Antioxidant activity of Alginate Oligosaccharides (AOS) from *Sargassum* sp. for Improving the Cutaneous Wound Enclosure in Zebrafish (*Danio rerio*)

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Abstract

Sargassum sp. classified as brown seaweed which is known as an alginophyte (alginate producer). Alginate has undergone a depolymerization process called alginate oligosaccharides (AOS) and has been shown to have antioxidant activities to increase wound tissue recovery. This study aimed to determine the antioxidant activity of Alginate oligosaccharides (AOS) and their ability to improve the cutaneous wound enclosure in Zebrafish (*Danio rerio*). The IC_{50} value was used to calculate the ability of extract to inhibit free radicals using DPPH (516 nm). Zebrafish were immersed 12 hours before the injury and shortly after injury with a two factorial design, i.e., alginate concentration and immersion time. Zebrafish were immersed for 1 hour, 3 hours, and 5 hours with serial concentration of 200 ppm, 400 ppm, and 600 ppm, respectively. Morphological observations were carried out at the the first day, fourth day, tenth day, and twenty-first-day post wounding. The results of this study showed that alginate from *Sargassum* sp. has a yield of 40.5 ± 1.125% with a purity level of 89.95%. Based on antioxidant activity, alginate is categorized as moderate (178,377 ppm) and evidently has the ability to increase wound recovery compared to control. It has indicated by the formation of the wound enclosure.

Keywords: Alginate Oligosaccharides, Antioxidant, *Danio rerio*, Wound recovery

INTRODUCTION

Sargassum sp. is one of the most abundant biological resource seaweed from Indonesia as alginophyte (alginate producer). Alginate is a natural polysaccharide commonly found in all brown algae species (Szekalska *et al*., 2016) and yielded up to 40% of the total dry weight (Yudiati and Isnansetyo, 2017). Alginate plays an essential role in maintaining the structure of algal tissues. The application of biotechnology to produce natural products from the sea is increasing rapidly due to the tendency of humans paradigm "back to nature". This tendency occurs because the products made from natural product do not cause side effects, non-toxic (Lin *et al*., 2015), biodegradable and valuable renewable products (Sylvia *et al*., 2020). In processing fishery products, alginate is used as glazing in freezing fish to avoid oxidation reactions (Laksanawati *et al.,* 2017). Antioxidants are compounds or substances that can inhibit, delay, prevent or slow down oxidation reactions (Hidayati *et al*., 2019) and has the potential to be used in the wound healing process (Gong *et al*., 2013; Yang *et al*., 2016; Pandey *et al*., 2020; Comino-Sanz *et al*., 2021).

The wound disrupts normal conditions on the skin that causes loss of organ function,

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blood clotting, bacterial contamination, and cell death (Kozier, 1995). Wound healing occurs in three distinct and continuous phases, namely the inflammatory phase, the tissue formation phase (proliferation/reepithelization), which consists the formation of granulation tissue and fibroblasts, and the remodeling/recovery phase (Wang *et al.,* 2017). Many antiseptic agents are commonly used to treat wound infections but can be toxic to the cells involved in the wound healing process. According to Nagoba *et al.* (2013), antiseptic agents such as iodine, chlorhexidine, hydrogen peroxide, alcohol, and betadine (povidone-iodine) can interfere the normal healing process, negatively impact fibroblasts and allow microbes to dominate. While 70% alcohol can damage healthy skin cells around the wound because the material has a mechanism of action as an antiseptic that damages cell walls, denatures proteins, and lyses cells in the alcohol-treated cells (McDonnel and Russel, 1999).

Alginate has a proinflammatory activity (Kezia *el al.*, 2013) and also has antioxidant activity (Yudiati *et al*., 2018) that is needed to balance the levels of reactive oxygen species (ROS) in the body caused by stepping of wound inflammation stage (Arief & Widodo, 2018). The inflammatory cells produce excess ROS and impacts the wound healing process (Kurahashi & Fujii, 2015). According to Angelina *et al.* (2021), alginate can improve the wound enclosure process one day faster because it can increase pro-inflammatory cytokines, namely proteins that play an essential role in increasing the phagocytic function in the inflammatory process. The increment of phagocytosis in the inflammatory process will lead to the formation of fibroblasts and increased wound healing process (Kim & Moudgil, 2017).

Zebra fish (*Danio rerio)* are used as models in wound recovery testing. According to Ko *et al*. (2011), zebrafish are used in many bioactivity screening studies due to their short life span, genome similarity to mammals, comparatively small size, and the ability of the female fish to produce a large number of eggs. The major steps and principles of cutaneous wound healing are conserved among adult mammals and adult zebrafish, it makes zebrafish a valuable model for studying vertebrate skin repair (Richardson *et al*., 2013) and cutaneous wound closure (Richardson *et al*., 2016). This study aimed to determine the antioxidant activity of Alginate oligosaccharides (AOS) and their ability to improve the cutaneous wound enclosure in Zebrafish (*Danio rerio*).

MATERIALS AND METHODS

Alginate Oligosaccharides (AOS) extraction

The *Sargassum* sp. sample was collected from Teluk Awur Waters, Jepara. Soon as came in to the laboratory, the samples were washed using fresh water and dried up at room temperature. The dried *Sargassum* sp. were extracted according to the methods by Yudiati & Isnansetyo (2017). The alginate was then dried at 140 °C for 5 hours to get the oligosaccharide (Yudiati *et al*., 2018).

Fourier-Transform IR (FT-IR) spectroscopy

The FT-IR spectra of alginates were recorded in the 4000–500 cm^{-1} region using a Thermo Nicolet 380 FTIR (Germany). Preparation was done by mixing the samples with KBr in pellets formation (10% w/w) (Yudiati *et al.,* 2018).

Determination of DPPH Maximum Absorbance

DPPH was weighed for 4 mg and dissolved with 100 mL of methanol (0.1 mM). The solution is homogenized and incubated for 120 mins in a dark place. After that, DPPH solution was taken 4 mL, then put into the cuvette and observed the absorbance using a spectrophotometer (Shimadzu UV-1280) at 400-800 nm (Hidayati *et al*., 2017). The highest absorbance will then be used to measure antioxidant activity.

The Assessment of Antioxidant Activity

Antioxidant activity determination was carried out using spectrophotometric methods (Moubayed *et al*., 2017). A total of 4 mL of the test solution was added with 1 mL of DPPH 0.1 mM. The solution was incubated for 30 minutes and then measured the absorbance at the maximum wavelength. The percentage of inhibition is calculated using the formula (Yudiati *et al*., 2018).

The inhibition percentage data was plotted to constructed the linear regression equation and determined the IC_{50} value (Hidayati *et al*., 2020).

Determination of Lethal Concentration 50 (LC_{50}-24 Hours)

Alginate were dissolved in various concentrations (125, 250, 500, and 1000 ppm) using a magnetic stirrer and administered in 5 L. Thirty adult zebrafish were added to each aquarium. All treatments were applied in two replications. Observations on the mortality of the test animals were carried out at 0 hours, 0.5 hours, 1 hour, 2 hours, 4 hours, 6 hours, 8 hours, 16 hours, and 24 hours. Probit analysis was carried out in this study to determine the value of LC_{50} using IBM SPSS 20 software.

Skin injury and alginate immersion

Twenty zebrafish were immersed in each treatment before being injured; then, the zebrafish were put into an aquarium containing clove oil (10%) used as an anesthetic (Rahim, 2017). The fish is then cut with a hot looped iron and attached to the area around its dorsal fin. The zebrafish were then put back in the immersion medium according to the treatments. After the immersion treatment, zebrafish were maintained on standard media for 21 days (Richardson *et al*., 2013).

RESULT AND DISCUSSION

Alginate Extraction

Extraction of alginate from *Sargassum* sp. shows the average percentage alginate yield is 40.5% of the total dry weight (Table 1).

According to Yudiati and Isnansetyo (2017), the yield obtained is relatively high because alginate yield of the *Sargassum* sp. from other researchers ranges from 3.3 to 40.34%. The percentage of alginate yield is influenced by several factors: the type of brown algae, habitat conditions, the temperature at extraction, and the concentration of materials used for the alginate extraction process (Yudiati & Isnansetyo, 2017). The yield of alginate produced in this study was higher than the alginate extraction using the calcium method (11.70±0.41%) and the acid method (9.95±0.31%) (Maharani *et al*., 2018). The high percentage of alginate yield obtained was due to EDTA in the extraction process. EDTA is a water-soluble polyamine carboxylic acid and functions as a chelating agent (Yudiati *et al*., 2018). The addition of EDTA can increase the absorption capacity of metal ions (Sari *et al*., 2016), and resulting in more bonds of alginate compounds than alginate extraction without the addition of EDTA (Latifi *et al.,* 2015). Polysaccharides generally have large molecules and are more complex than mono and oligosaccharides, making them difficult to dissolve in water. Therefore, oligosaccharides are carried out to facilitate the dissolution of alginate with water and increase its antioxidant activity (Rizfa *et al*., 2020).

FT-IR Spectroscopic Analysis

Alginate purity level was determined through Infrared Spectroscopy analysis using FTIR to compare the value of the deacetylation degree of the sample alginate with the alginate standard (Bastman, 1989).

The FTIR results (Figure 1b) showed that the extracted alginate had the same functional group as the standard alginate (Figure 1a) and had a purity level of 89.95%. The FTIR results already meet the standards because alginate has three peak specifications: The spectra around 3200 cm^{-1} is signed of hydroxyl group (Bahar *et al*., 2012),

Table 1. Percentage alginate yield

No.	Dried *Sargassum* sp, (gram)	Dried Alginate (gram)	percentage yield of alginate (%)	Average
1.	20	8,27	41,35	
2.	20	8,20	41,00	40,5 ± 1,125
3.	20	7,85	39,25	

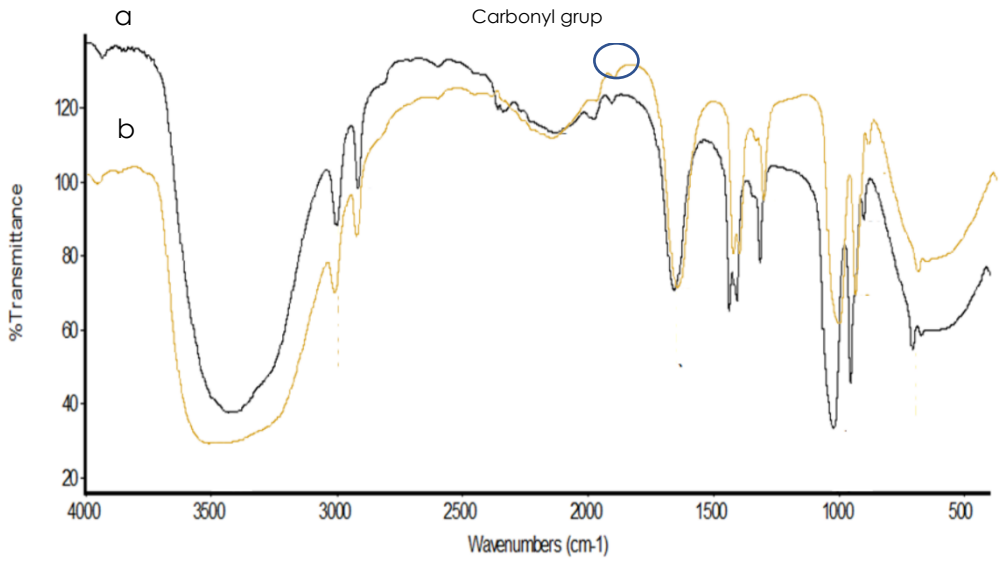

Figure 1. Standar alginate (a); Extraction alginate (b)

the spectra around 1600 cm^{-1} indicated of COO-asymmetry and 1400 cm^{-1} predicted of COO-symmetry (Maharani *et al.,* 2017). Based on Yudiati *et al*. (2018) in the heat treatment carbonyl group were positively formed. Oligosaccharides contain glycosidic linkages (acetal or ketal) that release two or more monosaccharide units upon hydrolysis and can be produced by splitting of the glycosidic bonds. Alginate was heated at 60 °C to break the glycosidic bonds. It was suggested that the attack of a hydrogen atom in position linked polysaccharides caused a division of the glycosidic bond, and consequently the formation of carbonyl groups (Soukaina *et al*., 2020).

DPPH radical scavenging activity

The maximum absorbance of DPPH was obtained at a wavelength of 516 nm (Rizfa *et al*., 2020). While, Hidayati *et al.* (2017) reported that the maximum DPPH absorbance was occurred at a wavelength of 514 nm. This maximum wavelength was then used to test the antioxidant activity.

The results showed that alginate had an IC_{50} value of 178.377 ppm and was classified as a moderate antioxidant (Jun *et al*., 2003). In this research, The IC_{50} value was slightly higher than heat-degraded alginate (40.86 ppm) and lower than heat-degraded alginate (196.15 ppm) (Rizfa *et al*., 2020). Therefore, the lower value of IC_{50} indicates a higher antioxidant activity (Sivaraman *et al.,* 2013). The process of inhibiting free radicals is carried out by taking electrons from antioxidant compounds so that free radicals become stable (Sayuti & Yenrina, 2015). Antioxidants are compounds or substances that can inhibit, delay, prevent or slow down oxidation reactions (Hidayati *et al*., 2019) and has the potential to be used in the wound healing process (Comino-Sanz *et al*., 2021).

Lethal Concentration 50 (LC_{50}-24 Hours)

Determination of Lethal Concentration 50 (LC_{50}-24 Hours) was carried out to determine the concentration of the extract that could kill 50% of the test organisms and determine the concentration to be used for further testing. The results showed that the highest mortality of Zebrafish was found at a concentration of 1000 ppm alginate, with 17 deaths within 24 hours. The probit analysis results showed that the proper concentration range for alginate immersion was <955.714 ppm with an immersion time of <24 hours. Based on this data, we have chosen the immersion concentration applied were 200 ppm (A), 400 ppm (B) and 600 ppm (C) with an immersion time of 1 hour (I), 3 hours (II) and 5 hours (III), respectively.

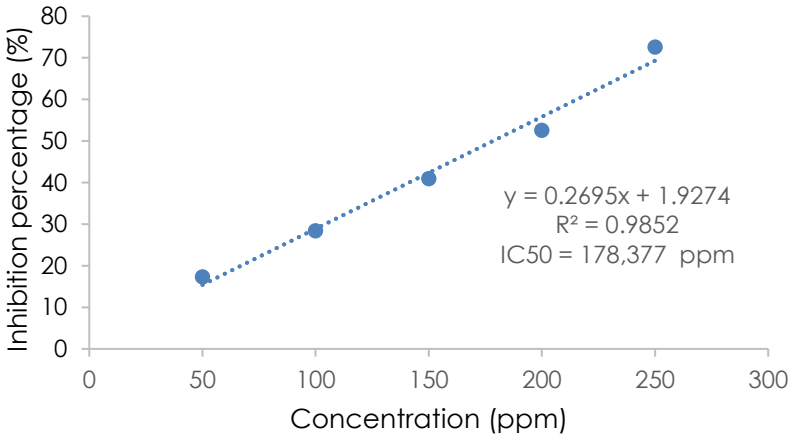

Figure 2. Antioxidant activities of Alginate oligosaccharide (AOS) from *Sargassum* sp.

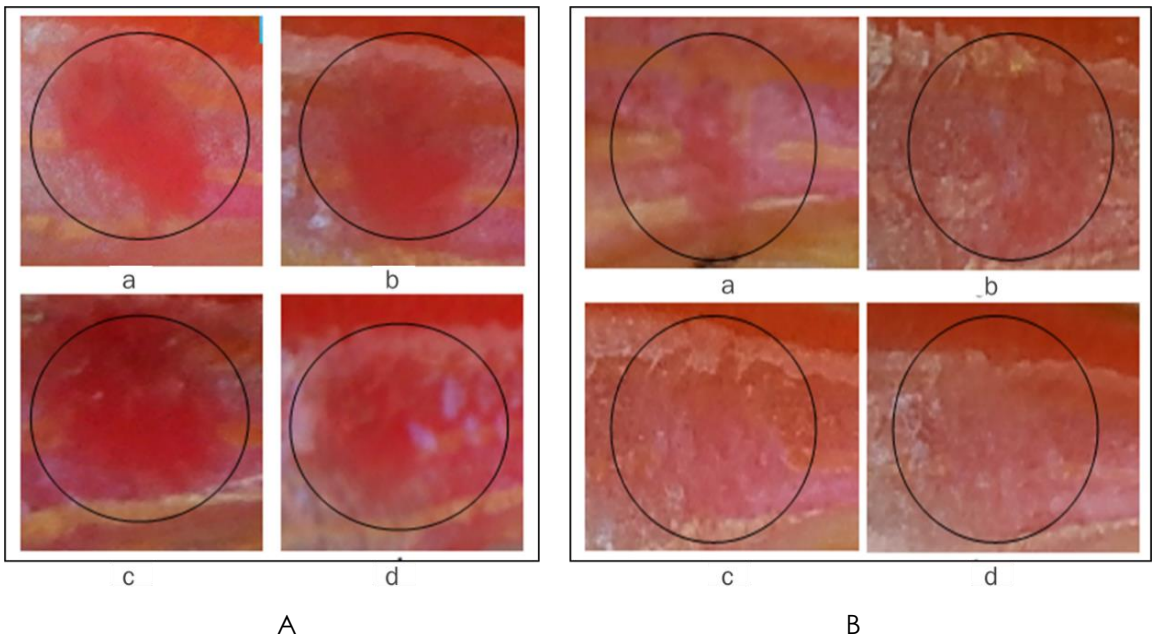

Figure 3. Cutaneous wound healing models in adult zebrafish A (First day post wounding) B (21 days post wounding) (a = Negative Control; b = Immersed for 5 hours in alginate with a concentration of 200 ppm; c = Immersed for 5 hours in alginate with a concentration of 400 ppm; d = Immersed for 5 hours in alginate with a concentration of 600 ppm.)

Cutaneous wound healing

The results showed that the samples were immersed for 5 hours with a concentration of 200 ppm (AIII), 400 ppm (BIII), and 600 ppm (CIII) treatments had the best results and used to represent the description of wound healing with alginate morphologically. Morphological observation of wounds was carried out as an indicator of wound healing as seen from wound closure in zebrafish (Figure 3).

Morphologically, the appearance of wounds for all treatment groups was still exposed and showed widening of the wound due to the inflammatory response on the first day. However, the wound edges for all treatments began to get narrower in the next day, though wound enclosure has not appeared yet. Twenty-four hours after injury, the control and treatment groups (immersed for 5 hours) in each concentration (200 ppm (AIII), 400 ppm (BIII), and 600 ppm (CIII)), did not

Table 2. Percentage of wound enclosure (%)

Immersion time	Concentration (ppm)		
	200 (A)	400 (B)	600 (C)
1 hour (I)	28 ± 0,035 %	65 ± 0,071 %	70 ± 0,141 %
3 hours (II)	50 ± 0,141 %	28 ± 0,212 %	65 ± 0,071 %
5 hours (III)	53 ± 0,177 %	70 ± 0,141 %	83 ± 0,035 %

show any difference. In addition, the wound on the zebrafish skin was still clearly visible. Morphological observation of the wound on the last day of maintenance showed that the treatment group AIII, BIII and CIII showed scab formation covering the scar. In contrast, the wound was still open in the control group, and the scab did not appear to cover the wound completely. However, the scars in the controls were minor in size compared to the previous days. In addition, there was no sign of infection in the wound group until the 21st day of observation.

Table 2 showed that samples immersed for 5 hours with a concentration of 600 ppm (CIII) had the best results based on the wound enclosure indicators. After 21 days, it can cover 83% of its wounds, compared to samples immersed for 5 hours with a concentration of 200 ppm (AIII), which only covers 28% of its wounds. The wound healing process in aIII treatment had taken longer time than CIII treatment. According to Aponno *et al*. (2014), the active ingredients' concentration, including antioxidant properties, is essential in wound healing.

Wound healing is a complex dynamic process. In the early phase of the inflammatory reaction, neutrophils and macrophages will enter the damaged or injured tissue, and these cells will produce Reactive Oxygen Species (ROS). ROS are also produced in the proliferative phase and play an essential role in intracellular signalling in response to various extracellular stimuli (Arief & Widodo, 2018). However, excessive ROS production can cause tissue damage and interfere with the wound healing process because it can interfere with communication between cells. Therefore, antioxidants play an important role in removing ROS in wound healing.

Alginate is known to have the ability to induce monocyte cells and stimulate macrophages so this will increase the production of pro-inflammatory cytokines (Kezia *et al.,* 2013) to accelerate the wound healing process. According to Angelina *et al*. (2021), alginate with a concentration of 0.1% was succeeded to accelerate the wound healing process. Furthermore, according to Wang *et al*. (2015), alginate can fasten the wound healing by increasing type I collagen. Thus, the alginate in this study was managed to accelerate the wound healing process. Alginate is a promising agent as an alternative for the new types of natural wound medicine.

CONCLUSION

The results of this study showed that alginate from *Sargassum* sp. has a yield of 40.5 ± 1.125% with a purity level of 89.95%. Alginate has a moderate category of antioxidant activity (178,377 ppm) and has the ability to increase wound recovery compared to control. It is indicated by the enclosure of the wound formed.

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