Jurnal Akuakultur Indonesia 18 (2), 120–129 (2019)

Original article

DOI: 10.19027/jai.18.2.120-129

Seaweed extract of *Gracilaria verrucosa* as an antibacterial and treatment against *Vibrio harveyi* infection of *Litopenaeus vannamei*

Ekstrak rumput laut *Gracilaria verrucosa* sebagai antibakteri dan pengobatan terhadap infeksi *Vibrio harveyi* pada udang vaname

Mad Rudi[1], Sukenda[1], Dinamella Wahjuningrum[1], Wesly Pasaribu[1], Dendi Hidayatullah[1]

[1]Department of Aquaculture, Faculty of Fisheries and Marine Sciences, IPB University
*Corresponding author: sukenda@ipb.ac.id

(Received June 6, 2018; Accepted May 22, 2019)

ABSTRACT

The objectives of this study were to investigate the antibacterial activity of *G. verrucosa* extract in test inhibitory zone with different concentrations (500, 1000, 1500, and 2000 mg/L) and to examine *G. verrucosa* extract with different dosage (0.5, 1.0, 1.5, 2.0 g/kg) in feed on immune responses (total hemocytes count, phagocytic activity, phenoloxidase activity, respiratory burst) and survival rate in the *Litopenaeus vannamei* against the pathogenic *Vibrio harveyi*. Pacific white shrimp with an initial body weight of 5.25 ± 0.55 g was reared in the aquarium (60×30×30 cm^3) with a density of 10 shrimp/aquarium. Pacific white shrimp had been fed three times a day as much as 3% in at satiation for 14 days after challenged with *V. harveyi*. The first results of the inhibitory test showed that all the concentration of *G. verrucosa* extract was able to inhibit the growth of *V. harveyi* and the second result showed that the extract of *G. verrucosa* can increase the immune responses of shrimp. In the result of survival showed that shrimp fed with 0.5, 1.0, 1.5, and 2.0 g/kg has 80, 73, 70, and 70%, respectively. In conclusion, the seaweed extract of *G. verrucosa* has antibacterial activity and can induce the immune responses and resistance of Pacific white shrimp against *V. harveyi* infection.

Keywords: *Gracilaria verrucosa*, seaweed, *Vibrio harveyi*, vibriosis, *Litopenaeus vannamei*

ABSTRAK

Tujuan dari penelitian ini adalah untuk menguji aktivitas antibakteri ekstrak *G. verrucosa* dalam uji zona hambat dengan konsentrasi yang berbeda (yaitu 500, 1000, 1500, dan 2000 mg/L) dan studi perlakuan pengobatan untuk menguji ekstrak *G. verrucosa* pada pakan dengan dosis yang berbeda (yaitu 0,5; 1,0; 1,5; dan 2,0 g/kg) pada respons imun (jumlah total hemosit, aktivitas fagositik, aktivitas fenoloksidase, *respiratory burst*) dan tingkat kelangsungan hidup pada udang vaname terhadap bakteri patogen *Vibrio harveyi*. Udang vaname dengan berat badan awal 5,25 ± 0,55 g dipelihara di akuarium (60×30×30 cm^3) dengan kepadatan 10 udang/akuarium. Udang vaname pasifik diberi makan tiga kali sehari 3% *at satiation* selama 14 hari setelah di uji tantang *V. harveyi*. Hasil pertama dari uji zona hambat menunjukkan bahwa semua konsentrasi ekstrak *G. verrucosa* mampu menghambat pertumbuhan *V. harveyi* dan hasil kedua menunjukkan bahwa pemberian ektrak *G. verrucosa* dapat meningkatkan respon imun udang. Hasil tingkat kelangsungan hidup menunjukkan bahwa perlakuan pakan udang dengan dosis 0,5; 1,0; 1,5; dan 2,0 g/kg memiliki tingkat kelangsungan hidup masing-masing 80, 73, 70, dan 70%. Kesimpulannya, ekstrak rumput laut *G. verrucosa* memiliki aktivitas antibakteri dan dapat menginduksi respons imun & ketahanan udang terhadap infeksi *V. harveyi.*

Kata kunci: *Gracilaria verrucosa*, rumput laut, *Vibrio harveyi*, vibriosis, udang vaname

INTRODUCTION

The Pacific white shrimp *Litopenaeus vannamei* is one of the most important marine aquaculture species in the world. The diseases that most often impact intensive shrimp cultivation are bacterial, viral, and co-infectious diseases (Teixeira-Lopes *et al*., 2011). Vibriosis is a major disease in Pacific white shrimp culture caused by *Vibrio harveyi* infection (Widanarni *et al*., 2012). Shrimp infected by vibriosis experienced symptoms such as browning-skin damage, reddish tail, and swimming legs, necrosis, black lymphoid organ, brown gills, brownish muscle, empty intestine, and weak movement (Cano-Gomez *et al*., 2009). Antibiotic treatment of bacterial diseases in fish culture has been applied for many years. But antibiotics have been banned because it can cause pathogenic bacteria to be resistant to antibiotics, leaving residues on shrimp & aquatic environment, and harmful to consumers healthy (Zhang *et al*., 2014).

The natural compound originated from plants have potential in fish farming as an alternative to antibiotic use (Van Hai *et al*., 2015). The medicinal plant also has an antimicrobial effect on the aquatic organism (Citarasu, 2010). Seaweeds are known to be an important source of secondary metabolites for the pharmaceutical industry in drug development. Seaweed from genus *Gracilaria* is a potential source for alternative natural medicines for fish because the potential had been reported as an antioxidant, antibacterial, antitumor and many others (Chen *et al*., 2012; Wongprasert *et al*., 2014; Vienna *et al*., 2015).

Maftuch *et al*. (2012) and Vienna *et al*. (2015) reported that seaweed *Gracilaria verrucosa* have antibacterial activity and can enhance innate immune of shrimp. Sirirustananun *et al*. (2011) demonstrated that the supplementation of *G. tenuistipitata* in shrimp feed at a dose of 0.5–2.0 g/kg for 14 days treatment can increase the immune system of shrimp that infected by WSSV. Jasmanindar *et al.* (2018a) reported that *G. verrucosa* extracts increasing the immune system of shrimp and increased survival rate against *Vibrio harveyi* bacteria, up to 66%. This study aim to investigate the antibacterial activity of *G. verrucosa* extract and evaluating different doses of *G. verrucosa* in shrimp feed to increase the immune response of shrimp challenged with *V. harveyi.*

MATERIALS AND METHODS

Research procedures

Extraction of seaweed Gracilaria verrucosa

Gracilaria verrucosa seaweed was obtained from cultivation farming area in Muara Gembong, Bekasi, Indonesia. Seaweed cultivated for 1–1.5 months after being planted. Extraction was done by using Zahra *et al*. (2017) method which had been modified. The seaweed was washed with both seawater and freshwater to remove dispose of salt, microorganisms, and other unwanted materials, and dried under the sun. Afterward, seaweed was finely ground and sieved by mean of a fine sieve (60 mesh size). Extraction was performed by adding ethyl acetate solvent at a ratio of 1:3 (w/v), shaking for 24 hours using thermoshaker 130 rpm at 40°C, precipitating, and finally filtering. The obtained filtrate was then evaporated using a vacuum rotary evaporator at a temperature of 50°C to acquire a crude extract.

Experimental fish

The Pacific white shrimp *Litopenaeus vannamei* came from a shrimp farm located in Fisheries Academy of Ministry of Marine and Fisheries Affairs Indonesia, Serang Province. Shrimp with an average body weight of 6.07 ± 0.10 g/shrimp were used and acclimatized in fiber tanks for two weeks at 28°C, and shrimp were stocked at a density of 10 shrimp/aquarium.

Bacteria preparation

Vibrio harveyi culture was harvested and re-cultured in 25 mL of SWC broth consisting 5 g bactopeptone, 1 g yeast extract, 3 mL glycerol, 750 mL seawater, and 250 mL aquades) and incubated in waterbath shaker at a temperature of 37°C for 24 hours. Suspension of bacteria was moved into microtube of 2 mL and serial dilution was performed until the bacterial density of 10^8 CFU/mL was obtained. Approximately 50 µL of bacterial suspension was spread on TCBS agar media at 28℃ for 24 hours.

Shrimp feed preparation

G. verrucosa extract based on doses all of the treatment previously determined, was dissolved in a 100 mL of distilled water that was previously mixed with egg white as a binder at a concentration of 2%. The obtained solution was then evenly spraying with 1 kg commercial shrimp feed and dried in an oven at 37°C.

Screening phytochemical test

The phytochemical test was performed through the color visualization method by Harborne (2006), which included a test of flavonoid, alkaloid, tannin, saponin, quinone, steroid, and triterpenoid.

Alkaloid test. A total of 0.1 g extract of *G. verrucosa* has added 1 mL of HCI 2 N and 9 mL of hot distilled water and then heated for 2 minutes. After the cold, the filtrate then shares and is divided into two small tubes. The first tube was added with Bauchardat reagent and the second tube was added by Dragendrauf reagent. Positive results based on the formation of chocolate to blackness in reagent Bauchardat and white deposits in Dragendrauf reagent.

Saponin Test. 0.1 g extract of *G. verrucosa* added 5 mL of distilled water and then heated for 5 minutes. Then the extract is filtered and the filtrate is shaken. Positive results are indicated by the presence of foam for 10 minutes.

Tanin Test. 0.1 g extract of *G. verrucosa* added 5 mL of distilled water and then heated for a few minutes. The filtrate is then filtered and added 1% $FeCl_3$. Positive results are indicated by a change in color to dark blue or greenish-black.

Triterpenoid and steroid tests. 0.1 g extract of *G. verrucosa* added 2 mL ethanol then heated and filtered. The resulting filtrate is evaporated until thick and 1 mL of ether is added, 3 drops of anhydrous acetic acid, and 1 drop of concentrated H_2SO_4. Triterpenoid positive results are indicated by the presence of red or purple, and a positive result of steroids with the formation of green.

Phenolic and flavonoid tests. As much as 0.1 g extract of *G. verrucosa* boiled ambon banana are added with 2 mL of methanol and then heated and filtered. The resulting filtrate is divided into two tubes, the first tube is added 10% NaOH and the second tube is added with concentrated H_2SO_4. The reddish-orange color shows the presence of phenolic compounds, and the red to brownish color indicates the presence of flavonoid.

Evaluation inhibitory zone test through the agar diffusion method

Inhibitory zone test through the agar diffusion method was performed to observe the inhibition zone around the paper disk. Seaweed extract was made into various concentrations, those were 500, 1000, 1500, and 2000 mg/L and positive control (K+) using 50 mg/L chloramphenicol antibiotic. The extract was dissolved in 10 mL of sterile aquades and dripped 20 µL with a micropipette on a paper disk. The paper disk is placed on the surface of a medium that has been spread by *V. harveyi* with a concentration of 10^6 CFU/mL and incubated at 28°C. The bacterial inhibition zone is measured based on the diameter of the clear zone formed using caliper with a precision of 0.01 mm.

Evaluation of G. verrucosa extract dose in shrimp feed

A completely randomized design was used in the present research. *G. verrucosa* extract doses in shrimp feed consisted of six treatments (with three replicates each) as follows: K- (without *G. verrucosa* extract), K+ (without feed extract + *V. harveyi* infection), A (0.5 g/kg feed extract + *V. harveyi* infection), B (1.0 g/kg feed extract + *V. harveyi* infection), C (1.5 g/kg feed extract + *V. harveyi* infection), and D (2.0 g/kg feed extract + *V. harveyi* infection). Shrimp were fed at satiation and feeding treatment fed after the challenge test was 3 times a day for 14 days.

Hemolymph collection

As much as 0.2 mL hemolymph, was collected from the bottom swimming leg of the shrimp using 1 mL syringe (previously filled with 0.3 mL of Na-citrate anticoagulant) and homogenized by a handshake to shape number 8 for 5 minutes. Hemolymph was collected in order to determine parameters such as total haemocyte count (THC), phagocytic activity (PA), phenoloxidase activity (PO), and respiratory burst (RB). Data collection on parameters were done by gathering hemolymph in each replication pre-treatment, first, second and fourth-day after *V. harveyi* challenge test, and then 14 days post-treatment or challenge test.

Parameters

Total hemocyte count

Total hemocyte count for each treatment was determined according to the method of Immanuel *et al*. (2012). An anticoagulant-hemolymph mixture was mixed gently and then a drop of mixture placed and counted with a hemocytometer (Neubauer chamber) using a light microscope at 100 times magnification.

Phagocytic activity (PA)

As much as 0.1 mL shrimp hemolymph was placed into a microplate, mixed with 25 µL of Staphylococcus aureus (107 CFU/mL) bacteria and incubated for 20 minutes. Afterward, 5 µL was dropped on a preparation glass to form preparation pads. The preparations were fixed

with absolute methanol for five minutes and stained with Giemsa (10%) for 15 minutes. Phagocytic activity was measured based on the percentage of phagocytic cells that carried out the phagocytic activity.

Phenoloxidase activity (PO)

Phenoloxidase activity was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA) based on the procedures of Liu and Chen (2004) with slight modification. The optical density of the shrimp's phenoloxidase activity for all test conditions was expressed as dopachrome formation in 50 µL of hemolymph.

Respiratory burst (RB)

Respiratory burst (RB) activity of hemocytes was quantified using the reduction of nitroblue tetrazolium (NBT) to formazan as a measure of superoxide anion production following the method of Cheng *et al.* (2004) with slight modifications. The optical density of formazan was measured at 630 nm using a 96-well microtiter plate and microplate reader Respiratory burst was expressed as NBT-reduction in 10 µL of hemolymph.

Survival rate

Survival rate (SR) was calculated at the end of the feeding experiment and 14 days post-challenge test. The survival rate was measured by the following formula:

$$SR\ (\%) = \frac{Nt}{No} \times 100$$

Note:
SR = survival (%)
Nt = the number of shrimp at the end of the rearing
No = the number of shrimp at the beginning of rearing

Data analysis

Data analysis was carried out using ANOVA (SPSS 17) methods with 95% (a= 0.05) of confidence level. ANOVA was used to analyze total hemocytes, phagocytic activity, phenoloxidase activity, respiratory burst, and survival rate. An ANOVA test was followed by Duncan's post-hoc comparison test if differences were found in software SPSS 22.

RESULTS AND DISCUSSION

Results

The bioactive compounds contained in *G. verrucosa* extracts such as alkaloids, saponins, phenolics, flavonoids, triterpenoids, steroids, and glycosides. Seem liked the result of the research Siregar *et al*. (2012), seaweed *G. verrucosa* contained alkaloid compounds, flavonoids, and

Table 1. The result of the phytochemical test of *G. verrucosa* bulbs extract

Bioactive compounds	Results
Alkaloid	Positive
Saponin	Positive
Tannin	Negative
Phenolic	Positive
Flavonoid	Positive
Triterpenoids	Positive
Steroids	Positive
Glycosides	Positive

Table 2. The result of inhibitory zone *G. verrucosa* extract

Treatment	The diameter of inhibitory zone (mm)
Concentrations of extracts *G. verrucosa* A (500 mg/L)	7.13 ± 0.03[a]
Concentrations of extracts *G. verrucosa* B (1000 mg/L)	7.34 ± 0.19[a]
Concentrations of extracts *G. verrucosa* C (1500 mg/L)	7.42 ± 0.03[a]
Concentrations of extracts *G. verrucosa* D (2000 mg/L)	8.65 ± 0.20[b]
Concentrations of chloramophenicol (50 mg/L)	12.43 ± 0.82[c]

Note: Mean values in the same column with a different superscript letter are differed significantly (P<0.05)

steroids. The results of the phytochemical analysis of *G. verrucosa* extract are presented in Table 1.

The results of in vitro showed that *G. verrucosa* extract is able to inhibit the growth of *V. harveyi* bacteria and the higher the dose used the greater the inhibit zone (P<0.05). The test results of antibacterial activity *G. verrucosa* extract presented in Table 2.

The total hemocytes increased the first, second and 14th day after challenge *V. harveyi* on all of the dose treatment of *G. verrucosa* extract compared to the control (P<0.05) are presented in Figure 1.

Total hemocytes on all of the extract dose were higher compared with positive control on the first day after challenge (Figure 1). The highest total hemocytes on extract dose B was significantly higher on the first and fourth days after challenge (P<0.05). Total hemocyte on extract dose B was the highest significantly on the second days after challenge (P<0.05) both all of extract dose and control. Total hemocytes on the dose of extract A was the highest significantly of all doses extract and control at the 14 days (P<0.05).

Phenoloxidase activity on all of the dose extract on the first day after challenge increased and significantly different from the positive control (P>0.05) (Figure 2). Phenoloxidase activity on dose extract C was significantly higher on the second day after challenge (P<0.05). Phenoloxidase activity on all of the dose extract was higher than control and dose extract experiment A highest significantly at the 14 days challenge (P<0.05).

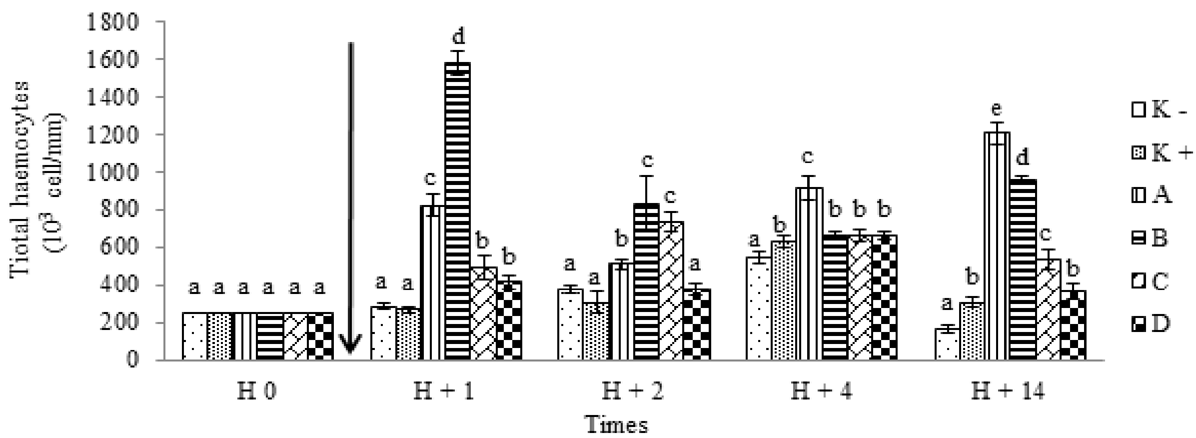

Figure 1. Total hemocytes (THC) of *L. vannamei* shrimp at the initial, the first, second, fourth and 14th day after challenge *V. harveyi*. Different letters at the top of each bar showed significant differences among treatments (P<0.05). Description: K-: negative control; K +: positive control; A: dose extract 0.5 g/kg of feed; B: dose extract 1.0 g/kg of feed; C: dose extract 1.5 g/kg of feed; D: dose of 2.0 g/kg of feed extract; ↓: challenge test.

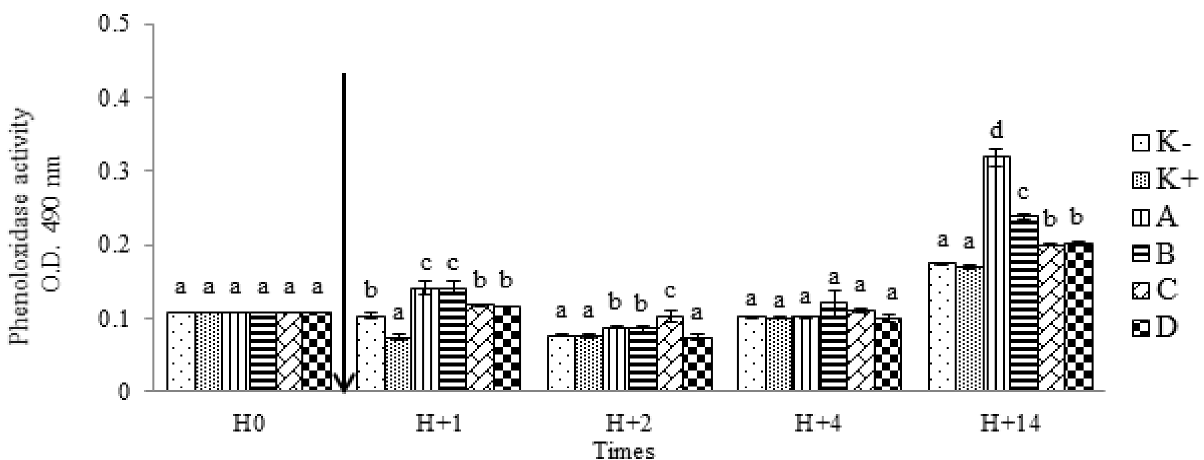

Figure 2. Phenoloxidase activity of *L. vannamei* shrimp at the initial, the first, second, fourth and 14th day after challenge *V. harveyi*. Different letters at the top of each bar showed significant differences among treatments (P<0.05). Description: K-: negative control; K +: positive control; A: dose extract 0.5 g/kg of feed; B: dose extract 1.0 g/kg of feed; C: dose extract 1.5 g/kg of feed; D: dose of 2.0 g/kg of feed extract; ↓: challenge test.

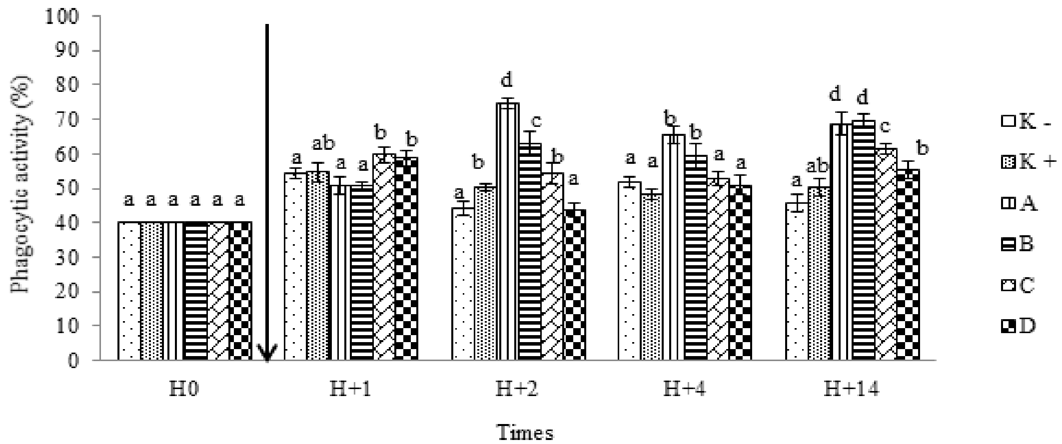

Figure 3. Phagocytosis activity of *L. vannamei* shrimp at the initial, the first, second, fourth and 14th day after challenge *V. harveyi*. Different letters at the top of each bar showed significant differences among treatments (P<0.05). Description: K-: negative control; K +: positive control; A: dose extract 0.5 g/kg of feed; B: dose extract 1.0 g/kg of feed; C: dose extract 1.5 g/kg of feed; D: dose of 2.0 g/kg of feed extract; ↓: challenge test.

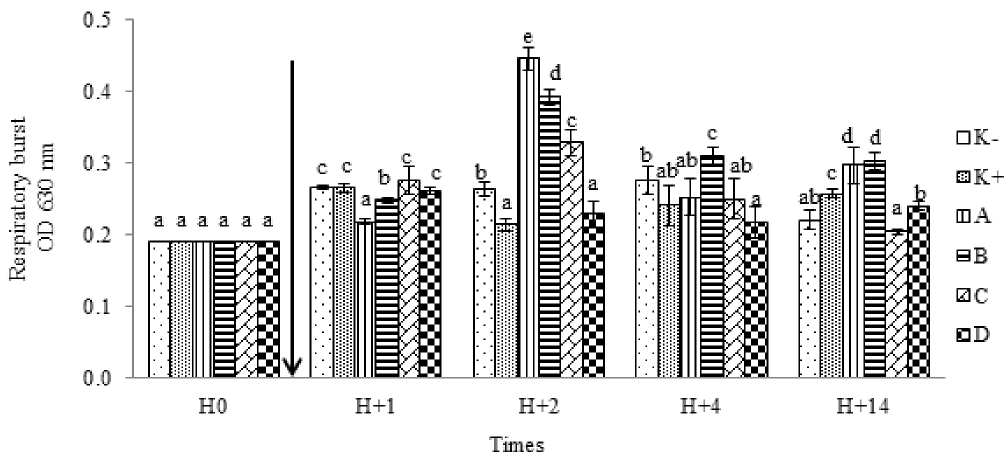

Figure 4. Respiratory burst of *L. vannamei* shrimp at the initial, the first, second, fourth and 14th day after challenge *V. harveyi*. Different letters at the top of each bar showed significant differences among treatments (P<0.05). Description: K-: negative control; K +: positive control; A: dose extract 0.5 g/kg of feed; B: dose extract 1.0 g/kg of feed; C: dose extract 1.5 g/kg of feed; D: dose of 2.0 g/kg of feed extract; ↓: challenge test.

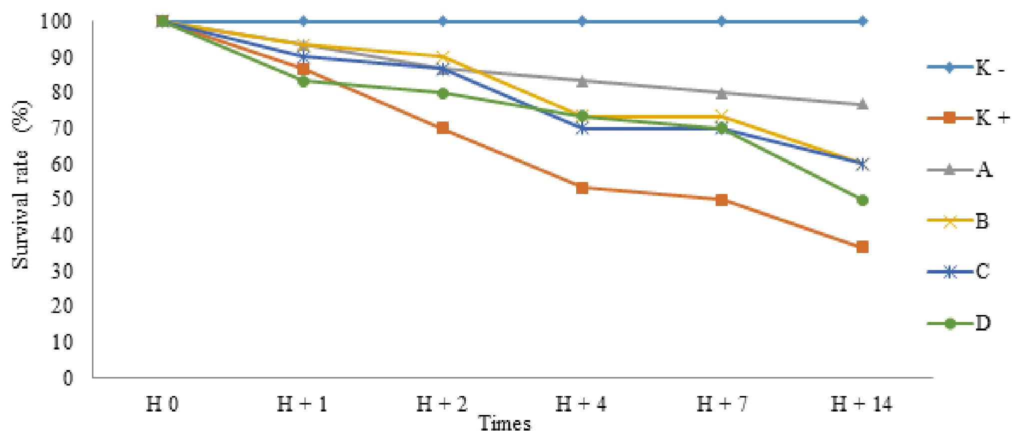

Figure 5. The survival rate of *L. vannamei* shrimp at the initial, the first, second, fourth, seventh and 14th day after challenge *V. harveyi*. Description: K-: negative control; K +: positive control; A: dose extract 0.5 g/kg of feed; B: dose extract 1.0 g/kg of feed; C: dose extract 1.5 g/kg of feed; D: dose of 2.0 g/kg of feed extract; ↓: challenge test.

The phagocytosis activity on all of the dose extract was higher than positive control at the first, second, fourth and 14 days after challenge (Figure 3). The phagocytosis activity on dose extracts C and D was higher with positive control on the first day after challenge (P<0.05). Phagocytosis activity on dose extract A was highest at 2 and 4 days after challenge (P<0.05). Phagocytosis activity on dose extract A and B was higher (P<0.05) compared with others at 14 days after challenge.

Respiratory burst activity was increased both dose extract and control (P<0.05) on the first day after challenge (Figure 4). The respiratory burst activity on dose extract A was significantly higher than positive control on the second day after challenge (P<0.05). The respiratory burst activity on dose extract B was higher than other dosage extract and control on the fourth day after challenge (P<0.05). The respiratory burst activity on dose extracts A and B was higher with other dose extracts and control at the 14 days after challenge (P<0.05).

The survival rate on dose extract was compared with the positive control treatment presented in Figure 5. The survival rate was decreased in all of the dose extract and positive control at the first, second and fourth day after challenge (P<0.05). The highest survival rate after challenge *V. harveyi* on the seventh day is the dose extract A (P<0.05).

Discussion

G. verrucosa can be used as an immunostimulant because it contained polysaccharide compounds (Vienna *et al.*, 2015). *G. verrucosa* extraction resulted in contained sulfate and galactose compounds (Jasmanindar *et al.*, 2018b). The bioactive compound affected the immune system in the shrimp. The bioactive compounds in *G. verrucosa* extract through the mechanism of molecular interaction with receptor surfaces in shrimps an important role in the immune system (Zahra *et al.*, 2017).

Hemocyte is an important role in both cellular and humoral immune responses in immune defenses of crustaceans (Xu *et al.,* 2014). The results of the first, second, fourth and 14 days after challenge showed that total hemocytes of *G. verrucosa* extract were higher than the positive control. That was thought to be due to the effect of *G. verrucosa* extract as immunostimulant during maintenance to improve the Pacific white shrimp immune system.

Changes in the increase and decrease in the amount of hemocyte are the health status and stress indicator on the shrimp. This increase in total hemocytes indicates that the increased defense reaction in the shrimp body due to the presence of foreign particles entering the body of the shrimp, that *V. harveyi* bacteria. Antigen entering the body of the shrimp will be recognized by the hemocyte cell receptor to produce cellular responses such as phagocytosis (Lin *et al.*, 2013). An increasing number of hemocytes is a response to resistance to a pathogen invasion caused by an increase in the number of pathogens in the host.

Phenoloxidase activity is an ability or activity of the body’s defense of Pacific white shrimp in recognized foreign objects entering (Costa *et al.*, 2009). Phenoloxidase activity after the first-day challenge experienced an increase in treatment and control. Total hemocyte is closely related to the activation of proPO to produce phenoloxidase activity. Phenoloxidase is an enzyme that plays a role in the melanization process. This enzyme is produced through a proPO (prophenoloxidase) system that can be activated by immunostimulant. Activation of the prophenoloxidase system results in the production of melanin, a dark brown pigment responsible for several processes, including to inactivate foreign particles, and to protect its spread on the host, and to repair cuticle damage. The proPO system plays an important role in the introduction of foreign bodies including phagocytosis, melanization, cytotoxic reactant production, particle encapsulation, and the formation of nodules and capsules (Amparyup *et al.*, 2013).

The phagocytosis activity continued to increase at the first, second, fourth and 14 days after challenge. During the process of phagocytosis, the foreign particles or bacteria will be recognized by the receptors on the cell surface, then deleting by cells that rearrange the cytoskeleton for the formation of phagosomes. The previous study has suggested an increase in phagocytosis activity in shrimp also occurs after the challenge test as a shrimp defense mechanism (Febriani *et al.*, 2013). Wongprasert *et al.* (2014) that polysaccharides of seaweed can stimulate non-specific immune systems, in this case, phagocytosis and respiratory burst activity through the interaction mechanisms of polysaccharide molecules with receptor-mediated surfaces.

Phagocytosis activity was associated with an increased respiratory burst. Respiratory burst

activity is a mechanism of particle removal by phagocytic hemocyte cells involving the release of degradative enzymes into the phagosome (an oxygen-dependent killing mechanism) and generating reactive oxygen intermediates (Sirirustananun *et al*., 2011). Respiratory burst of the dose extract has increased compared to the initial measurement at first and second day after challenge. The respiratory burst was a decrease between treatment and control on the fourth day after challenge.

Extract of *G. verrucosa* has antibacterial activity derived from flavonoid & saponin compounds as well as sulfated galactan compounds as immunostimulants in white shrimp. Mechanisms to stimulate the immune system in shrimp through sulfated galactan compounds. Sulfated galactan compound in *G. verrucosa* extract is a type of carbohydrate element that is lipopolysaccharide. The introduction of pathogens through molecular patterns by an identifiable protein called pattern recognition protein (PRRs). The protein can recognize carbohydrates from bacterial cell walls or microorganisms such as lipopolysaccharide (LPS) or peptidoglycan (Cochet & Peri, 2017). Receptors in shrimp that are pattern recognition receptors (PRRs) have an important role in the immune system of shrimp consisting of lipopolysaccharide and β-1.3-glucan binding protein (LGBP) and toll receptors. The introduction of pathogens is triggered by PRRs activation via the serine protease pathway which is then broken down into proPO to produce phenoloxidase (Li & Xiang, 2013). Sulfated galactan in *G. verrucosa* extract binds to LGBP on the hemocytic membrane. Sulfated galactan capable of stimulating the immune system in shrimp is assumed through the mediation or interaction of sulfated galactans with receptors on the surface of the hemocytes (Wongprasert *et al*., 2013). The presence of a bond between sulfated galactan and receptor activates signal signaling to increase the proliferation of hemocytes and stimulates immune system activity.

The best survival rate that *G. verrucosa* dose extract has a higher compared with positive control. This suggests that *G. verrucosa* extract is capable of controlling *V. harveyi* infections that are supported through the enhancement of non-cellular and humoral specific immune systems. The best survival is the treatment of the dose extract A (0.5 g/kg of feed). Previous research has shown that *G. verrucos*a extract is to maintain the survival of white shrimp against diseases caused by *Vibrio* bacterial infection (Kanjana *et al*., 2011; Sirirustananun *et al*., 2011; Jasmanindar *et al.,* 2018). Seaweed *Gracilaria* genus is a potential source for alternative natural medicines for the fish cause this species had been reported had potential as anti-oxidant, anti-bacterial, anti-tumor, anti-viral, antibacterial activity and enhances innate immune of shrimp (Maftuch *et al.,* 2016; Saraswaty *et al*., 2015).

CONCLUSION

The effective dose extract *G. verrucosa* which gives the best results in enhancing the immune response and also the white shrimp resistance to *V. harveyi* infection is a dose of 0.5 g/kg of feed. The application of *G. verrucosa* extract in white shrimp feed has antibacterial properties and can increase immune responses and resistance to vibriosis disease caused by *V. harveyi* infection.

REFERENCES

Amparyup P, Charoesapsri W, Tassanaajon A. 2013. Prophenoloxidase system and its role in shrimp immune responses against pathogens. Fish & Shellfish Immunology 34: 990−1001.

Cano-Gomez A, Bourne DG, Hall MR, Owens L, Hoj L. 2009. Molecular identification, typing and tracking of *Vibrio harveyi* in aquaculture systems: current methods and future prospects. Aquaculture 287: 1−10.

Cheng W, Liu CH, Yeh ST, and Chen JC. 2004. The immune stimulatory effect of sodium alginate on the white shrimp *Litopenaeus vannamei* and its resistance against *Vibrio alginolyticus*. Fish & Shellfish Immunology 17: 41−51.

Chen YY, Sim SS, Chiew SL, Yeh ST, Liou CH, Chen JC. 2012. Dietary administration of a *Gracilaria tenuispitata* extract produces protective immunity of white shrimp *Litopenaeus vannamei* in response to ammonia stress. Aquaculture 370−371: 26−31.

Citarasu T. 2010. Herbal biomedicines: a new opportunity for aquaculture industry. Aquaculture International 18: 403−414.

Cochet F, Peri F. 2017. The role of carbohydrates in the lipopolysaccharide (LPS)/toll-like receptor 4 (TLR4) signalling. International Journal of Molecular Sciences 18: 2318.

Costa AM, Buglione CC, Bezerra FL, Martins PCC, Barracco MA. 2009. Immune assessement of farm-reared *Penaeus vannamei* shrimp naturally infected by IMNV in NE Brazil. Aquaculture 291: 141−146.

Febriani D, Sukenda, Nuryati S. 2013. Kappa-carrageenan as immunostimulant to control infectious myonecrosis (IMN) disease in white shrimp *Litopenaeus vannamei*. Jurnal Akuakultur Indonesia 12: 77–85.

Harborne JB. 2006. Metode fitokimia: Penuntun Cara Modern Menganalisis Tumbuhan. Edisi IV. Kokasih P. dan I. Soediro. (penerjemah). Insitut Teknologi Bandung, Bandung.

Immanuel G, Sivagnanavelmurugan M, Marudhu PT, Radhakrishnan S, Palavesam A. 2012 The effect of fucoidan from *Sargassum wightii* on WSSV resistance and immune activity in shrimp *Penaeus monodon* (Fab). Fish & Shellfish Immunology 32: 551−564

Jasmanindar Y, Sukenda S, Zairin JrM, Alimuddin A, Utomo NB. 2018a. Dietary administration of *Gracilaria verrucosa* extract on *Litopenaeus vannamei* immune response, growth, and resistance to *Vibrio harveyi*. Aquaculture, Aquarium, Conservation & Legislation Bioflux 11: 1069−1080.

Jasmanindar Y, Sukenda S, Zairin JrM, Alimuddin A, Utomo NB. 2018b. The chemical composition of *Gracilaria verrucosa* extract and its utilization on survival and growth of *Litopenaeus vannamei*. Omni-Akuatika 14: 1−9.

Kanjana K, Radtanatip T, Asuvapongpatana S, Withyachumnarnkul B, Wongprasert K. 2011. Solvent extracts of the red seaweed *Gracilaria fisheri* prevent *Vibrio harveyi* infections in the black tiger shrimp *Penaeus monodon*. Fish & Shellfish Immunology 30: 389−396.

Li F, Xiang J. 2013. Signaling pathways regulating innate immune responses in shrimp. Fish & Shellfish Immunology 34: 973−980.

Lin YC, Chen JC, Morni WZ W, Putra DF, Huang CL, Li CC, Hsieh JF. 2013 Vaccination enhances early immune responses in white shrimp *Litopenaeus vannamei* after secondary exposure to *Vibrio alginolyticus*. PloS ONE 8: 1−7.

Liu CH, Chen JC. 2004. Effect of ammonia on the immune response of white shrimp *Litopenaeus vannamei* and its susceptibility to *Vibrio alginolyticus*. Fish & Shellfish Immunology 16: 321−334.

Maftuch, Toban MH, Risjani Y. 2012. Administration of marine algae *Gracilaria verrucosa* immunostimulant enhances some innate immune parameters in black tiger shrimp (*Penaeus monodon* Fabricus) against *Vibrio harveyi* infection. Journal of Applied Sciences Research 8: 1052−1058.

Maftuch, Kurniawati I, Awaludin A, Zamzami I. 2016. Antibacterial effect of *Gracilaria verrucosa* bioactive on fish pathogenic bacteria. Egyptian Journal of Aquatic Research 17: 407−414.

Saraswaty V, Mozef T, Risdian C, Rasyid A. 2015. Bioactivity of polysaccharide of *Gracilaria verrucosa* as *a-Glucosidase* inhibitor. Procedia Chemistry 16: 687−693.

Siregar AF, Sabdono A, Pringgenies D. 2012. Seaweed extract antibacterial potential against bacterial skin diseases *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, and *Micrococcus luteus.* Journal of Marine Research 1: 152–160.

Sirirustananun N, Chen JC, Lin YC, Yeh ST, Liou CH, Chen LL, Sim SS, Chiew SL. 2011. Dietary administration of a *Gracilaria tenuistipitata* extract enhances the immune response and resistance against *Vibrio alginolyticus* and white spot syndrome virus in the white shrimp *Litopenaeus vannamei*. Fish & Shellfish Immunology 31: 848–855.

Teixeira-Lopes MA, Vieira-Girão PRN, da Cruz Freire JE, Rocha ÍRCB, Costa FHF, Rádis-Baptista G. 2011. Natural co-infection with infectious hypodermal and hematopoietic necrosis virus and infectious myonecrosis virus in *Litopenaeus vannamei* in Brazil. Aquaculture 312: 212−216.

Van Hai N. 2015. The use of medicinal plants as immunostimulants in aquaculture: A review. Aquaculture 446: 88−96.

Vienna S, Tjandrawati M, Chandra R, Abdullah R. 2015. Bioactivity of polysaccharide of *Gracilaria verrucosa* as *a-Glucosidase* inhibitor. Procedia Chemistry 16: 687–693.

Widanarni, Widagdo P, Wahjuningrum D. 2012. Oral application of probiotic, prebiotic, and synbiotic in Pacific white shrimp *Litopenaeus vannamei* challenged with *Vibrio harveyi*. Jurnal Akuakultur Indonesia 11: 54−63.

Wongprasert K, Rudtanatip T, Praiboon J. 2013. Immunostimulatory activity of sulfated galactans isolated from the red seaweed *Gracilaria* fisheri and development of

resistance against white spot syndrome virus (WSSV) in shrimp. Fish & Shellfish Immunology 36: 52–60.

Xu D, Liu W, Alvarez A, Huang T. 2014. Cellular immune responses against viral pathogens in shrimp. Development & Comparative Immunology 47: 287–297.

Zahra A, Sukenda S, Wahjuningrum D. 2017. Extract of seaweed *Gracilaria verrucosa* as immunostimulant to controlling white spot disease in Pacific white shrimp *Litopenaeus vannamei*. Jurnal Akuakultur Indonesia 16: 174−183.

Zhang Q, Yu H, Tong T, Tong W, Dong L, Xu M. 2014. Dietary supplementation of *Bacillus subtilis* and fructooligosaccharide enhance the growth, non-specific immunity of juvenile ovate pompano, *Trachinotus ovatus* and its disease resistance against *Vibrio vulnificus*. Fish & Shellfish Immunology 38: 7−14.