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Toxicity Test of Bioactive Red Alga Extract *Eucheuma spinosum* on Shrimp *Artemia Salina* Leach

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ABSTRACT

Ameloblastoma is a benign epithelial neoplasm and ranges from 10% of all odontogenic tumors. Utilization of the bioactive compound red algae extract, which acts as an anti-carcinoma and improves the activity of abnormal cell metabolism, has the potential to suppress ameloblastoma recurrence. The Brine Shrimp Lethality Test (BSLT) method using Artemia salina Leach shrimp larvae as a test animal as an effort to search for novel anticancer compounds derived from plants. The purpose of this study was to find out the minimal dose of red algae extract of Eucheuma spinosum which has a toxic effect, the level of red algae toxicity of Eucheuma spinosum obtained in the variation of solvents, the number of mortality in the shrimp larvae of Artemia salina Leach after giving Eucheuma spinosum red extract, the potential of Eucheuma spinosum red algae extracts as an antitumor. Based on the result of this study, we obtained a straight line equation that showed a positive correlation. The concentration to kill 50% or LC50 larvae of Artemia salina Leach shrimp is 58.82 ppm that suggested the potential for toxicity in BSLT methods in experimental animals. Based on the study conducted, ethanol extract of red algae Eucheuma spinosum has the potential for toxic activity against shrimp larvae Artemia salina Leach by using the BSLT method with LC50 <1000 ppm. The LC50 values indicate that red algae Eucheuma spinosum extract has potential as an antitumor.

Keywords: Ameloblastoma, Artemia salina Leach, Eucheuma spinosum, toxicity.

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INTRODUCTION

Ameloblastoma is a benign odontogenic tumor originates from epithelial tissue and the most common odontogenic tumor. The incidence is 10% - 11% and accounts for about 1% of all tumors in the head and neck region. Ameloblastoma has a high recurrence rate when treated because ameloblastoma is invasive, so rational treatment is complete surgery or resection. The relatively high recurrence rate in ameloblastoma is influenced by the molecular etiology, management approach, and initial treatment of the patient (Effiom, Ogundana, Akinshipo, & Akintoye, 2018). In addition, ameloblastoma can lead to malignancy. Complications that can occur postoperatively is that it can cause loss of stomatognathic function, e.g., phonetics, and mastication, as well as facial deformities that affect appearance. Patients can experience complications in the form of neurofacial paraesthesia due to the effects of postoperative anesthesia (McClary et al., 2016).

Seaweed has the ability as an antioxidant, immunostimulant, and antibacterial activity. Seaweed, especially red algae, contains secondary metabolites in different forms, such as terpenoids, steroids, coumarin, flavonoids, and alkaloids, which can inhibit free radical activity (Aramanadka, Kamath, & Kudva, 2018). Bioactive compounds in red algae have a role as a high antioxidant which can suppress degenerative diseases, tumor/cancer. In the study of Hu X et al. in 2006, oligosaccharide carrageenan from the red algae Kappaphycus striatum was given orally for 14 days into mice inoculated with tumor cell suspension S180. This results in inhibition of the growth of transplanted sarcoma cells, increased phagocytosis of macrophages, increased antibody production, increased lymphocyte proliferation, more potent NK cell activity, and increased levels of IL-2 and TNF-α. These results indicate that the oligosaccharides studied exert their antitumor effect by promoting the immune system (Fedorov, Ermakova, Zvyagintseva, & Stonik, 2013). *Eucheuma spinosum* is seaweed from the Rhodophyceae (red algae), which is capable of producing carrageenan. At present, *Eucheuma spinosum* is commercially cultivated in the Philippines, China, Indonesia, Malaysia (Sabah), Tanzania, and Kiribati (Tamaheang, Makapedua, & Berhimpon, 2017).

Natural materials have long been traditionally used by the community to maintain health even for the treatment of diseases (Sugrani, Natsir, Djide, & Ahmad, 2019). People prefer to use natural materials because they are believed to cause no side effects on health (Rao & Rao, 2007). But unmeasured concentrations will cause toxic effects on individuals. Toxicity testing is important to estimate the degree of damage caused by a compound of biological or non-biological material.

The Brine Shrimp Lethality Test (BSLT) method using *Artemia salina* Leach shrimp larvae as a test animal is one method that is widely used to search for novel anticancer compounds derived from plants (K. Duraikannu*, 01 August, 2014). Toxicity test results with this method have been shown to correlate with cytotoxic compounds of anticancer. This method is easy to do, cheap, fast, and quite accurate.

Seeing the many uses of herbal medicines in the community whose concentration is not measured as well as the high recurrence rate of ameloblastoma, the authors intend to make a study aimed at exploring the potential of red algae extracts that contain flavonoid bioactive substances, alkaloid steroids, and terpenoid compounds that have the potential as antioxidants that can suppress

oxidation process of free radicals causing tumors by testing the toxicity of bioactive extracts of red algae *Eucheuma spinosum* on *Artemia salina* Leach shrimp larvae, thus the alternate drugs from natural materials can be utilized in the field of dentistry that can suppress ameloblastoma recurrence.

MATERIALS AND METHODS

Algae Extraction Procedure

Red algae, *Eucheuma spinosum*, was found in the waters of Punaga Beach and Putondo in Takalar Regency, South Sulawesi. Red algae were cleaned first using seawater then washed again with clear running water to remove salt, moss, and dirt that sticks to the thallus. Algae that have been cleaned were then dried by the sun. Dry algae were then cut into small pieces, then blended and put into glass jars.

A total of 500 grams of the dry weight of *Eucheuma spinosum* was extracted with ethanol solvent by maceration for three days while stirring occasionally. The maceration container was tightly closed and kept in a cool place and not exposed to direct sunlight. After three days by shaking or stirring to get better extract results, maceration results were filtered, and the filters were collected. The filtrate was collected, concentrated with a rotary evaporator until a thick extract is obtained, and the amount obtained is recorded.

Hatching of Artemia salina Leach Eggs

Artemia salina Leach eggs were obtained from fish feed shops. The hatching of eggs *Artemia salina* Leach was done by preparing the culture media, seawater with salinity ranging from 33-35 ppt. Furthermore, weighing *A. salina* cysts as much as 2.5 grams and put into seawater that has been prepared as a culture medium. Mixing must be done carefully so that the cyst is not damaged. Aeration was given to increase dissolved oxygen in the culture container. After 48 hours, the eggs become larvae that are ready to be used as test animals. Harvesting the culture results was done by keeping the container from the light and turn off the aeration. The positive phototaxis nature of *Artemia salina* Leach will make it difficult to separate the shells and larvae if done in bright conditions. The eggshells will float, and the artemia larvae settle down so that the larvae can be removed using a suction pipette or opening the faucet under the culture container.

Experimental procedure on test animals

The experimental procedure was carried out with four treatment groups and two replications per treatment using 80 test animals, which were divided into 10 test animals per treatment. Each treatment was consist of:

a. The control group (K): 2 ml, the concentration of the test solution 0% + 2 ml of seawater
b. Treatment group 2 (P2): 2 ml, the concentration of the test solution 10% + 2 ml of seawater
c. Treatment group 3 (P3): 2 ml, the concentration of the test solution 100% + 2 ml of seawater
d. Treatment group 4 (P4): 2 ml, the concentration of the test solution 1000% + 2 ml of seawater.

Determination of Toxicity Value

The algae extraction product was taken 30 mg each, then diluted in 3 ml of ethanol solvent according to the solvent used in maceration. Dilution is carried out to obtain concentrations (10, 100, and 1000 ppm). Determination of this concentration refers to the study by Nurhayati et al. (2006) with a duration of treatment for 24 hours. The test was carried out by inserting *Artemia salina* Leach larvae into vial bottles that had been determined. Then 2 ml was taken from each concentration and mixed in 2 ml of seawater. Then the vial bottle was placed under the lighting. After 24 hours, the number of larvae was observed with the help of a magnifying glass, and the number of mortalities was calculated as a percentage.

The data obtained in this study were displayed in the form of tables and graphs and statistical tests, then explained descriptively.

RESULT

The research resulted in a thick extract of red algae *Eucheuma spinosum* obtained by maceration as much as 30 grams, then dry weight as much as 26 grams. Meanwhile, the total extract obtained was 18.7 grams. Percentage of water content obtained by 8.67%, according to the requirements of the water content, i.e., ≤10% with a moisture content of less than 10% can prevent mold growth and enzyme activity so that the material is more durable and the content of its active ingredients is not reduced.

Statistically, the most significant number of larvae mortalities was seen at a concentration of 1000 ppm. However, at a minimum concentration of 10 ppm, the concentration showed 2 *Artemia salina* Leach larvae mortality. This showed that mortality is influenced by the large concentration of the red algae extract. The average larvae mortality is obtained by dividing the total larvae mortality at each concentration by the number of replications carried out, which is twice the percentage of larvae mortalities calculated from the average mortality at each concentration multiplied by 100%.

Determination of LC50 Value

LC50 calculations using Microsoft Office Excel by making a graph to get the straight line equation Y = mX + b. The figure 2 shows the log concentration of probit values obtained from larvae mortality. Obtained a straight line equation Y = 1.64x + 2.0667, there is a positive correlation because the value of R^2 = 0.8818. The results of the BSLT test on ethanol extract, obtained LC50 values of 1.788 µg / ml with% larvae mortality at a concentration of 1000 ppm = 95% and probit value = 6.64; % of larvae mortality at a concentration of 100 ppm = 85% and probit value = 6.04; and% of larvae deaths at a concentration of 10 ppm = 5% and probit value = 3.36.

Eucheuma spinosum red algae extract test showed that the concentration to kill 50% or LC50 larvae of *Artemia salina* Leach shrimp was 58.82 ppm so that it can be said that red algae extract of *Eucheuma spinosum* in this study had the potential to kill the BSLT method ie, in treatment with experimental animals Artemia larvae of Leach Salina Leach.

Eucheuma spinosum red algae extract test showed that the concentration to kill 50% or LC50 larvae of *Artemia salina* Leach shrimp was 58.82 ppm. It can be said that the red algae extract of *Eucheuma spinosum* in this study had the potential for toxicity according to the BSLT method in treatment with experimental animals Artemia larvae Salina Leach.

DISCUSSION

Ameloblastoma treatment varies from curettage to extensive bone resection, with or without reconstruction. Radiotherapy is not indicated because this lesion is radioresistant. Re-examination (postoperative follow-up) is important because almost 50% of recurrence cases occur in the first five years postoperatively. Conservative treatments include curettage, enucleation, cryosurgery, and enucleation with curettage or dredging methods. Epidemiology, treatment, and complications of ameloblastoma, about 35 patients were treated conservatively (62.5%), and 21 patients were treated radically (37.5%). Most patients treated conservatively underwent enucleation and curettage (62.8%); the rest only received enucleation (37.25%). According to the radically treated patients, ten patients received marginal resection (47.6%), and ten patients received segmental resection (47.6%), while only one patient underwent maxillectomy (4.8%). It was swelling as a pre-operative complication, as many as 56 cases (100%). Of the 56 patients, with pain complications in eight cases (10%), numbness in two cases (2%), respiratory obstruction in one case (1%), and swallowing problems, there were two cases (2%), no complaints regarding speech difficulty (Hartati, Masrukhan, & Cahyana, 2014). Hanapi et al. (2013) extracted the red algae *Eucheuma spinosum* to test the effectiveness of antioxidant content. The results of high antioxidant activity obtained from methanol extract of red algae *Eucheuma spinosum* against DPPH were EC50 values of 22.13 ppm. Therefore, the research carried out the extraction of active compounds in red algae *Eucheuma spinosum* with ethanol solvent, then proceed with toxicity testing as an effort to reveal the biological and medical properties of marine flora and to optimize the utilization of marine natural material (Fatimawali, 2013). The toxicity test with the BSLT method can find out the toxic effects of a compound that is determined in a short time, which is a period for 24 hours after administration of the extract. The toxic effect is known by determining LC50 from the activity of active plant components against larvae of *Artemia salina* Leach. Something is said to be toxic based on the BSLT method if the LC50 value <1000 ppm, so it correlates with this acute toxicity test with a cytotoxic test. This method correlates the number of shrimp larvae mortality with the test concentration (Afif, Fasya, & Ningsih, 2016).

In this study, different variations of concentration were carried out; concentrations of 10, 100, and 1000 ppm to compare the toxic effects caused by each of these concentrations and to look at what concentration of shrimp larvae underwent LC50. Seawater use as control is intended to see whether the mortality response is from the sample and not from seawater. Seawater used has been measured by pH, and the results are suitable for larvae growth media, namely pH around 8-9. Shrimp larvae are used in this method because these animals are general bioassays so that all substances can penetrate the larvae cell walls, and these animals have a high sensitivity to toxic substances (Mutia, 2010).

Average larvae mortality is obtained by dividing the total larvae mortality at each concentration by the number of replications performed, which is twice. Then calculate the percentage of larvae mortality from the average mortality at each concentration multiplied by 100%. Factors that influence the high toxic effect are the higher the extract concentration causing the higher toxic properties (Mart, 2016).

A plant genus in one species will generally exhibit similar chemical contents. Eucheuma has chemical contents, including alkaloid compounds, flavonoids, triterpenoids, and steroids that have biological activity (Saleem et al., 2017). Based on other studies regarding acute toxicity tests on one of the genera Eucheuma, namely ethanol extract from red algae, *Eucheuma cottoni* has a high level of toxicity against shrimp larvae *Artemia salina* Leach, indicated by an LC50 value <1000 ppm ethanol extract with an LC50 value of 58,0128. The research with this research has similarities in the use of red algae from the genus Eucheuma extracted with methanol solvent and BSLT method on larvae of *Artemia salina* Leach shrimp. LC50 value in the study with the study conducted is not much different and has the potential for acute toxicity (Fatimawali, 2013).

Red algae *Eucheuma spinosum* extract test showed that the concentration to kill 85% of *Artemia salina* Leach shrimp larvae was 100 ppm so that it can be said that the red algae extract of *Eucheuma spinosum* in this study had the toxicity potential of the BSLT method, namely in the treatment with experimental animals *Artemia salina* Leach larvae. Meyer et al. (1982) stated that the determination of bioactive potential was carried out by comparing the LC50 of each extract with the provisions of McLaughin (1991) said to be toxic when the LC50 value <1000and not toxic when the LC50 value> 1000. From the results of the research, the BSLT test on ethanol extract, obtained LC50 value of 1.788 µg / ml with% larvae mortality at a concentration of 1000 ppm = 95% and probit value = 6.64; % of larvae mortality at a concentration of 100 ppm = 85% and probit value = 6.04; and % of larvae mortality at a concentration of 10 ppm = 5% and probit value = 3.36. This shows that LC50 values have potential as an antitumor as for the class of compounds contained, namely flavonoids, steroids, alkaloids, and terpenoids.

CONCLUSION

Ethanol extract of red algae *Eucheuma spinosum* has toxic activity towards Shrimp Larvae (*Artemia salina* Leach) according to the BSLT method with LC50 <1000 ppm. LC50 value of *Eucheuma spinosum* red algae ethanol extract indicate that the extract of red algae *Eucheuma spinosum* has potential as an antitumor.

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Table 1 : The Frequency of Mortality of *Artemia salina* Leach Larvae Toward Red Algae Extract *Eucheuma spinosum*

Variable	Mortality of *Artemia salina* Leach larvae in each treatment and control group
Concentration solution 10 ppm	5 %
Concentration solution 100 ppm	85 %
Concentration solution 1000 ppm	95 %
Control group	5 %

(Data source: Primary data processed, 2019)

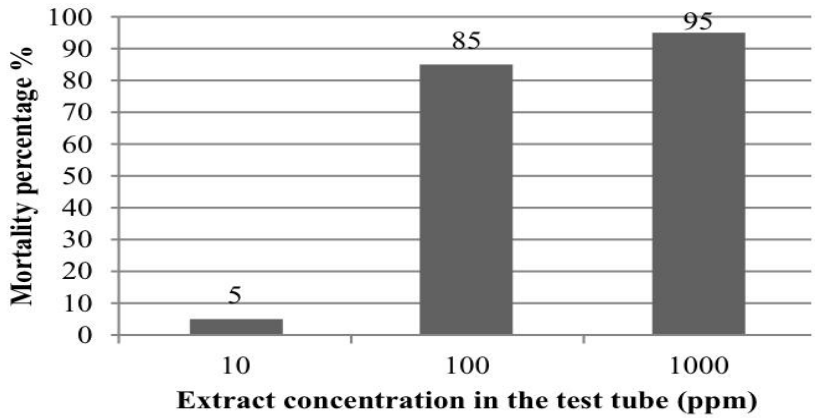

Figure 1 : Chart of the effect *Eucheuma spinosum* extract concentration on the mortality of *Artemia salina* Leach Larvae.

Table 2 : Calculation of LC50 with the Probit method

Extract concentration in the test tube(ppm)	Log Concentration (X)	Mortality	Probit (Y)
10	1	5%	3,36
100	2	85%	6,04
1000	3	95%	6,64
Total	6		16,04

(Data source: Primary data processed, 2019)

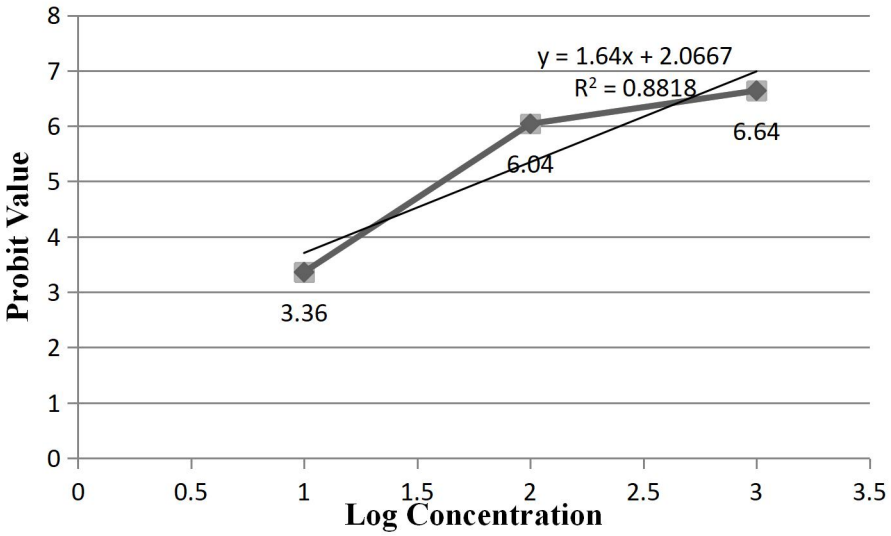

Figure 2 : Linear Regression Comparison Chart