytic and hemagglutination activities of *Clathria vulpina* and *Stylissa carteri* extracts were also done. **Results:** Bioactive compound profiles from *Clathria vulpina* extract were found as derivatives of palmitic acid, oleic acid, stearic acid, arachidonic acid, and DHA. Meanwhile, bioactive compound profiles from *Stylissa carteri* extracts as derivatives fatty acids, alcohol and steroids. Both *Clathria vulpina* and *Stylissa carteri* extracts had hemolytic activities, but did not have hemagglutination activity. Molecular docking analysis showed that *Clathria vulpina* bioactive compounds cannot bind ecto-ATPase better than control positive, and *Stylissa carteri* bioactive compounds can bind ecto-ATPase better than positive control. **Conclusion:** Bioactive substances from Indonesian sponges *Clathria vulpina* and *Stylissa carteri* were known as ecto-ATPase inhibitors. These findings suggest that *Stylissa carteri*'s bioactive substances may be ecto-ATPase inhibitors and should be further studied for myocardial infarction treatment.

Keywords: *Clathria vulpine* ; *Stylissa carteri* ; Myocardial infarction ; Inhibitory agent ; Hemolysis ; Hemagglutination

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INTRODUCTION

In the rapidly developing 21st century, the impact of science and technology is profound, leading to a fast-paced and instant lifestyle. Unfortunately, this lifestyle has resulted in a decline in health quality and increased susceptibility to diseases [1]. One such disease that poses a significant threat to human health is myocardial infarction, characterized by the presence of blood clots that obstruct blood circulation to the heart. This condition is caused by ecto-ATPase interfering with the action of tissue plasminogen activator (tPA), which normally breaks down plasminogen into plasmin. Plasmin is responsible for breaking down fibrin and dissolving the clots that impede blood flow [2]. Currently, antiplatelet agents are commonly used to treat myocardial infarction [3]. However, these medications come with side effects such as blood vomiting, clot-induced cough, nosebleeds, and increased risk of hemorrhages [4]. Therefore, it is crucial to explore new drug candidates derived from natural biological compounds to prevent or mitigate these side effects. Marine sponges offer a rich source of natural bioactive compounds. They have been extensively studied for their diverse array of primary and secondary metabolites, making them a valuable resource for medical and pharmaceutical applications [1]. Two notable sponge species, *Clathria vulpina* and *Stylissa carteri*, belonging to the Demospongiae class, are of particular interest. Demospongiae is the largest and most common class in the sponge phylum, encompassing approximately 85% of all known sponge species [5]. *Clathria vulpina* is recognized for its antioxidant,

anti-inflammatory, and antihypertensive activities [6], while *Stylissa carteri* is known for its anti-inflammatory and anti-tumor properties [7]. Marine sponges exhibit a high diversity of natural compounds, including alkaloids, terpenoids, peptides, steroids, and many others [8]. The objective of this research is to investigate the potential of bioactive compounds derived from *Clathria vulpina* and *Stylissa carteri* as inhibitory agents for ecto-ATPase.

MATERIALS AND METHODS

Materials
The materials used in this research included *Clathria vulpina*, *Stylissa carteri*, methanol, human erythrocytes, 0.13 M NaCl in 0.02 M pH 7.4 Tris-HCl buffer, and 1% Triton X-100. The equipment used consisted of a mortar and pestle, vacuum oven, Gas Chromatography-Mass Spectrophotometry (GC-MS), and a microplate reader. The research involved several steps, including sample preparation, extraction of *C. vulpina* and *S. carteri*, GC-MS analysis, hemolytic assay, hemagglutination assay, and in silico analysis using molecular docking Pyrx.

Sample Preparation
Sample preparation followed the procedure described by Hutagalung et al. The dried *Clathria vulpina* and *Stylissa carteri* samples were freeze-dried for three days. The dried samples were then ground to a powder using a mortar and pestle. The powdered samples were placed in a beaker glass and covered with plastic wrap.

Sample Extraction
Extraction was performed based on the procedure outlined by Hutagalung et al., with a slight modification. Methanol extracts were obtained by macerating 2 g of each sponge sample in 40 mL of methanol (1:20 ratio) for 5 hours. The extracts were filtered using a microfilter and syringe to separate the filtrate from the residue. The methanol extracts were then evaporated using a vacuum oven at 50 ºC for 1 h [9].

Gas Chromatography-Mass Spectrophotometry (GC-MS) Analysis
GC-MS analysis followed the procedure described by Prasasty et al. [10]. The methanol extracts were analyzed using Thermo Fisher Scientific Trace™ 1310 Gas Chromatograph with an Ultra Alloy +- 5 capillary column (30 m x 0.25 mm x 0.25 µm). Helium was used as the carrier gas at a constant flow rate of 1.0 mL/min. The injector temperature was set to 260 ºC. The oven temperature was programmed from 140 ºC (held for 2 minutes) up to 250 ºC with a 10 ºC/min interval. Mass spectra were recorded in the range of 45 – 450 Da with an ionization energy of 70 eV. The interpretation of GC-MS analysis results was conducted by comparing the data with the National Institute Standard and Technique (NIST) database.

Hemolytic Assay
The hemolytic assay followed the procedure outlined by Hutagalung et al. Human erythrocytes were used for this analysis. Erythrocytes were obtained by centrifuging blood at 4 ºC, 2000 rpm, for 4 minutes. The supernatant was discarded, and the pellet was washed three times with a 9% NaCl solution at a ratio of 1:9 by mass. The washed pellet was then diluted with a buffer containing 0.13 M NaCl in 0.02 M pH 7.4 Tris-HCl to achieve a concentration of 0.5% w/v. The methanol extracts used for GC-MS analysis were evaporated again using a vacuum oven at 50 ºC until no methanol remained. Then, 1 mg of dried extract was diluted in 1 mL of buffer solution to create an extract solution with a concentration of 1000 ppm. Serial dilutions were performed to obtain solutions with concentrations of 500 ppm, 250 ppm, and 125 ppm. The hemolytic assay was conducted using a Tecan Infinite 200 Pro Microplate Reader and a round bottom Costar 96-well plate. In each well, 20 µL of the sample extract and 100 µL of erythrocyte suspension were added. This process was repeated for each concentration in triplicate. As a negative control, 20 µL of buffer solution and 100 µL of erythrocyte suspension were used, while 1% Triton X-100 was used as the positive control. The absorbance was measured at a wavelength of 655 nm every 5 minutes for a duration of 20 minutes. The microplate was agitated at a moderate speed and maintained at 25 ºC. The percentage of hemolytic activity was calculated using the following equation:

$$\%\text{hemolysis} = \frac{(\text{abs. sample} - \text{abs.negative control})}{(\text{abs.positive control} - \text{abs.negative control})} \times 100\%$$

Hemagglutination Assay
The hemagglutination assay was carried out following the procedure described by Hutagalung et al. In a Costar 96-well microplate, 100 µL of erythrocyte suspension was added to each well, followed by the addition of 20 µL of the sample solution for each concentration in triplicate. The plate was incubated at 25 ºC for 45 minutes, followed by 1 minute of agitation at a moderate speed.

Molecular Docking Molecular docking was conducted to investigate the inhibition of ecto-ATPase by the biological compounds derived from *Clathria vulpina* and *Stylissa carteri*, as identified through GC-MS analysis. PyMOL 2.5.2 [11], AutoDock Tools [12], and PyRx [13] were utilized as the programs for molecular docking. The receptor used in this research was *Rattus norvegicus* NTPDase2 (ecto-ATPase) retrieved from the Protein Data Bank (PDB ID: 3CJ1). The ligands used were the secondary metabolite compounds obtained from the GC-MS results of the sponge extracts. These compounds, including 4,7,10,13,16,19-docosahexaenoic acid, methyl ester; 5,8,11,14-eicosatetraenoic acid, methyl ester; methyl stearate; trans-13-octadecenoic acid, methyl ester; hexadecanoic acid, methyl ester; 10-octadecenoic acid, methyl ester; oleic acid, 3-(octadecyloxy)propyl ester; ethyl iso-allocholate; spirost-8-en-11-one, 3-hydroxy-, (3á,5à,14á,20á,22á,25R)-; and tricyclo[20.8.0.0(7,16)]triacontane, 1(22),7(16)-diepoxy-, were obtained from PubChem and converted to PDB format. Ligands were optimized by removing hydrogen atoms and stored in pdbqt format using AutoDock Tools. The docking interaction between NTPDase2 as the receptor and the bioactive compounds as ligands was performed using PyRx. Prior to docking, both the ligands and the macromolecule target were converted to pdbqt format and tested for energy minimization. The 2D and 3D structures were visualized using

BIOVIA Discovery Studio Visualizer. The conformation with the lowest ΔG value and closest binding to the receptor area was considered the potential biological compound. The docking area was defined using BIOVIA, and the coordinates for the docking were determined as 28.975130 (x), 33.036783 (y), and 26.243696 (z). The docking analysis provided information on the binding affinity and interactions between the ligands and the target proteins. The binding energy and the inhibition constant (Ki) of the protein were calculated based on the docking results [14]. The visualized docking results allowed for the examination of the specific interactions between the ligands and the receptor.

RESULTS

GC-MS Analysis

Bioactive profiles using GC-MS analysis performed the significant findings regarding the bioactive compounds present in the extracts of *Clathria vulpina* and *Stylissa carteri*. These compounds exhibit biological activities associated with hemolytic and hemagglutination processes. The identification of these compounds was based on the top 5 GC-MS peaks observed in each sample, sorted by retention time. Please refer to Figure 1, Table 1, and Table 2 for a detailed overview of the findings.

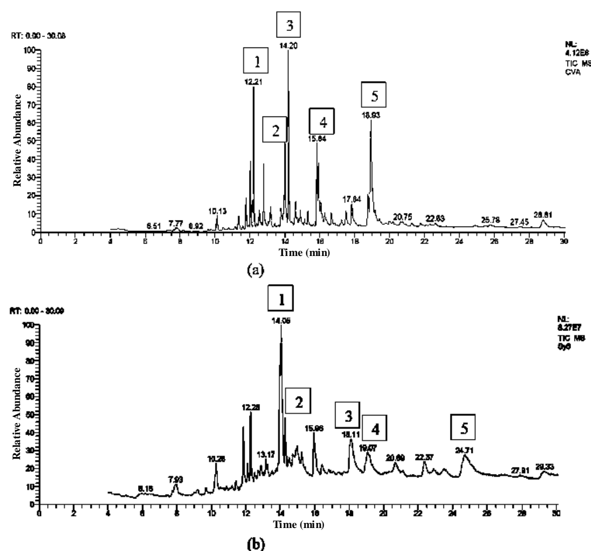

Figure 1: GC-MS chromatogram of (a) *Clathria vulpina*, and (b) *Stylissa carteri*.

Table 1: Bioactive compounds from top 5 GC-MS peak of *Clathria vulpina*.

No.	Compound	Molecular Formula	Category	Retention Time	Similarity Index
1	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	Palmitic acid, saturated	12.21	927
2	Trans-13-octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	Oleic acid, unsaturated	14.01	922
3	Methyl stearate	$C_{19}H_{38}O_2$	Stearic acid, saturated	14.21	930
4	5,8,11,14-Eicosatetraenoic acid, methyl ester,0 (all-Z)-	$C_{21}H_{34}O_2$	Arachidonic acid, unsaturated	15.84	913
5	4,7,10,13,16,19-Docosahexaenoic acid, methyl ester, (all-Z)-	$C_{23}H_{34}O_2$	DHA, unsaturated	18.94	916

Table 2: Bioactive compounds from top 5 GC-MS peak of *Stylissa carteri*.

No.	Compound	Molecular Name	Category	Retention Time	Similarity Index
1	10-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	Fatty acid	14.05	860
2	Tricyclo[20.8.0.0(7,16)]triacontane, 1(22),7(16)-diepoxy-	$C_{30}H_{52}O_2$	Alcohol	14.97	742
3	Oleic acid, 3-(octadecyloxy)propyl ester	$C_{39}H_{76}O_3$	Fatty acid	18.11	732
4	Spirost-8-en-11-one, 3-hydroxy-, (3á,5à,14á,20á,22á,25R)-	$C_{27}H_{40}O_4$	Steroid	19.08	690
5	Ethyl iso-allocholate	$C_{26}H_{44}O_5$	Steroid	24.72	768

Hemolytic Activity

The hemolytic analysis result of *Clathria vulpina* and *Stylissa carteri* contained the presence of hemolytic activity and percentage of hemolytic activity from each sample (Figure 2; Table 3, Table 4). Based on these results, it was shown that *Clathria vulpina* extract with concentration 250 ppm and time of 15 min and *Stylissa carteri* extract with concentration 125 ppm and time of 15 min gave the best results, respectively.

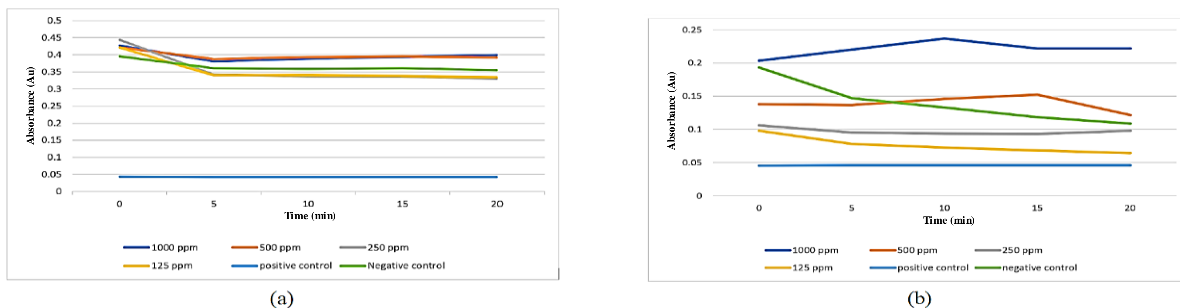

Figure 2: Hemolytic activity by various concentration of (a) *Clathria vulpina*, and (b) *Stylissa carteri*.

Table 3. Percentage of hemolytic activity from *Clathria vulpina* extracts.

Concentration (ppm)	% Hemolytic activity				
	0 minute	5 minutes	10 minutes	15 minutes	20 minutes
1000	-	-	-	-	-
500	-	-	-	-	-
250	-	6.062	6/6582	8.064	8
125	-	6.878	5.549	7.123	6.624

Table 4. Percentage of hemolytic activity from *Stylissa carteri* extracts.

Concentration (ppm)	% Hemolytic activity				
	0 minute	5 minutes	10 minutes	15 minutes	20 minutes
1000	-	-	-	-	-
500	37.585	10.109	-	-	-
250	59.362	51.338	45.381	35.359	17.28
125	64.722	68.483	69.630	69.613	70.88

Hemagglutination Activity The hemagglutination analysis result of *Clathria vulpina* and *Stylissa carteri* contained the presence of hemagglutination activity from each sample. Concentration used was in ppm unit, started from 1000 ppm, 500 ppm, 250 ppm, and 125 ppm (Figure 3). Based on these results, it was shown that both extracts did not have hemagglutination activity.

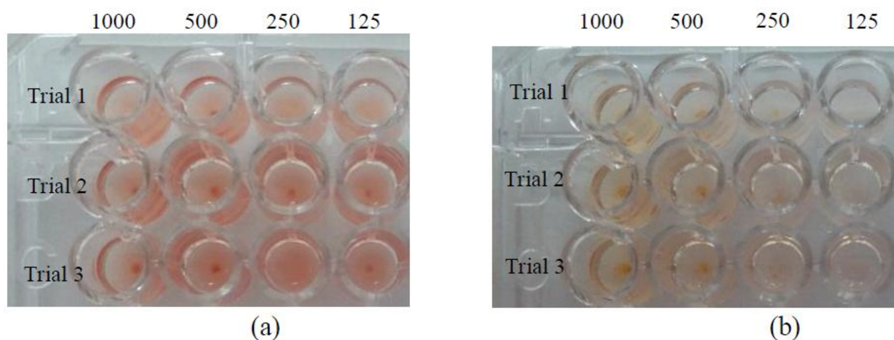

Figure 3: Haemagglutination activity by various concentrations of (a) *Clathria vulpina*, and (b) *Stylissa carteri.*

Molecular Docking Analysis

The results of the molecular docking analysis for *Clathria vulpina* and *Stylissa carteri* included the binding energy values of each bioactive compound and the positive control. A lower binding energy value indicates a stronger binding affinity (Table 5). The findings indicated that the top 5 bioactive compounds from *Clathria vulpina* extracts did not exhibit lower binding energy values compared to the control. However, in the case of *Stylissa carteri* extracts, two bioactive compounds demonstrated lower binding energy values than the control (Table 6).

Table 5. Molecular docking analysis result on *Clathria vulpina* extra.

No.	Compound	Category	Binding Energy (kcal/mol)
1	4,7,10,13,16,19-Docosahexaenoic acid, methyl ester, (all-Z)-	DHA, unsaturated	-4.9
2	5,8,11,14-Eicosatetraenoic acid, methyl ester,0 (all-Z)-	Arachidonic acid, unsaturated	-4.4
3	Methyl stearate	Stearic acid, saturated	-4.1
4	trans-13-Octadecenoic acid, methyl ester	Oleic acid, unsaturated	-3.9
5	Hexadecanoic acid, methyl ester	Palmitic acid, saturated	-4.1
6	ARL67156 (trisodium salt, control positive)	Ecto-ATPase inhibitor	-8.2

Table 6. Molecular docking analysis result on *Stylissa carteri* extracts.

No.	Compound	Category	Binding Energy (kcal/mol)
1	10-Octadecenoic acid, methyl ester	Fatty acid	-4.7
2	Oleic acid, 3-(octadecyloxy)propyl ester	Fatty acid	-5.0
3	Ethyl iso-allocholate	Steroid	-8.3
4	Spirost-8-en-11-one, 3-hydroxy-, (3á,5à,14á,20á,22á,25R)-	Steroid	-9.7
5	Tricyclo[20.8.0.0(7,16)]triacontane, 1(22),7(16)-diepoxy-	Alcohol	-9.4
6	ARL67156 (trisodium salt, control positive)	Ecto-ATPase inhibitor	-8.2

DISCUSSION

Extraction is a process that involves separating biological compounds from living organisms using solvents. The selection of the extraction solvent is based on the principle of "like dissolves like," where polar solvents attract polar compounds and non-polar solvents attract non-polar compounds. In this study, a vacuum oven with a temperature of 50ºC was employed for solvent extraction to prevent the evaporation and degradation of bioactive compounds [15]. The GC-MS analysis revealed the presence of several bioactive compounds in *Clathria vulpina* and *Stylissa carteri* extracts. The top 5 GC-MS peaks from each sample's chromatogram included compounds such as 4,7,10,13,16,19-docosahexaenoic acid, methyl ester (all-Z), 5,8,11,14-eicosatetraenoic acid, methyl ester (all-Z), methyl stearate, trans-13-octadecenoic acid, methyl ester, and hexadecanoic acid, methyl ester for *C. vulpina*. For *S. carteri*, the extracts contained 10-octadecenoic acid, methyl ester, oleic acid, 3-(octadecyloxy)propyl ester, ethyl iso-allocholate, spirost-8-en-11-one, 3-hydroxy-, (3á,5à,14á,20á,22á,25R)-, and tricyclo[20.8.0.0(7,16)]triacontane, 1(22),7(16)-diepoxy- [16]. Hemolytic activity was assessed by measuring absorbance at a wavelength of 655 nm. The results of the hemolytic assay showed that *Clathria vulpina* extracts at a concentration of 250 ppm demonstrated better results compared to other concentrations. In the case of Stylissa carteri, a concentration of 125 ppm exhibited the best hemolytic activity. The presence of hemolytic activity indicates the potential cytotoxic activity of these extracts, suggesting their possible use as anticancer drugs [9]. The absence of lectin in the sample extracts might contribute to the observed hemolytic

activity [17]. Hemagglutination activity was evaluated by examining the presence of blood clot formation at the bottom of the well. The results of the hemagglutination assay indicated the absence of hemagglutination activity in the sample extracts, indicating no blood clot formation. Hemagglutination activity in sponges is typically associated with lectins, which are proteins found in sponges. Several factors could contribute to the lack of hemagglutination activity in the sample results, including the low concentration of lectin in the crude extract, which may not be sufficient to trigger hemagglutination. Additionally, certain types of lectins may not induce hemagglutination activity [9, 18]. The molecular docking analysis compared the binding energy of the sample extracts with a positive control. The results showed that none of the Clathria vu1pina extracts exhibited a lower binding energy than the positive control. However, in the case of Stylissa carteri, spirost-8-en-11-one, 3-hydroxy-, (3á,5à,14á,20á,22á,25R)-, and tricyclo[20.8.0.0(7,16)]triacontane, 1(22),7(16)-diepoxy- demonstrated lower binding energy values compared to the positive control. A lower binding energy indicates a more stable and stronger interaction between the protein and ligand, suggesting a potential for better inhibition of the

protein's activity (Xing et al., 2016). The receptor used in this study was Rattus norvegicus NTPDase2. The extracts with the most negative ΔG values were spirost-8-en-11-one, 3-hydroxy-, (3á,5à,14á,20á,22á,25R)-, and tricyclo[20.8.0.0(7,16)]triacontane, 1(22),7(16)-diepoxy-. These results indicate that these compounds have the potential to act as inhibitors for ecto-ATPase activity. They show better binding affinity than ARL67156 or trisodium salt [19, 20].

CONCLUSION

The extraction approach involves separating biological compounds from living organisms using appropriate solvents based on their polarity. The GC-MS analysis identified bioactive compounds present in *Clathria vulpina* and *Stylissa carteri* extracts. Hemolytic activity assays demonstrated cytotoxic potential, while hemagglutination assays revealed the absence of lectin-induced clot formation. Molecular docking analysis highlighted specific compounds with promising binding energies, indicating their potential as inhibitors for ecto-ATPase activity. These findings contribute to the understanding of the bioactive properties of the studied extracts and their potential applications in biomedical fields.

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