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Toxicity and Antibacterial Activity of ascidian *Eudistoma* cf. *purpuropuctatum*

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ABSTRACT

Ascidians are invertebrates found in coral reef ecosystems that produce bioactive compounds for pharmacological purposes where these animals can associate with microbes and have a great deal of molecular potential due to their bioactive secondary metabolites that they produce.

Using the ascidian extract *Eudistoma* cf. *purpuropunctatum* collected from Manado Bay, this study aims to determine its toxicity and antibacterial activity. Analyses showed the LC_{50} for ascidian *Eudistoma* cf. *purpuropunctatum* is 7.31 ppm and it is classified as extremely toxic. Further bioactivity testing of this ascidian shows that it has very strong activity against Gram-negative strain *Escherichia coli* DSM498 and Gram-positive strain *Bacillus megaterium* DSM32^T. In addition, the related species has a Minimum Inhibitory Concentration (MIC) value against *B. megaterium* at a concentration of 1000 ppm, whereas the Minimum Bactericidal Concentration (MBC) was greater than 1000 ppm.

KEYWORDS: Ascidian, Antibacterial, *Bacillus megaterium* DSM32^T, BSLT, Cytotoxic, *Escherichia coli* DSM498, *Eudistoma* cf. *purpuropunctatum*, , MBC, MIC.

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INTRODUCTION

The Indonesian Waters is the center of the world's coral reef diversity and is a rich source of natural products with unique structures and biological activities. The content of secondary metabolites possessed by marine organisms has attracted the attention of researchers, because these compounds have unique chemical structures and very interesting pharmacological activities, such as anticancer, antimicrobial, anti-inflammatory, antiviral, antifouling, and inhibiting enzyme activity [1-7]

Ascidian live sessile, which is attached to the substrate so that they carry out a self-defense mechanism by producing bioactive compounds (secondary metabolites) to control attachment sites, compete for space, prevent predator and pathogenic microbes. Although known as invasive animals, ascidian have benefits for humans such as being a source of protein. In addition, it is stated that ascidians are widely developed for marine natural products for biomedical applications. The existence of ascidian is unique because these organisms are found in various shapes, colors and sizes on various types of substrates, such as those found in the waters of North Sulawesi [8]. Several studies of marine organisms from Indonesia have been reported to have antimicrobial, antibacterial, anticancer/antitumor, antiviral activity [9-21]

The marine biodiversity of North Sulawesi mirrors the chemical diversity of the marine natural materials that inhabit it. The abundance and chemical diversity of marine organisms can be used as a research tool to develop the next generation of drug candidates. Therefore, the study aimed to determine the cytotoxicity and antibacterial properties of the ascidian *Eudistoma* cf. *purpuropunctatum* extract and fractions collected from Manado Bay.

MATERIALS AND METHODS

The tools used in this research are sample bottles, test tubes, Erlenmeyer, petri dishes, inoculation needles, paper discs, Whatman filter paper grade 3, Bunsen lamps, spatula, evaporator, micropipette, freezer, falcon tubes , ultrasonic shaker, orbital shaker, tweezers, paper duct tape, aluminum

foil, oven, autoclave, camera, tropical pacific invertebrates book, cotton and gauze, sterile cotton swab, analytical balance, shaker, aquarium, separatory funnel, incubator, vortex, incubator, laminar air flow, refractometer, filtered seawater, Nutrient Agar, Brain Heart Infusion (BHI), Müller Hinton Agar (MHA), aquadest, *Escherichia coli*, *Bacillus megaterium*, *Artemia* sp. Larvae, Amoxicillin, several solvents such as ethyl acetate, methanol, ethanol and n-octane and ascidian *Eudistoma* cf. cf. *purpuropunctatum.*

Sampling

Ascidian sample was collected from Manado Bay, Malalayang District, is located at 1°27'39.72"N and 124°47'31.47"E. (Figure 1). Sampling was carried out by diving at a depth of about 6-8 meters and a temperature of 29°C using SCUBA. The sample was collected directly from the substrate using a knife and placed in a plastic sample. Furthermore, the samples were documented and brought to the lab for further analysis. The book "Tropical Pacific Invertebrates" [22], including correspondence with an expert was used in order to assure the identification of the collected specimens (de Voogd, N 2021, pers. com. (personal communication).

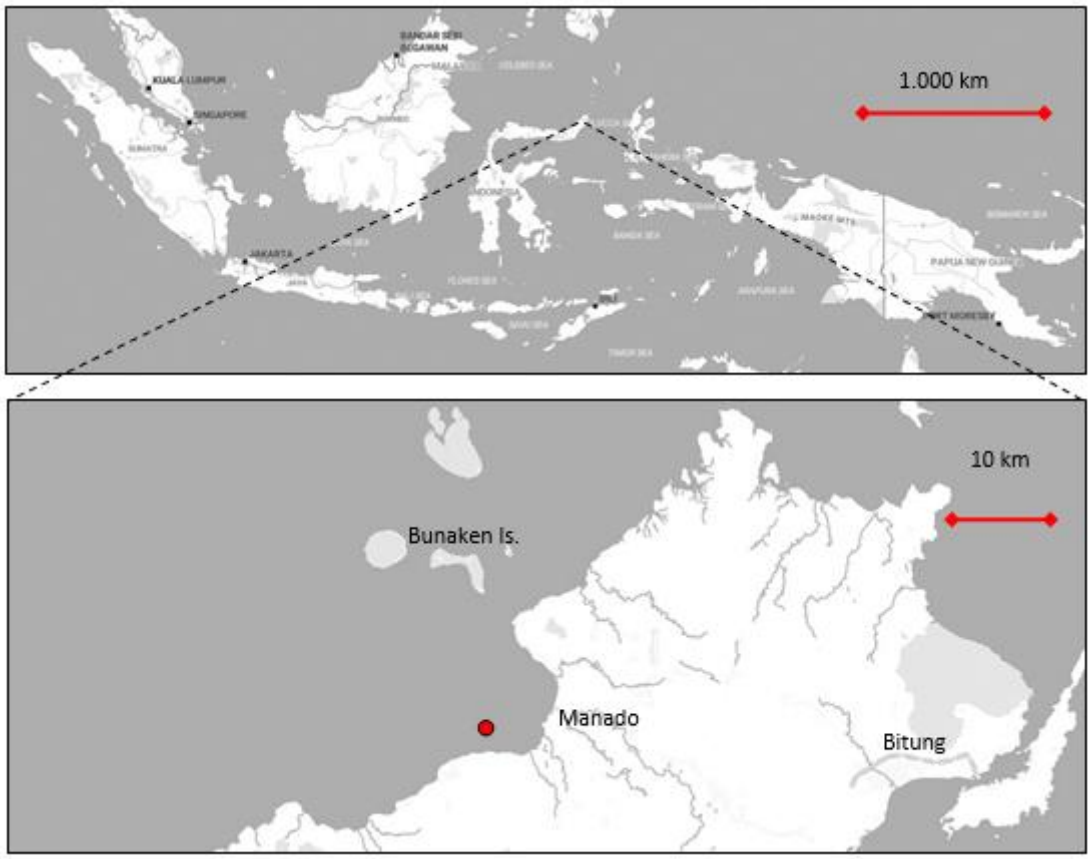

Figure 1. Sampling site of Ascidians *Eudistoma* cf. *purpuropunctatum*, Manado, Indonesia. Red dot = sampling point

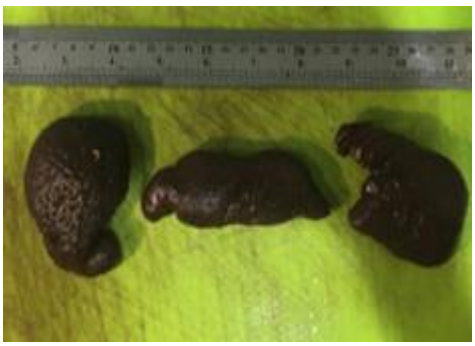

Figure 2. Ascidian *Eudistoma* cf. *purpuropunctatum* samples

Extraction

Extraction was carried out by the maceration method using ethyl acetate as the solvent. Samples of ascidian *Eudistoma* cf. *purpuropunctatum* (Figure 2) was rinsed with fresh water to remove salt concentration, weighed then sliced into small pieces and then filled into sample bottles. Furthermore, the

sample was soaked in ethyl acetate solvent and performed in a dynamic extraction using an orbital shaker. After 24 hours, samples were filtered using Whatman Paper Grade 3 followed by evaporating at a temperature of 40°C until lyophilized. The extract was inserted into a 50 mL of falcon tube for further analysis.

Cytotoxicity

The hatching of *Artemia* sp. larvae

Media for hatching the Artemia cyst was using filtered seawater with 34 ppt of salinity. This was done in a glass container which is separated compartment in the middle. One part was for the lightroom and the other for the darkroom. 0.5 gram of Artemia cyst was added in the dark side left for 24 hours for hatching and after 48 hours, the larvae were ready to be used for testing.

Cytotoxicity assay

Toxicity test with *Artemia* sp., following the modified method of [23]. The cytotoxic test was repeated three times at each concentration. Extract of ascidian *Eudistoma* cf. *purpuropunctatum*. with an initial concentration of 100,000 ppm was diluted into a series of dilutions using filtered seawater. The dilution series was made at 1000 ppm, 100 ppm, 50 ppm, 10 ppm, 5 ppm, 1 ppm, respectively. 5 ml of each concentration was put into each test tube followed by 10 larvae. A blank was made in a test tube containing filtered seawater and 10 larvae, then covered with aluminum foil. The observation was carried out every hour for 24 hours. The observations were made for death of *Artemia* sp. The mortality criterion for measuring the larvae is if the larvae are immobilized during observation [24].

DATA ANALYSIS

Toxic effects were obtained from observations by calculating the percent mortality (mortality) of *Artemia* sp. at each concentration. Number of *Artemia* sp. The dead in each tube for 24 hours were counted. Percentage of death larvae mortality obtained by the formula:

$$\% \text{ Dead} = \frac{\text{Number of dead larvae of } \textit{Artemia} \text{ sp.}}{\text{Number of test larvae}} \times 100\%$$

From the percent of deaths, the value of each concentration of test animals was sought regarding the Probit table. The treatment concentration of each test group is converted into the logarithmic value as the value of x and is made into a graph of a function with a linear equation, namely; y= a + bx. Where y is the Probit number and x is the concentration log, then a line is drawn from the Probit price to the x-axis, we get the concentration log. The logarithmic concentration is converted into antilogarithmic to get the value of y = 5 (Probit of 50% of test animal deaths) so that x is produced as the concentration log value. calculated and obtained from the antilog of the x value [25]

Antibacterial Assay

Sterilization

The equipment and materials used in this study were sterilized to avoid unwanted microbial contamination. Materials such as bacterial growth media were sterilized in an autoclave at 121°C for 15 minutes. Equipment such as petri dishes, test tubes, Erlenmeyer, tweezers, inoculum needles was sterilized using a hot air oven at 160°C for around 2 hours.

Nutrient Agar (NA) Media

Nutrient Agar (NA) was prepared by dissolving 23 grams of media in 1 L of demineralized water. Sterilized by autoclaving at 121ºC for 15 minutes. The media was poured into sterile petri dishes to solidify. For slant agar, 10 ml of media was transferred into a test tube and covered with sterile cotton and allowed to solidify at a slope of 15°.

Brain Heart Infusion (BHI) Media

Brain Heart Infusion (BHI) was prepared by dissolving 37 grams of media in 1 L of demineralized water, sterilized by autoclaving at 121ºC for 15 minutes.

Müller Hinton Agar (MHA) Media

Müller Hinton Agar (MHA) was prepared by dissolving 38 grams of media in 1 L of demineralized water. Sterilized by autoclaving at 121ºC for 15 minutes. The media was kept at 50 ºC to prevent solidification. Bacteria culture was added to it to reach a final density of $1x10^5$ cells/ml, followed by pouring into petri dishes to solidify.

Antibacterial Assay

Gram-negative bacteria *E. coli* DSM498 and Gram-positive bacteria *B. megaterium* $DSM32^T$ strains obtained from The Leibniz Institute-German Collection of Microorganisms and Cell Cultures GmbH (Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ)), Braunschweig, Germany. Both strains were cultured on nutrient slope agar overnight. The cultures were harvested and measured for their density. The initial bacterial density was checked by using a UV-Vis spectrophotometer (λ = 600 nm). The value of OD_{600} of 1 is equivalent to a bacterial density of $8x10^8$ cells/ml. The cultures were diluted until $1x10^6$ cells/ml prior to use, 1 ml of the bacterial culture was subjected and seeded onto the MHA agar and after solidification, 20 µl of the extract of each sample or partition fractions were spotted onto the plates. Chloramphenicol (1mg/ml) and 96% ethanol served as positive and negative controls, respectively. Plates were incubated at 37°C for 24 to 48 hours and checked for inhibition zones. All were repeated in triple.

Partition

Ascidian extract was partitioned using water, ethyl acetate, ethanol and n-octane with the final result obtained in 3 fractions, namely water (polar), ethanol (semipolar). and octane fractions (non-polar). The dried extract was dissolved with ethyl acetate and water and then put into a separatory funnel and shaken slowly until two eluents were well mixed and then allowed to stand still until fractional separation occurs. When the two fractions had separated into 2 layers, the lid of the separatory funnel was opened and the

separatory funnel tap was slowly opened and collected into separate flasks. Both fractions were then evaporated.

The partitioning was continued by dissolving the dried ethyl acetate fraction with 96 % of ethanol into a separatory funnel. The n-octane solvent was added to reach the final volume of 1:1, then shaken until dissolved well and then allowed to stand still until the fraction separation occurred. Followed the procedure mentioned above. All fractions were then reperformed for their antibacterial property in order to see which fraction had antibacterial activity following the same test procedure previously described. The fraction that shows the largest inhibition zone in the test will then further investigate their MIC and MBC value.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Assays

The MIC and MBC methods are used to determine the smallest concentration of antimicrobial agents to inhibit (MIC) or to kill (MBC) the tested microorganisms after 18 - 24 hours [26]. The MIC test was carried out using the microdilution method. The active fraction whose initial concentration was 100 mg/ml (100,000 ppm) was made in a series of dilutions of 1000, 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.9 ppm, respectively. A total of 10 sterile test tubes were prepared and labeled. Bacterial suspension of *B. megaterium* with the concentration of $1x10^3$ cells/ml was added to the series of concentrations mentioned previously. Negative control was a test tube containing bacterial concentration no active fraction of ascidian *Eudistoma* cf. *purpuropunctatum* was added. Furthermore, all tested tubes were incubated at 37°C for 1 x 24 hours. After 1 x 24 hours, observations were made to check whether bacteria grow on the media which is characterized by clearness to turbidness of the growth media in comparison to negative and blank controls.

On the other hand, the MBC test follows the agar dilution method. After the MIC concentration had been determined, 50 ul was taken from a test tube that showed no turbidity from the MIC test and dripped onto NA medium and scratched in a zigzag pattern. Then the petri dishes were then incubated for 1 x 24 hours at 37°C. After 24 hours, the media is observed. If no bacterial colonies are growing on the media, then this indicates the Minimum bactericidal concentration (MBC) value.

RESULTS AND DISCUSSION

Extraction

89 g of ascidian *Eudistoma* cf. *purpuropunctatum* were extracted by maceration method giving 34,8 g of crude extract.

Brine Shrimp Lethality Assay

Table 1. Mortality results of Artemia larvae towards ascidian *Eudistoma* cf. *purpuropunctatum* extract

Repetition	*Eudistoma* cf. *purpuropunctatum* extract Conc. (ppm)					Negative control
	1	5	10	100	1000	
I	1	1	7	10	10	0
II	0	2	9	9	10	0
III	2	0	9	10	10	0
Total Deaths	3	3	25	29	30	0
Average	1	1	8.3	9.6	10	0
% Larvae Death	10	10	83	96	100	0
Log Conc.	0	0.6	1	2	3	0
Probit Value	3.72	3.72	5.95	6.75	8.95	0
Calculated LC_{50}	**7.31**					

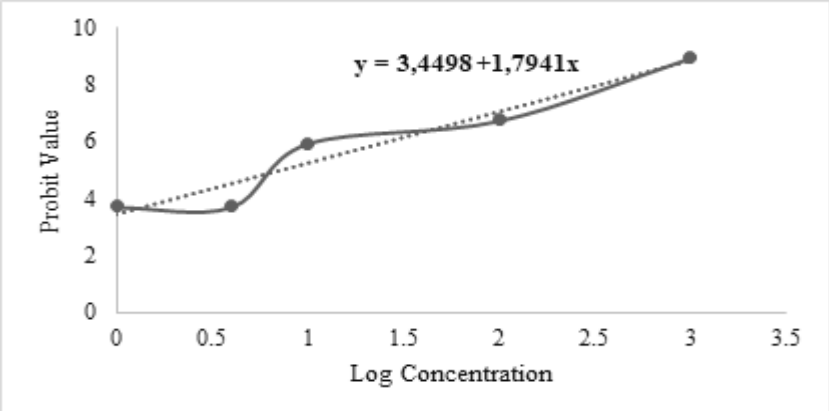

Figure 3. ascidian *Eudistoma* cf. Linear Regression. *purpuropunctatum*

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Based on the BSLA test on the ascidian *Eudistoma* cf. *purpuropunctatum* extract (Table 1), the following linear regression equation was obtained (Figure 3):

$y = 3.4498 + 1.7941x$
$5 = 3.4498 + 1.7941x$
$x = (5-3.4498)/1.7941$
$x = 0.8640$
LC_{50}= Antilog 0.8640
LC_{50} = 7.31 ppm

Hence, a higher concentration of the extract resulted in a higher mortality rate. The negative control showed no dead *Artemia* sp. This control was performed to check whether the death of the test animals was caused by the extract tested. According to the test, the mortality of Artemia sp., larvae was exclusively caused by the active content in the *Eudistoma* cf. *purpuropunctatum* extract, with the LC_{50} value is 7.31 ppm.

[27] stated if the value is less than 1000 ppm, the extract is toxic, and if the value is greater than 1000 ppm, the extract is non-toxic. Clarkson and Tanamatayarat have characterized it more precisely. According to Clarkson, toxicity is classified as non-toxic if it is above 1000 ppm, low toxic if it is between 500 to 1000 ppm, moderate toxic if it is 100-500 ppm, and very toxic if it is 0-100 ppm [28]. However, the toxicity classification according to Tanamatayarat is extremely toxic if the LC_{50} value is less than 10 ppm, moderately toxic, when the value ranging from 10 to 100 ppm, weakly toxic when it between 100 to 1000 ppm, and non-toxic when the result is beyond 1000 ppm [29]. Based on BSLA result, it can be concluded that the extract of ascidian *Eudistoma* cf. *purpuropunctatum* is categorized as extremely toxic according to the Tanamatayarat classification.

The BSLA test is a rapid way in predicting an extract or compound, and there is a correlation between BSLA toxicity and cytotoxicity of a compound in a variety of cell lines [30]. Since the particular extract obtained was less than 10 ppm, the LC_{50} value of ascidian extract *Eudistoma* cf. *purpuropunctatum* demonstrated that the extract has very strong cytotoxic effects and potential as an anticancer drug development. Several Eudistoma species have been reported around the globe contain anticancer property. Rajesh and Annapan (2015) revealed that ascidians *Eudistoma viride* which is found along the India's Southeast Coast, was tested for its in vitro cytotoxic activity against human cervical carcinoma (HeLa). The crude methanolic extract of the related species displays an IC_{50} of 53 µg/ml [31]. Two new staurosporine derivatives are produced by *E. vannamei*, an endemic species on Brazil's Northeast Coast. These chemicals have been found to be cytotoxic in a variety of tumor cell lines [32]. Other compound such as rigidin derived from *E. rigida*,is an antimitotic agent that targeting to destabilize microtubule which in turn will inhibit the process of mitosis in cancer cells [33]. Cholesta-4,6-dien-3-ol, isolated from the marine ascidian *E. kaverium*, possesses cytotoxic effect in HeLa and MCF-7 cell line through apoptosis induction [34]. No report elsewhere has been recorded regarding bioactive compounds derived from *Eudistoma purpuropunctatum.*

Artemia sp., was selected as cytotoxicity because the related species has biological similarities with mammal cells, for example, the type of DNA-dependent RNA polymerase of the species is similar to that found in mammals [35, 36]. Correspondingly, it also has ouabain sensitive Na+ and K+ dependent ATPase, an enzyme that is also found in mammal cells. The compounds and extracts that show inhibition activity on both systems prior stated in *Artemia* usually will show similar effects on mammalian cells [37-39].

Antibacterial Activity of *Eudistoma* cf. *purpuropunctatum* extract

The results of the observation of antibacterial activity on the test bacterial media after being incubated for 1 x 24 hours show that the crude extract showed inhibition on the growth of *E. coli* and *B. megaterium* bacteria with various diameters of inhibition zones. The classification of the strength criteria of an antibacterial agent is as follows: the diameter of the inhibition zone <5 mm is categorized as weak, and the zone of inhibition 5-10 is categorized as moderate, while the diameter of the inhibition zone of 10-20 mm is categorized as strong and beyond 20 mm is declared a compound or extract has very strong antibacterial activity [40]. The clear zone of ascidian *Eudistoma* cf. *purpuropunctatum* extract against *E. coli* bacteria showed an inhibition zone of 23 mm which characterized as very strong and *B. megaterium* showed an inhibition zone of 17.3 mm which characterized as very strong based on Davis and Stout classification.

After 2 x 24 hours of incubation, the inhibition zone of *Eudistoma* cf. *purpuropunctatum* extract against *E. coli* showed an inhibition zone of 5.3 mm (moderate) and *B. megaterium* showed an inhibition zone of 17 mm (strong).

Antibacterial Activity Test of *Eudistoma* cf. *purpuropunctatum* Fractions

The results of the antibacterial Assay of fraction *Eudistoma* cf. *purpuropunctatum* towards *B. megaterium* only the

ethanol fraction showed activity. No clear zone was observed from both octane and water fractions .

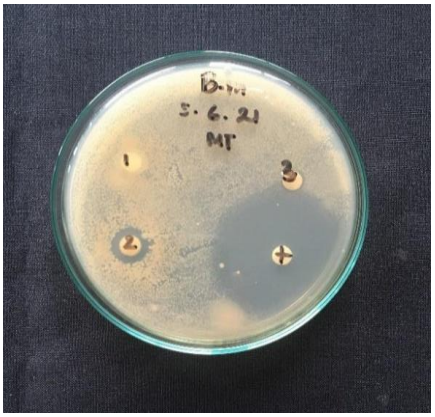

Figure 4. Antibacterial activity of *Eudistoma* cf. *purpuropunctatum* fractions towards *B. megaterium* in 1 x 24 hours of incubation

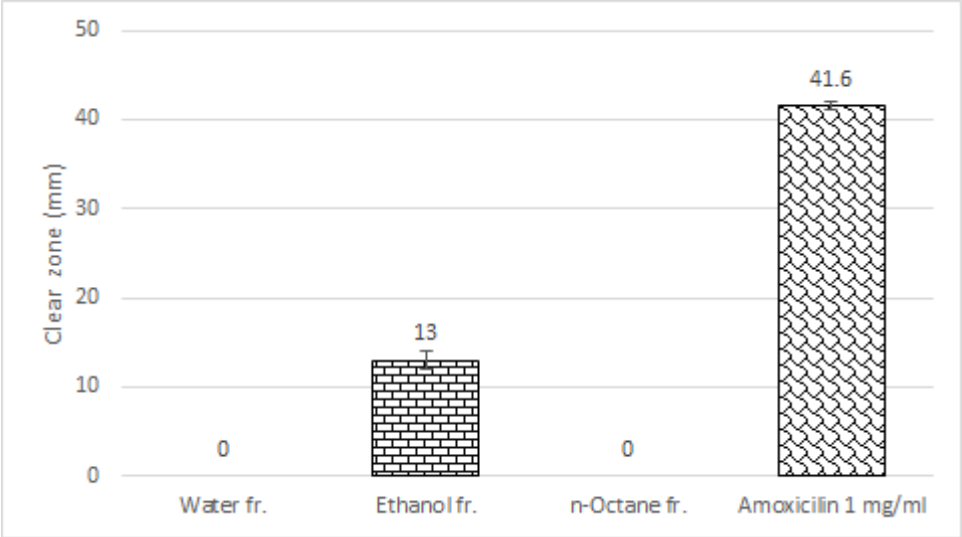

Figure 5. The average inhibition of *Eudistoma* cf. *purpuropunctatum* fraction towards *B. megaterium* in 1 x 24 hours of incubation

The measurement data is presented in Figure 5. It can be explained that only the ethanol fraction showed to be active after 24 hours of incubation, with an average of 13±1 mm and as a comparison, the positive control showed an average of 41.6±0.5 mm. The results of the antibacterial activity test of the test fraction against *B. megaterium* bacteria is presented in Figure 6. The result shows only the ethanol fraction showed to be active towards *B. megaterium* after 48 hours of incubation.

Figure 6. Antibacterial activity test of *Eudistoma* cf. *purpuropunctatum* fraction after 48 hours of incubation

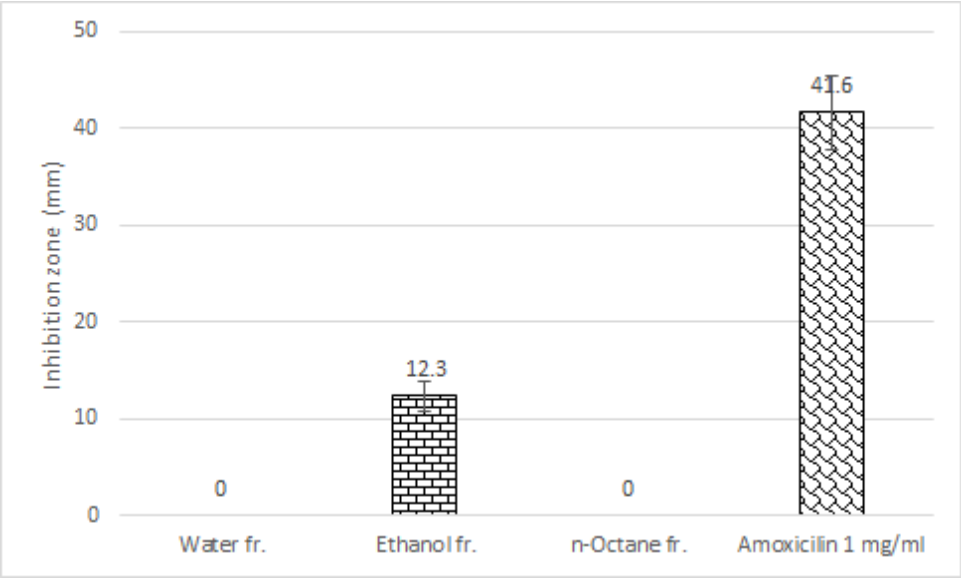

Figure 7. The average inhibition of *Eudistoma* cf. *purpuropunctatum* fraction towards *B. megaterium* for 2 x 24 hours

The observation in Figure 7, can be explained by the fact that only the ethanol fraction, with an average of 12.3±1.5 mm, displayed inhibition towards *B. megaterium* after 48 hours of incubation. The antibacterial ingredient from the extract of ascidian *Eudistoma* cf. *purpuropunctatum* is located in the semipolar section, according to the findings of the antibacterial activity test, because only the ethanol fraction can suppress the growth of *B. megaterium*. In a previous study by [41] revealed that ascidian *Eudistoma* sp., collected from Pangalisang Waters, Bunaken Island, was active against *E. coli* and *S. aureus* in both the polar and semipolar fractions . Meanwhile, antibacterial activity has been reported for ascidian *Lissoclinum* sp., extract from Pangalisang waters on Bunaken Island [42]. Eudistomin Y6, a beta-carboline-based metabolite isolated with 6 other related compounds from *Eudistoma* sp., collected near Tong-Yeong City, South Sea, Korea, only exhibit mild antibacterial activity toward Gram-positive bacteria *Staphylococcus epidermis* and *Bacillus subtilis* [43]. Other eudistomin type compound, eudistomin X derived from *Eudistoma* sp., growing in Micronesian Waters also exhibited antibacterial activity toward *Bacillus subtilis*, *Staphyloccocus aureus*, and *Escherichia coli* and was also found to be fungicidal against *Candida albicans* [44].

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Assays

Following the procedures described above, the ethanol fraction of *Eudistoma* cf. *purpuropunctatum* was tested to determine its Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). Figures 8 and 9 show the results of the MIC and MBC after 24 hours of incubation.

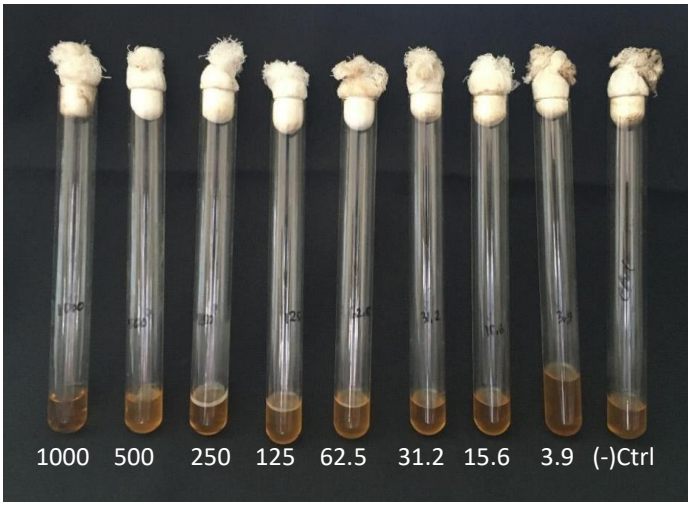

Figure 8. the MIC Test Result

Figure 9. The MBC Test Result

In comparison to other series of dilutions, the 1000 ppm growth medium was still clear to the sightedness. The MIC test results were carried over to the MBC test to determine the lowest concentration at which tested bacteria are killed. The MBC test results showed the growth of bacterial colonies on solid NA medium incubated for 24 hours, indicating that the MBC value of the ethanol fraction of ascidian *Eudistoma* cf. *purpuropunctatum* is greater than 1000 ppm.

At a concentration of 1000 ppm, the ethanol fraction of ascidian *Eudistoma* cf. *purpuropunctatum* only exhibits bacteriostatic properties, as it only inhibits the growth of the tested strain without having the ability to kill it. Antimicrobial agents are categorized as bacteriostatic if it can only inhibit bacterial growth when the extract, fraction or compound is continuously administered, but if it is stopped or exhausted, bacterial growth resumes, as evidenced by the growth of bacterial colonies. On the contrary, if the clarity remains constant during the next incubation period, the extracts, fractions, or even compounds are classified as bactericidal. This is due to the fact that it can still kill bacteria and stop their physiological activity even when the administration of the interesting agents is stopped.

The findings of this study are very intriguing because the related ascidian fraction have a significant activity array against Gram-negative *E. coli.* Gram-negative infection control has so far become a major issue in modern medicine. This is due to the characteristics of the specific group, such as their dense and compact peptidoglycan layer, which inhibits antibiotics' ability to reach bacterial cellular mechanisms. It also has a "efflux-pump mechanism," which is a compound removal mechanism that isn't required in their cellular biotransformation processes via their secretion system [45, 46].

CONCLUSIONS

1. A cytotoxic test using the Brine Shrimp Lethality Assay (BSLA) was carried out on the ascidian crude extract of *Eudistoma* cf. *purpuropunctatum.* The result shows that the extract of a particular species was extremely toxic with an LC_{50} value of 7.31 ppm.
2. Crude extract of the ascidian *Eudistoma* cf. *purpuropunctatum* inhibits the growth of bacteria *E. coli* DSM498 and *B. megaterium* DSM 32T, with inhibition zones ranging from strong to very strong.
3. Only the ethanolic fraction from the *Eudistoma* cf. *purpuropunctatum* extract was demonstrated to be active towards *B. megaterium*.
4. The Minimum Inhibitory Concentration (MIC) value of *Eudistoma* cf. *purpuropunctatum* ethanolic fractions is at a concentration of 1000 ppm, while the Minimum Bactericidal Concentration (MBC) value is beyond 1000 ppm.

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