Conference Paper

Potency of Endo-Exopolysaccharide from *Porphyridium cruentum* (S. F. Gray) Nägeli as Antioxidant (DPPH) and Biological Toxicity (BSLT)

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Abstract

Sources of antioxidants are abundant in nature, one of which is derived from microalgae. Microalgae species that have potentially to be developed as a producer of antioxidant compounds is *Porphyridium cruentum* (S. F. Gray) Nägeli. *P. cruentum* is a red microalga (Rhodophyceae) and has been known as polysaccharides producer (exopolysaccharide and endopolysaccharide) and as an antioxidant. This study aims to determine antioxidant activity of the extracts and biological toxicity of endo- and exopolysaccharide *P. cruentum*. The antioxidant activity use reduction method of free radicals (DPPH), whereas toxicity uses Brine Shrimp Lethality Test. (BSLT). The result shows that there is an antioxidant activity with IC_{50} value of 50.586 5 mg · kg^{-1} (exopolysaccharide) and 145.998 8 mg · kg^{-1} (endopolysaccharide). In addition, the result indicates the toxic nature of the *Artemia salina* Leach. with LC_{50} values of 513.175 1 mg · kg^{-1} (exopolysaccharide) and 521.823 3 mg · kg^{-1} (endopolysaccharide). Therefore, endo- and exopolysaccharide produced by *P. cruentum* suitable to be used as an alternative source of natural antioxidants and potential for further developed as antitumor drugs.

Keywords: antioxidant; biological toxixity; endo-exopolysaccharide; *Porphyridium cruentum* (S. F. Gray) Nägeli.

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1. Introduction

Here today, the world of medicine and health care much about free radicals (free radical) and antioxidants. It happens because most diseases are initiated by the presence of excessive oxidation reactions in the body. Oxidation reactions can occur at any time, even when breathing oxidation reaction ensued. This oxidation reaction can trigger the formation of free radicals that can damage the structure and function of cells [1].

Free radicals are atoms or molecules that contain one or more electrons that are not paired in the outer orbitals. Free radicals are highly reactive because of losing one or more electrons are electrically charged, and to restore the balance of free radicals trying to gain electrons from other molecules or remove the unpaired electrons.

Excessive amounts of free radicals in the body are very dangerous because it causes a breakdown of cells, nucleic acids, proteins and fat tissue [2].

Antioxidants are substances that may delay, impede, and prevent oxidation, can be either enzymes (eg. superoxide dismutase (SOD), catalase and glutathione peroxidase), vitamins (eg. vitamin E, vitamin C, vitamin A and β-carotene), polysaccharides (β-glucan) and other compounds (eg. flavonoids, albumin, bilirubin, ceruloplasmin and others). In the body there are antioxidants and free radical mechanisms are endogenous, but when the number of free radicals in the body of excess is needed antioxidants derived from natural or synthetic sources from outside the body [1, 3].

Natural source of antioxidants in the very abundance, one of which comes from microalgae. Microalgae species that have the potential to be developed as one of the antioxidants are *P. cruentum*. This microalga is cultured and potential as a source of polysaccharides as well as a source of fatty acids, pigments, and other chemical compounds. Polysaccharides from *P. cruentum* mainly consisting of xylose, glucose, and galactose. The complexity of the cell wall also *Porphyridium* contains sulfuric, glucuronic acid and glycoproteins. Based on the literature, it is known that the polysaccharide sulfate of *Porphyridium* sp. potential as an antioxidant. The last few years, polysaccharide of marine organisms including polysaccharides sulfate has been shown to have antioxidant activity [4–6].

The potential biological activity of the extract can be performed through Brine Shrimp Lethality Test (BSLT) using larval shrimp *A. salina*. This method is widely used for environmental studies, toxicity and screening bioactive compounds in plant extracts. The toxicity test with BSLT method can be seen from the number of dead larvae shrimp *A. salina*. According to Meyer et al. [7] an extract said to be toxic to *A. salina*. if the price has Lethal Concentration 50 (LC_{50}) < 100 0 ρg · mL^{-1}.

Therefore, in this research was done the antioxidant activity of the polysaccharides from *P. cruentum* using methods curbs free radical DPPH (1,1-diphenyl-2-picrylhydrazyl) and toxicity with BSLT (Brine Shrimp Lethality Test) to determine the biological activity.

2. Material and Methods

2.1. Extraction endo- and exopolysaccharide of Porphyridium cruentum [8]

One liter culture of *P. cruentum* obtained from stationary phase cultures were centrifuged at a speed of 3 500 rpm (1 rpm = 1/60 Hz) for 15 min. Results of centrifuge will be obtained biomass and supernatant. Biomass used to extract endo-polysaccharide extract and supernatant for extracting exo-polysaccharide.

2.1.1. Endo-polysaccharide

One gram of biomass was added 20 mL of 1% calcium chloride and solved its cell using a sonicator with a power of 40 HTZ for 15 min. After that was done freezing with

put in the freezer for 24 h and then in thawing so perfect melt, then centrifuged at a speed of 3 500 rpm for 15 min. This work was repeatedly done until the pigment (phycoerythrin) extracted perfectly. After that, biomass is washed with distilled water three times. Then the biomass obtained was added 15 mL of 0.75 N sodium hydroxide and centrifuged at 3 500 rpm for 15 min, then add 0.5 N of acetic acid and centrifuged again. Then the precipitate was added 15 mL of aquades and centrifuged again. After that, the precipitate was added 15 mL of ethanol. The precipitate obtained was dried at a temperature below 50°C for 24 h. The precipitate obtained is endo-polysaccharide crude.

2.1.2. Exo-polysaccharide

Supernatant (25 mL) was added with 50 mL of ethanol, centrifuged at a speed of 3 500 rpm for 15 min, the supernatant separation section. The precipitate obtained is exopolysaccharide products. Exopolysaccharide dried in an oven at 50°C for 24 h. Weight exopolysaccharide obtained from the difference between the empty and dry test tube containing the exopolysaccharide.

2.2. Characterization of endo-exopolysaccharides from Porphyridium cruentum

The analysis of carbohydrates by the phenol-sulfuric acid method with glucose as standard, while the analysis of proteins using Biuret method with Bovine Serum Albumin as standard.

2.3. Antioxidant activity of free radicals reduction method using 1,1-Diphenyl-2-Picrylhydrazyl (DPPH)

2.3.1. Preparation of extract of test solution

The sample is weighed approximately 10 mg, then dissolved in 10 mL of methanol pro-analysis (1 000 mg $\cdot$ L^{-1}), this solution is the mother liquor. Pipette mother liquor as much as 25 mL, 50 mL, 125 mL, 250 mL and 500 mL, was put into a test tube which has been given a limit of up to 5 mL. DPPH solution was added 1 mL into each tube and added with methanol pro-analysis up to the mark 5 mL, to obtain the concentration of sample (5, 10, 25, 50 and 100) mg $\cdot$ mL^{-1}. The mixture was homogenized and mouth closed tube of aluminum foil.

2.3.2. Preparation of vitamin C as a positive control

Vitamin C is weighed approximately 10 mg, then dissolved in 10 mL of methanol pro-analysis (1 000 mg $\cdot$ L^{-1}), this solution is the mother liquor. Mother liquor pipette 10 mL , 20 mL , 30 mL, 40 mL, and 50 mL into a test tube which has been given a limit up

to 5 mL. Into each tube was added 1 mL of DPPH (0.4 mM) and added with methanol pro-analysis up to the mark 5 mL, to obtain the concentration of (2, 4, 6, 8 and 10) mg · mL^{-1}. The solution is homogenized and mouth tube covered with aluminum foil.

2.3.3. Absorbance measurements

Test solution and vitamin C with several concentrations were incubated in an incubator at 37°C for 30 min. Absorption was measured at a wavelength of 516 nm using a ultraviolet-visible light spectrophotometer.

2.3.4. Formula calculates percentage of Inhibition (1)

$$\text{Barriers (inhibition)} = \frac{(\text{absorbance blanks} - \text{absorbance sample})}{\text{absorbance blank}} \times 100\% \quad (1)$$

IC_{50} is an antioxidant concentration (μg/mL) which is capable of providing deterrence percent free radicals by 50% compared to controls through a line equation. IC_{50} values obtained from the intersection of the lines between the power constraints and the concentration axis, then put into the equation y = a + bx, where y = 50 and x indicate IC_{50} values. Extracts declared active as an antioxidant when the IC_{50} value of less than 200 μg · mL^{-1} [8].

2.4. Toxicity tests using the method brine shrimp lethality test

2.4.1. Hatching eggs of artemia salina

Synthetic sea water was prepared by dissolving 38 g of salt without iodine in 1 000 mL of water, then filtered through Whatman paper. Brooders sealed vessel that has two sides of the room, the light, and dark sides. Then enter the amount of approximately 20 mg of *A. salina* eggs into the vessel (dark) already contains seawater. Seawater is synthetic and irradiated with 18-watt fluorescent lamp. The eggs hatch after 24 h into nauplii and moved to another place (light), 24 h after the nauplii can be used as test animals.

2.4.2. Preparation of test solution

The test solution is made with a concentration of 1 000 mg · L^{-1}, 100 mg · L^{-1}, 10 mg · L^{-1} and each made three repetitions. A total of one vial is used for control. The mother liquor is made by weighing 20 mg of the extract were dissolved in 2 mL of a suitable solvent. If the sample is difficult to dissolve, added dimethyl sulfoxide (DMSO) 1% as much as 0.1mL to 50 mL.

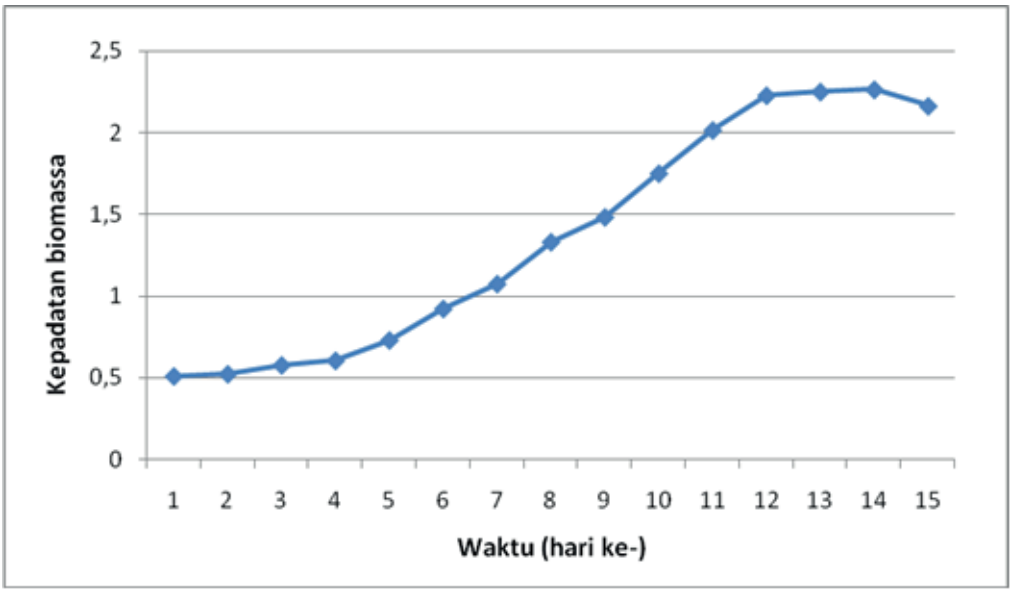

Figure 1: Porphyridium cruentum growth curve.

2.4.3. Toxicity test

The mother liquor pipetted successively 500 mL, 50 mL and 5 mL, and each put in a vial and then evaporated to dryness. Each concentration was made with three repetitions, then into each vial inserted 3 mL of seawater and 10 tails nauplii and seawater are added to 5 mL. If the sample is difficult to dissolve in seawater, was added dimethyl sulfoxide (DMSO) 1%. The solution was stirred until homogeneous, for each concentration was performed three repetitions. Mortality was calculated by comparing the number of dead larvae and the total number of larvae. LC_{50} used to calculate the curve that states the log concentration as the x-axis and y-axis% mortality as. LC_{50} results obtained from the intersection of the lines on both axes. A substance said to be active or toxic when the $LC_{50} < 1\,000$ mg $\cdot L^{-1}$ [7].

3. Result and Discussion

Observation of the growth curve aims to determine the growth phase of *P. cruentum* and to determine appropriate harvest time. In this study, the manufacture of the growth curve is based on the method of turbidimetry on a spectrophotometer with a wavelength of 680 nm. This method is done because although *P. cruentum* is microalgae unicellular but sometimes cells bound by the mucilage that will be visible colonies, therefore, the spread of the cells under a microscope into the cell deployment is uneven and the result becomes inaccurate. Optical density measurement results can be seen in Figure 1.

In Figure 1 indicates that the 1 d to 4 d cultivation is an adaptation phase, characterized by slow growth because of energy allocation focused on adaptation to a new culture medium. Cells that have a high tolerance can live and grow. On 5 d to 11 d is a logarithmic phase, in this phase of rapid growth occurs, characterized by increasing

Extract	Absorbant (λ 490 nm)	Average of Absorbant	Content (mg · L^{-1})	Glucose (%)
Exopolysaccharide	0.603	0.602	57.495	0.558
	0.600			
	0.602			
Endopolysaccharide	0.517	0.516	49.226	0.483
	0.514			
	0.518			

Table 1: Results of the glucose contained in the extract polysaccharides *Porphyridium cruentum*.

the number of cells. After the number of cells reached the top, it can not grow again because increasing the number of cells growth rate equal to the rate of death. This phase is called the stationary phase. Stationary phase occurs on the 12 d, at this phase optimal content of secondary metabolites are formed. Carbohydrates as primary metabolites are polysaccharides contained in the cells used to grow *P. cruentum*, while the sulfate-polysaccharide is secondary metabolites that are excreted by the cell into the medium. In the stationary phase, cultures can be seen viscosity increases. It indicates the amount of polysaccharide in the medium increases. According to Arad [6], production will increase viscosity soluble polysaccharide and polysaccharide excreted into the medium in the stationary phase is greater than the logarithmic phase.

3.1. Glucose analysis

Research conducted by Percival & Foyle [9], the composition of the polysaccharides of *P. cruentum* consisting of xylose, glucose, and galactose. Based on this, then in this study conducted an analysis of one type of polysaccharide *P. cruentum* monosaccharides, ie glucose levels to prove that the extract used is a polysaccharide extract which one constituent is glucose. Becker medium used does not contain any carbohydrates so that we can be sure the medium does not affect the measurement of glucose.

Analysis of glucose used is phenol sulfate method. This method is used because it is more stable over a relatively long period. Sulfuric acid is used to destroy the cell and degrade polysaccharides. Phenol under acidic conditions will condense with the monomers glucose resulting from the degradation process and form compounds orange and absorbance were measured at a wavelength of 490 nm. The regression equation obtained from the standard curve of glucose is then used for the calculation of glucose contained in the polysaccharide of *P. cruentum*. Results of glucose can be seen in Table 1.

Results obtained glucose contained in exopolysaccharide amounted to 0.558 2% and the extract endopolysaccharide amounted to 0.482 6%.

3.2. Protein analysis

Extract polysaccharides produced by *P. cruentum* is still a rough polysaccharide extract, in which still contained impurities such as proteins. Based on this, the analysis is done

Extract	Absorbance (λ 550 nm)	Average of Absorbance	Content (mg · L^{-1})	Protein (%)
Exopolysaccharide	0.077	0.074	298.237	2.895
	0.073			
	0.072			
Endopolysaccharide	0.104	0.104	406.968	3.989
	0.101			
	0.107			

Table 2: Results of the analysis of protein as an impurity contained in the extract polysaccharides *Porphyridium cruentum*.

Extract	IC_{50} (mg · L^{-1})
Exopolysaccharide	150.586
Endopolysaccharide	145.998
Vitamin C	5.004

Table 3: Results of the antioxidant activity of endo-exopolysaccharides from *P. cruentum* and vitamin C by the method of reduction of free radicals (DPPH).

to determine the protein content of the protein contained in the extract polysaccharides from *P. cruentum*. Based on the literature, the polysaccharide sulfate of *P. cruentum* protein binds to the 5% [8].

Analysis of proteins was done by using the Biuret method. The formation of a complex between of Cu^{2+} ion in the Biuret reagent with a peptide bond in the sample produces a bluish purple color. Biuret method is selective because it only reacts with the nitrogen atom incorporated in the peptide bonds.

The regression equation obtained from the standard curve of BSA (Bovine Serum Albumin) and then used for calculating the glucose contained in polysaccharides *P. cruentum*. Results of the analysis of protein levels can be seen in Table 2.

3.3. Activity of antioxidant

Antioxidant activity using the method of reduction of free radicals (DPPH) was conducted to determine the activity of endo-exopolysaccharide of *P. cruentum* in reducing free radical DPPH by looking at the value of IC_{50}. DPPH (1,1-diphenyl-2-picrylhydrazyl) is a stable free radical compound and can only be muted by antioxidants which can provide hydrogen atom. When the radical compound is reacted with an antioxidant compound that is endo-exopolysaccharide extract of *P. cruentum*, antioxidant compounds will contribute its H atoms, so DPPH no longer be as free radicals, which are marked by a color change to yellow. The rest of the DPPH radical are not bound by H atoms remain free radical antioxidant. The remainder of these radicals is measured, then determined IC_{50} of the endo-exopolysaccharide extract of *P. cruentum*.

In this study, vitamin C used positive control. The result of the antioxidant activity of the extract of endo-exopolysaccharide *P. cruentum* and vitamin C by using the method of reduction of free radicals (DPPH) can be seen in Table 3.

Extract	LC_{50} (mg · L^{-1})
Exopolysaccharide	513.175
Endopolysaccharide	521.823

Table 4: The result of biological activity in extracts endo- and exopolysaccharide by BSLT (Brine Shrimp Lethality Test) Method.

Based on Table 1, vitamin C as a positive control IC_{50} values of 5.003 8 mg · L^{-1}, while the exopolysaccharide extract IC_{50}value of 150.586 5 mg · L^{-1} and extract endopolysaccharide IC_{50}value of 145.998 8 μg · mL^{-1}. Endo-exopolysaccharide extract of *P. cruentum* has the ability as an antioxidant because it has IC_{50} values < 200 mg · L^{-1}. It is according to research conducted by Spitz et al. [6] that the solution of the polysaccharide of *Porphyridium* belonging to the polysaccharide sulfate showed their antioxidant activity using the ferrous oxidation (FOX) [6].

Based on the function of antioxidants in the body, polysaccharides of *P. cruentum* a secondary antioxidant that can capture free radicals and prevent a radical chain reaction.

3.4. Biological activity

The principle of a plant or microorganism can be used as an anticancer drug that is when the plant or microorganism contains compounds that are cytotoxic. BSLT (Brine Shrimp Lethality Test) is one method for screening cytotoxic compounds using *A. salina*. Namely to get LC_{50} values by calculating the rate of death or mortality by comparing the number of larval shrimp *A. salina* divided by the total number of dead shrimp larvae. LC_{50} is the concentration of compounds that provide a level of mortality (death) by 50% of the test animals. The result of biological activity endo-exopolysaccharide extract is BSLT can be seen in Table 4.

According to Meyer [7], a sample of otherwise showed biological activity against the larvae of shrimp if it has a value of LC_{50} < 1 000 mg · L^{-1}. Observation of the endo-exopolysaccharide extract of *P. cruentum* LC_{50} value is 513.175 1 mg · L^{-1} (exopolysaccharide) and 521.823 3 mg · L^{-1} (endopolysaccharide), this indicates the biological activity against *A. salina*.

Based on this, it can do further research on endo-exopolysaccharide extract the active compounds that are known to be toxic or even a compound having antitumor activity due to the toxicity test method BSLT often have a positive correlation to its function as an antitumor.

4. Conclusions

- The antioxidant activity by the method of reduction of free radicals (DPPH) shows that the exopolysaccharide and endopolysaccharide extract of *P. cruentum* have antioxidant activity with IC_{50} values of each of 150.586 mg · L^{-1} and 145.998 mg · L^{-1}.

- Biological activity in BSLT showed that the exopolysaccharide and endopolysaccharide extract of *P. cruentum* have biological activity with LC_{50} values respectively 513.175 mg · L^{-1} and 521.823 mg · L^{-1}.
- Extract endo-exopolysaccharide of *P. cruentum* can be used as an alternative source of natural antioxidants and developed as a drug.

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