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Brine Shrimp Lethality Test of Methanolic Extracts from Four Different Marine Biota in Lampung Province, Indonesia

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Abstract

This study aims to test the LC50 toxicity activity of brine shrimp using four samples of marine biota. The LC50 toxicity activity has the potential as an anti-bacterial, anti-fungal, and anti-cancer agent that needs to be studied further before being used as an industrial-scale natural product, this study is basic research on the brine shrimp lethality test. The test was carried out using the Brine Shrimp Lethality Test (BSLT), which analyzes the number of deaths of shrimp larvae to determine the level of toxicity of an ingredient. The marine biota samples used were Rhizopora sp., Sargassum sp., Halimeda sp., Diadema setosum. The manufacturing stage begins with making extracts and hatching artemia, then toxicity tests are carried out within 24 hours with different concentrations of sample solutions. The results of this research show that the sample extracts that have the highest to lowest toxicity values respectively are Rhizopora sp., Sargassum sp., Halimeda sp., Diadema setosum. with a value of 870.96 µg/mL; 745.05 µg/mL; 697.97 µg/mL; 575.4 µg/mL.

Keywords: *LC50, BSLT, Rhizopora sp., Sargassum sp., Halimeda sp., Diadema setosum*

1. Introduction

Antibiotics are medicines used to treat bacterial infections, which have the properties of destroying bacteria (bactericidal) or inhibiting bacterial growth (bacteriostatic) which can be grouped based on their mechanism of action, chemical structure and spectrum of activity (Indonesian Ministry of Health Regulation 28, 2021), for more than 70 years antibiotics have been used to treat infectious diseases such as micro bacterium, staphylococcus, streptococcus, enterococcus, to the treatment of postoperative patients (Desrini, 2015).

The usage of antibiotics that are not appropriate for the procedure can trigger the presence of antibiotic resistance. Antibiotic resistance is the effect of the improper application of antibiotics or the mutation of bacterial genes that cause resistance to antibiotics given (Putra et al., 2019). Bacteria that are resistant to antibiotics will be immune to the treatment given, which has the impact of increasing mortality, morbidity, and excessive treatment costs (Rukmini et al., 2019). Antibiotic resistance is reported by the world health agency to have become a major threat to human health the world (Putra et al., 2019), several case studies of antibiotic resistance reported in Indonesia include the presence of MRSA or methicillin-resistant Staphylococcus aureus and extended-spectrum βlactamase at RSUD Soetomo Surabaya in 2000-2004 (Hadi et al., 2008), and Dr. Kariadi Hospital in 2001-2005, germ resistance to ampicillin, gentamicin and cefotaxime antibiotics at H. Adam Malik Hospital in 2008-2010 (Sianturi, 2012), resistance to acute appendicitis and peritonitis at Raden Mattaher Hospital in Jambi in 2016-2018 (Ambasari et al, 2020).

It is widely known that contents such as alkaloids, flavonoids, terpenoids, and steroids have been widely studied as secondary metabolite compounds that can inhibit the growth of gram-positive or gram-negative bacteria. In the field of fisheries, the utilization of secondary metabolite compounds is also

commonly applied by utilizing mangrove species (Rhizophora sp., Soneta alba), seaweed (brown and green), and some marine biota such as sea urchins or *Diadema setosum*.

Mangroves have been widely applied in the medical field, for the therapy of various diseases such as eye diseases, skin diseases, asthma, rheumatism, diabetes, hepatitis, malaria, cholera, dysentery, fever, tumors, leukemia, as analgesics, antiseptics, and antimicrobials/antibiotics (Bandaranayke, 1998; 2002), as well as seaweed which has been widely studied for the medical field such as anticoagulant, anticancer, antithrombotic, anti-inflammatory, antiviral, antibacterial (Burtin, 2003; Shiratori et al., 2005) to the treatment of calcium deficiency risk (Fitton, 2005) and sea urchins in the medical field have the potential as antifungal, antibacterial and anti-inflammatory compounds.

The potential of mangrove, seaweed, and sea urchin as alternative antimicrobial agents should be the preliminary study of toxicity assay with the brine shrimp lethality test (BSLT) method. The BSLT method is a technique to determine the bioactivity of compounds contained in natural resources using Artemia salina larvae by analyzing the total shrimp larvae mortality to assess the toxicity of the tested natural substances (Kusmiati, 2014). The BSLT study of mangrove extracts (*Rhizopora* sp), seaweed (*Sargassum* sp and *Halimeda* sp) and sea urchins (*Diadema setosum*) aims to determine the effective doses of extracts in the utilization of their bioactive compound content so as to provide a reference dose for the application of mangrove extracts (*Rhizopora* sp), seaweed (*Sargassum* sp and *Halimeda* sp) and sea urchins (*Diadema setosum*) for further studies on the utilization or manufacture or engineering of natural products as antimicrobials.

2. Materials And Methods

Collection of Material

The fresh mangrove (*Rhizopora* sp), seaweed (*Sargassum* sp and *Halimeda* sp), and sea urchins (*Diadema setosum*) were collected from Ketapang Beach, Batu Menyan Village, Teluk Pandan District, Pesawaran Regency, Lampung Province. Sampling was carried out on the beach at low tide, then the samples were washed with pure unsalted water to remove any debris and epiphytes, and the samples were stored in sample bottles.

Extraction of Materials

200gram samples from *Rhizopora* sp., *Sargassum* sp., *Halimeda* sp., and 10.08gram samples of sea urchins (*Diadema setosum*) were macerated using 100 ml methanol solvent in Erlenmeyer for 24 hours at room temperature (25°C), and filtered through filter paper to separately filtrate and solvent residue, which was repeated till the maceration was colorless. The study Flow Diagram can be seen in Figure 1. The maceration products from each sample were extracted separately with Soxhlet Extractor for two days. The clear filtrate was evaporated with a rotary evaporator at 40°C to the dried residue.

Determination of Cytotoxicity

Brine Shrimp

Brine Shrimp, *Artemia salina* Leach, commonly called Artemia, is a genus of the family Artemiidae, first identified in Lymington, England (1755). *Artemia salina* is one of the organisms that is often used to assay bioactivity. The assay based on Artemia is widely recognized as the method of the Brine Shrimp Lethality Test (BSLT).

Hatching of Brine Shrimp

Freeze-dried cysts are readily found in aquarium outlets around Lampung Province. They last for several years and can be hatched without special equipment. The method is 0.5 grams of artemia eggs added with 700 mL of seawater and placed into each hatching tank, the tank was provided with TL lamps and aerated by aerators. After 24 hours, the artemia eggs will hatch into larvae and be kept in the media to be used as inoculation material after 48 hours of hatching.

Brine Shrimp Lethality Test

The cytotoxic activity of the samples was evaluated using the brine shrimp mortality method by conducting a preliminary test to be compared with the primary test in which 6 graded doses in the preliminary test of *Rhizopora* sp., Sargassum sp., *Halimeda* sp., and *Diadema setosum* samples (viz. 1000 µg/mL, 100 µg/mL, 10 µg/mL, 1 µg/mL, 0.1 µg/mL, and 0 µg/mL) were used. While for the primary test the LC_{50} of *Rhizopora* sp. samples (*viz.* 1999.5 µ/mL, 891.84 µg/mL, 239.88 µg/mL, 83.17 µg/mL, 28.84 µg/mL, and 0 µg/mL), *Sargassum* sp. and *Halimeda* sp. samples. (*viz*. 2000 µ/mL, 1500

µg/mL, 700 µg/mL, 500 µg/mL, 300 µg/mL, and 0 µg/mL), *Diadema setosum* samples (*viz*. 2000 µ/mL, 1700 µg/mL, 1500 µg/mL, 1300 µg/mL, 1140 µg/mL, and 0 µg/mL).

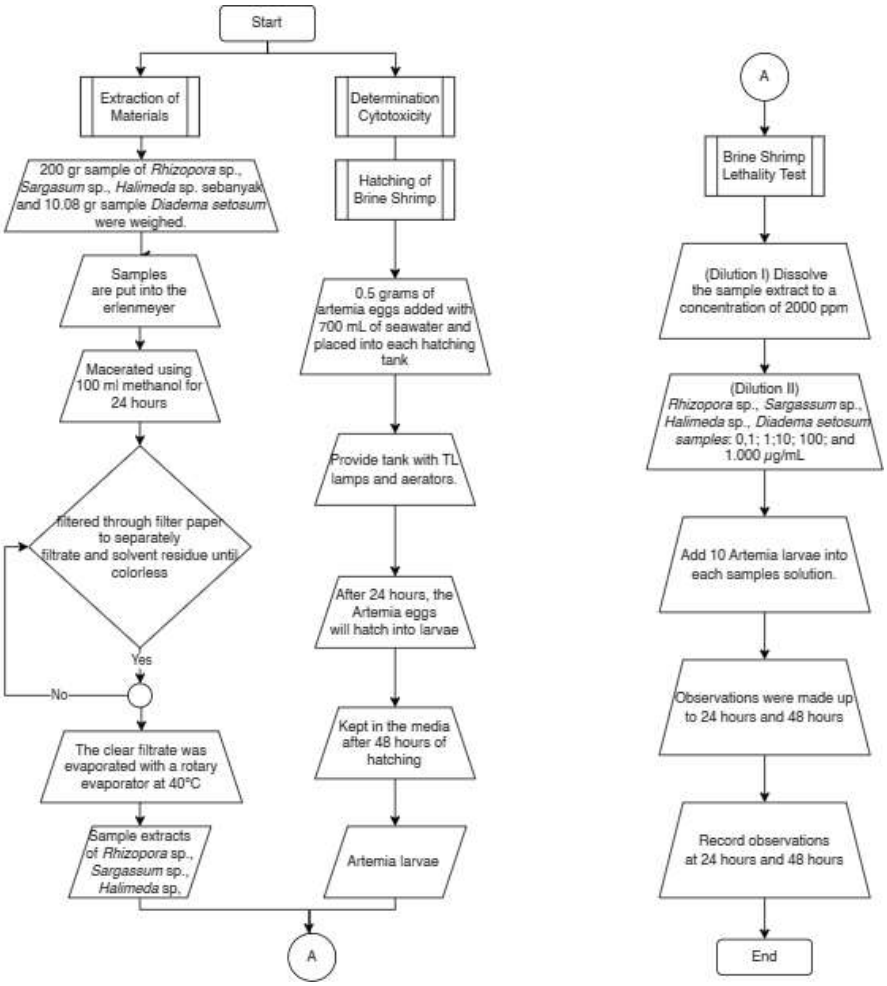

Figure 1. Research Flow Chart

Source: Authors

The number of survivors was counted after 24 hours. Larvae were considered dead if they did not exhibit internal or external movement for several seconds of observation. Larvae were not fed. To ensure that the mortality observed in the BSLT could be attributed to the bioactive compound and not to starvation; we compared the larvae that died in each treatment with the larvae that died in the control.

3. Results and Discussion

Extract Material

The maceration results of the samples used were evaporated using a rotary evaporator at 48°C, 200 rpm for 24-48 hours, the yield results of the sample extracts are shown in Figure 2. Based on Figure 2, the maceration results from each sample have different extract weights. It is shown in Table 1.

Table 1. Result of the maceration of each sample

Tested Sample	Initial of Weight (Gram)	Extract of Weight (Gram)	Yield of Extract (%)
***Rhizopora* sp.**	200	12.20	6.1
***Sargassum* sp.**	200	24.0	0.008195
***Halimeda* sp.**	200	22.6	11.3
Diadema setosum	200	22.84	24.66

Source: Authors

Based on Table 1, two samples produce yields of more than 10%, such as *Halimeda* sp. and *Diadema setosum*. The yield is the ratio between the number of metabolites after the evaporation process and the weight of the sample used, whereas if the resulting yield is greater than 10%, it is indicated that the metabolite results from an extracted sample are good (Wardiningrum *et al*. 2019).

Figure 2. Extraction results from each sample

Source: Authors

On the other side, *Rhizopora* sp. and *Sargassum* sp. samples had less than 10% yields. The low yield percentage indicates the lower the bioactive compounds contained therein and is directly proportional to their antioxidant activity (Lantah *et al*., 2017). The percentage of yield obtained from the extraction results is affected by several factors, such as type of solvent, ratio of material weight to solvent volume, temperature, mixing, extraction time, solid size, and length of soaking (Gazali *et al*., 2018). Based on several variables, the factor that triggers the low yield in this study is the ratio of the weight of the material to the solvent volume.

Results of *Brine Shrimp Lethality Test* (BSLT)

The LC_{50} values from the brine shrimp mortality test obtained for these extracts are shown in Table 2 and Table 3. All extracts showed significant results of toxicity to brine shrimp. The results of this study compare the preliminary test with the primary toxicity test. The LC_{50} values of the extracts of each sample were in the range of 575.4 to 870.96 µg/mL. So these extracts can be considered as promising candidates for antibacterial compounds derived from marine biota. The highest LC_{50} value was the extract from *Rhizopora* sp., which was 870.96 µg/mL and showed more cytotoxic effects, while *Diadema setosum* was the least among the other biota tested with an LC_{50} value of 575.4 µg/mL.

Table 2. Toxicity activity of methanolic extract of fourth different marine biota from Lampung Province on brine shrimp *Artemia salina*.

Tested materials	Concentration tested (µg/mL)	Probit	Mortality (%)
Rhizopora sp.	1999.5, 691.84, 239.88, 83.17, 28.84	5.74, 4.56, 4.39, 3.12, 3.12	43, 33, 26, 20, 10
Sargassum sp.	2000, 1500, 700, 500, 300	6.08, 5.48, 5.16, 5.08, 4.36	87, 70, 57, 33, 27
Halimeda sp.	2000, 1500, 700, 500, 300	5.84, 5.33, 5.25, 4.9, 4.16	80, 63, 60, 47, 20
Diadema setosum	2000, 1700, 1500, 1300, 1140	6.48, 6.08, 6.02, 5.95, 5.84	93, 83, 81, 80, 73

Source: Authors

Based on Table 2 used 5 different concentrations. In the sample of *Rhizopora* sp. concentrations of 28.84 and 83.17, the percentage of mortality was 10 and 20% and the same probit was 3.12. At a concentration of 239.88, the mortality percentage was 26.7 and the probit was 4.39. At a concentration of 619.84, the mortality percentage was 33.3 and the probit was 4.56. The last concentration was 1999.5 with a mortality percentage of 76.7 and a probit of 5.74. Analysis of LC_{50} was done by constructing a linear graph of the relationship between the probit value and the Log_{10} concentration. Based on the equation graph, the LC_{50} was found to be 870.96 µg/mL. Therefore, the *Rhizopora* sp. extract will cause 50% mortality at a concentration of 870.96 µg/mL.

In the sample of *Sargassum* sp. the concentration of 300 µg/mL has a percentage of 27% and the probit was 4.36. At a concentration of 500 µg/mL, the percentage of mortality is 33% and the probit was 5.08. At a concentration of 700 µg/mL, the percentage of mortality is 57% and the probit was 5.16. At a concentration of 1500 µg/mL, the percentage of mortality is 70% and the probit was 5.48. The last concentration is 2000 µg/mL with a mortality percentage is 87% and a probit was 6.08. With the results of the calculation of the effect of log concentration on probit mortality of artemia larvae, LC_{50} was obtained at 745.05 µg/mL. So *Sargassum* sp. extract will cause 50% mortality at a concentration of 745.05 µg/mL.

The samples of *Halimeda* sp. used similar concentrations as *Sargassum* sp. The concentration of 300 µg/mL had a percentage mortality rate of 20% and a probit of 4.16. At a concentration of 500 µg/mL has a percentage mortality rate of 47% and a probit of 4.9. At a concentration of 700 µg/mL has a percentage of the mortality rate of 60% and a probit of 5.25. At a concentration of 1500 µg/mL has a mortality percentage of 63% and a probit of 5.33. The last concentration is 2000 µg/mL with a mortality percentage of 80% and a probit of 5.84. With the results of the calculation of the effect of log concentration on probit mortality of artemia larvae, an LC_{50} of 697.97 µg/mL was obtained. So *Halimeda* sp. extract will cause 50% mortality at a concentration of 697.97 µg/mL.

The *Diadema setosum* sample used different concentrations with all samples. At a concentration of 1140 µg/mL has a mortality percentage of 73% and a probit of 5.84. At a concentration of 1300 µg/mL has a mortality percentage of 80% and a probit of 5.95. At a concentration of 1500 µg/mL has a mortality percentage of 81% and a probit of 6.02. At a concentration of 1700 µg/mL has a mortality percentage of 83% and a probit of 6.08. The last concentration is 2000 µg/mL with a mortality percentage of 93% and a probit of 6.84. With the results of the calculation of the effect of log concentration on probit mortality of artemia larvae, an LC_{50} of 575.4 µg/mL was obtained. So *Diadema setosum* extract will cause 50% mortality at a concentration of 575.4 µg/mL. The difference in concentration of each sample was determined from preliminary tests.

Preliminary tests in toxicity tests are intended to obtain a range of levels of a toxic substance that will be used in the main test. Based on the results of preliminary tests at concentrations of 0.1, 1, 10, 100, and 1000 ppm there was no evidence of toxicity or death of Artemia salina, so the initial concentration was increased for the main test by 1140, 1300, 1500, 1700, 2000 ppm. According to the OECD (Organization for Economic Cooperation and Development) procedure, the concentration can be increased if there is no evidence of toxicity or death. Preliminary tests are stopped if there is evidence of toxicity in the form of changes in the autonomic activity of test animals such as the absence of signs of life (moving Artemia salina larvae), so that the dose used in the main test can be determined (OECD, 2001).

Table 3. LC_{50} value of methanolic extract of fourth different marine biota from Lampung Province on brine shrimp *Artemia salina*.

Tested materials	LC_{50} (µg/mL)
Rhizopora sp.	870.96
Sargassum sp.	745.05
Halimeda sp.	697.97
Diadema setosum	575.4

Source: Authors

The compounds that can be used in the pharmaceutical field should be tested through toxicity tests that can determine the effectiveness of the compound. The toxicity test in this study used the BSLT method with the LC_{50} value which determines whether a compound will be good or bad. Based on the results of the toxicity test, the LC_{50} value in the *Rhizopora* sp extract is 870.96 µg/mL, 745.05 µg/mL in *Sargassum* sp., 697.97 µg/mL in *Halimeda* sp. and 575.4 µg/mL in *Diadema setosum* which shows that the extract tested has toxic material because it causes the death of artemia. According to the research of Ungcharoenwiwat. *et al*., (2023) the BSLT method of an extract is highly toxic if it has an LC50 <30 µg/mL, is toxic if it has an LC50 of 31-1000 µg/mL, and is not toxic if it has an LC50 > 1000 µg/mL. Based on the LC50 values obtained, all samples tested could be considered as good compounds for antibacterial, antifungal, and anticancer.

To confirm the potential of the four tested samples to be antibacterial, anti-fungal, and anti-cancer agents, studies on the content of bioactive compounds possessed by these four samples must be included. *Rhizopora* sp. is known to contain bioactive compounds from the alkaloid, flavonoid, phenolic, and saponin groups (Mutik *et al*., 2022), *Sargassum* sp. contains bioactive compounds

consisting of polyphenolic, terpenoid, carotenoid, polysaccharide, phenolic acid, streroid, chlorophyll, and glycolipid groups (Rohim *et al*., 2019), 2019), *Halimeda* sp. contains bioactive compounds that are almost the same as *Sargassum* sp. namely the steroid and alkaloid groups (Nugraha *et al*., 2022) as well as *Diadia* sp, 2022) as well as *Diadema setosum* contains bioactive compounds from the steroid, flavonoid, terpenoid and alkaloid groups, where it is known that the contents of alkaloids, flavonoids, terpenoids, and steroids are bioactive compounds that have benefits or have been widely used as antioxidants, anticancer, antitumor, antiallergic, anti-inflammatory to antibacterial or antimicrobial.

Alkaloids are bioactive compounds derived from secondary metabolites that can be found in various parts of plants such as flowers, seeds, leaves, twigs, roots to tree bark (Ningrum *et al*., 2016), naturally in plants alkaloids have a function as a storage compound that can supply the elements needed by plants such as nitrogen (Wink, 2008). Alkaloids can inhibit the constituent components of peptidoglycan in bacterial cells (Compean and Ynalvez, 2014), and inhibit the formation of protein synthesis that can interfere with bacterial metabolism (Robinson, 1995), making the alkaloid content benefits as an antibacterial.

Flavonoids are like alkaloids which are bioactive compounds derived from secondary metabolite compounds in plant tissues (Rajalakshmi and Narsimhan, 1985). Flavonoids are present in all parts of plants including leaves, roots, wood, bark, pollen nectar, flowers, fruits, and seeds, flavonoid bioactive compounds are not present in algae, microorganisms, bacteria, lichens, and fungi. Flavonoids have the potential as anti-inflammatory, antitumor or anticancer, antiviral, antiallergic, cardiovascular disease, estrogen and osteoporosis, anticholesterol (Heliawati, 2018), and also as antibacterial (Parubak, 2013). As antibacterial content, flavonoids form complex compounds with soluble extracellular protein components that will cause damage to the bacterial cell membrane and the release of intracellular compounds (Amalia *et al*., 2017).

Terpenoids are dehydrogenated and oxygenated derivatives of terpene compounds commonly referred to as isoprenoids, terpenoids can be found in plants as secondary metabolite compounds, insects, and some marine animals. Apart from being a secondary metabolite content, terpenoids are also the building blocks of a number of important compounds in living things. Terpenoids have been widely used in the medical world as antiseptics, diabetes treatment, menstrual disorders, antitoxins, and even as antibacterial (Heliawati, 2018). The mechanism of action of terpenoids as an antibacterial is by reacting with transmembrane proteins on the outer membrane of the bacterial cell wall to form strong polymer bonds that damage the transmembrane and reduce the permeability of the bacterial cell wall which will trigger nutrient deprivation in bacteria and cause inhibition of bacterial growth and even death of bacteria (Cowan, 1999).

Steroids are non-hydrolyzed sterol fatty organic compounds that are widely found in plants and animals. Steroids have been widely utilized in both plants and humans (Heliawati 2018) and have also been shown to function as an antibacterial against the growth of bacterial *Escherichia coli* (Anggraini, 2019). Steroids will inhibit bacterial growth, reduce membrane integrity, make fragile and cell lysis so that it will cause leakage in bacterial liposomes.

4. Conclusion

This study showed the LC_{50} toxicity activity of the four marine biota samples ranged from 575.4-870.96 µg/mL. In this study, *Rhizopora* sp recorded the highest LC_{50} toxicity activity of 870.96 µg/mL, while *Diadema setosum* was 575.4 µg/mL. The LC_{50} toxicity activity has the potential as an anti-bacterial, anti-fungal, and anti-cancer agent that needs to be studied further before being used as an industrial-scale natural product, this study is basic research on the brine shrimp lethality test of methanolic extracts from four different marine biota, this study has limited data as well as complex and detailed studies on how the extracts of the four samples can be made natural products with effective doses. Marine natural products can be increased in the future by expanding the quantity of extracts from various biota in the sea which currently has not been evenly explored.

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Conflict of Interest:

The authors declare no conflict of interest

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