



Antimalarial Activity of Sea Sponge Extract of *Stylissa massa* originating from waters of Rote Island

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Abstract

Research on the isolation, toxicity test, antimalarial test, and identification of the active compound from the ethyl acetate fraction of *Stylissa massa* sponge from Oenggae waters, Rote Island, has been conducted. This study aimed to investigate the antimalarial activity of the ethyl acetate fraction of the *Stylissa massa* sponge. Isolation was carried out by the extraction method using a mixed solvent of methanol: dichloromethane of 3: 2 (v/v), then the extract was partitioned in a solvent mixture of ethyl acetate: water of 1: 2 (v/v). The ethyl acetate extract obtained was separated by column chromatography using the gradient polarity system method. The toxicity test of each fraction was carried out by the Brine Shrimp Lethality Test (BSLT) method, and the antimalarial test was carried out by the haematin polymerization inhibition method. Identification of compounds from the active fraction in the antimalarial test was carried out using Liquid Chromatography–Mass Spectrometry (LC–MS). The extraction yield was 1.14 g (0.23%) of the ethyl acetate extract in the form of a dark brownish–yellow oily solid. Separation by column chromatography resulted in 15 fractions. Toxicity test results showed the four most active fractions with LC₅₀ values, which are very promising for new drug discovery. The IC₅₀ value in the antimalarial activity test of the four fractions indicated that the *Stylissa massa* sponge ethyl acetate extract was more active than the standard chloroquine compound (115 µg/mL). The LC–MS analysis indicates that fraction 11 contains two compounds that have been reported, and 1 compound is unknown. In contrast, fraction 14 indicates that it contains three compounds that have been reported and one unknown compound.

1. Introduction

Malaria is still a problem that needs attention. The resistance of *Plasmodium falciparum* to antimalarial drugs is the cause of the spread of malaria to new areas. It causes the re-emergence of malaria in areas that have been eradicated. Based on a World Malaria Report 2019 report, there are 228 million malaria cases globally, and 405,000 have died [1]. Thus, the discovery of new compounds as candidates for antimalarial drugs is essential.

Exploration of antimalarial compounds from marine materials, especially sponges, has been relatively developed in this decade. Manzamine A from the *Haliclona oculata* sponge, Diacarnuperoxide M from the *Diacarnus megaspinothabdosus* sponge, Psammaphysin H, and Tsitsikammamine C from the *Zyzya sp.* sponge are some of the compounds reported to have antimalarial activity [2, 3, 4, 5].

Stylissa massa sponges are widespread in tropical seas such as the Banda Sea, Sulawesi Sea, Mergui Islands, and Papua New Guinea [6]. Several compounds that have potential as medicinal substances have been isolated

from sponges of this species, such as Stylissatin A (cyclic peptide) as an anti-inflammatory agent, Stylissatin B as an antitumor agent, Spongiacidin C as a USP7 inhibitor, and aldisine alkaloid as a MEK-1 inhibitor [7, 8, 9, 10]. Currently, the exploration of bioactive compounds and antimalarial activity tests of the *Stylissa massa* sponge has not been widely carried out. This sponge is commonly found in the waters of the island of Rote (the southern border island of Indonesia) but has not been widely studied.

Recent studies have shown that ethyl acetate extract from *Stylissa carteri* sponge has antimalarial activity with an IC_{50} of 20.56 $\mu\text{g/mL}$ [11]. The *Stylissa carteri* sponge under the same genus as *Stylissa massa* allows a similar compound in the *Stylissa massa* sponge. Other studies have shown that some of the antimalarial compounds that have been reported are less polar isolated from less polar solvents, such as dichloromethane and ethyl acetate. So, this research was conducted to provide information about the antimalarial activity of the *Stylissa massa* sponge extract.

2. Methodology

This research was conducted in several stages, starting with sampling. Furthermore, activities are carried out in the laboratory, such as extraction, phytochemical testing, component separation, toxicity testing, and antimalarial testing. LCMS data were used to predict the compound content in each fraction.

2.1. Sponge Sampling

The *Stylissa massa* sponge was taken from Oenggae Waters, Rote Island, East Nusa Tenggara, at a depth of 7 meters, in the distance of 600 meters from the coastline. The dive was carried out at 10.00–12.00 Central Indonesian Time on June 27, 2020, then cleaned of foreign material and other organisms and then put into a temporary storage box containing an ice pack. The samples were then freeze-dried and stored.

2.2. Extraction

500 g of the sponge was cut into small pieces and then macerated with the solvent mixture methanol: dichloromethane of 3: 2 (v/v) for 2×1 hour. The maceration results were filtered and separated with a separating funnel to obtain dichloromethane extract. An evaporator evaporated the dichloromethane extract at a temperature below 35°C until a concentrated dichloromethane extract was obtained. The concentrated dichloromethane extract was then partitioned with ethyl acetate: water of 1: 2 (v/v). This process is carried out in a separating funnel with three repetitions. The ethyl acetate layer was separated and evaporated by an evaporator at 40°C until ethyl acetate extract (concentrated extract) was obtained.

2.3. Phytochemical Test

The alkaloid test in this study used Wagner's reagent. This reagent was prepared by mixing 2.5 g of iodine with 2 g of KI in 200 mL of distilled water. The alkaloid test was carried out by dropping 2 M H_2SO_4 solution into a vial containing ethyl acetate extract, then dropping Wagner's

reagent. The ethyl acetate extract was positive for alkaloid compounds if a brown precipitate was formed [12].

The terpenoid test in this study used a vanillin- H_2SO_4 reagent. This reagent was prepared by dissolving 0.1 g of vanillin in a mixture of 16 mL concentrated H_2SO_4 and 4 mL of ethanol. The terpenoid test was carried out by dissolving the ethyl acetate extract in methanol, then dropping the vanillin- H_2SO_4 reagent. Ethyl acetate extract was positive for terpenoids if there was a change in color to purple [12].

The saponin test was carried out by dissolving the ethyl acetate extract with distilled water in the vial, then shaking the vial. The saponin-containing compounds were characterized by a stable foam for approximately 15 minutes [12].

The steroid test in this study used the Liebermann-Burchard reagent. The steroid test was done by dissolving ethyl acetate extract with 2 mL of chloroform in a vial, adding ten drops of acetic anhydride and three drops of concentrated H_2SO_4 . The steroid-containing ethyl acetate extract was characterized by a color change from red to green/blue [12].

2.4. Separation of Ethyl Acetate Extract Components by Column Chromatography

The separation of ethyl acetate extract components was carried out using column chromatography. The column was attached to the stative vertically, and cotton was added to the bottom of the column. Silica gel 60 (mobile phase) before use was activated by heating at 120°C for 2 hours. Before using, the column was filled with n-hexane to be rinsed. The activated Silica gel 60 was made a slurry with n-hexane. The slurry was then put into the column and made homogeneous. After filling finished, the top of the column was wrapped with aluminum foil to prevent drying on the silica gel surface. The column was then conditioned for one night.

Ethyl acetate extract was dropped on the surface of the column and then eluted using a solvent system with step gradient polarity (SGP). The included solvent system ranged from nonpolar to polar solvents, which were 100% n-hexane, a mixture (n-hexane: dichloromethane) with a ratio (v/v) of 9:1; 7:3; 5:5; 3:7; and 2:8, dichloromethane 100%, a mixture (dichloromethane: ethyl acetate) with a ratio (v/v) of 9:1; 7:3; 5:5; 3:7; and 2:8, 100% ethyl acetate, a mixture (ethyl acetate: methanol) with a ratio (v/v) of 7:3; 5:5; and 3:7, 100% methanol, respectively. The eluate produced was then put into a weighed vial (5 mL). Eluate in vials was analyzed by thin-layer chromatography (TLC). Eluate, which had the same spot pattern, was merged into one fraction.

2.5. Brine Shrimp Lethality Test (BSLT) for ethyl acetate extract

The toxicity test was carried out using the brine shrimp lethality test (BSLT) method by Meyer *et al.* [13].

2.5.1. Hatching *Artemia salina* eggs

The egg hatching started with preparing a container ($30 \times 10 \times 15$ cm) filled with seawater. A glass separator

with a hole was inserted into the container to form a bulkhead. The container made consisted of two parts, which were dark and light. The hatchery was then equipped with a lamp and aerator, which was located on the bright side. Eggs were put in a dark container. The eggs were hatched for ± 24 hours with the help of heat. Shrimp larvae that were 48 hours old were used in the toxicity test.

2.5.2. Test and control material preparation

A 250 µg/mL stock sample solution was prepared by dissolving 2 mg of the test material in 50 µL DMSO in an Eppendorf tube. The solution was then rotated with a vortex mixer to get the maximum solubility. The solution was then added by seawater to a total volume of 8 mL. The control stock solution was prepared by infusing 50 µL of DMSO into Eppendorf without any test material. The solution was then added by seawater to a total volume of 8 mL.

2.5.3. Toxicity test

Ten larvae of *Artemia salina* aged 48 hours were inserted into 1.5 mL Eppendorf tubes, then added with stock sample solutions with volume variations of 1200; 600; 300; 150 and 75 µL. The tube was then put in seawater until the limit mark so that the final concentration of the sample was 200; 100; 50; 25, and 12.5 µg/mL. 1200 µL of control stock solution was put into a 1.5 mL Eppendorf tube, then 10 *Artemia salina* larvae were added, and seawater was added to the limit mark. Each treatment was repeated three times. The observation of the number of dead larvae was carried out after 24 hours using a magnifying glass.

2.5.4. Toxicity Analysis

Toxicity represents %mortality (percentage of deaths) which is then analyzed to obtain LC₅₀. The percentage of death (%mortality) at each concentration variant was calculated using equation 1 [14]. The %mortality data from each concentration variant were analyzed using the probit analysis method at SPSS 23. The LC₅₀ was determined from the analysis results with a probit value of 0.500 (50%).

$$\%mortality = \frac{(\text{dead larvae on the test} - \text{dead larvae on the control})}{\text{dead larvae on the test}} \times 100\% \quad (1)$$

2.6. Antimalarial test

The antimalarial test was carried out using the haematin polymerization inhibitory activity method by Basilico *et al.* [15].

2.6.1. Preparation of 1 mM haematin solution

A total of 7.59 mg of haematin crystals (Mr = 632.5 g/mol) was put into a conical bottle, and 3 mL of 0.2 M NaOH solution was added. Then shaking it until dissolved and added with another 0.2 M NaOH solution to a volume of 12 mL.

2.6.2. Haematin standard curve creation

A series of haematin solutions in 0.1 M NaOH was prepared in five different concentrations, of which 187.5; 125; 62.5; 31.25, and 15.6 µM were diluted from the mother

liquor of 1 mM haematin. Each concentration of the haematin solution was made by inserting 1 mM of haematin solution into the Eppendorf tube. Then each added 0.1 M NaOH solution until the total solution became 400 µL. The calculation of the dilution volume from the primary solution of haematin to the respective solution levels can be seen in table 1.

Table 1. Volumes of haematin mother liquor for dilution

the volume of haematin1 mM (µL)	Haematin concentration (µM)
75.00	187.50
50.00	125.00
25.00	62.50
12.50	31.25
6.25	15.60

A total of 100 µL of each concentration was added to the 96 microculture wells. The absorbance value of each level was read using a spectrophotometer Elisa reader at λ = 405 nm. The standard curve for haematin y = a + bx was made by plotting the x-axis as concentration and the y-axis as absorbance.

2.6.3. Preparation of stock solution for each fraction

The stock solution for each fraction with a 5.0 mg/mL concentration was prepared by weighing 2 mg of the sample in an Eppendorf tube. 40 µL of DMSO 100% was added, shaken until the sample dissolved and homogeneous. 360 µL of distilled water was added and homogenized.

2.6.4. Preparation of concentration series for each fraction

Each fraction was prepared with a concentration of 1.25; 0.625; 0.312; 0.156; and 0.078 mg/mL as much as 200 µL. The concentration series was made by diluting the stock solution of each fraction by a certain volume in an Eppendorf tube. 10% DMSO solution was added to the total volume of 200 µL and homogenized. The volume of stock solution for each fraction needed can be seen in Table 2.

Table 2. The volume of stock solution for each fraction for the preparation of the concentration series

Stock solution volume (µL)	Fraction concentration (mg/mL)
50.000	1.250
25.000	0.625
12.500	0.312
6.250	0.156
3.125	0.078

2.6.5. Haematin polymerization inhibition test

This test is based on the Basilico *et al.* [15] method with related modifications to the concentration of the solution and the test material [16]. 100 µL of 1 mM haematin solution in 0.1 M NaOH was put into the Eppendorf tube, then added 50 µL of test material with various levels of levels, namely 1.25; 0.625; 0.312; 0.156; and 0.078 mg/mL. To start the haematin polymerization

reaction, 50 μL of the glacial acetic acid solution was added to the Eppendorf tube, which already contained the haematin solution and samples, then incubated at 37°C for 24 hours. The positive control was chloroquine, while the negative control was distilled water and 10% DMSO solution. After the incubation ended, the Eppendorf tube was rotated using a centrifuge at a speed of 8000 rpm for 10 minutes. The supernatant obtained was discarded, and the precipitate was washed three times with 200 μL DMSO. Each washing was carried out using centrifugation at a speed of 8000 rpm for 10 minutes. Then, the precipitate obtained was added with 200 μL 0.1 M NaOH. Every 50 μL of each solution was put into a 96 well microplate, and the absorbance was read with an Elisa reader at $\lambda = 405$ nm. The results of the absorbance value were used to calculate the hemozoin level. The value of the inhibitory activity of haematin polymerization was expressed in IC_{50} , which is a number that indicates the concentration of the test material capable of inhibiting 50% of haematin polymerization. The IC_{50} value of haematin polymerization inhibitory activity was calculated using the probit analysis method at SPSS 23.

2.7. Liquid chromatography–mass spectrometry (LC-MS)

Screening each fraction using LC-MS/MS, with the following conditions: column ACQUITY UPLC @ BEH C18 1.7 μm ; eluent (A = double distilled water - THF 0.1%, B = CH_3CN); ionizing electrospray ionization (ESI); flow 0.8 mL/min; M/z start = 100, end = 1200 ES+; Injection volume: 2 μL .

2.8. Statistical analysis

Determination of the LC_{50} and IC_{50} values was carried out by entering the percentage of inhibition data as observed responses, 100 as the number of subjects, and the concentration as the concentration on SPSS 23 using the probit analysis method.

3. Results and Discussion

The results of this study are presented based on the treatment of the method used in this study.

3.1. Extraction

500 g of a wet sponge was dried with a freeze dryer to obtain a dry sponge weighing 71 g. The result of sponge maceration with solvent of methanol: dichloromethane was a very sticky yellow-brown extract. The yellow extract was partitioned with a mixture of ethyl acetate and water. This partitioning process aims to separate polar compounds and salts remaining in viscous extracts using a water solvent, while less polar compounds can be partitioned into ethyl acetate solvent. The results of the partitioning process showed three layers inside the separating funnel. The top layer is a layer of ethyl acetate which is dark yellow-brown. The middle layer is a light-yellow emulsion, while the bottom layer is a yellow water layer. The overall extraction process resulted in the form of ethyl acetate extract weighing 1.14 g (0.23%).

3.2. Phytochemical Profile

Phytochemical tests were carried out to obtain information about the class of compounds in the *Stylissa massa* sponge. A brown precipitate was seen in the alkaloid test, indicating that the ethyl acetate extract of *Stylissa massa* sponge was positive for alkaloid class compounds. Alkaloid compounds such as spongicidin c have been reported from *Stylissa massa* sponge [8]. The results of the terpenoid test also showed that the ethyl acetate extract of *Stylissa massa* was positive for the terpenoid class compounds. This is confirmed by a change in the color of the test solution to purple color. Terpenoids such as 8-isocyanato-15-formamidoamphilect-11 (20) -ene and 8-isothiocyano-15-formamidoamphilect-11 (20) -ene have been reported from this sponge [17]. Saponin test results on ethyl acetate extract of *Stylissa massa* did not show any foam, so it could be said that the *Stylissa massa* ethyl acetate extract did not contain saponin class compounds. In the steroid test, the ethyl acetate extract of *Stylissa massa* was indicated to contain steroid class compounds in which there was a change in the color of the test solution to green.

3.3. Separation of Ethyl Acetate Extract Components

Table 3. Column chromatography results of ethyl acetate extract

Fraction	Eluent	Mass (mg)	Description
1	<i>n</i> -hexane 100%	47.1	Oily (moss green)
2	<i>n</i> -hexane/chloroform (9: 1)	4.5	Oily (moss green)
3	<i>n</i> -hexane/chloroform (9: 1)	60.0	Oily (Dark yellow)
4	<i>n</i> -hexane/chloroform (7: 3)	6.4	Crystal (light yellow) has a fishy smell
5	<i>n</i> -hexane/chloroform(5: 5)	98.7	Oily (orange)
6	Chloroform 100%	28.0	Crystals (orange)
7	Chloroform/dichloromethane (9: 1)	35.8	Oily (dark green)
8	Chloroform/dichloromethane (7: 3)	6.8	Oily (yellow)
9	Chloroform/dichloromethane (3: 7)	3.1	Oily (yellow)
10	Dichloromethane 100%	2.0	Oily (yellow)
11	Dichloromethane/ethyl acetate (9: 1)	10.3	Oily (yellow)
12	Dichloromethane/ethyl acetate (5: 5)	20.1	Solid (brown)
13	Ethyl acetate 100%	29.3	Solids (brown)
14	Ethyl acetate 100%	38.5	Solids (green)
15	Ethyl acetate/methanol (9: 1)	20.3	Solids (moss green)

Separation of the components of the ethyl acetate extract of *Stylissa massa* sponge resulted in 43 eluents that came out of column chromatography. The combination of 43 eluates was carried out by looking at the similarity of spots in the TLC test so that 15 fractions were obtained that were simpler to facilitate the next research steps. The complete column chromatography results of ethyl acetate extract are presented in Table 3. During the separation process, vial 1 (7 mL) took 36 minutes to fill entirely. Vial 4 took 13 minutes to fully charge, while vial 43 (last vial) only took 5 minutes. The increase in the rate of drops coming out of the column occurred as the eluent polarity increased. Each eluate that came out had a different color except for eluate 1 (colorless).

3.4. Toxicity Test Results

In this study, BSLT was used as a preliminary test to determine the bioactivity of the ethyl acetate extract of *Stylissa massa* sponge. The DMSO used in this study was only 0.63%, still below the allowable limit, so that it did not poison *Artemia salina* larvae [14]. The results of the SPSS 23 calculation show that the LC₅₀ value of ethyl acetate extract is 934.395 µg/mL. According to Meyer *et al.* [13], toxic (active) compounds have an LC₅₀ value <1000 µg/mL. Thus, ethyl acetate extract can be classified as toxic because it has an LC₅₀ value <1000 µg/mL. The toxicity LC₅₀ value of each fraction is presented in Table 4. Table 4 shows that the toxic compounds are in the middle fraction. As in general isolation, compounds that have the potential to have high toxicity are less polar compounds. This is consistent with the principle of medicinal chemistry that a drug must not be too polar or too nonpolar to enter the target organ [18]. Calculations using SPSS 23 show that fractions 8, 11, 12, and 14 are a series of fractions classified as very toxic. These fractions were then tested for antimalarial.

Table 4. LC₅₀ value of each fraction

Fraction	LC ₅₀ (µg/mL)	Description
1	786.12	Toxic
2	4463.47	Non-toxic
3	977.52	Toxic
5	167.14	Toxic
6	-	Non-toxic
7	174.74	Toxic
8	13.08	Toxic
11	1.01	Toxic
12	11.33	Toxic
13	243.68	Toxic
14	3.59	Toxic
15	448.73	Toxic

3.5. Antimalarial test results

In the antimalarial test, the addition of acetic acid aims to initiate the polymerization process of haematin into hemozoin. The washing of hemozoin deposits is the most sensitive of the whole series of antimalarial tests. If the hemozoin deposits formed after 24 hours of incubation are lost during washing, the absorbance value on the Elisa reader is poor, and the hemozoin concentration data is inaccurate. This washing aims to remove the supernatant containing haematin which does not polymerize due to inhibition of the test material. The results of the hematin polymerization inhibition test for each test material are presented in Table 5.

Table 5. Results of Haematin Polymerization Inhibition Test

No.	Test Material	IC ₅₀ value(µg/mL)
1	Chloroquine	115
2	Fraction 8	82
3	Fraction 11	93
4	Fraction 12	105
5	Fraction 14	96

Based on the results of calculations using the probit analysis method at SPSS 23, the IC₅₀ value of all fractions tested showed a smaller number than chloroquine (positive control). A compound can have antimalarial activity if it has an IC value of less than chloroquine [19]. This indicates that fractions 8, 11, 12, and 14 of the ethyl acetate extract from the *Stylissa massa* sponge had antimalarial activity.

3.6. LC-MS Results of Fraction 11

LC-MS analysis of fraction 11 produces the chromatogram as shown in Figure 1. Figure 1 shows the rudimentary separation, especially after 5 minutes of separation. This may occur because the eluent system and the flow rate are not yet suitable, or in other words, the separation conditions are not optimal.

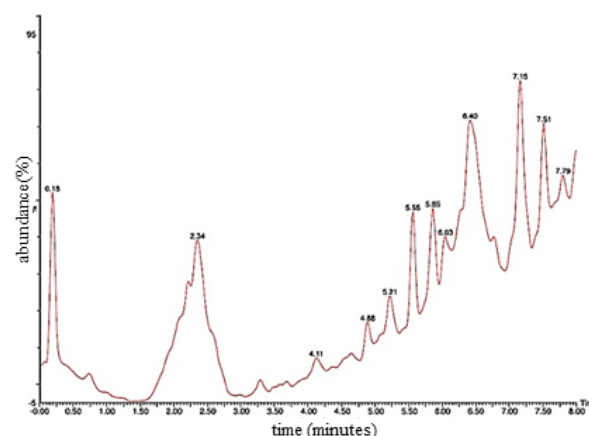


Figure 1. LC-MS chromatogram of fraction 11 with acetonitrile: water eluent (THF 0.1%)

3.6.1. Identification of compounds at a retention time of 0.179 minutes

The mass spectrum at the 0.179 minute retention time in Figure 2 shows the positive ion at the peak of m/z of 442.49 [M+Na]⁺, m/z of 458.73 [M+K]⁺ and m/z of 420.44 [M+H]⁺, so that [M]⁺ is 419.44 amu. Buchanan *et al.* [20] discovered a compound from the *Stylotella agminata* sponge from the waters of Caroline Island, the Republic of Belau, with a theoretical mass of 419.87 amu. The study revealed that a compound with a molecular mass of 419 amu was identified as Palau'amine with the molecular formula C₁₇H₂₂ClN₉O₂. Thus, the compound with m/z of 419 in this study is thought to be a Palau'amine compound with the molecular formula C₁₇H₂₂ClN₉O₂. The molecular formula is consistent with the nitrogen rule because it has an odd number of nitrogen atoms.

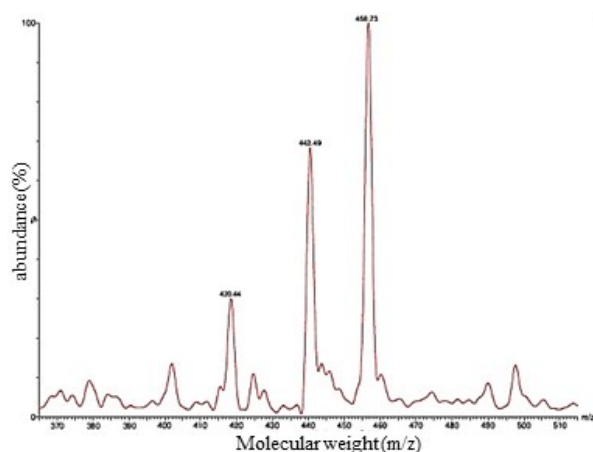


Figure 2. Mass spectrum for the peak at 0.179-minute retention time of fraction 11

Palau'amine is a pyrrole-imidazole hexacyclic alkaloid that contains a chain with eight chiral centers [20]. The compound, which was isolated from the *Stylotella agminata* sponge, has cytotoxic and immunosuppressive activities [21]. Palau'amine was successfully synthesized for the first time in 2010. This synthesis was carried out again by another researcher in 2015, where this synthesis route could explain the pharmacophores and the details of the mechanism of immunosuppressive activity [22, 23]. The structure of the Palau'amine compound is presented in Figure 3.

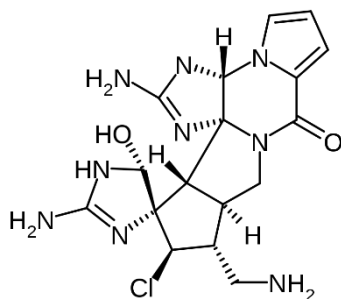


Figure 3. Structure of the Palau'amine compound

3.6.2. Identification of compounds at a retention time of 2.323 minutes

The ESI-MS mass spectrum at a retention time of 2.323 minutes in Figure 4 shows positive ions at the m/z peaks of 1097.10 $[M-Na+K+H]^+$, m/z of 1113.07 $[M+Na]^+$ and m/z of 1080.93 $[M+H]^+$, so that $[M]^+$ is 1079.07 amu. Based on literature studies, compounds with a mass of 1079 amu have never been published regarding the isolation of marine natural materials. This compound with a mass of 1079 is thought to be an unknown compound from the sponge.

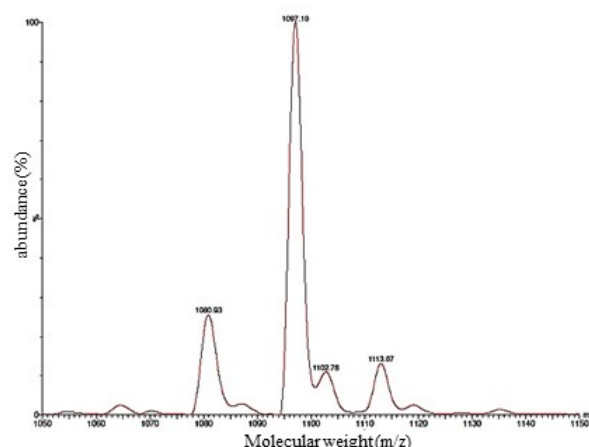


Figure 4. The peak mass spectrum at the retention time of 2.323 minutes from fraction 11

3.6.3. Identification of compounds at a retention time of 7.149 minutes

The mass spectrum at the retention time of 7.179 minutes in Figure 5 shows positive ions at the m/z peaks of 346.39 $[M+Na]^+$ and m/z of 324.35 $[M+H]^+$, so that $[M]^+$ is 323.35 amu. Cimino *et al.* [24] discovered a compound from the *Axinella verrucosa* sponge from Mediterranean waters with a theoretical mass of 323.00 amu. The study revealed that a compound with a molecular mass of 323 amu was identified as *hymenialdisine* with the molecular formula $C_{11}H_{10}BrN_5O_2$. Based on the above literature, the compound with m/z of 323 in this study was thought to be a hymenialdisine compound with the molecular formula $C_{11}H_{10}BrN_5O_2$. The molecular formula is consistent with the nitrogen rule because it has an odd number of nitrogen atoms.

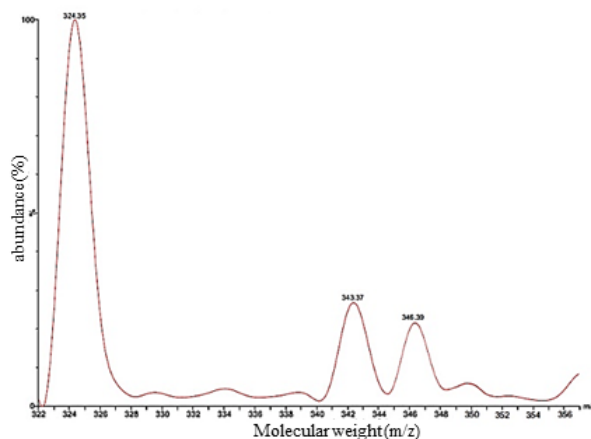


Figure 5. Mass spectrum for the peak at a retention time of 7.149 minutes of fraction 11

The hymenialdisine compound is a compound of the alkaloid group. This compound was first isolated in 1982 from the *Axinella verrucosa* sponge. This compound has also been isolated from the *Stylissa massa* sponge from the waters of Surigao, Philippines, and was reported to have MEK-1 (a cancer target) inhibitor activity with an IC_{50} value of 3 nM [10]. This compound has also been reported as an inhibitor of interleukin-2 production ($IC_{50} = 2.4 \mu M$) and tumor necrosis factor α (IC_{50} of 1.4 μM) [25]. Several researchers have synthesized this compound, but a recent

study succeeded in synthesizing hymenialdisine in 6 stages with a 44% yield [26]. Feng *et al.* [27] stated that hymenialdisine has antifouling activity against *P. viridis* with EC_{50} of 31.77 $\mu\text{g/mL}$, *B. neritina* with EC_{50} of 3.43 $\mu\text{g/mL}$, and *U. prolifera* with EC_{50} of 8.31 $\mu\text{g/mL}$. The structure of the hymenialdisine compound is presented in Figure 6.

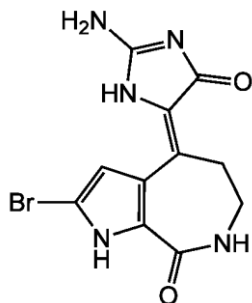


Figure 6. Structure of the hymenialdisine compound

3.7. LC-MS Results of Fraction 14

The LC-MS analysis of fraction 14 produced the chromatogram, as shown in Figure 7. The chromatogram shows that the separation at fraction 14 is good enough. The acetonitrile: water eluent system (THF 0.1%) with a flow rate of 0.8 mL/min resulted in a fairly good separation condition. In the LC-MS chromatogram fraction 14, six peaks with the mass spectrum were taken.

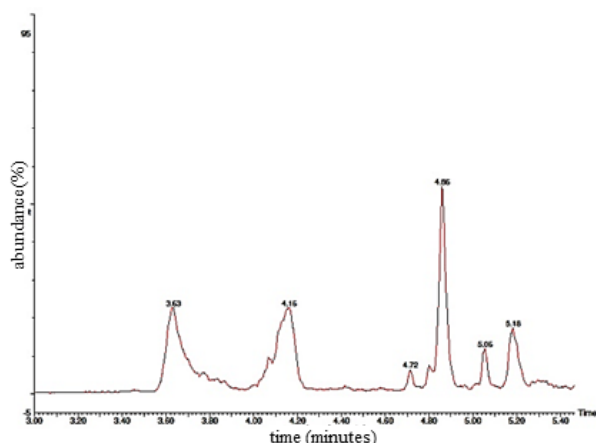


Figure 7. LC-MS chromatogram of fraction 14 with acetonitrile: water eluent (THF 0.1%)

3.7.1. Identification of compounds at a retention time of 3.626 minutes

The ESI-MS mass spectrum at a retention time of 3.626 minutes in Figure 8 shows positive ions at the m/z peak of 969.39 $[M+Na]^+$, m/z of 985.43 $[M+K]^+$ and m/z of 947.34 $[M+H]^+$, so that $[M]^+$ is 946.34 amu. Cafieri *et al.* [28] discovered a compound from the *Ectyoplasia ferox* sponge from the waters of the island of San Salvador, the Bahamas, with a theoretical mass of 946.07 amu. The study revealed that a compound with a molecular mass of 946 amu was identified as *ectyoplaside B* with the molecular formula $C_{46}H_{74}O_{20}$. Based on the above literature, the compound with m/z of 946 in this study was thought to be an *ectyoplaside B* compound with the molecular formula $C_{46}H_{74}O_{20}$.

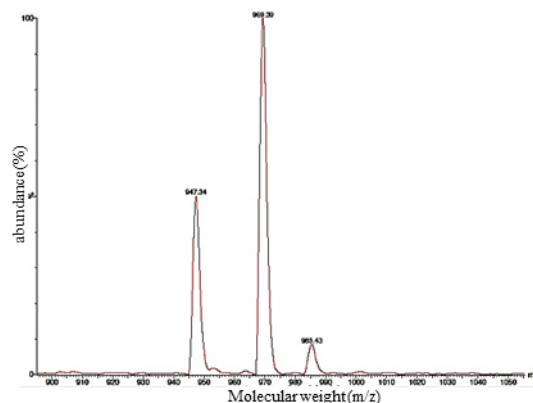


Figure 8. Mass spectrum for the peak at a retention time of 3.626 minutes of fraction 14

The *ectyoplaside B* compound was first isolated from the *Ectyoplasia ferox* sponge from the waters of the island of San Salvador, Bahamas. This white amorphous compound is a unique triterpene oligoglycoside with a sugar chain consisting of 2 β -galactose units and 1 α -arabinose unit. *Ectyoplaside B* was reported to have cytotoxic activity against cancer cells in the IC_{50} range of 8.5 to 11.0 $\mu\text{g/mL}$ [28]. The structure of the *ectyoplaside B* compound is presented in Figure 9.

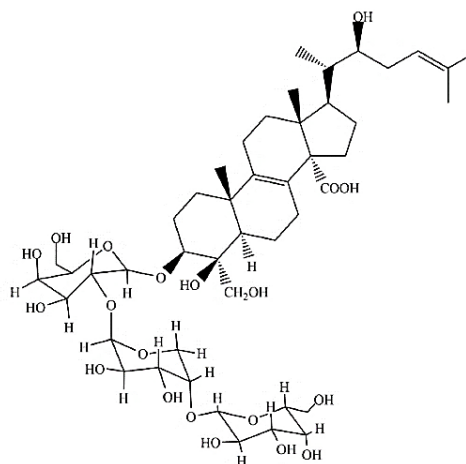


Figure 9. Structure of *ectyoplaside B* compounds

3.7.2. Identification of the compound at a retention time of 4.094 minutes

The ESI-MS mass spectrum at a retention time of 4.094 minutes in Figure 10 shows positive ions at m/z peak of 980.34 $[M+K]^+$, m/z of 964.37 $[M+Na]^+$ and m/z of 942.39 $[M+H]^+$, so $[M]^+$ is 941.39 amu. Based on literature studies, compounds with a mass of 941 amu have never been published regarding the isolation of marine natural materials. The compound with a mass of 941 is thought to be an unknown compound from the sponge.

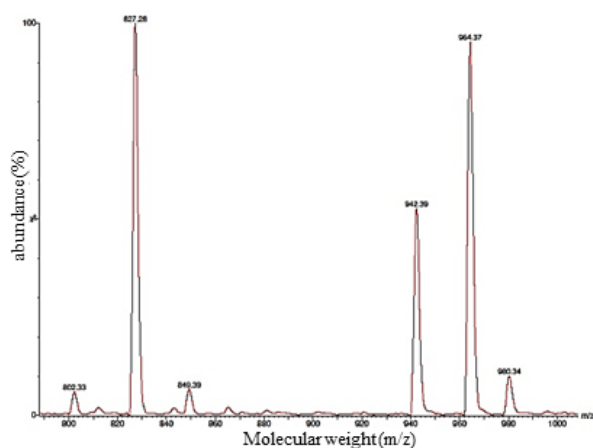


Figure 10. Mass spectrum for the peak at a retention time of 4.094 minutes of fraction 14

At the retention time of 4.094 minutes, there were also positive ions at the m/z peaks of 849.39 $[M+Na]^+$ and m/z of 827.28 $[M+H]^+$, so that $[M]^+$ is 826.28 amu. Tsuda *et al.* [29] found compounds from the *Hymeniacidon sp.* sponge from Okinawa Island, Japan, with a theoretical mass of 826.40 amu. The study revealed a compound with a molecular mass of 826 amu was identified as hymenamamide C with the molecular formula $C_{43}H_{54}N_8O_9$. Based on the above literature, the compound with m/z of 826 in this study was thought to be a hymenamamide C compound with the molecular formula $C_{43}H_{54}N_8O_9$. Hymenamamide C compound isolated from the *Hymeniacidon sp.* sponge was reported to have antifungal activity against *Cryptococcus neoformans* with a MIC value of 133 $\mu\text{g/mL}$. The structure of the hymenamamide C compound is presented in Figure 11.

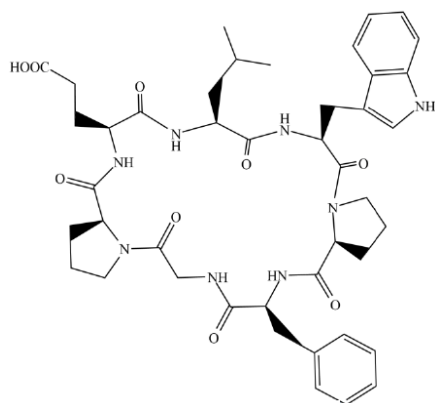


Figure 11. Structure of the hymenamamide C compound

3.7.3. Identification of compounds at a retention time of 4.860 minutes

The ESI-MS mass spectrum at a retention time of 4.860 minutes in Figure 12 shows positive ions at the m/z peak of 942.39 $[M+K]^+$, m/z of 926.35 $[M+Na]^+$, m/z of 904.44 $[M+H]^+$, so $[M]^+$ is 903.44 amu. Tsuda *et al.* [30] found a compound from the *Hymeniacidon sp.* sponge from Okinawa Island, Japan, with a theoretical mass of 903.52 amu. The study revealed that a compound with a molecular mass of 903 amu was identified as hymenamamide H with a theoretical mass of 903.52 amu and a molecular formula of $C_{47}H_{69}N_9O_9$. Based on the above literature, the compound with m/z of 903 in this study

was thought to be a hymenamamide H compound with the molecular formula $C_{47}H_{69}N_9O_9$. The molecular formula is consistent with the nitrogen rule because it has an odd number of nitrogen atoms.

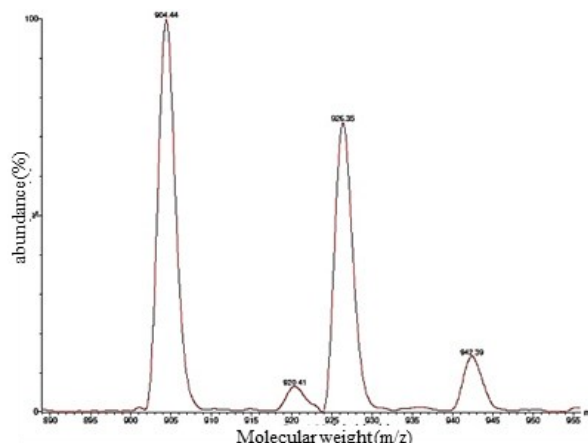


Figure 12. Mass spectrum for the peak at a retention time of 4.860 minutes of fraction 14

The hymenamamide H compound is a cyclopeptide group compound previously isolated from the *Hymeniacidon sp.* sponge from the waters of Okinawa Island, Japan, with cytotoxic activity against L1210 cells with an IC_{50} value of 6.3 $\mu\text{g/mL}$ [30]. The structure of the hymenamamide H compound can be seen in Figure 13.

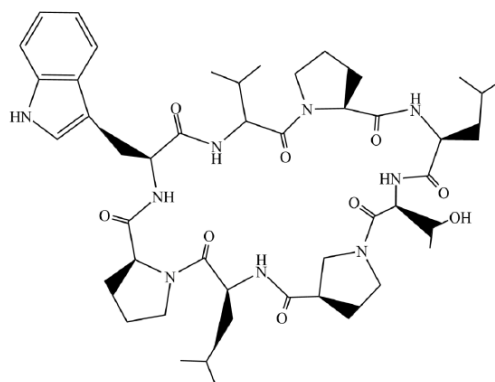


Figure 13. Structure of the hymenamamide H compound

Based on the results of LC-MS analysis, it can be predicted that the bioactive compounds contained in fraction 11 are alkaloid group compounds, which are Palau'amine and hymenialdisine compounds. Meanwhile, in fraction 14, it can be predicted that the bioactive compounds contained are alkaloid and terpenoid class compounds, which are ectyoplaside B, hymenamamide C, and hymenamamide H.

4. Conclusion

Based on this research, several things can be concluded. Isolation from *Stylissa massa* sponge resulted in 1.14 g (0.23%) ethyl acetate extract with 15 fractions separated by column chromatography. The toxicity test results for fractions 8, 11, 12, and 14 showed that the LC_{50} value was 13.080, 1.014; 11.332 and 3.595 $\mu\text{g/mL}$, respectively; giving indications of toxicity. The results of the antimalarial activity test showed that the fractions 8, 11, 12, and 14 are the most active with IC_{50} values of 82, 93,

105, and 96 µg/mL. Fractions 8, 11, 12, and 14 have potential as antimalarial agents with IC₅₀ values lower than chloroquine (115 µg/mL). The results of LC-MS analysis indicated that fraction 11 contained *Palau'amine*, *hymenialdisine*, and one unknown compound, while fraction 14 contained *ectyoplaside B*, *hymenamide C*, *hymenamide H*, and one unknown compound.

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