

Article

# Exopolysaccharide from *Porphyridium cruentum* (*purpureum*) is Not Toxic and Stimulates Immune Response against Vibriosis: The Assessment Using Zebrafish and White Shrimp *Litopenaeus vannamei*.

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**Abstract:** Exopolysaccharides or extracellular polysaccharides (EPS, sPS) represent valuable metabolite compound synthesized from red microalgae. It is a non toxic natural agent and can be applied as immunostimulant. Toxicity test of exopolysaccharides from *Porphyridium* has been done in-vivo using zebrafish (*Danio rerio*) embryonic model, or the ZET (Zebrafish Embryotoxicity Test). The administration of extracellular polysaccharide or exopolysaccharides (EPS) from microalgae *Porphyridium cruentum* (synonym: *P. purpureum*) on shrimps *Litopenaeus vannamei* was investigated to determine the effect of this immunostimulant on their non specific immune response and to test if this compound can be used as a protective agent for shrimp related to *Vibrio* infection. For immune response, exopolysaccharides was given to shrimps by immersion method on day 1 and booster on day 8. Shrimp hemocytes were taken on day 1 (EPS administration), day 7 (no treatment), day 8 (EPS booster) and day 9 (*Vibrio* infection) and tested for their immune response on each treatment. Result shows that the EPS is not toxic as represented by the normal embryonic development and the mortality data. In the Pacific whiteshrimps, it show an increase in values of all immune parameters in line with the increasing EPS concentration, except the Differential Haemocyte Count (DHC). In detail, an increase was noted in total hemocytes (THC) value, Phagocytotic Activity (PA), Respiratory Burst (RB) in line with the EPS concentration increase. These results and other previous studies indicate that EPS from *Porphyridium* is safe and it enhances immune parameters in shrimp rapidly and has the ability as an immunostimulant or an immunomodulator. It is a good modulator for the non-specific immune cells of Pacific white shrimps, and it can be used as a preventive agent against vibriosis.

**Keywords:** Hemocytes; innate immune cells; Phagocytic Activity; Respiratory Burst; whiteshrimp; microalgae; immunomodulator; toxicity; Extracellular Polysaccharide; *Vibrio harveyi*; *Danio rerio*

## 1. Introduction

Vaname, the white shrimp (*Litopenaeus vannamei*) is one type of the popular and easily cultivated shrimps. This shrimp is highly and quickly productive. Among other superiorities, it is able to live in a wide range of salinity (euryhaline) between 5 to 30 ppt, as well as being adaptable to high stocking densities, and growing well at low protein feeding levels. It entered Indonesia according to the Decree of the Minister of Marine and Fisheries of Republic of Indonesia Number 41 of 2001, as a substitute for the tiger shrimp commodity whose production declined due to disease attacks [1]. Nevertheless, along with the expansion of cultivation activities, the disease on white shrimp (vaname) became increasingly familiar, one of which is vibriosis caused by a pathogenic bacteria *Vibrio harveyi* [2; 3].

Vibriosis by *Vibrio harveyi* is still an important discussion in the development of vaname shrimp cultivation. The pathogenic bacteria often attack shrimp during nauplii, zoea, and mysis stages and

it sometimes occurs in the post larval stage, even during maintenance in ponds at the age of 1 to 1.5 months. This disease can spread directly through the waters, as well as direct contact between shrimps, killing the population shortly after 1 to 3 days from the initial infection [4]. Pathogen occurrences on shrimp occur faster because their immunity is different from fish. They do not have any adaptive immune system, and they only have a non-specific or innate/natural immune system, which on a cellular basis includes phagocytic activity, encapsulation, and nodule formation. They do not have certain antibodies induced by certain antigens, so they are more susceptible to outbreaks [2; 5]. The emergence of disease in shrimp is often associated with poor aquatic environmental conditions, especially in Asia [6].

White shrimps are susceptible to outbreaks of vibriosis because they do not have any adaptive immune system, they only have a non-specific innate immune system. In many invertebrates, the important component in an immune mechanism is the guarding process by organisms that can detect the presence of foreign objects or the emergence of foreign molecules originating from the outside of the organism. A good system must be able to stimulate defense responses from foreign molecules, including those mediated by cells. Invertebrates innate immunity resemble to vertebrates immune response [30]. They do not have specific epitope immune activities and immunoglobulins, but they are able to recognize and destroy invading or parasitic microorganisms [21]. Cellular response as biomarker for immune systems of aquatic animals has been studied as an effect not only associated to bacterial infection or disease, but also related to environmental changes [18; 39; 40]. Invertebrates cell wall have proteins such as lipopolysaccharide,  $\beta$ -1,3-glucans (BG) and a specific protein called beta glucan binding protein (BGBP). BGBP in shrimp appears to be the main plasma protein right after binding to  $\beta$ -glucans, where  $\beta$ -glucan is a compound composed of polysaccharides, which react with the surface of the hemocytes and stimulate the formation of granule cells. Granule cells are one of the hemocyte cells that are responsible for immune system in shrimps [21].

This study used exopolysaccharides (EPS, sPS) from *Porphyridium cruentum* as immunostimulant for the Pacific white shrimp. *Porphyridium cruentum* is a single-celled red microalga belonging to the class of Rhodophyceae, currently has another synonym: *P. purpureum* (Bory). It is capable of living freely or colonizing in marine waters, and can be cultivated in a fast life cycle. The round shaped *Porphyridium cruentum* cells is 4 to 9  $\mu\text{m}$ , and almost 60% is composed of carbohydrates. The cells are bound in mucilage, a compound constantly excreted by the cell forming a capsule, surrounding the cell, containing polysaccharide sulfate, known as EPS in general term or sPS in specific term. This metabolite is one of the important components in the function of *P. cruentum* as an antioxidant, antibacterial, antiviral, and anti-hyperglycemic substance [14; 15]. Various studies on the benefits of *P. cruentum* as an anti-bacterium have been widely carried out, but the use of exopolysaccharides from this species as immunostimulant in *Vibrio harveyi*-infected shrimp has not been done. Therefore, in addition to becoming the initial foundation of research related to future problems, this study is needed to determine the effectiveness of exopolysaccharides or the Extracellular Polysaccharide Sulfate (EPS, sPS) of *P. cruentum* as a source of immunostimulant.

The term EPS in this study is used based on the previous research [27]. In fact, it is a general term of exopolysaccharides that can also be called "Extracellular Polysaccharides", based on the source of the polysaccharides presence issued from the cells as metabolite product into water media [16; 27]. The term based on the type of polysaccharide, it can be EPS in general or goes into sulphated polysaccharides sPS [44]. Beside genus *Porphyridium*, in general, some strains of Cyanobacteria, i.e., *Cyanothece sp.*, *Oscillatoria sp.*, *Nostoc sp.*, and *Nostoc carneum* also secrete this compound [33].

In our previous study [16], we reported about *P. cruentum* growth and extracellular polysaccharides or exopolysaccharides production from this species. Microalgae growth was observed every day for 14 days of culture with the provision of 1ml/L of Fe, silicate, and vitamins and 24-hours continuous lighting. After 14 days of microalgae culture, with densities of 15%, 20% and 25%, respectively, had produced exopolysaccharides of 10,000 mg /L, 12,000 mg /L and 14,000 mg /L, respectively. The Fourier-transform infrared spectroscopy (FTIR) test reported in our previous study showed that exopolysaccharides of *P. cruentum* consist of dominant bonds, namely phenol bonds and polysaccharide bonds [16].

Polysaccharides, one of the ingredients that can be used to produce immunostimulant, are classified into carbohydrates. They are the combination of more than six monosaccharide molecules, which can be hydrolyzed back into many monosaccharide molecules. The main sugars in this bioactive compound are xylose and galactose. The percentages of sulphate are between 7.6% and 14.6 %, protein of 1% to 2 % and uronic acid is between 7.8 and 10% [45-47]. Exopolysaccharides is easily biodegradable, non-toxic, and can favour antioxidant limitations [20].

Although the exopolysaccharides have been reviewed to be the non toxic agent, the influence as immunostimulant in immunomodulation for shrimp disease application has not yet been illustrated comprehensively and it needed for assuredness. In this study we use the the ZET method to assess the toxicity of exopolysaccharies from *P. cruentum (purpureum)*. Toxicity test using zebrafish *Danio rerio* as model organism is increasing and widely developed. The ZET is a suitable approach for the toxicity assessment for various objectives, and for both toxicological risk in human and also ecotoxicological risk in environment. It has several characteristics that make it favorable and a good model organism, such as easy to handle, high egg production, they have sensitive responds and have a transparency body [48-53]. A study showed that zebrafish genome found strikingly similar to humans [11]. It can be used for acute aquatic toxicity testing in many types of chemical compounds [35]. We use different concentration of exopolysaccharides from *Porphyridium cruentum* as media for the ZET, including a simple qualitative morphological view to see the teratogenic potential of this metabolite compound.

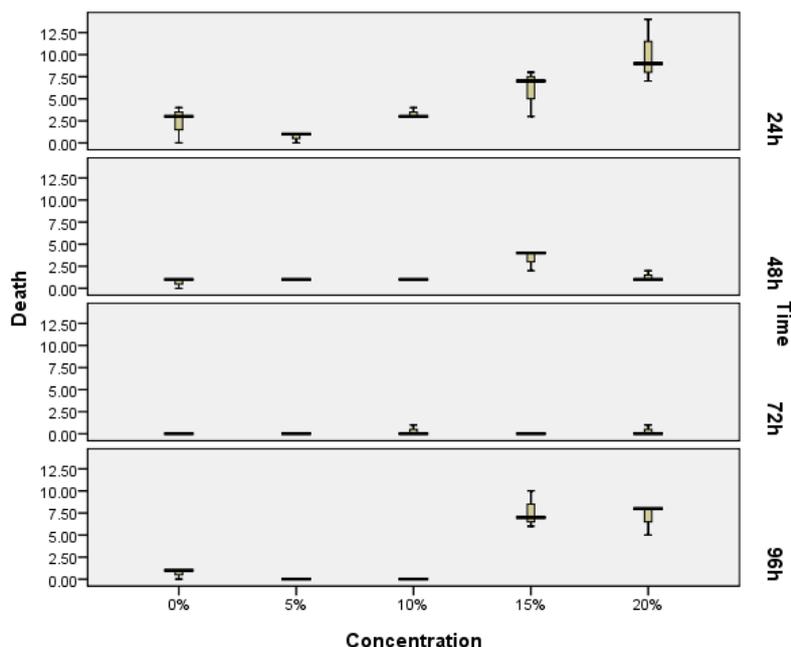
Return to the vibriosis problem of the Pacific white shrimp, various preventive efforts are done; one of which is the provision of immunostimulants. The use of immunostimulant is one of the alternatives to defend from pathogenic infections [7; 8; 9]. Virtually, immunostimulant compounds can be obtained from various sources known to contain lipopolysaccharide, vitamin C, glucan, levamisole, and some microalgae. Among the many species of microalgae that are scattered in the oceans [10], one type of microalgae containing extracellular polysaccharide or exopolysaccharides for anti-bacteria and potentially as a source of immunostimulant through antioxidant activity is *Porphyridium cruentum* [11; 12; 13]. In this study we suggest the provision of immunostimulant using *Porphyridium cruentum*, a red microalga to determine the effect of this bioactive compound on white Pacific shrimps non specific immune response and to test if this compound can be used safely as a protective agent against *Vibrio* infection.

## 2. Results

### 2.1. The Zebrafish Embryotoxicity Test (ZET).

Toxicity test of exopolysaccharides from *Porphyridium* has been done in-vivo using zebrafish (*Danio rerio*) embryonic model, or the ZET method. **Figure 1** show the data of the number of mortalities with the concentration up to 20% of the EPS bioactive compound and with the different exposure time. While, the statistical difference was noted on the **Table 1**. The data shows that concentration up to 15% of EPS is safe until 72 h of exposure time, or under this concentration for 96 h of exposure time thus, the concentration used in this study is appropriate. The highest mortality is shown immediately in 24 h. of exposure time which is associated to initial physiological adaptation and particularly in higher EPS concentration.

As shown in morphological development (**Figure 2**), in all concentration, 5, 10, 15 and 20 % of EPS, all zebrafish embryos developed normally and morphologically did not show body oddities and abnormality. Exopolysaccharides from *Phorphyridium* did not shows a teratogenic effect on the embryo.



**Figure 1.** Median number of individual death of zebrafish embryos observed following exposure to different exopolysaccharide concentrations according to the ZET method. Box represents 25th- 75th percentiles; bars represent minimum and maximum values; Twenty embryos were treated in each concentration treatment (N=20) with triplicate.

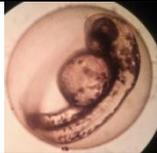
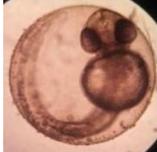
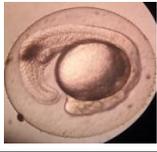
**Table 1.** Least significance difference test (LSD) of the ZET in the difference concentration of exopolysaccharides (EPS). Different letters notation show statistically significant difference at 0.05.

EPS (%)	Mean	5%	0%	10%	15%	20%	Notation
5%	1.7						a
0%	3.7	2					a
10%	4.7	3	1				b
15%	17	15.3	13.3	12.3			c
20%	18	16.3	14.3	13.3	1		c

**Table 2.** Heart beat of zebrafish embryos post fertilization hours in the different EPS concentration. N represent the number of hatching eggs. Twenty eggs were treated per plate of each concentration (N=20) with triplicate.

EPS. Concentration	Replication	ExposureTime							
		N	24h	N	48h	N	72h	N	96h
0%	1	0	0	0	0	12	81	19	76
	2	0	0	4	70	14	72	15	86
	3	0	0	0	0	7	83	15	84
5%	1	0	0	0	0	13	86	19	83
	2	0	0	4	85	17	85	18	84
	3	0	0	2	85	18	87	18	83
10%	1	0	0	2	72	17	70	17	84
	2	0	0	0	0	13	79	14	84
	3	0	0	4	78	16	76	16	85
15%	1	0	0	0	0	9	60	9	82
	2	0	0	1	80	8	79	8	87
	3	0	0	0	0	14	79	16	88
20%	1	0	0	0	0	8	53	10	78
	2	0	0	0	0	7	68	12	78
	3	0	0	0	0	0	70	5	77

**Table 2.** shows heart beat number of the zebrafish embryo post fertilization and the number of hatching eggs. The number of hatched eggs are varied according to time of immersion. In general, the eggs begin to hatch after 48 hours of fertilization (Hpf). Most eggs have hatched between 72 and 96 hours after fertilization. While, the heart beat of the embryo counted in 30 minutes shows the lowest beat is 53 and the highest value is 86 beats.

EPS (%)	Exposure Time (h)			
	24	48	72	96
0				
5				
10				
15				
20				

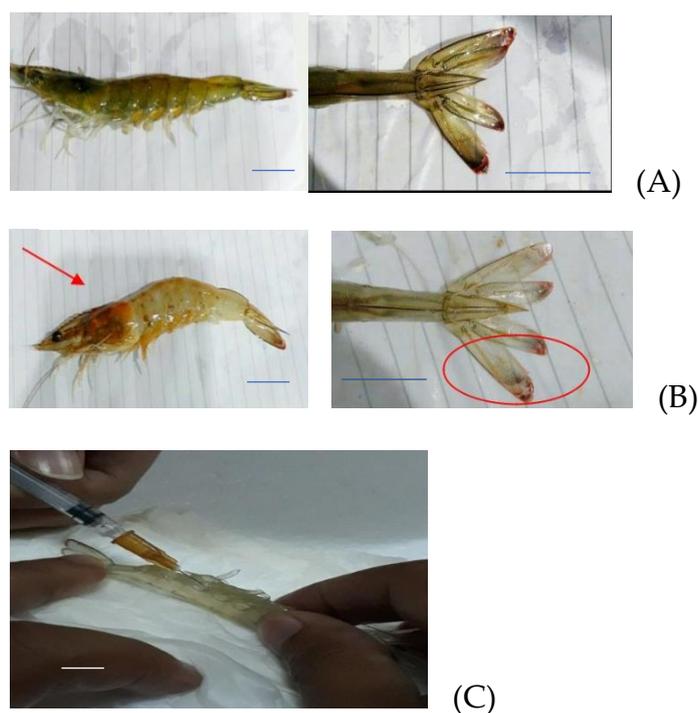
**Figure 2.** Zebrafish (*Danio rerio*) embryonic development exposed to exopolysaccharide from *Porphyridium cruentum* (*purpureum*) of the different EPS concentration (v/v). Photos taken under a microscope Olympus CE-21 with 100 magnification.

### 2.2. Morphological view of vibriosis in the Pacific white shrimp (*Litopenaeus vannamei*)

Vaname, the Pacific white shrimp (*Litopenaeus vannamei*) when it is infected by *Vibrio harveyi* showing vibriosis as shown in **Figure 3**. All treatments almost showed the same symptoms, where the shrimps experienced a change in color from the carapace – cephalotorax to the caudal parts. The whole body and the hepatopancreas organ show smoky-pale coloration and the lateral cephalotorax and caudal fin parts indicate reddish orange.

### 2.3. Shrimp Immune cells number

The result shows that the EPS modulates all immune cells, including hemocytes. In this paper they were expressed in Total Hemocyte Count (THC), Differential Hemocyte Count (DHC) including hyaline cells number, granular and semi granular cells number. Phagocytic activity (PA) and Respiratory Burst.

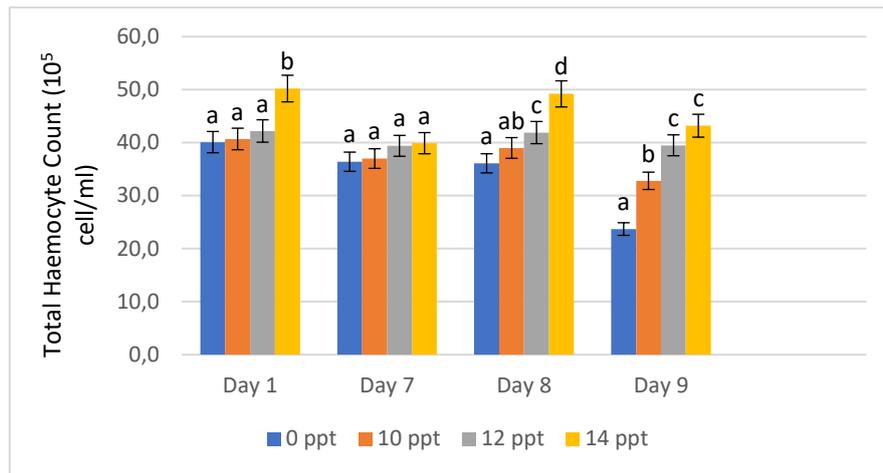


**Figure 3.** Morphology of Vaname (*Litopenaeus vannamei*). Health (A) and shrimp infected by *Vibrio harveyi* (B) showing vibriosis as indicated by smoky body and organ coloration and reddish color change at cephalothorax and caudal fin parts (arrows). Left: whole body, center: uropod. (C). Hemocyte preplacement with a needle. Scale bars: 1 cm. (Photos: Intan Hasanah).

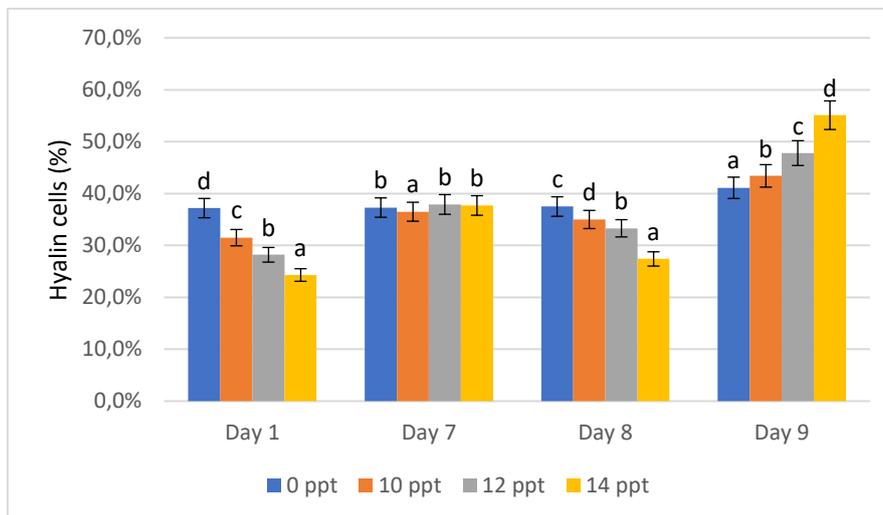
### 2.3.1. Total Hemocyte Count (THC)

Total Hemocyte Count (THC) was observed on the 1st day of EPS administration, day 7 (no treatment), day 8 and on the 9th day. EPS booster administration was conducted with immersion method using 10 ppt, 12 ppt, and 14 ppt. On the 9th day, THC values were obtained after infection with *Vibrio harveyi*  $10^7$  cells/ml.

After immunostimulant addition on day 1 and day 7 (without booster), we observed changes in THC value, where the highest value was obtained from 14 ppt EPS resulting in  $39.9 \times 10^5$  cells/ml, followed by 12 ppt EPS resulting the THC value of  $39.4 \times 10^5$  cells/ml, and the lowest THC value was obtained from the control (0 ppt) resulting in  $36.4 \times 10^5$  cells/ml. On the 8th day, after the booster was given, or through re-soaking with EPS, the highest THC value was obtained at the EPS treatment of 14 ppt resulting in  $49.2 \times 10^5$  cells/ml, followed by EPS treatment of 12 ppt resulting in  $41.9 \times 10^5$  cells/ml, and 10 ppt EPS treatment resulting in  $39.0 \times 10^5$  cells/ml. The lowest THC value was obtained from the control (without EPS) resulting in  $36.1 \times 10^5$  cells/ml. After 9 days of infection, there was a decrease in THC values in all treatments. The EPS treatment of 14 ppt resulted a decrease of THC value to  $43.2 \times 10^5$  cells/ml, while treatment of 12 ppt resulted in a decrease to  $39.5 \times 10^5$  cells/ml, and the treatment of 10 ppt decreased the value to  $32.8 \times 10^5$  cells/ml. For the control, without EPS, THC value decreased very rapidly to  $23.7 \times 10^5$  cells/ml (**Figure 4**).



**Figure 4.** Total Haemocyte Count (THC) of *Litopenaeus vannamei* after EPS administration on. Day 1: EPS administration, Day 8: EPS booster. Day 9: post-infection with *Vibrio harveyi*,  $10^7$  cells/ml. Bars represent mean with SD, different letters (a, b, c, d) between the bars indicate highly significant differences ( $p < 0.05$ ).



**Figure 5.** Percentage of Hyalin cells of *Litopenaeus vannamei* after EPS administration with different immersion concentration. Day 1: EPS administration; Day 7: no treatment; Day 8: EPS booster; Day 9: post-infection with *Vibrio harveyi*,  $10^7$  cells/ml. Bars represent mean with SD, different letters (a, b, c, d) between the bars indicate highly significant differences ( $p < 0.05$ ).

### 2.3.2. Differential Hemocyte Count (DHC)

Differential Hemocyte Count (DHC) consists of hyaline, which is the smallest type of hemocytes in shrimp and two other hemocyte cells types: semi-granular and granular cells.

#### 2.3.2.1. Hyaline cells

On day 1 the highest hyaline cell was obtained from the 0 ppt control (37.2%), followed by 10 ppt (31.5%), 12 ppt (28.2%), and 14 ppt (24.3%). On day 7, without immunostimulant administration, there was an increase in the number of hyaline cells in all treatments, where the highest value was obtained from 12 ppt (37.9%), followed by 14 ppt gives (37.7%), and the 0 ppt control (37.3%).

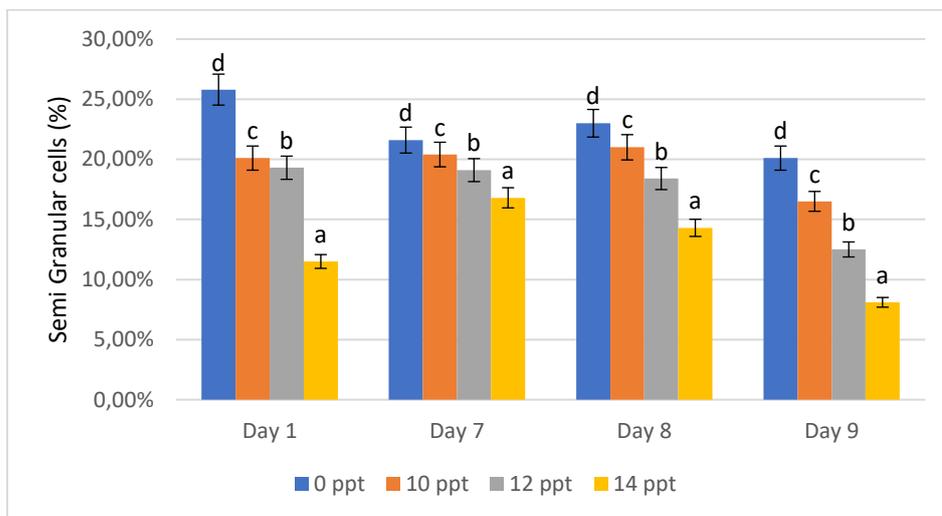
On the 8th day, immunostimulant booster was given, resulting in a significant decrease in the number of hyaline cells in three treatments. The decrease of 35%, 33.3%, and 27.4% occurred for 10, 12, and 14 ppt EPS treatment respectively. The hyaline of the control slightly increased for 37.5%. On the 9th day (after infection), the hyaline cell significantly increased. The highest hyaline cell value

was obtained from 14 ppt EPS treatment (55.1%), followed 12 ppt (47.8%), and 10 ppt (43.4%). The hyaline value of the control (no treatment, 0 ppt EPS) is 41.1%. (**Figure 5**).

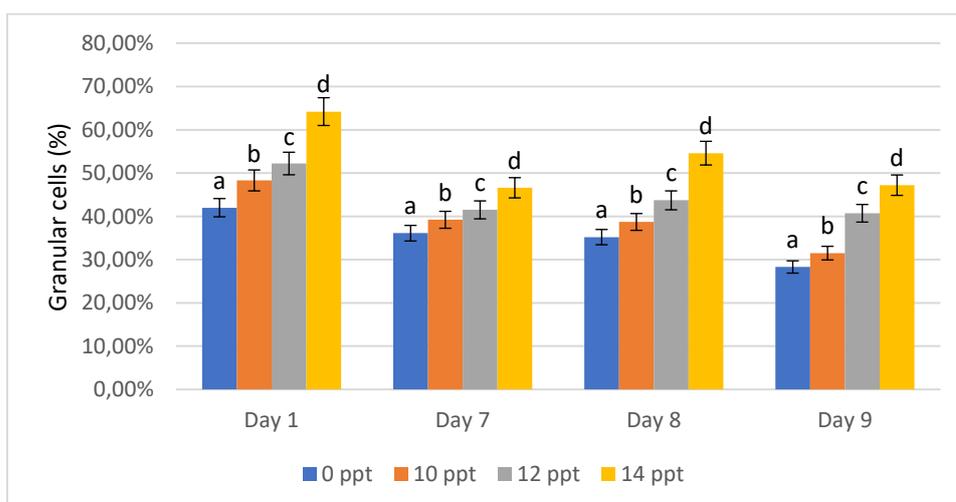
### 2.3.2.2. Semi-granular and granular cells

The lowest number of semi-granular cells for all days was obtained from the treatment of 14 ppt EPS; 11.50% on day 1, 16.80% on day 7, 14.30% on day 8, and 8.10% on day 9. (**Figure 6**). The decrease value of cell number were shown on all concentration treatments. On the other hand, treatment with higher concentration influenced on the decrease of semi granular cells.

In contrast with semi granular cells, granular cells increase as the exopolysaccharide concentration increase. The highest granular cell value was obtained from all 14 ppt EPS treatment: 64.20% on day 1 immunostimulant administration, 46.60% on day 7 without immunostimulant administration, 54.60% on day 8 after the booster, and 47.20% on day 9 after infection. On the 7th day without EPS addition, the value of granular cells appeared to decrease; this indicates a decrease in shrimp's immunity (**Figure 7**).



**Figure 6.** Semi Granular cells of *Litopenaeus vannamei* after EPS administration with different immersion concentration. Day 1: EPS administration; Day 7: no treatment; Day 8: EPS booster; Day 9: post-infection with *Vibrio harveyi*,  $10^7$  cells/ml. Bars represent mean with SD, different letters (a, b, c, d) between the bars indicate highly significant differences ( $p < 0.05$ ).



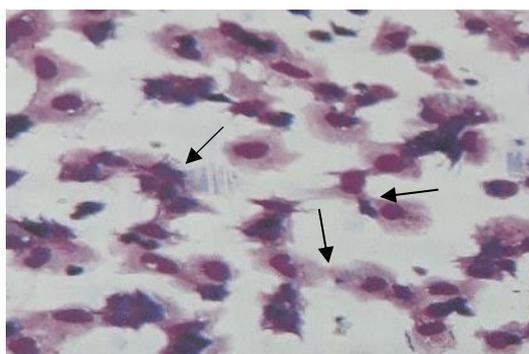
**Figure 7.** Percentage of granular cells of *Litopenaeus vannamei* after EPS administration with different immersion concentration. Day 1: EPS administration, Day 7: no treatment; Day 8: EPS booster. Day 9: post-infection with *Vibrio harveyi*,  $10^7$  cells/ml. Bars represent mean with SD, different letters (a, b, c, d) between the bars indicate highly significant differences ( $p < 0.05$ ).

## 2.4. Shrimp Immune Activity

### 2.4.1. Phagocytic Activity (PA)

**Figure 8** and **9** showed Phagocytic Activity (PA) of hemocyte cells of *L. vannamei* related to *Vibrio harveyi* infection. Phagocytosis increase in line with increasing concentration of exopolysaccharide. On day 1 after immunostimulant administration, the highest phagocytosis level was obtained from 14 ppt EPS treatment (22.9%), followed by 12 ppt EPS treatment (18.7%), and 10 ppt EPS treatment (14.6%). The lowest value was on the control, without EPS, which is 12.3%. On the 7th day without immunostimulant, values of 13.6 % and 13.3% were obtained from the control and 10 ppt EPS treatment. The treatment of 12 and 14 produced the value of 15.1% and 16.4%.

In all treatments, the phagocytosis level in day 7 is lower than that in day 1, except the control, probably due to no immunostimulant treatment. On day 8 after booster administration, the phagocytosis level is higher than the level in day 7, except for control (EPS= 0 ppt). The use of 10 ppt of EPS concentration increased the level to 15.5%, 12 ppt of EPS concentration increased the level to 18.8%, and 14 ppt treatment increased the level to 22.8%. A significant increase in phagocytic activity was on day 9 after infection, but the control and 10 ppt EPS treatment did not make any significant increase (26.3% and 27.8%). The highest phagocytic activity was from 12 and 14 ppt EPS treatment that produced 33.8% and 36.1% respectively.



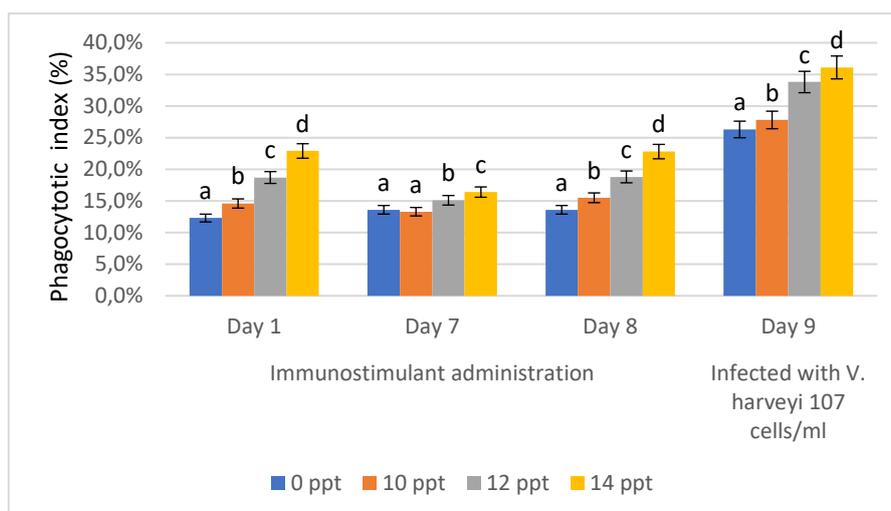
**Figure 8.** Phagocytic Activity (PA) of hemocyte cells of vanname post 24 h. *Vibrio harveyi* bacterial infection. Arrows showed the cells phagocyte yeasts actively. Photo was done under a light microscope (750 magnifications).

### 2.4.2. Respiratory Burst (RB) Activity

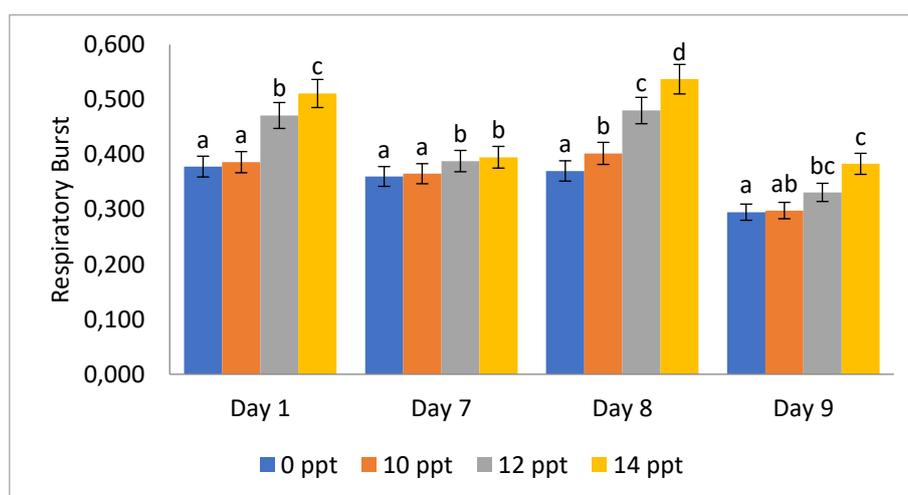
It was found that respiratory burst activity increases along the EPS concentration increasement. An interesting phenomenon was on the 9th day post-infectious treatment with *Vibrio harveyi*, where the respiratory burst (RB) value is lower than of the 1st, 7<sup>th</sup>, and 8<sup>th</sup> (0.511, 0.395, 0.537, and 0.383 respectively) (**Figure 10**).

## 3. Discussion

This paper showed the first study of exopolysaccharides application as a “stimulator” or “modulator” of immune system in *Litopenaeus vannamei* from *Vibrio harveyi* infection. We revealed that the EPS from *Porphyridium cruentum* (*P. purpureum*) modulates all immune cells rapidly, as shown by the number of Total Hemocyte Count (THC), Differential Hemocyte Count (DHC), Phagocytic Activity (PA) and Respiratory Burst (RB), where increasing EPS concentration will influence in a stronger effect of stimulation and this indicates that EPS is a good modulator for the non-specific immunity of Pacific white shrimps. Regarding the toxicity of the compound for the testing animals, several series of separate experiment using the ZET method also shows that the exopolysaccharides from *Porphyridium cruentum* (*purpureum*) is not toxic and can be safely administered to increase immune system in aquatic animal.



**Figure 9.** Phagocytosis activity on *Litopenaeus vannamei* after EPS administration with different immersion concentration. Day 1: EPS administration; Day 7: no treatment; Day 8: EPS booster. Day 9: post-infection with *Vibrio harveyi*,  $10^7$  cells/ml. Bars represent mean with SD, different letters (a, b, c, d) between the bars indicate highly significant differences ( $p < 0.05$ ).



**Figure 10.** Respiratory Burst Activity on *Litopenaeus vannamei* after EPS administration with different immersion concentration. Day 1: EPS administration; Day 7: no treatment; Day 8: EPS booster. Day 9: post-infection with *Vibrio harveyi*,  $10^7$  cells/ml. Different letters show statistically significant difference at 0.05.

It exists various methods, approaches and animal models to analyze toxicity, from biochemical, cellular or mortality assays [39]. Indeed, the toxicity test using rat model shows that *Porphyridium* biomass was not toxic [41]. A separate toxicity experiment has been carried out to test if the exopolysaccharides of red microalgae is the toxic compound or not. Moreover, EPS has been evaluated to be non toxic organic compound, our study using EPS didn't show any toxicity as has been revealed in our work.

This study reveals that exopolysaccharides from *Porphyridium* did not shows a teratogenic effect on the embryo. Morphological features of the embryo did not show any abnormality. It also shows that heart beat of the embryo is in normal health condition. Various studies of toxicity tests have been done using different animal models, and zebrafish has been widely applied for toxicity

studies [48-53]. Those studies show that the ZET method is a suitable approach for the toxicity assessment for various objectives, and for both toxicological risk in human and also ecotoxicological risk in environment. It has several characteristics that make it favorable and a good model organism, such as easy to handle, high egg production, they have sensitive responds and have a transparency body. A study shows that this species has about 70% of gene strikingly similar to human [11] that makes an alternative model also for human.

Based on our preliminary experiment of infection, we used a certain number of *Vibrio harveyi* ( $10^7$  cells/ml), so that the shrimps become infected. Other previous studies reported the same bacterial cells concentration [3; 9]. After infection with *Vibrio harveyi*, all individuals showed the same indicators of color change, where the shrimps infected experienced a change in color from the carapace to the caudal part, i.e., from a clear grey to a grey- reddish color on certain parts, and to the smoky-pale coloration on the whole body. However, no softened carapace was found until a day after infection. Softened shrimp carapace conditions occurred after three days post bacterial infection. In some cases, the sign of disease can be consisted one or more indicators i.e., lesions, reddish color change, melanization or discoloration and loss of function of affected parts. In general, vibriosis shows several clinical symptoms in the infection, among others, the hepatopancreas changes the color to brownish red from its original black color, the body's color changes to brownish red either on the uropod, carapace to the feet, and carapace becomes soft. If vibriosis is very severe, the body of the infected shrimp will look interrupted at night [34;35;36].

Generally, in all treatment, the higher concentration of EPS extracted from *Phorphyridium* influenced the modulation of immune cells. This effect can be interpreted as "immunostimulatory" effect caused by the exopolysaccharides. Although after infection can be assessed as unfavorable or, in other words, the infection attacks and reduces the number of immune cells, but the addition of EPS concentration give stimulatory effect on shrimp's health. Stimulation or modulation of immune cells occurred very rapidly, and this is the reason we conducted the experiments for nine days. This study is in line with other studies which had revealed that, due to bacterial infection, hemocyte in invertebrate changes in hours [23; 37]. Cellular immune response triggered within minute of bacterial introduction, and bacterial immune challenges to see hemocyte and phagocytosis assay is 24 hours [38].

Decreasing and increasing total hemocyte (THC) values after bacterial infection can be occurred rapidly due to the body's defense efforts. The pattern of immune system parameter increases and decrease in *Vibrio alginolyticus*-infected white shrimp was recorded in the previous study, where THC values began to decline from 0 to 24 hours and increased again after 36 hours as a form of shrimp natural immune system recovery [23]. By increasing the number of hemocytes, shrimp protect itself from infection, but when the infection occurs and the process of resistance to foreign matter is successful, the number of hemocytes decrease since a large number of them died after destroying foreign macromolecules.

Hyaline cells in shrimp are important for defense system. These cells are the smallest cell type with a ratio of high cytoplasmic nuclei and relatively a few cytoplasmic granules. The number of the semi-granular cells is related to the increase and decrease in the number of hyaline cells, where semi-granular cells are formed from the advanced stages of hyaline cell development. As a result, these cells cannot develop into semi-granular cells, so this makes the number of semi-granular cells decrease.

The increase of hyaline cells is associated with phagocytic activity; where in hyaline cells are infected, it will get a significant increase, as well as when immunostimulant, which can stimulate the body's defense activities, is given so that hyaline cells increase as the first defense response [22]. Hyaline cells decrease after being given immunostimulant. This can be caused by granular cell formation through the process of hyaline cell maturation. Semi granular cells are the cellular type between hyaline cells and granular cells that play an active role in the encapsulation of larger-size foreign bodies that cannot be phagocyted by hyaline [24]. The effect of EPS after infection can be assessed as unfavorable or, in the other words, the infection attacks and reduces the number of semi-

granular cells and granular cells increased at day 9, although in all treatment, the higher concentration of EPS influenced the modulation of immune cells.

Granular cells are the largest hemocyte cell type whose nucleus is active in the process of storing and releasing prophenoloxidase and cytotoxicity systems [24]. These cells are characterized by the presence of granules in their cytoplasm. They are able to respond to polysaccharides from bacterial cell walls or  $\beta$ -glucan derived from fungi [25]. They play a role in phenoloxidase enzymes production for non-specific body defense activities, which are driven by the influence of immunostimulatory components such as  $\beta$ -glucan, composed of polysaccharides [26]. They are involved in a fast defense response to the virus attack two hours post infection [31].

A significant increase of phagocytic activity was noted in shrimps post infected with *Vibrio harveyi* as seen in this study. The results above reveal that shrimp has phagocytic activity against *Vibrio harveyi* infection, where the phagocytic activity itself is a reaction of cellular defense and is an important process to maintain and eliminate microorganisms or other foreign particles that enter the body [23; 18]. In general, phagocytic activity and respiratory burst increase in line with the increasing EPS concentration.

The measurement of phagocytic activity aims to determine the level of phagocytosis that occurs through several treatments. The entry of microbial components in the body can activate the body's defense response cellularly [18]; this can be observed through phagocytic activity, which is the main activity in the process of defending against foreign infections. Respiratory bursts were related, and they are monitored to identify the body's defense level associated with the activity of superoxide anion ( $O_2^-$ ) which was characterized by the ability of blood cells to reduce NBT (nitrobluetetrazolium). In addition, the value of RB is related to the level of phagocytosis. The higher the respiratory burst value, the better the shrimp's defense system [24].

Respiratory burst is an advanced activity of the phagocytosis process, where particles planted in phagolysosomes will be destroyed by digestive respiratory burst enzymes until free radical release occurs in phagolysosomes. In line with this study, RB activity of turbot phagocytes increases in high concentration water soluble seaweed extracts [28]. While, RB activity decreased when the treatment is added with serum, it might cause suppressive effects on cellular parameter. In contrast, RB increased when aquatic animal is in under osmotic stress [29].

Various preventive efforts have been done; among others using macroalgae extracts, to overcome vibriosis in shrimps particularly in black tiger shrimps [32] and in white shrimps [42]. Algae has polysaccharides contents which is potential for various purposes [43], among other, EPS. EPS has been tested to have the antimicrobial activities, particularly for HSV virus, types 1 and 2, (*Vaccinia virus* and *Vesicular stomatitis virus*), two Gram-negative (*Escherichia coli* and *Salmonella enteritidis*) and one Gram-positive (*Staphylococcus aureus*) bacteria. All EPS extracts revealed a strong activity against *V. stomatitis virus*, higher than the activity of all chemical compounds tested [27]. In this study, we revealed that the EPS stimulates or modulates all immune biomarkers rapidly, and this indicates that EPS from microalgae is a good modulator for the non-specific immunity of Pacific white shrimps. In spite of the fact that the EPS can modulate immune response of white shrimps rapidly, there is still a need for further research on the function of EPS not only as an immunostimulant for preventive objective but also as curative material with the addition of higher doses of EPS or with a longer time treatment post *Vibrio* infection.

Exopolysaccharides from *Porphyridium cruentum* (*purpureum*) is very promising for health of the Pacific white shrimps related to their immune system. Although our previous study has shown their chemical contents [16] and other studies revealed the main sugars component which are composed of xylose and galactose, with other chemical components like sulphate, protein and uronic acids content in these metabolite products have been quantified [45-47], but the information of their structure information is still need to be deeply explored. It is very important to note that for these polysaccharides, a great structure-activity dependence occurs, being of great relevance to do a structural characterization of them.

## 4. Materials and Methods

### 4.1. General

This study uses experimental method with a simple Complete Randomized Design (CRD) and three different dosage treatments and one control, each with three replications. Red microalgae (*Porphyridium cruentum*) were obtained from Situbondo Brackish Aquaculture Center (BBAP) in East Java. The test animals are Pacific white shrimp (*Litopenaeus vannamei*) obtained from UPT Brackish Water Aquaculture Center of Bangil, East Java. The research was conducted at Fish Reproduction Laboratory of Parasites and Fish Diseases Laboratory and Marine Sciences Laboratory of Fisheries and Marine Sciences Faculty of Universitas Brawijaya, Malang, Indonesia.

### 4.2. Culture condition

The Pacific white shrimps or vanname (*Litopenaeus vannamei*) of the young stage, aged 45 days or more, with the length of 7 cm and weight of 5 grams are maintained in aquariums. Acclimatization was done one day before treatment, using pellet feeding and cultured in laboratory condition. Temperature, pH, dissolved oxygen (DO) and salinity were controlled during the study. Daily temperature was between 25 and 26°C, pH was 7.5-7.8, DO value was between 5.64 and 6.5 mg/L, and salinity was 35 ppt.

### 4.3. Extracellular Polysaccharides Extraction

Extracellular Polysaccharides (EPS) can be obtained from microalgae as a supernatant. The EPS supernatant was obtained from the centrifugation process of *Porphyridium cruentum* together with the medium of its life. Centrifugation was carried out at a speed of 10,000 rpm for 15-20 minutes. The acquired supernatant was separated using microalgae pellets; only the supernatant was used. The combination of all *P. cruentum* supernatants was carried out by maceration using Ethanol 96% solvent with a solvent and media ratio of 1: 0.75 v/v. The samples were stored and allowed to stand at room temperature for 72 hours until white EPS deposits were formed. After 72 hours, the sample was put in a water bath for 1 hour at 80°C. The samples were filtered using simple filter paper, and precipitation was carried out using cold ethanol. EPS suspensions obtained in the freeze dryer and dialysis were carried out by resuspending the dry EPS into distilled water, and this process was carried out several times.

We have reported the Chemical functional group of EPS in our previous study which used the Fourier Transformed Infrared (FTIR) method to determine the chemical groups of the compounds contained in EPS as initial information about the chemical composition of EPS [16].

### 4.4. Shrimp Treatment

The test animals were white shrimps (*Litopenaeus vannamei*), 7 cm in length and 5 gram in weight. The shrimps were acclimatized before treatment at laboratory in a controlled condition. The test treatment was carried out by challenging the white shrimp (*L. vannamei*) using *Vibrio harveyi* with the density of  $10^7$  cells/ml using immersion method. The test shrimp samples were maintained by administering EPS as immunostimulant with variations in dosages of 10 ppt, 12 ppt, and 14 ppt. Immunostimulant addition was carried out on the 1st day. The shrimps were then left alive without immunostimulant until the 7th day and were given a booster immunostimulant on the 8th day. On the 8th day (3-4 hours after immunostimulant was given), the shrimps were infected with  $10^7$  cells/ml of *V. harveyi* for 24 hours. Hemolymph was taken from the shrimps at each treatment as the parameter test material for the study of immune cells.

### 4.5. Immune parameters

The parameters which included Total Hemocyte Count (THC), Differential Hemocyte Count (DHC), Phagocytotic Activity (PA) and Respiratory Burst (RB) were calculated using a hemocytometer with the help of a light microscope with 400x magnification. Total Hemocyte Count

(THC), Differential Hemocyte Count (DHC), and Phagocytotic Activity (PA) were based on the procedure as described on the previous studies [17, 18]. The respiratory burst activity was measured using the reduction of nitro-blue tetrazolium (NBT) assay [19].

#### 4.6. Toxicity test using the ZET method

To test the toxicity of EPS, other separated experimental study has been performed previously. Mortality test is indicated as the number embryonic zebrafish mortality immersed in various EPS concentration: control, 5%, 10%, 15%, and 20% (v/v) respectively during 24, 48, 72 and 98 h of exposure time. Each treatment was done with 20 embryos in triplicate. All experiments including the morphological features and heart beat frequency data were taken using Olympus microscope CE-21 with 40 to 100 magnification. Heart beat frequency was counted using hand tally counter and a stop watch during 30 seconds.

#### 4.7. Data Analyses

The data was analyzed using one-way Analysis of Variance (ANOVA) with a confidence interval of 95%. This analysis was used to analyze differences in the average value between groups of treatments or variations obtained between test groups. Thus, if  $F_{\text{count}} > F_{\text{table 5\%}}$  and  $F_{\text{count}} > F_{\text{table 1\%}}$ , it can be concluded that the results of this research are significantly different. Then, the test was continued with the Smallest Significant Difference test (LSD) and Tukey test. Data of toxicity test were analyzed separately by using descriptive statistics (SPSS version 16) for median and interquartile range.

#### 4.8. Ethical Consideration

The approval according to the regulation on the use of animals in our study is not necessary because our research used a limited number of common shrimps frequently consumed by the wider community.

### 5. Conclusions

This study conclude that the exopolysaccharide synthesized from *Porphyridium cruentum* is a non-toxic metabolite compounds. The ZET method shows that the concentration used in this study is relatively safe, supported strongly by the mortality, morphological features and heart beat frequency data. Moreover, this biodegradable organic metabolite is very valuable as the preventive agent for the Pacific white shrimp from vibriosis infection. The provision of exopolysaccharides (EPS, sPS) immunostimulant from *Porphyridium cruentum* can rapidly stimulate the non-specific immune activity of *Litopenaeus vannamei*, with the best dose being 14 ppt. Immune activity responses were characterized by an increase in THC value, DHC (hyaline and granular cells), Phagocytotic Activity (PA), and Respiratory Burst (RB) particularly before being infected with *Vibrio harveyi*. Although post-infection, decreases were identified in parameters, except PA, but in general all immune parameters show the increase responses as the EPS concentration increases. All these results indicate that the EPS from *P. cruentum* is a good modulator for the non-specific immune cells of Pacific white shrimps, and it can be used as a preventive agent against vibriosis.

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