Application of black cumin *Nigella sativa* as immunostimulant for the prevention of White Spot Disease in Pacific whiteleg shrimp *Litopenaeus vannamei*

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Abstract. White spot disease (WSD) is a serious problem for Pacific whiteleg shrimp (*Litopenaeus vannamei*) production in the world. Therefore, some efforts to prevent WSD are needed, including through the application of immunostimulants. This study aims to obtain the best dose of black cumin for the duration of 4 and 6 weeks as an immunostimulant in *L. vannamei*. The experiment used a Completely Randomized Design, with a control and 3 treatments: black cumin with a dose of 0% (control - K), 0.1%, 0.5%, and 1%. The shrimp received black cumin through feed before being infected with white spot syndrome virus (WSSV) and observed for 7 days. The parameters observed included immunity parameters and production performance parameters. The use of black cumin was able to increase the resistance of the tested shrimp against WSSV infection. The doses of 1% for a duration of 4 weeks and 0.1% for a duration of 6 weeks showed the best results. Black cumin increased the total haemocyte count value of the tested shrimp compared to the control. The application of black cumin at a dose of 1% for 6 weeks did not have a negative impact on the production performance of Pacific whiteleg shrimp.
Key Words: disease prevention, herbs, immunity, production performance.

Introduction. Disease outbreaks pose major issues in Pacific whiteleg shrimp *Litopenaeus vannamei* farming activities. This has an impact on the growth of the shrimp industry and causes huge economic losses, reaching billions of dollars every year (Shinn et al 2018). Of all these diseases, white spot disease (WSD) remains a serious threat to Pacific whiteleg shrimp production in the world (Thitamadee et al 2016). This virus attack can even result in 100% of shrimp mortality within 7-10 days after infection (Wiyoto et al 2017). The efforts to treat this disease in shrimp by using chemotherapy, including with antibiotics, hardly produce any satisfactory results. For this reason, some measures to prevent the disease need to be taken, including using imunostimulants. The administration of imunostimulants to increase the shrimp resistance to diseases is deemed effective since their immunity is dominated by non-specific defense systems. One of many ingredients that are often used as immunostimulants are herbs (Ding et al 2020). Herbs are rich in substances that can increase the body’s natural resistance to infection and prevent various diseases.

Black cumin (*Nigella sativa*) has long been used all over the world as a traditional medicine for preventing and treating a number of diseases. Various other benefits of black cumin have been reported, with properties like antioxidant, anti-inflammatory, antibacterial, antifungal, antiparasitic, antiviral, cytotoxic, anticancer, analgesic, neuroprotective, gastroprotective, cardioprotective, hepatoprotective, and nephroprotective (Islam et al 2017; Ijaz et al 2017). Black cumin is widely used as an immunostimulant because of its ability to increase the non-specific and specific immune systems (Dorucu et al 2009; Shewita & Taha 2011). Several studies of black cumin on aquatic commodities have shown that its administration can increase the immunity of rainbow trout *Oncorhychus mykiss* (Awad et al 2013; Bektaş et al 2019), tilapia *Oreochromis niloticus* (Elkamel & Mosaad 2012; Dey et al 2020), carp *Cyprinus carpio* (Khondoker et al 2016; Yousefi et al 2021), climbing perch *Anabas testudineus* (Khatun et al 2015), rohu *Labeo rohita* (Ali et al 2020), and Pacific whiteleg shrimp (Lei & Xiao-en 2019; Nur et al 2020).

The use of black cumin as an immunostimulant to treat WSD needs to be evaluated. In its application, it is necessary to pay attention to the dose administered since black cumin can also be immunosuppressive. The research of Haq et al (1999) showed that the administration of black cumin *in vitro* at 1 μg mL^{-1} increased lymphocyte proliferation by more than 50%, whereas, at a dose of 10 g mL^{-1} it suppressed lymphocyte proliferation. This study aimed to obtain the best dose and duration to administer black cumin as an immunostimulant to prevent WSD, as well as its effects on the production performance of *L. vannamei*.

Material and Method

Research design. The experiment used a completely randomized design with three replications. The black cumin treatment was used at a dose of 0% (control - K), 0.1%, 0.5%, and 1%. Black cumin is then mixed into shrimp feed by using the coating method. The test shrimp (3 g) were fed with the treatment for 4 weeks and 6 weeks. At the end, the parameters for production performance were measured, followed by a challenge test with white spot syndrome virus (WSSV) infection to measure the parameters of immune response. The observation of these parameters was conducted for 7 days.

Tank preparation. The tanks used for the research were 60x40 cm aquariums. Each aquarium was washed and disinfected using chlorine at a dose of 100 ppm and filled with water (24 ppt salinity). The height of the water was 25 cm (with a volume of 72 L). Each aquarium was installed with 1 point of aeration at a distance of 3 cm from the bottom. The water temperature was maintained between 29 and 31°C by utilizing a room heater (1000 watt power). The room was assisted by 2 fans to homogenize the temperature.

Feed treatments preparation. Black cumin in various doses, based on each treatment, was mixed with a binder (gelatin), which weighted 1% of the feed. This was mixed into the feed until it was evenly coated. The treatment feed was dried in an oven at 40°C for 60 minutes (until dry). The treatment feed was made every three days to maintain the quality of the feed.

Feeding treatments and parameters measurements. Post-larvae 12 stage shrimp were transported from Anyer. They were first kept in a concrete tank for 6 weeks until they had an average weight of 3 g. The test shrimp were transferred to the aquaria. Each aquarium had 15 individuals (1 shrimp 5 L^{-1}). The treatment feed was given 4 times a day at 7 AM, 11 AM, 3 PM, and 7 PM with a feeding rate of 3-6%, adjusted to the average weight of the shrimp. The treatment feed was given to the test shrimp with a duration of 4 weeks and 6 weeks. During the maintenance of the test shrimp, water replacement was carried out every 2 weeks.

At the end of rearing, the final length and weight of the shrimp were measured to calculate the production performance. Production performance parameters such as survival rate (SR), average weight (ABW), growth rate (GR), specific growth rate (SGR), feed conversion ratio (FCR), feed efficiency (FE), and production, based on the formulae performed by Ponce-Palafox et al (2019), were determined. Afterwards, the test shrimp were infected with WSSV and their immune response was then observed for 7 days post-infection. The infection of WSSV was performed with method of Escobedo-Bonilla et al (2005). 10 test shrimp from each tank were infected with WSSV using a 200 μL injection, conducted intramuscularly on shrimp's back between the 2^{nd} and 3^{rd} segments. Two controls were used to observe the immune response, namely the WSSV-infected control (K+) and the WSSV-uninfected control (K-).

The parameters for measuring immune response that were observed included mortality, relative percentage of survival (RPS), total haemocyte count (THC), phenoloxidase activity (PO), respiratory burst activity (RB), and hemolymph clotting time. The mortality of the test shrimp was measured every day until day 7 post-infection. THC parameters were observed on one day before infection (D-1), day 1 post-infection (D1), day 4 post-infection (D4), and day 7 post-infection (D7). The parameters for PO activity, as well as for RB activity, were measured on D1 and D4, while parameters for hemolymph clotting time were measured on D1. Measurement of PO activity was carried out based on Liu & Chen (2004), RB activity was measured in accordance to Song & Hsieh (1994), while hemolymph clotting time was based on Jussila et al (2001).

Shrimp blood (hemolymph) was collected using a 1 mL syringe that had been rinsed and filled with 200 µL of anticoagulant. Hemolymph was collected from the ventral sinus at the base of the 5^{th} leg. The collected hemolymph was placed into a 2 mL Eppendorf, whose volume was recorded as a dilution factor. To preserve the hemolyph samples during the analysis process, the samples were stored in a cool box filled with ice cubes.

Data analysis. The data acquired from this study are primary data taken from the main and supporting parameters. Data analysis was performed using analysis of variance (ANOVA) at a 95% confidence level. If any differences between treatments were found, the data analysis was then carried out by using the Tukey test. Data analysis was conducted using the SPSS 22 program.

Results and Discussion. White spot syndrome virus infection resulted in 83.3% mortality rate of the control shrimp (K+) with 4-week duration treatment and 45.8% in shrimps with a 6 week duration treatment. At the 4-week duration, the best dose of black cumin was 1% and in the longer duration (6 weeks), the best dose was the lower one, of 0.1%. The mortality rate (MR) and RPS of treated shrimp were better than the positive control's (p<0.05). Furthermore, the 4-week and 6-week feeding treatments on the test shrimp did not affect the hemolymph clotting time. The data on mortality, RPS, and hemolymph clotting time are presented in Table 1.

Table 1

Mortality rate (MR), relative percentage of survival (RPS), and hemolymph clotting time of Pacific whiteleg shrimp that was treated with various doses of black cumin for 4 weeks and 6 weeks

Treatment		*Parameters*		
Duration	*Doses (%)*	*MR (%)*	*RPS (%)*	*Hemolymph clotting time (seconds)*
4 weeks	0.1	58.3 ± 14.4^{b}	30 ± 17.3^{b}	42.7 ± 9.5^{a}
	0.5	50 ± 12.5^{b}	40 ± 15^{b}	35 ± 3.5^{a}
	1	37.5 ± 12.5^{b}	55 ± 15^{b}	29 ± 7^{a}
	K(+)	83.3 ± 14.4^{c}	0 ± 17.3^{a}	28 ± 2.6^{a}
	K(-)	4.2 ± 7.2^{a}	95 ± 8.7^{c}	36.3 ± 1.2^{a}
6 weeks	0.1	25 ± 12.5^{b}	45 ± 27.3^{b}	34 ± 4.4^{a}
	0.5	33.3 ± 7.2^{bc}	27.3 ± 15.7^{ab}	28 ± 3.6^{a}
	1	33.3 ± 7.2^{bc}	27.3 ± 15.7^{ab}	33 ± 4^{a}
	K(+)	45.8 ± 7.2^{c}	0 ± 15.7^{a}	31 ± 6.1^{a}
	K(-)	4.2 ± 7.2^{a}	90.9 ± 15.7^{c}	36.3 ± 1.2^{a}

Note: K+ - positive control; K- - negative control; MR - mortality rate; RPS - relative percentage of survival; different superscripts show significant differences (p<0.05).

Both test shrimp, from the 4 week and 6 week treatments, showed an increase in total haemocyte count (THC) after receiving black cumin mixed with the feed. This was significantly different from the positive control (Figures 1A and 1B). The highest THC values were obtained at 0.5% and 1% doses with a duration of 4 weeks, and also at a dose of 0.1% for the 6 weeks treatment. Moreover, WSSV infection caused a change in the THC value of the tested shrimp when being compared to the uninfected shrimp (K-), which was relatively stable. Although some changes in THC values tended to vary among treatments, in general, WSSV infection caused a decrease in THC values on day 1 (D1) and increased again on day 4 (D4).

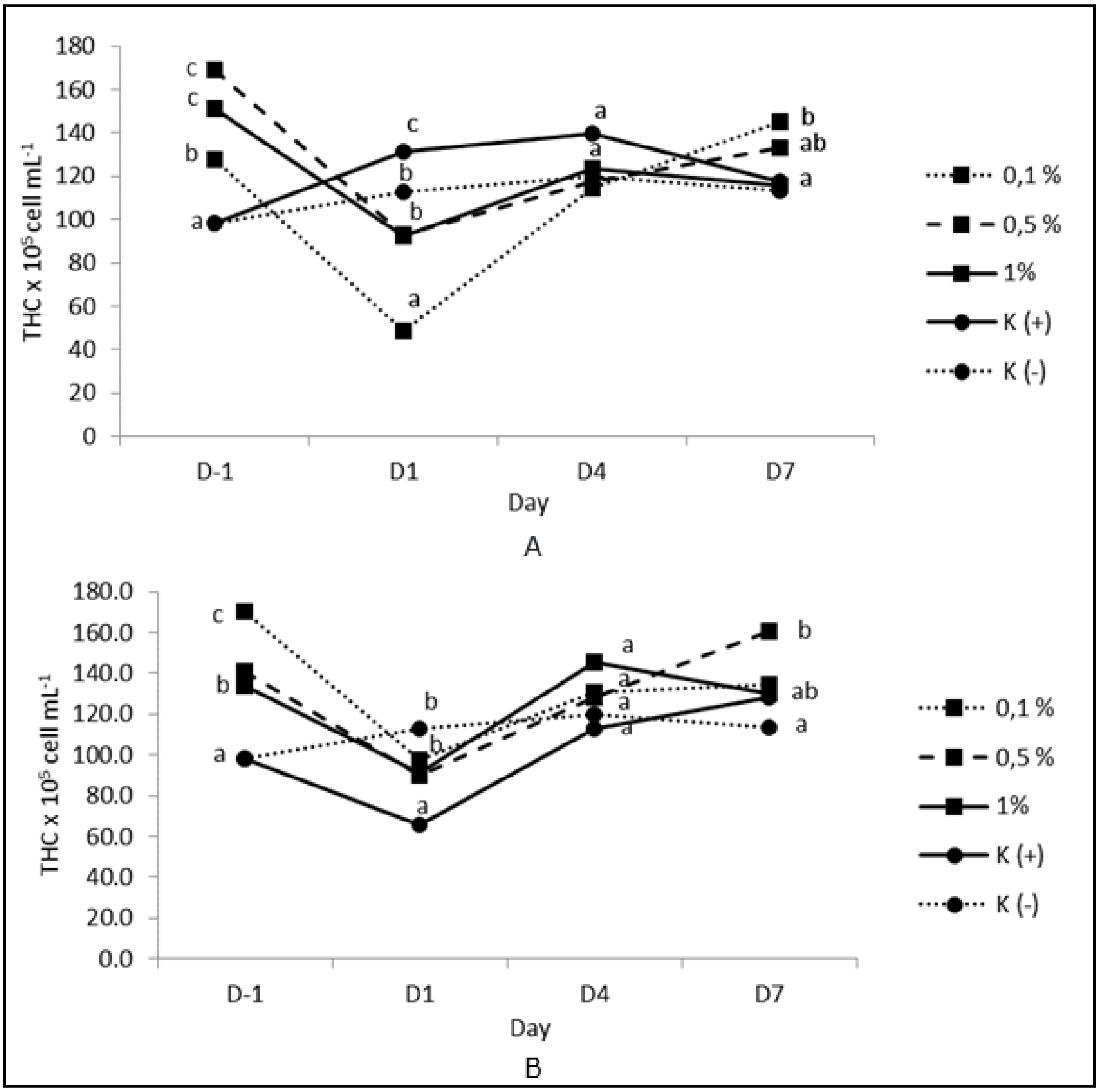

Figure 1. Total haemocyte count of Pacific whiteleg shrimp plasma that were given feed treatments with black cumin for the duration of: (A) 4 weeks and (B) 6 weeks; before WSSV infection on day 1 (D-1) and after the infection on day 1 (D1), day 4 (D4), and day 7 (D7); different letters on lines show significant differences ($p<0.05$).

There was no significant difference when it comes to the value of PO activity between treatments and controls both on D1 and D4 post-infection, except for the 0.5% dose treatment in the 4-week duration treatment (Figure 2A and 2B). This indicates that the administration of black cumin relatively did not affect PO activity. In the 4-week duration treatment, the PO activity of the treated shrimp plasma was relatively higher than K(-). Like the PO activity, the RB activity of the shrimp treated with black cumin was also not significantly different among treatments and controls in both 4-week or 6-week administration (Figure 3). Furthermore, WSSV infection caused a decrease in RB activity of *L. vannamei* on D4, as well as PO activity, especially in the 6-week duration treatment.

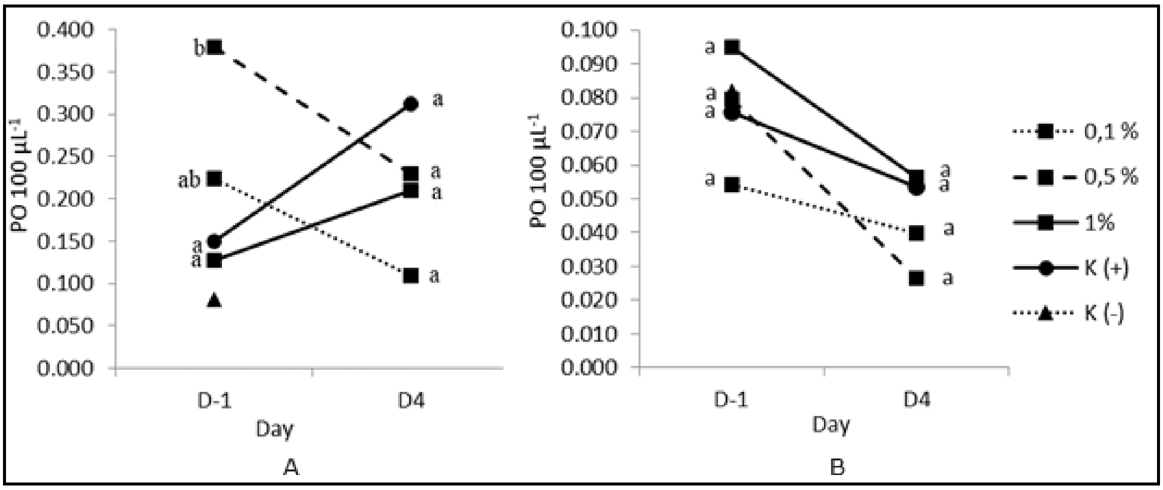

Figure 2. Phenoloxidase (PO) activity of Pacific whiteleg shrimp plasma that were given feed treatment with black cumin for the duration of: (A) 4 weeks and (B) 6 weeks; before WSSV infection on day 1 (D-1) and after the infection on day 4 (D4); different letters on lines show significant differences ($p<0.05$).

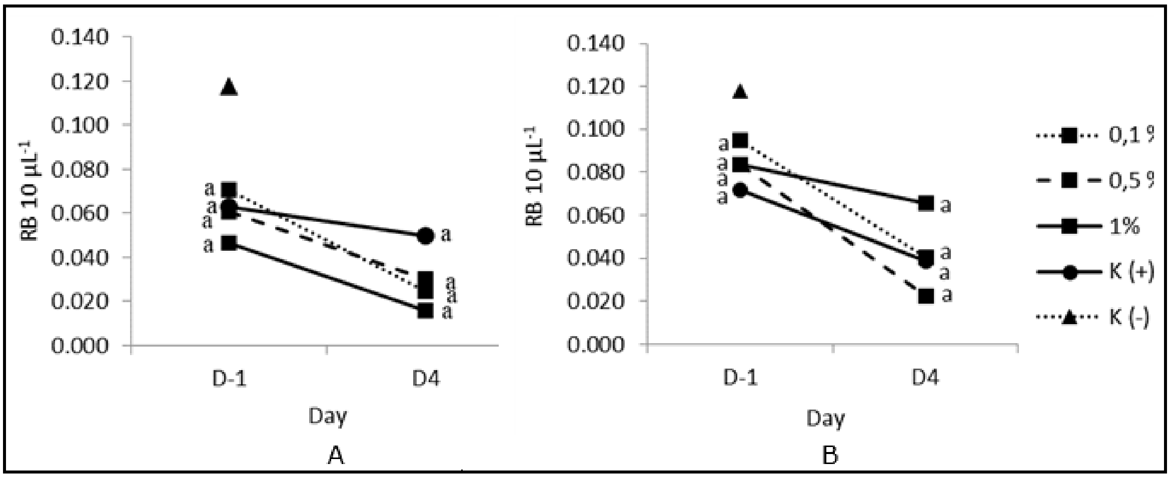

Figure 3. Respiratory burst (RB) activity of Pacific whiteleg shrimp plasma that were given feed with black cumin for the duration of: (A) 4 weeks and (B) 6 weeks; before WSSV infection on day 1 (D-1) and after the infection on day 4 (D4); different letters on lines show significant differences ($p<0.05$).

The administration of black cumin in feed up to 1%, for 6 weeks, did not produce any significant differences compared to controls in all growth and survival parameters (Table 2). However, the highest production parameters were obtained at a dose 1% for the 4 week duration and at a dose of 0.1% for the 6 week duration treatments.

Immune response. The use of black cumin can increase the immunity and decrease the mortality of the test shrimp infected with WSSV, though the best doses will depend on the duration of the administration. The best treatments were the ones with a dose of 1% for 4 weeks and a dose of 0.1% for 6 weeks. The increase in immunity of Pacific whiteleg shrimp that was treated with black cumin is predicted to be caused by an increase in the THC value of the tested shrimp. In crustaceans that only rely on nonspecific defense mechanisms, haemocytes play an important role. They eliminate foreign particles that enter the body through phagocytosis, encapsulation, nodule formation, and humoral components stored in haemocyte granules, including the phenoloxidase enzyme (Jiravanichpaisal et al 2006). Therefore, THC is often used as an indicator of immune response in shrimp. Nur et al (2020) reported an increase in the amount of THC and a decrease in the mortality of shrimp fed with black cumin extract and infected with *Vibrio harveyi*. The best result was shown by shrimp who received the highest dose of black cumin, which was 0.75%.

Table 2

Survival rate (SR), average body weight (ABW), growth rate (GR), specific growth rate (SGR), feed convertion ratio (FCR), feed efficiency (FE), and production of pacific whiteleg shrimp that was treated with various doses of black cumin for 4-week and 6-week duration treatment

Treatments		*Parameters*						
Duration	*Doses (%)*	*SR (%)*	*ABW (g)*	*GR (g day⁻¹)*	*SGR (% day⁻¹)*	*FCR*	*FE (%)*	*Production (g m⁻²)*
4 week	0.1	88.89±3.85[a]	14.98±0.04[a]	0.23±0[a]	2.28±0.03[a]	0.73±0.05[a]	137.71±8.5[a]	832.46±38.16[a]
	0.5	88.89±10.18[a]	14.73±0.18[a]	0.22±0[a]	2.13±0.03[a]	0.76±0.06[a]	131.89±9.93[a]	818.21±92.79[a]
	1.0	93.33±6.67[a]	15.25±0.75[a]	0.24±0.02[a]	2.31±0.19[a]	0.74±0.11[a]	137.8±18.45[a]	887.82±36.06[a]
	K	88.89±3.85[a]	14.69±0.75[a]	0.22±0.02[a]	2.21±0.17[a]	0.8±0.09[a]	125.98±12.91[a]	815.1±10.75[a]
6 week	0.1	84.44±7.7[a]	17.46±1.02[a]	0.24±0.02[a]	2.24±0.11[a]	1.02±0.01[a]	97.56±0.5[a]	918.35±28.7[a]
	0.5	82.22±10.18[a]	16.26±1.29[a]	0.22±0.02[a]	2.07±0.08[a]	1.15±0.09[a]	87.72±7.28[a]	830.21±36.67[a]
	1.0	77.78±7.7[a]	17.07±0.96[a]	0.23±0.02[a]	2.17±0.1[a]	1.14±0.13[a]	88.8±10.12[a]	831.5±116.63[a]
	K	84.44±3.85[a]	16.68±1.13[a]	0.23±0.03[a]	2.16±0.22[a]	1.05±0.06[a]	95.45±4.98[a]	879.01±35.34[a]

Note: K - control; SR - survival rate; ABW - average weight; GR - growth rate; SGR - specific growth rate; FCR - feed conversion ratio; FE - feed efficiency; different superscripts show significant differences ($p<0.05$).

White spot syndrome virus infection caused a decrease in THC values on day 1 (D1). The decrease in total haemocytes can be caused by several factors that pose as defense mechanisms, such as hemocytic infiltration in infected tissue or hemocytic cell death due to apoptosis (Hauton 2012). Despite being caused by the effects of phagocytic activity, encapsulation, and nodule formation, the decrease in THC values can also be caused by degranulation process for the activation of the proPO system and other body defense mechanisms (Smith et al 2003).

The use of black cumin improves the percentage of granular cells (granulocytes and semigranulocytes) as the dose of black cumin is increased, though the percentage of hyaline cells decreases (Nur et al 2020). Hyaline cells play a role in the phagocytosis process, while granular cells play a role in encapsulation, melanization, coagulation, and AMP production processes. They are also involved in cytotoxic reactions (Hauton 2012).

A decrease in the percentage of hyaline cells can decrease phagocytic activity. Phagocytosis is the most common cell defense mechanism that digests and destroys pathogens and foreign particles that enter the body. The destruction of the phagocytic material involves the intracellular production of free radicals. These free radicals can directly kill organisms that attack or combine with nitrogen compounds or synergize with lysozyme (Rodríguez & Le Moullac 2000). Respiratory burst, also called oxidative burst, is the activity of releasing free radicals, especially from immune cells. It has an important role in the immune system, as it is an important reaction occurring in phagocytosis process (Yang et al 2016). The results of this study showed that RB activity of the test shrimp was not affected by the black cumin treatment. This is in contrast with the results of Altunoglu et al (2017), which showed that an administration of black cumin methanol extract to rainbow trout infected with *A. hydrophila* did not affect phagocytic activity, but increased respiratory burst activity, lysozyme activity, and myeloperoxidase activity.

PO activity was used to measure proPO system activation. This immunity parameter is the dominant part of the crustacean’s defense system that plays a role in cell behavior, important functional molecule’s releases and/or activation, and neutralization of infectious agents (Smith et al 2003). Like the RB activity, the administration of black cumin relatively did not affect PO activity. Giving black cumin also did not affect the clotting time of hemolymph. The clotting system itself is the first line of defense and is associated with the activation of antimicrobial peptides (AMP). The speed of blood agglutination is very important for the immune system, for example in preventing blood loss during injury or wound recovery (Maningas et al 2013). Thus, black cumin enhancing shrimp immunity may be related to the role of other granulocyte cells.

Black cumin can increase the immune response in humans and terrestrial animals (Gholamnezhad et al 2016; El-Hack et al 2016). Thymoquinone (2-Isopropyl-5-methylbenzo1,4-quinone) is the main active ingredient in black cumin (Islam et al 2017). The immunomodulatory mechanisms of black cumin and thymoquinone, along with the signaling pathways involved in it, have been reviewed comprehensively by Majdalawieh & Fayyad (2015). Apart from being an immunomodulator, black cumin also has antiviral properties. The application of *Nigella sativa* as an antiviral agent in humans showed an increase in the ratio of helper-T-cell and suppressor-T-cell (T4/T8), natural killer (NK) cell activity, and in the number and function of M-phi and CD4+ve T cells with interferon-gamma (INF-γ) production (El-Hack et al 2016; Islam et al 2017). Black cumin therapy can also reduce the amount/load of the virus, improve oxidative stress and clinical conditions in patients infected with the Hepatitis C virus (Barakat et al 2013).

Black cumin also has an ability to increase the immune response of aquatic animals when infected with pathogens. However, information regarding the mechanism of black cumin as an immunomodulator as well as an antiviral agent in controlling viral diseases in aquatic animals, especially in Pacific whiteleg shrimp, is still very limited. Black cumin at a dose of 3% increased the white blood cell count and phagocytic activity of tilapia infected with *Aeromonas hydrophila*, but did not affect serum immunoglobulins and phagocytic index compared to control fish (Elkamel & Mosaad 2012). In contrast, research by Altunoglu et al (2017) showed that an administration of black cumin methanol extract to rainbow trout infected with *A. hydrophila* did not affect phagocytic activity, but increased respiratory burst activity, lysozyme activity, and myeloperoxidase activity. Furthermore, the administration of black cumin methanol extract did not induce pro-inflammatory cytokine genes (IL-1b, IL-8 and TNF-a), but instead suppressed the IL-12 gene that controls cell-mediated immune responses and induced IFN-g production from Th1 and natural killer cells, as a protection against pathogens. Several other immunity parameters showed different results, which were influenced by the dose of black cumin administration. At a dose of 0.1 g kg^{-1}, the administration of black cumin extract suppressed the IL-1β gene and TGF-β gene, but did not affect the IL-10 gene. On the contrary, at a dose of 0.5 g kg^{-1}, it did not affect the IL-1 gene, but increased the TGF-β gene and suppressed the IL-10 gene (Altunoglu et al 2017).

The application of black cumin (doses of 0.25%, 0.5%, and 1%) to carp for 60 days, which was then exposed to glyphosate at sublethal concentrations for 14 days, showed higher superoxide dismutase and glutathione peroxidase levels than the control, while catalase and malondialdehyde did not show any significant differences (Yousefi et al 2021). Blood biochemical parameters such as total protein, albumin, cholesterol, triglycerides, low-density lipoprotein cholesterol, and high-density lipoprotein cholesterol were higher than in the control. Alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and cortisol were lower. Serum immunity in the form of lysozyme activity and total immunoglobulin was higher than in the control, while the alternate complement activity was not significantly different (Yousefi et al 2021).

Production performance. At the highest dose treatment and the longest duration of administration, the application of black cumin as an immunostimulant for Pacific whiteleg shrimp did not have a negative impact on production performance. The best treatments showed a tendency for better production performance. This is in line with the research findings of Niroomand et al (2020), who showed that an administration of black cumin up to a dose of 3% for 90 days did not affect the growth of Pacific whiteleg shrimp. However, it did begin to inhibit growth at a dose of 5%. The administration of black cumin to rainbow trout, up to 0.5 g kg^{-1} dosage of feed for 30 days (Altunoglu et al

2017), or up to a dose of 2% for 40 days (Bektaş et al 2019), and to tilapia, up to a dose of 4% for 28 days (Dey et al 2020), also did not affect growth. However, different results were reported by Lei & Xiao-en (2019), who showed an increase in the growth performance of Pacific whiteleg shrimp that were given a dose of 2% black cumin for 35 days. Administration of black cumin at 0.5% and 1% for 60 days showed an increase in the activity of digestive enzymes such as proteases, lipases, and carp amylase resulting in better SGR and FCR (Yousefi et al 2021).

Conclusions. Black cumin can increase the body resistance of *L. vannamei* infected with white spot syndrome virus, demonstrated by a decrease in the mortality rate of the tested shrimp. The best application of black cumin was at a dose of 1% given for 4 weeks, or at a dose of 0.1% given for 6 weeks. Giving black cumin through feed increased the total haemocytes of the tested shrimp, but relatively did not affect PO activity, respiratory burst activity, and blood agglutination time. The application of black cumin up to a dose of 1% for 6 weeks did not have a negative impact on the performance of *L. vannamei* production.

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Conflict of Interest. The authors declare that there is no conflict of interest.

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