Oral Administration of Alginate Oligosaccharide from *Padina* sp. Enhances Tolerance of Oxygen Exposure Stress in Zebrafish (*Danio rerio*)

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Abstract

Alginate is rich in bioactive compounds and has been known to act as a stimulator on the innate immune system. The objective of this study is to determine polysaccharide and oligosaccharide alginate yield, that percentage inhibition with a different type of extraction, to evaluate growth performance as well as immune response by oxygen stress tolerance. Thermal heating with oven laboratory at 140^{o}C for 4.5 hr was done to breakdown the polysaccharide into oligosaccharide. The extraction was conducted by maceration, filtration, precipitation, and centrifugation. Factorial design with two factors was implemented to 260 Zebrafish and reared in thirteen aquariums (20 fish per aquarium) for 12 days. Zebrafish was fed at different dose (4.0g; 6.0g; 8.0g.kg^{-1}) and different type of extraction [noEDTA/noKCl; KCl; EDTA and (EDTAandKCl)]. The evaluation of radical scavenging activity was done spectrophotometrically at 515 nm. Results showed that the highest alginate yield either polysaccharide or oligosaccharide was gained from KCL treatments, percentage inhibition (82.61%), growth performance as well as tolerance of stress (P<0.05). The best growth performance was reached in oligosaccharide supplementation at 6.0g.kg^{-1} treatment. It can be concluded that alginate oliogosaccharide produced by thermal heating enhanced the antioxidant activity, boost the fish's immune system, proofed by better growth performance and more tolerant to the low oxygen stress.

Keywords: *alginate, Padina sp., Zebrafish, stress tolerance*

Introduction

Brown macroalgae, including *Padina* sp. cell wall are rich in polysaccharides known as alginate. Lately, this alginate research has gained a high interest due to their unique physico-chemical properties. Moreover, alginate is also easy to extract (Jork *et al*., 2000; Kim, 2004; Yudiati *et al*., 2018a). Sodium alginate is a linear polysaccharide extracted from phaepophycae is made of α-L-1,4 guluronate (G) dan C5 epimer β-1,4 D-mannuronate (M) (Pawar and Edgar, 2011).

Antioxidants counteract the damage caused by oxydation and mediated by free radicals (Fawzy *et al*., 2017; Hifney *et al*., 2018; Yudiati *et al*., 2018b). A large number of synthesized chemicals may result in the dangerous side effect which act as a free radical scavenger (Melo-Silveira *et al.*, 2014). Nowadays, researchers are trying to use antioxidants with natural origin, including alginate. Researchers have also shown that alginate polymer has the antioxidant properties and this property increases by breaking the polymer chain into oligosaccharide (Falkeborg *et al*., 2014; Fawzy *et al*., 2017; Yudiati *et al*., 2018b).

Immunostimulants, especially from the natural product have evidently increased the innate immune response of penaeid shrimps (Yudiati *et al*., 2016; Yudiati *et al*., 2019) and Catfish (Isnansetyo *et al*., 2014) as well as Zebrafish (Yousefi *et al*., 2018). Those researchers applicated their trials by oral dietary administration and feed

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ijms.undip.ac.id
DOI: 10.14710/ik.ijms.25.1.7-14
Received : 29-01-2020
Accepted : 21-02-2020

supplementation. Yudiati *et al.* (2018b) had proven that Low Molecular Weight of Sodium Alginate (LMWSA) managed to boost the antioxidant activity to scavenge the free radicals in the mechanism at resporatory burst which produces Reactive Oxygen Species (ROS). Some stress tolerance including oxygen exposure was also clearly shown that this high tolerate is related to the adequate immune system (Sudaryono *et al*., 2018).

This present research is aimed to find out whether the LMWSA will boost the antioxidant activity, improve the growth performance and enhance the survival rate of Zebrafish (*Danio reiro*) by low oxygen-stress tolerance.

Material and Methods

Padina sp. was collected from Kupang Waters, Indonesia. Extraction was done from the method by Jork *et al*. (2000). Seaweed was macerated by overnight extraction with 5% Na_2CO_3/50 mM EDTA. Filtration of the pellet was administered and 0.13M KCl was added. This followed by precipitation with 96% ethanol in 1:1 vol and stirred. Centrifugation at 3500 rpm for 5 min was then performed. There were four treatments of alginate extraction methods. The first treatment, namely No EDTA/no KCl treatment was merely macerated with 5% Na_2CO_3, without any EDTA and KCl addition. The second, KCl treatment was done by adding 5% Na_2CO_3, no EDTA, with 0.13 M KCl. EDTA as the third treatment was executed by pouring 5% Na_2CO_3/ 50µM EDTA, no KCl. The 4th treatment was done by adding 5% Na_2CO_3/ 50µM EDTA and 0.13 M KCl, completely. At the end of the extraction, all treatments were heated for 4.5 hours thermal oven heated at 140^{o}C (Yudiati *et al.,* 2018b). These to be considered as oligosaccharide alginate or Low Molecular Weight Sodium Alginate (LMWSA).

The DPPH radical scavenging activity assay

This activity was determined spectro-photometrically The concentration of samples was one w/v. An aliquot of each sample (100 µL) was mixed with 100 µL of 0.1 mM DPPH and then followed by incubation for 30 min. The absorbance of each sample was read at 515 nm (Hidayati *et al*., 2019) using a microplate reader (R-Biopharm Well Reader, Germany). The percentage of scavenged DPPH was calculated using the following equation (Banerjee *et al*., 2005).

DPPH scavenging activity (%) = [(Ac /As) /Ac] × 100

Note : Ac refers to an absorbance of the control (100 µL of ethanol with 100 µL of the DPPH solution) and As refer to an absorbance of the sample.

Preparation of experimental diets

The diet was produced by Brackishwater Aquaculture Development Centre, Jepara, Indonesia as a basal diet. Supplementation of alginate was done by dilution alginate with aquadest. On the other side, Progol® (PT. Indosco, Surabaya, Indonesia) was also diluted with aquadest. These two solutions were then mixed together and sprayed over the top of the feed pellets. Finally, those pellets was dried up at room temperature, stored in a plastic bag at 4^o C and ready to use (Yudiati *et al*., 2016).

Zebrafish (Danio rerio) feeding trial

This study was factorial designed with two factors and one control group. The first factor was the dose of alginate. There were four doses of alginate supplementation treatments in the experimental diet namely control (non-alginate-supplemented) and alginate supplementation in the diet at a dose of 4.0, 6.0 and 8.0 g.kg^{-1}. The second factor a was type of extraction. The four treatments of extraction methods were (no EDTA/no KCl; KCl; EDTA; EDTA & KCl). Thirteen aquariums (30 L) were prepared and set up in the Laboratory of Biology, Department of Marine Science, Diponegoro University. 160 Zebrafish were bought from the ornamental fish farm nearby. Furthermore, 20 fish (2,8±0.09 g) were stocked in each aquarium, randomly. There were three Zebrafish in every treatment which replicate individually.

All aquariums were maintained with aeration in order to keep the good water quality. The monitoring of water quality was conducted daily. Water temperature noted was 27.3±1.2°C, pH 7.5±0.15, dissolved oxygen 7.08±0.2 mg.L^{-1}. The feeding trial was reared for twelve days. During this rearing period, Zebrafish (*D. rerio*) were fed up 4% of biomass at three times.day^{-1} (Seo *et al*., 2017).

Specific growth rate and survival rate

The specific growth rate of Zebrafish was determined by weight out all the fish at the end of the experiment. The formula was used to calculate the specific growth rate and survival parameters:

Specific growth rate (SGR)=100 [In W2−In W1]/T.
Survival=(Nf/No)×100

Note W1: initial weight (g); W2: final weight (g); N0: initial number of fish; NF: final number of fish.

Tolerance of low oxygen exposure stressor in Zebrafish

This method was basically adapted from Supamattaya *et al*. (2005) with slight modification.

On the 14th day, ten zebrafish were kept in beaker glass and maintained in 500 mL of media without any aeration. The top of each chamber was wrapped with a plastic sheet. There were four treatments and replicated three times. The unoxygen exposure was executed until five hours. The level of DO was checked using the DO meter (YSI Inc., USA). The mortality of each treatment was then counted every hour.

Statistical analysis

The statistical analysis of the data was performed by One-Way analysis of variance (ANOVA) then followed by multiple comparison (LSD) tests to examine significant differences among treatments. The software used for this data was SPSS ver. 20.0 (SPSS Inc., Chicago, IL, USA).

Results and Discussion

Yield of alginate extraction

All four treatments were extracted in Na_2CO_3. Based on the result, the lowest yield (23.50%) obtained from treatment without EDTA and KCl addition (Figure 1). It is shown from Figure 1, that all the addition of EDTA, KCl, and combination of both gave the higher results in yield (%). *Ethylendiaminetetraacetic acid (*EDTA) is well known as a chelating agent and, in fact this has improved the alginate extract yield. This finding is in agree with Rahelivao *et al (*2013) and his research about three species of seaweed from Madagascar. Even though, the KCl addition was even produced a higher yield compared to EDTA chelating agent.

The percentage of antiradical scavenger DPPH activity

The activity in each treatment was performed in percentage and represented in Figure 2. The higher inhibition percentage of DPPH was reached from all KCl addition treatments [polysaccharide (73.24%) and oligosaccharide (82,61 %)], in contrast to the Treatment 1 (no EDTA/no KCl). Nevertheless, the increment of this treatment is high (28.22%). The results of radical scavenging activity in this research are inline to Figure 1 above. The addition of EDTA and/or KCl succeed to improve the antiradical scavenging activity. Thermal heating allowed the donation of electron or hydrogen atoms from alginate oligosaccharide (Falkeborg *et al*., 2014). Oligosaccharide of LMWSA which has adequate kation will contribute the Hydrogen atoms to raise the antioxidant activity. Certain high thermal heating and time exposure technique were allowed the degradation of the hydrochemical compound (Holme *et al*., 2008; Kelishomi *et al*., 2016; Yudiati *et al*., 2018b).

Based on data from Figure 1 and 2, it can be seen that the KCl treatment produced the highest antioxidant activity. Moreover, these KCl treatments were also able to produce a high yield of sodium alginate. This research has proven that the addition of KCl is highly suggested due to the effect of alginate production as well as antioxidant activity.

The effect of different alginate dose supplementation on the specific growth rate

The specific growth rate of every treatment was varied along the days of rearing (Figure 3). The Zebrafish initial weight were 1.2 g $\pm$ 0.2 and the final weight were 2.0g$\pm$0.2. Zebrafish with supplementation of 8 g alginate.kg^{-1} was leading until 8 days of rearing (76.74%), but then defeated by the supplementation of 6g alginate.kg^{-1} (105.71%). Six g of alginate was sufficient to raise growth performance and this due to the fact that the lower molecule of sodium alginate affected growth significantly. In their research, Yousefi *et al*. (2018) reported that oligosaccharide is a digestible dietary compounds oligosaccharide also provide beneficial effects to the host health and act by stimulating the growth, selectively. On the other hand, the control group was reached at the lowest percentage of specific growth rate (20.00%). Sudaryono *et al*. (2016) reported that *Sargassum cristaefolium* hot water extract can be a functional growth promoter agent for shrimp. Our findings on alginate supplementation on 14 days *L. vannamei* growth has also supported the statement (Yudiati *et al*., 2016). Furthermore, enrichment of diet with oligosaccharide improved growth performance in snakehead (*Channa striata*) fingerlings (Talpur *et al*., 2014). In contrast, 30 days of shrimp rearing with alginate supplementation orally wasn't given higher growth (Yudiati *et al*., 2019). The similar growth performance was found from Yousefi *et al*. (2018) and Ahmadifar *et al*. (2019) in zebrafish, in hybrid striped bass (*Morone chrysops* × *M. saxatilis*) (Burr *et al.,* 2010), rainbow trout (*Oncorhynchus mykiss*) (Hoseinifar *et al*., 2015), kutum (*Rutilus kutum*) (Hoseinifar *et al*., 2016). The supplementation of alginate on the diet of Zebrafish in this present research enhanced the innate immune. Moreover, LMWSA succeed to improve the antioxidant activity. Some researchers proofed that lower molecular weight produced better activity (Burr *et al*., 2010; Falkeborg *et al*., 2014; Yudiati *et al*., 2018b), including nutrient absorbtion (Yousefi *et al*., 2018). Our suggest, these contradictory results probably due to the duration of the experiment which influences the physiological process, life stage,

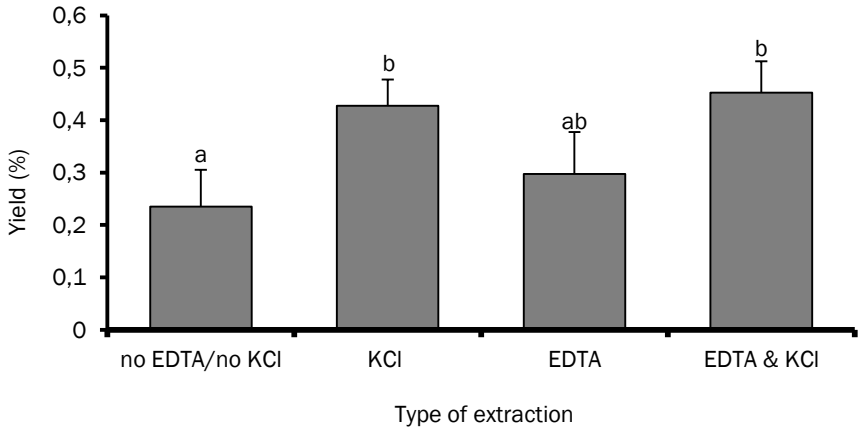

Figure 1. The yield of four treatments from a different type of sodium alginate extraction. Values in bar assigned with different letters denote a significant difference (*P*<0.05)

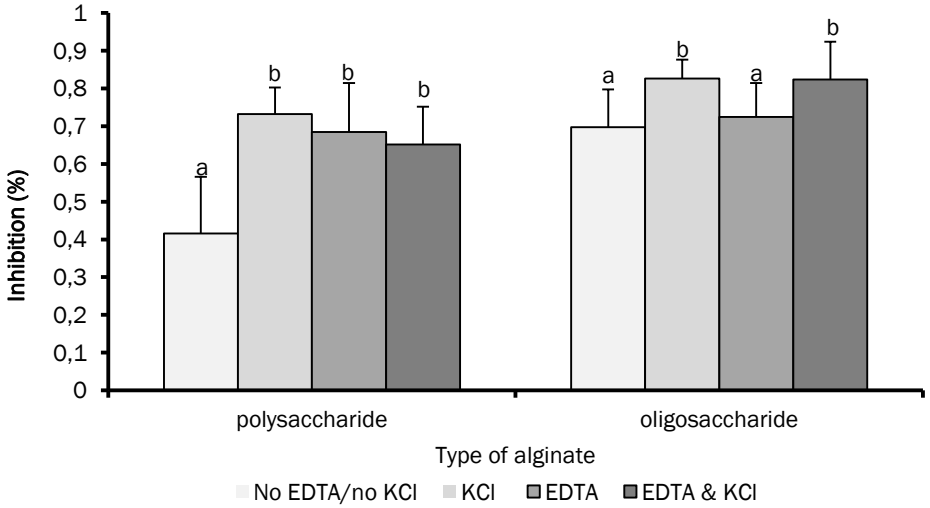

Figure 2. The inhibition percentage of free radical scavenging acitivity at different type of extraction, in alginate polysaccharide and oligosaccharide. Values in bar assigned with different letter denote a significant difference (P<0.05).

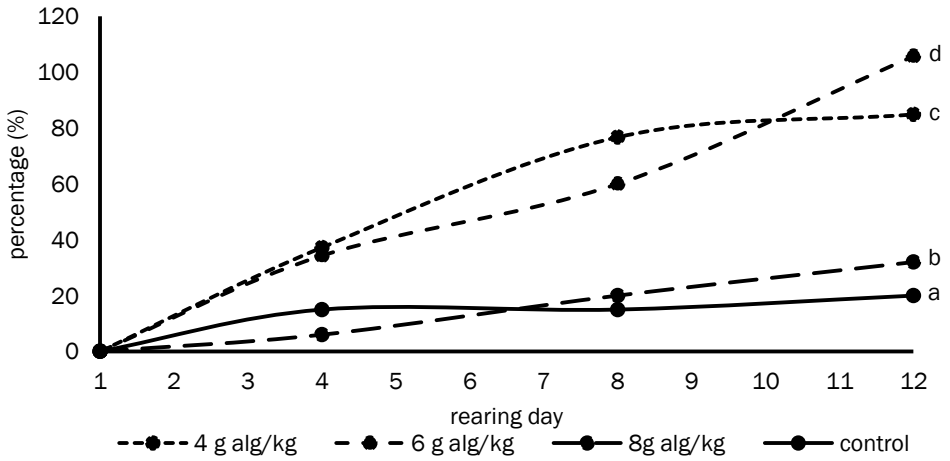

Figure 3. The effects of different doses of alginate supplementation on the specific growth rate of Zebrafish at 12 days of rearing. Values in bar assigned with different letters denote a significant difference (P < 0.05).

species, and experimental conditions (Yousefi *et al*.,2018).

The mechanism of the non specific immune system of fish/shrimp requires the recognition of LMWSA monosaccharide by a set of well defined receptors from host and foloed by the upregulating of the immune-related gene expression (Yudiati *et al*, 2019). Furthermore, this will eventually improve the non specific defence system (Yudiati *et al*., 2016, Yudiati *et al*., 2016, Purbomartono *et al*., 2019). As the defence system reach at higher level, Zebrafish become tolerate to oxygen exposure stress tolerance.

The effect of different alginate dose supplementation on stress tolerance

The stress tolerance was performed as the survival rate after unoxygen exposure. During the low DO stress tolerance trial period, different doses of alginate supplementation (control, 4g.kg^{-1}, 6g.kg^{-1}, 8 g.kg^{-1}) had a significant effect (Figure 4). Similar to growth performance, the highest survival rate has obtained a group of 6g alginate.kg^{-1} dose (88.87%). The DO content was dropped from 7.08 ± 0.2 mg to 3.01±0.11 mg. Sudaryono *et al* (2018) performed similar results in shrimps fed orally with Sargassum hot water extract. Based on the results clearly shown that optimum alginate supplementation was at 6 g.kg^{-1}. Alginate can effectively improve the immune response of shrimps (Yudiati *et al*., 2016; Yudiati *et al*., 2019; Santos *et al*., 2019) and fish including Catfish (*Clarias* sp) (Isnansetyo *et al.,* 2014). The high immune system will impact the fish's health, more tolerable to the stress and finally will improve the number the Zebrafish survivors. The average DO level during experiment was 4,21 ppm $\pm$ 0.05. It's better to show the DO of the water during the treatment.

The effect of different type of extraction on the specific growth rate

At the end of the experiment, the specific growth rate of (EDTA&KCl) (98.65%) and KCl group were leading (88.24%) (Fig 5). The specific growth rate of control (un-supplemented diet) was the lowest one. This strengthens our results before in Pacific White Shrimp (Yudiati *et al*., 2016; Yudiati *et al*., 2019). The LMWSA which have adequate ability on antiradical scavenging activity was able to improve the immune system and promote better growth performance.

The antioxidant serum will protect Zebrafish from damage caused by Reactive Oxygen Species (ROS) at The Respiratory Burst mechanism (Tassanakajon *et al*., 2013). At the final step, this will influence growth performance (Hoseinifar *et al*., 2015; Hoseinifar *et al*., 2016; Yudiati *et al*., 2016). Nevertheless, these results are still contradictory.

The effect of different type of extraction on stress tolerance

During the low DO stress tolerance trial period, control (un-supplemented alginate diet) was survived at the lowest number of fish (43.33%). The LMWSA diet was higher. The highest number of fish survivors was reached at KCl and EDTA groups (Figure 6). This, again clearly proofed that alginate groups were successfully managed to enhance the

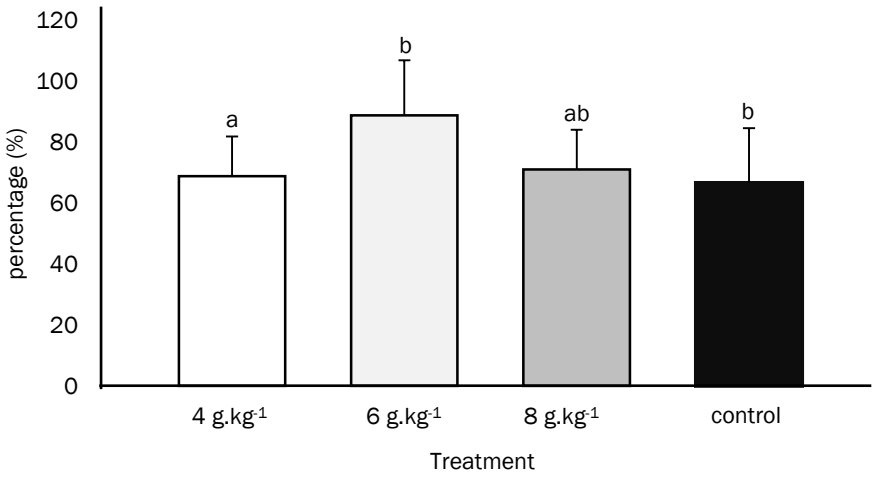

Figure 4. The effects of different dose of alginate supplementation on the survival rate of Zebrafish at 5 hrs of unoxygen exposure. Values in bar assigned with different letters denote a significant difference (P < 0.05).

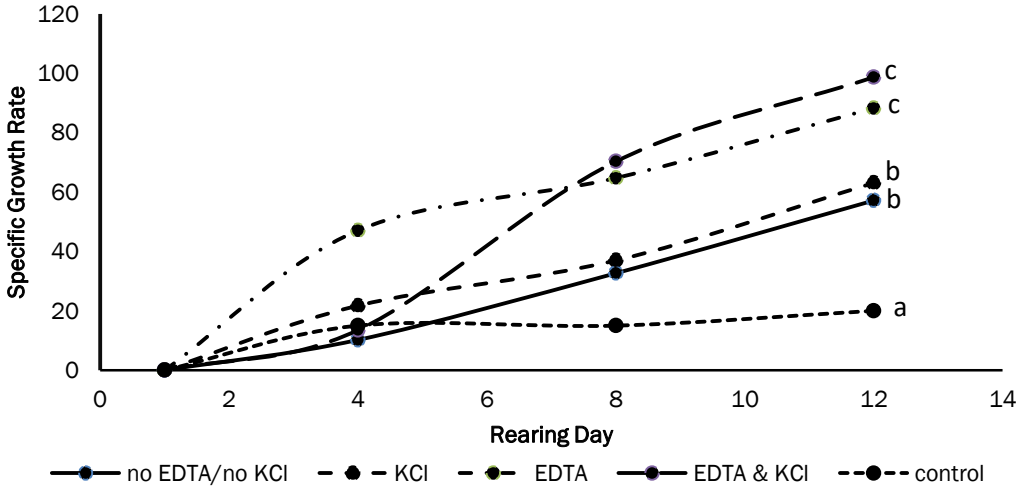

Figure 5. The effects of different doses of alginate supplementation on the specific growth rate of Zebrafish at 12 days of rearing. Values in bar assigned with different letters denote a significant difference (P < 0.05)

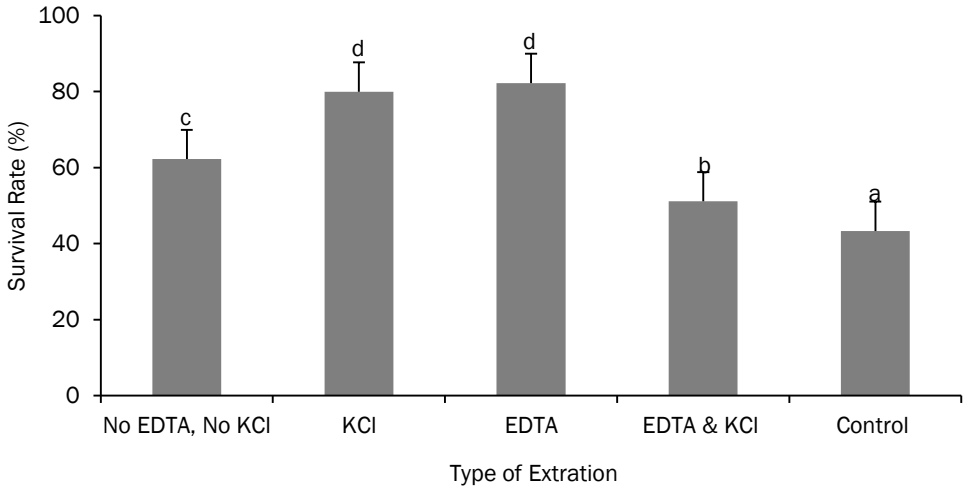

Figure 6. The effects of different type extraction on alginate supplementation on the survival rate of Zebrafish at 12 days of rearing. Values in bar assigned with different letters denote a significant difference (P<0.05).

Zebrafish immune system. At 0.32 ppm DO content, Zebrafish fed with alginate was still survived. This finding is in agreement with Sudaryono *et al*. (2018) results with their trials.

Conclusion

In conclusion, this study clearly showed that the KCl type of extraction produces the best alginate yield and the highest inbihition percentage to counter the radical scavenging activity. Moreover, KCl treatment also resulted the best growth performance and better resistance of stress. Supplementation of 6.0 g LMWSA.kg^{-1} with KCl addition on extraction technique was succeeded to manage the good health on Zebrafish.

Acknowledgment

This research was financially supported by a research grant from the Ministry of Research and Higher Education (contract number 101-98/UN7.P4.3/PP/2018) through Applied Superior Research dated 5th of February 2018, 2018 funding year.

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