Original Article

Assessment of antibacterial and immunostimulating activity of black cumin (*Nigella sativa*) extract against vibriosis in white shrimp (*Litopenaeus vannamei*)

Indriyani Nur[1*] Linianti[2] Waode Munaeni[1] La Ode Baytul Abidin[1] Maulidiyah[3]

Abstract

It is assumed that the active components of Black cumin seed (*Nigella sativa*) contain beneficial effects on shrimp health as in humans. Therefore, this study aims to explore the antibacterial and immunostimulant potential of black cumin extract for disease control across *V. harveyi*. Treatments applied include the administration of ethanol extract mixed into shrimp feed at varying dosages (2500, 5000, 7500 ppm), hereinafter referred to as treatments A, B, C, respectively. All shrimps were exposed to pathogen *V. harveyi* at a density of 10^6 CFU ml^{-1} after 14 days of testing duration excluding the negative control group. The immune responses of the shrimps were recognized as Average Total Haemocyte Count (THC) and Differential Haemocyte Count (DHC). In addition, the vibrio challenge test was applied to assess the resistance of shrimp and the population of bacteria and Relative Percent Survival (RPS) were consequently determined. The results indicated that the highest THC, the best proportion in both granula cells and hyalin cells, and the highest RPS (68%) was found in the treatment of 7500 ppm compared to other treatments at all sampling times. Also, it was able to suppres the proliferation of *V. harveyi* bacteria more prominently in the shrimp gut. Extract addition in the diet can control vibriosis especially at a dose of 7500 ppm, indicated by a decrease in the bacterial population and a better immune response. Therefore, extract of black cumin seeds has antibacterial as well as immunostimulant activity.

Keywords: Black cumin, *Vibrio harveyi*, antibacterial, immunostimulant, survival

[1]*Department of Aquaculture, Faculty of Fisheries and Marine Science, Halu Oleo University, Kendari, South East Sulawesi, Indonesia, 93232*

[2]*Graduate School, Study Program of Fishery Science, Halu Oleo University, Kendari, South East Sulawesi, Indonesia, 93121*

[3]*Department of Chemistry, Faculty of Mathematics and Natural Sciences, Halu Oleo University, Kendari, South East Sulawesi, Indonesia, 93232*

***Correspondence**: indriyani_nur@uho.ac.id* (I. Nur)

Received June 20, 2020.
Accepted September 21, 2020.

Introduction

Vibriosis outbreak is one of the principal causes of loss to vannamei shrimp, *Litopenaeus vannamei* production. *Vibrio harveyi* is a species of main pathogenic bacteria to vannamei shrimp disease shown by a "white tail" and is usually followed by mass mortality due to its high virulence (Zhou *et al*., 2012).

A variety of antibiotics have also been used to control the disease in vannamei shrimp, but in fact the usage of antibiotics for disease control is considered deal with the surrounding environment and the associated species. To prevent the adverse consequences of antibiotic practices, immunostimulants are certain choices to vaccines and antibiotics for the defense and prevention of disease outbreak. Immunostimulants comprise drugs, chemical compounds or other substances that can improve the non-specific animal defence and produce antigenic reactions that have not been formerly stimulated (Labh *et al*., 2014). Immunostimulant from medicative plants are effective to improve the immune responses and resistance to disease of *Vibrio* pathogenic in vannamei shrimp (Munaeni *et al*., 2020), and improve growth in shrimp (Labrador *et al*., 2009; Munaeni *et al*., 2019). The practice of medical plants are relatively affordable, simple methods to use, and not contaminate the around environment when applied over the long period (Jian and Wu, 2004; Citarasu, 2010).

Black cumin (*Nigella sativa*) has been known for a long time and is used as a medicinal plant for humans. The dominant chemical content contained in this plant is thymoquinone on of whose functions is as a hepatoprotector. Thymoquinone causes bacterial protein inactivity by forming an inversible complex with nucleophilic amino acids so that the protein loses its function (Musa, 2004). The black cumin seedling is a herb which can be used as an immunostimulant because it is capable of enhancing the non-specific and specific immune system (Dorucu *et al*., 2009; Shewita *et al*., 2011). Additionally, several studies have shown that black cumin has various pharmacological anti-viral (Zaher *et al*., 2008), anti-fungal (Suthar, 2010), anti-bacterial (Hosseinzadeh *et al*., 2007; Hannan *et al*., 2008; Dorucu *et al*., 2009), and anti-parasitic effects (Ayaz *et al*., 2007).

Application of extracts from *N. sativa* in the fish diet have been reported. A study conducted by Khatun *et al*., (2015) showed that the diet enriched with *N. sativa* oil greatly improved the immunity and resistance of *Anabas testudineus* facing *Aeromonas hydrophila*. A similar result was also achieved when *N. sativa* oil was administrated to the feed of Nile tilapia at 2% to control *Pseudomonas fluorescens* infection (Dey *et al*., 2020). Immunological parameters including bactericidal activity and phagocytic action of test fish increased after treatment. However, fish and shrimp differ significantly in immune response. Further studies dealing with extract of *N. sativa* as an effective therapeutic agent for pathogen infections and improving the general immunological system of shrimp need to be done.

Considering the features of the black cumin seed, it is assumed that this herb extract is useful in improving non-specific protection of shrimp, as well as inhibiting bacterial growth. Therefore, the present study aims to evaluate the antibacterial and immunostimulant potential of black cumin seed extract for disease control against *V. harveyi* in vannamei shrimp.

Materials and Methods

Study Area: This study was conducted from March to July 2016, placed in the Laboratory of Pharmacology at the Faculty of Pharmacy, and the Laboratory of Fish Health, and the Laboratory of Fish Breeding and Production at the Faculty of Fisheries and Marine Science, Halu Oleo University, Kendari, South East Sulawesi, Indonesia.

Preparation of black cumin extract and feed: The black cumin seed was obtained from a local farmer and then washed with tap water and dried for 1 week at room temperature. Using a grinding tool, the dried seeds were then ground until mild and sieved to gain a very fine simple powder.

The extraction method done by fine powder of black cumin as much as 2900 g was macerated treating ethanol solvent of 96% for 24 hours, then filtered to receive the filtrate with a Buchner funnel. The filtrate collected was then concentrated using a rotary vacuum evaporator at a maximal heat of 60 °C up to the ethanol solvent has evaporated and a paste-shaped concentrated have been produced that was left on the pumpkin surface. Then the extract of the paste was taken with a spatula, weighed and saved in a dark phial flask. The total result obtained from extraction process was 438 g. Extract was diluted with sterile water according to the dose tested.

The test feed used was commercial pellet feed containing 30% protein, 5% fat, 5% crude fiber, 13% ash and 11% water. The feed was mixed with black cumin seed extract by spraying as much of the tested dose homogenously.

Median Lethal Dose (LD50) Test: The LD50 test was carried out to resolve the density of bacteria that can make 50% of vannamei shrimp population perish in 72 after exposure.

The data will be applied for the challenge test. Robust vannamei shrimp (*L. vannamei*) was used as test shrimp with the average weight was 7 ± 0.5 g of 10 shrimps per aquarium. The type of bacteria applied was *V. harveyi* Rf^R (bacteria that was resistant to rifampicin antibiotics) generated from the isolation of vannamei shrimps from the Laboratory of Fish Health, Bogor Agricultural University, Indonesia which was afterwards re-cultured for pathogenicity raised in the Laboratory of Fish Health, Faculty of Fisheries and Marine Science, Halu Oleo University, Kendari, Indonesia.

The LD50 test was performed by injection of *V. harveyi* bacteria into the test shrimp at different concentrations, namely10^3, 10^4, 10^5, 10^6, 10^7 and 10^8 CFU ml^{-1} per shrimp. Each treatment was made with 10 shrimps. The concentration of each bacterium that was to be used in serial dilution was as much as 0.1 ml per shrimp administered intramuscularly. 3 days

observation was performed by counting the number of dead shrimps.

Experimental design and shrimp rearing: Shrimp were reared in aquariums with a size of 60 x 50 x 40 cm as many as 15 aquariums. The clean aquarium was filled up with sterilized seawater by 50% of its volume, and was then aerated. Ten juveniles of vannamei shrimps were then introduced to the media. Shrimps were adapted for 1 week with commercial feed prior to feeding, as an acclimatization process. Administration of feed test was carried out 4 times in a day for 14 days with feeding rate 10% of shrimp biomass.

Since the best dose obtained from the in vitro test conducted by Grandiosa (2010) was 5000 ppm, this study utilized three different doses, with two additional doses adopted from below and above the optimum dose mentioned with an interval of 2500 ppm. The research was designed as an in vivo test with a completely randomized 5 treatments and 3 replicates, namely: black cumin extract mixed in feed at doses of 2500 ppm (Treatment A), 5000 ppm (Treatment B), 7500 ppm (Treatment C) and without the administration of black cumin extract (control groups). The control groups consisted of a positive control (K +) and a negative control (K-). The (K+) is the shrimp group not administered with black cumin extract but challenged with *V. harveyi* infection, while the (K-) is the shrimp group not administered with black cumin extract or challenged with *V. harveyi* infection.

Challenge Test: Application of black cumin extract on vannamei shrimp was treated for 14 days, and on the 15th day the shrimp was infected with *V. harveyi* with a concentration of 10^6 CFU ml^{-1}. Infection was performed by injection method for all treatment, except negative control. Negative control was administered with 0.85% Sodium Chloride (NaCl). In addition, the shrimp was retained for 6 days by still feeding based on the treatment and then being observed daily.

Observed parameters*:* The observed parameters were median lethal dose (LD50), hemocytes profile as immune response, relatif percent survival (RPS), and number of intestinal bacteria *V. harveyi*.

The calculations for LD50 were built upon the formula provided by Reed and Muench (1938).

$$\text{Proportional Distance (PD)} = \frac{50\% - \text{mortality at concentration next below}}{\text{mortality next above} - \text{mortality next below}}$$

$$\text{LD50} = \text{Log concentration at mortality next below 50\%} + \text{PD}$$

Parameters for immune response were observed at 0, 18, and 21 d of treatment period or at 3 and 6 d after challenge test comprised Total Hemocyte Count (THC) and Differential Hemocyte Count (DHC). The THC was measured as the number of hemocytes according to the Blaxhall and Daishley method (1973), whereas DHC was calculated using Martin and Graves method (1985). On the 18th day (3 d post the challenge test) and the 21st day (6 d post the challenge test) bacterial population was observed in the intestine (Fig. 1).

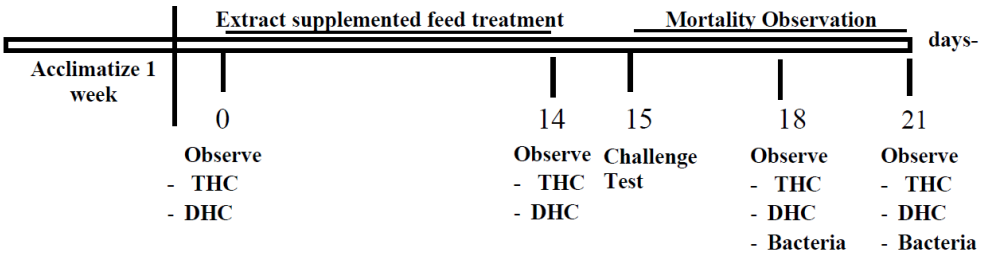

Figure 1 Experimental step of the study

Relatif Percent Survival (RPS) versus control was determined at the 6 d of contagion using the Amend (1981) equation in which RPS (%) = 1- (Number of shrimp mortality in the treatment group/number of shrimp mortality in the positive control group) x 100.

Data analysis: Data in all treatment groups were evaluated using ANOVA, when the results were significantly different, the Smallest Real Difference (SRD) test then was performed.

Results

LD50 Test: Based on data in Table 1 and LD50 calculation, the LD50 test results for *Vibrio* bacteria that can kill 50% of the vannamei shrimp population (*L. vannamei*) within 72 hours were bacteria with a density of 10^6 CFU ml^{-1}.

Bacterial population in the gut of white shrimp: The observation of the population of *Vibrio* bacteria in the gut of vannamei shrimp fed with the black cumin extract-supplemented and control diets is presented in Table 2.

Based on the data in Table 2, both on the 18th day (3 days after the challenge test) and on the 21st day (6 days after the challenge test) the population of *Vibrio* bacteria in the gut of vannamei shrimp for all treatments was significantly different. The results on the bacterial population demonstrated that treatment C (7500 ppm) exhibited the lowest total bacteria both on the 18th day and on the 21st day at 5.7 x 10^3 and < 10^3 CFU g^{-1}, respectively. Meanwhile, positive control resulted in the highest *Vibrio* population on both sampling occasions: 5.97 x 10^5 and 4.22 x10^4 CFU g^{-1}.

Hemolymph Response Parameters: Total haemocyte count (THC) of vannamei shrimp before and after the challenge test are presented in Fig. 2. Moreover, Differential Hemocyte Count (DHC) observations are presented in Fig. 3 and Fig. 4. DHC can be divided into two types of cells, namely hyalinocyte and

granulocyte. Semi-granulocyte cells are classified as granulocyte cells.

The highest THC after 14 days of treatment was demonstrated in the shrimp group with the highest dose of extract (C=7500 ppm). Treatments B and C (5000 and 7500 ppm, respectively) were significantly different in comparison to both control groups (K+ and K-). However, the response of shrimps in treatment C (2500 ppm) did not differ from the control. Three days after the challenge test (18th day), all shrimp groups showed a decrease in THC, though, the highest THC was found in treatment C, followed by treatment B, A and (K +), in sequence. Six days after the challenge test (21st day) data trends remained the same as before, though THC values in all groups increased, indicating that the shrimps had entered a recovery period (Fig. 3).

The DHC data in Fig. 4 implies that the highest granulocyte and semi-granulocyte cells were found in treatment C at each measurement time, both before and after the challenge test. The reverse trend is seen in the percentage of hyalinocyte cells.

Relative Percent Survival (RPS): The relative percent survival rates of vannamei shrimp post challenge test is presented in Table 3. Based on the results, the highest RPS was found in the 7500 ppm dose (treatment C) and the lowest was 2500 ppm (treatment A) at 68% and 45%, respectively. Mortality rate was recorded after 6 days of the challenge test and it was found that higher doses permitted lower mortality, while the (K+) shrimp group had the highest percentage of mortality. However, statistical analysis showed that no dose treatments significantly affected mortality and RPS ($P > 0.05$). No mortality data and RPS were obtained from the (K-) group because this group did not receive *V. harveyi* infection.

Table 1 LD50 Bacteria *Vibrio harveyi* in Vannamei Shrimp

Bacterial Density (CFU mL^{-1})	∑ shrimps die	∑ shrimps alive	Accumulation			Mortality percentage (%)
			Death	Alive	Ratio	
10^8	10	0	30	0	30/30	100
10^7	8	2	20	2	20/22	90,91
10^6	5	5	12	7	12/19	63.16 *
10^5	3	7	7	14	7/21	33.33 *
10^4	2	8	4	22	4/26	15.38
10^3	2	8	2	30	2/32	6.25

The superscript (*) indicates the range of bacterial density caused 50% mortality.

Table 2 Total bacterial population of *Vibrio harveyi* (CFU g^{-1}) in the intestine.

Days	Total number of *V. harveyi* (CFU g^{-1}) in the intestine in all treatments				
	K-	K+	A	B	C
18	0	$^c5.97 \times 10^5$	$^b3.63 \times 10^4$	$^b1.88 \times 10^4$	$^a5.7 \times 10^3$
21	0	$^c4.22 \times 10^4$	$^b7.6 \times 10^3$	$^b1.7 \times 10^3$	$^a < 10^3$

Different letters on the same line indicate significant differences ($P < 0.05$).

The total population of *Vibrio* bacteria in the intestine at 3 days after the challenge test (18th day) and 6 days after the challenge test (21st day) for each treatment: K- (negative control), K + (positive control), A (2500 ppm), B (5000 ppm), C (7500 ppm). Different superscript letters indicate significant differences ($P < 0.05$) between groups of means at each sampling time.

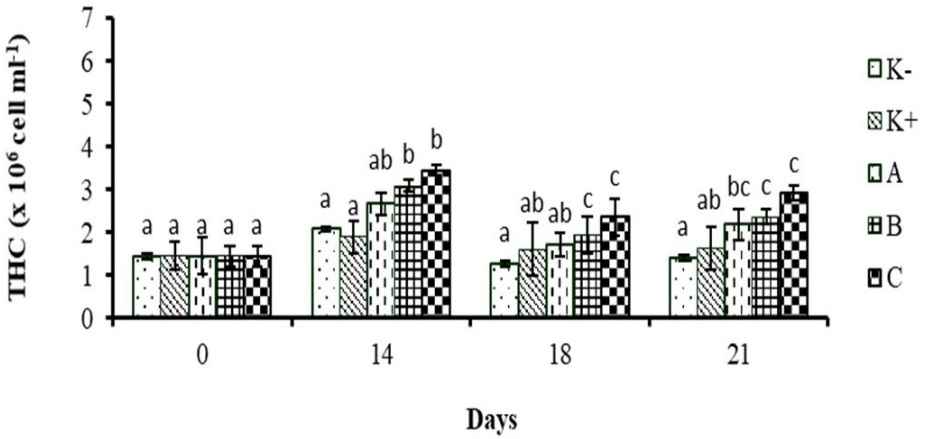

Figure 2 Total hemocyte count (THC). The number of hemocyte cells per volume unit (ml) of hemolymph in white shrimp *L. vannamei*, fed with the three levels of black cumin seed extract dose 2500 ppm (A), 500 ppm (B), 750 ppm (C), positive control (K+), and negative control (K-) at days 0, 14 (before challenge test), 18 and 21 (post challenge test). Different superscript letters illustrate significant differences between groups of means ($P < 0.05$) at each sampling time.

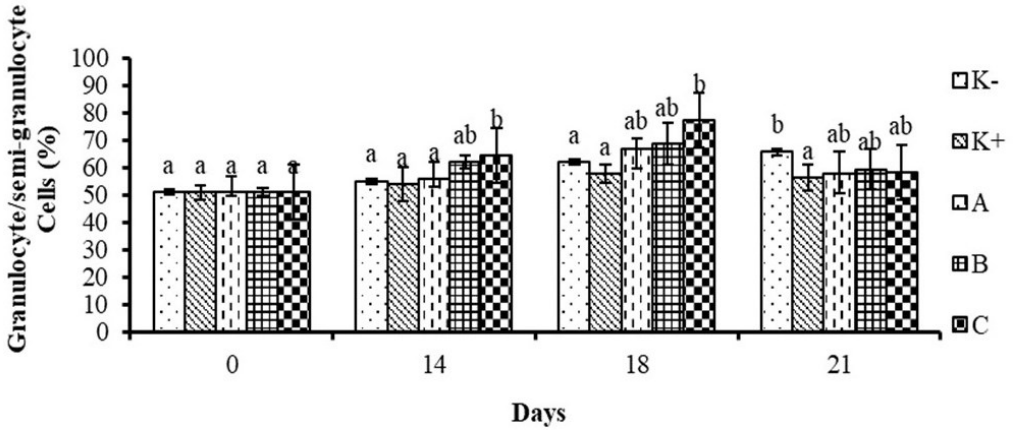

Figure 3 Differential cell count. Granulocyte/semi-granulocyte cells of hemolymph in white shrimp *L. vannamei*, fed with the three levels of black cumin seed extract dose 2500 ppm (A), 500 ppm (B), 750 ppm (C), positive control (K+), and negative control (K-) at days 0, 14 (before challenge test), 18 and 21 (post challenge test). Different superscript letters illustrate significant differences between groups of means ($P < 0.05$) at each sampling time.

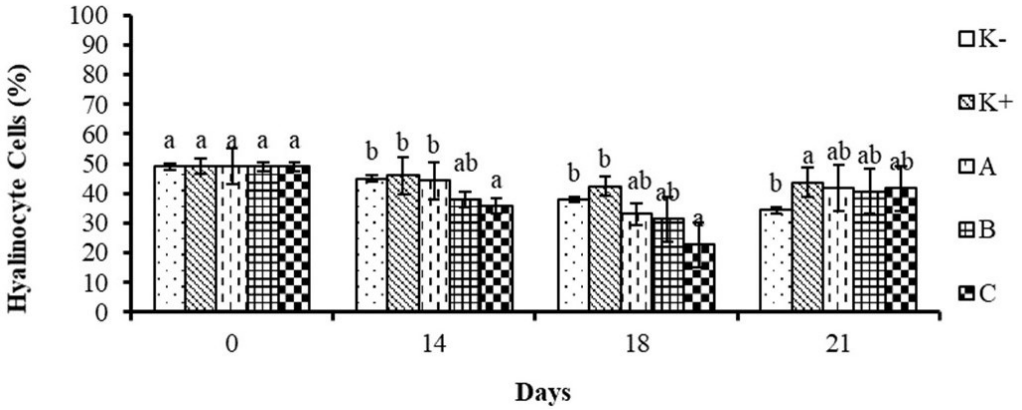

Figure 4 Differential cell count. Hyalinocyte cells of hemolymph in white shrimp *L. vannamei*, fed with the three levels of black cumin seed extract dose 2500 ppm (A), 500 ppm (B), 750 ppm (C), positive control (K+), and negative control (K-) at days 0, 14 (before challenge test), 18 and 21 (post challenge test). Different superscript letters illustrate significant differences between groups of means ($P < 0.05$) at each sampling time.

Table 3 Relative Percent Survival (RPS) of vannamei shrimp post challenge test

No	Treatment	Mortality (%)	RPS (%)
1	A (2500 ppm)	23,33	45[a]
2	B (5000 ppm)	16,66	62[a]
3	C (7500 ppm)	13,33	68[a]
4	(K+) (Positive Control)	43,33	-

Discussion

The use of medicinal plants and their derivatives as immunostimulants and antimicrobial in aquaculture is an efficacious and secure method in enhancing defense system to pathogens during stressful periods, such as intensive farming, grading, vaccine administration and reproduction. (Bektaş *et al*., 2013; Bulfon *et al*., 2013). Some researchers have found that various herbal diets helped to decrease the *Vibrio* bacteria load in shrimps after bacteria exposing, such as Indian herbal significantly inhibited *V. harveyi* population in *Penaeus monodon* (Velmurugan *et al*., 2010), seaweed *Ulva fasciata* extracts inhibited *V. harveyi* population in black tiger shrimp (Selvin *et al*., 2011), and guava leaf crude extract inhibited *V. harveyi* population in vannamei shrimp (Nur *et al*., 2019). Another work also showed that the usage of medical plant *Eleutherine bulbosa* (Mill.) Urb. could decrease the population of *V. parahaemolyticus* (Munaeni *et al*., 2020). In line with those studies, present work revealed that the administration of black cumin seed extract gave a significantly better effect on the shrimp immunity for protection and antibacterial responses. Study on cumin

extract for shrimps via oral administration found improved hemocytes, suppressed pathogenic bacteria, and decrease in mortality rate of shrimps after challenge test.

Prior to the challenge test using *V. harveyi* pathogenic bacteria, the adequate number of bacteria for assessment purposes needed to be determined. The results of the LD50 imply that *V. harveyi* bacteria applied in this work is within virulent pathogen type. Santos *et al*. (1988); Rico *et al*. (2008) grades the level of bacterial virulence derived from the value of LD50. LD50 between 10^4-10^7 CFU ml^{-1} belonging to the virulent bacteria group whereas LD50 > 10^8 CFU ml^{-1} are the avirulent bacteria.

The decrease in the population of *V. harveyi* in intestine of vannamei shrimp reveal that the dietary administration of black cumin crude extract can suppress pathogenic bacterial growth. This is because black cumin contains active compounds can intrude bacterial growth. This *in vivo* test in line with our previous study in which the result study showed that black cumin extract has capability to inhibit the growth of *V. harveyi* by *in vitro* (Linianti *et al*., 2017). The lowest concentration of extracts tested (2500 ppm) showed the ability to inhibit bacteria. As confirmed, it was found that the most successful concentration for inhibiting the *V. harveyi* was the highest concentration of extracts tested (7500 ppm). In general, different crude extracts reveal different levels of antibacterial among the various microbes tested. Several factors may influence its inhibition such as the amount and purity of active ingredients in the extract, pathogenicity bacteria, and the method of administration. Hosseinzadeh *et al*., (2007) reported that through *in vitro* and *in vivo* experiments, *N. sativa* chloroform and methanol seed extracts as well as its essential oil have dose dependent antibacterial activities effective on the gram-positive and gram-negative bacteria, while its aqueous extract shows low inhibitory effect. Additionally, Yasni *et al*., (2009) found that ethanol extract, essential oil, and ethyl acetate extract of *N. sativa* have a broad antimicrobial spectrum, especially in inhibiting food pathogens and spoilage bacteria.

The active pharmaceutical ingredients in black cumin seed were mentioned in our article before containing alkaloids, tannin, quinon, saponin, flavonoids, terpenoid (Linianti *et al*., 2017). In addition, Hosseinzadeh *et al*., (2007) and Hameed *et al*., (2019) found that thymoquinone is the active constituent of black cumin seed with making up around 27.8%–57.0%. Hosseinzadeh *et al*., (2007) reported that there are four alkaloids found to constitute *N. sativa* seeds. There are indazole nuclei of nigellicine and nigellidine, while nigellimine and its N-oxide are isoquinolines.

Seaweed *Sargassum* sp extracts protected vannamei from vibriosis (Mulyadi *et al*., 2020) due to they have bioactive comprising of saponin, tannin, β-carotene, alkaloids, flavonoids, phenolic, glycose and steroid (Mulyadi *et al*., 2019). The primary functions of secondary herb metabolites by nature are intended to afford protection to plants from attack by pathogens and other organisms, or to withstand such biotic and abiotic stress. According to Akiyama *et al*., (2001) the mechanism of inhibition of tannin against bacteria is through cell membrane reaction, deactivate important enzymes and degradation or deactivate genetic material expressions. The existence of a pyrogallol group and a galloyl group induces the antibacterial activity of tannin that can inhibit or kill bacterial growth by reacting with bacterial protein cells so that denaturation of proteins occurs. Denaturing proteins in the wall of bacterial cells trigger bacterial metabolism disruption which ultimately causes damage to the cell wall and finally lysis (Cowan, 1999). However, the secondary metabolite compounds of spices are dependent upon the type of solvent used in their extraction. Among the solvents, methanol showed a better performance compared to water (Hameed *et al*., 2019).

Akter *et al*. (2019) notes that saponins can inhibit bacterial proliferation as these compounds can minimize the surface tension of cell walls, and the walls split or lysis when interacting with bacterial walls. Saponins interact with the surface tension of the cell wall, so that when the surface tension is disrupted the antibacterial material quickly reaches the cell and interferes with the metabolism before bacterial death is eventually observed.

Flavonoids are one of the compounds which play a key role in strengthening the immune system. Flavonoids are plant secondary metabolites that have a protective impact on human cancer, heart disease and retinal inflammation (Pérez-Cano and Castell, 2016; Widodo and Pratiwi, 2018). Panche *et al*. (2016) notes that flavonoids have an antibacterial activity due to their potential to generate complex chains with extracellular proteins from the bacterial cell wall, this will compromise the integrity of the cell wall and ultimately weaken the cell wall and cause lysis.

Hemolymph is the circulating blood that perform decisive works in the immune system of shrimp. It contains both oxygen-carrying molecules (hemocyanin) and immunoactive molecules known as lectins. One of the characteristics of lectins is that once it is bound to sugar by a foreign agent, the complex is easily phagocytized. The phagocyte cell is known as hemocyte, which in this case is responsible for killing ingested microbes. Hemocytes are also involved in other physiological process as innate immune system such as encapsulation, coagulation, and melanization (Johansson *et al*., 2000). Encapsulation is a mechanism in which hemocytes are stimulated to form a multilayer capsule around a foreign entity, such as pathogenic bacteria. The activated hemocytes generate melanin and reactive oxygen species (ROS) which destroy the bacteria within. The coagulation mechanism is a proteolytic cascade, which is triggered by components of microbial cell wall. Gram negative bacteria and fungi invading the hemolymph activate factor C and factor G, respectively. This results in the subsequent activation of the proclotting enzyme and the subsequent coagulation enzyme catalyzes the conversion of a soluble protein (coagulogen) into an insoluble aggregate (coagulin) (Iwanaga, 1993). Meanwhile melanization is executed by phenoloxidase (PO) and regulated by prophenoloxidase (proPO) activation cascade. It plays an essential part in the invertebrate innate immunity in enabling quick response to fight foreign microbes in invertebrates (Amparyup *et al*., 2013).

There was an improvement in the immune response in the total number of hemocyte cells generated by the test shrimps (Fig. 3). In group with a dosage of 7500 ppm (treatment C), the number of hemocytes has a greater amount so that the shrimp is well prepared against pathogens. Rodriguez and Le Moullac (2000) state that crustacean hemocytes play an significant role in the body's mechanism of protection against pathogens such as bacteria, fungi, viruses, protozoa, and metazoa. Many studies are being carried onthe effectiveness of herbal black cumin for human health. Thymoquinone from black cumin can help improve the immune system, while its thymohidroquinone has antibacterial effects (Marlinda, 2015). Black cumin seed exhibited a better antioxidant profile in comparison with other spices such as coriander seed and fenugreek seed (Hameed *et al*., 2019). However, information related to supplementation of extract black cumin seed, especially in shrimp feed to manage diseases and produce healthy shrimp, is still scarce. Niroomand *et al*., (2020) revealed that black cumin seed had positive effects on body fatty acid composition, lowered the haemolymph cholesterol and triglyceride concentrations of Pacific white shrimp *L. vannamei* after feeding at a rate of 5% body weight for 90 days. In this study, the extract was administrated into shrimps within a relative short term of only 14 days. Another research revealed that the use of *N. sativa* given to rainbow trout as feed additives at the doses of 1.0 and 2.5 g/kg for 60 days feeding experiment resulted in a decrease in growth, feed conversion rate, and a deterioration of the blood (Bektaş *et al*., 2013). Therefore, more studies on the use of dosage over the long term are required.

This study revealed that medium quantity of black cumin extract (5000 ppm) had a preferable result on juvenile shrimp hemolymph profiles after administration in diet compared to shrimp groups without extract. The highest number of shrimp THCs fed with extract showed that the concentration was sufficient of *N. sativa*. This concentration might have bioactive compounds can enhance the shrimp immune system because of the increased occasion of phagocytic cells being formed, which is very useful for controlling pathogenic microorganism attacks (Smith *et al*., 2003). Van de Braak (2002), Wang and Cheng (2004) and Maftuch *et al*. (2013) reported that the rising number of THCs suggested an increase in both humoral and cell mediated immune response. In the recent study, shrimp obtaining the black cumin extract through supplemented feed have shown an increased number of haemocytes indicating haemocyte proliferation was occurred. This meant that the immune system of juvenile white-legged shrimps was primed for pathogenic infections, thus inflicting in being resistant toward pathogens that eventually leads to high survival.

The hemocyte is produced in hematopoietic shrimp tissues. There are two/three types of hemocyte in general, distinguished by cell size, nucleus and cytoplasmic (N/C) ratio, and the presence of granules in cells (Söderhäll, 2016). Different hemocyte cells also have different roles in fighting against invading pathogens and foreign particles that invade host tissues.

Observation of DHC in this study can be separated into two groups, named as hyalin cells (Fig. 4) and granular cells (Fig. 5). Semi-granular cells are classified as granular cells. The percentage of hyaline cells is conversely equivalent to the percentage of granular/semi-granular cells. Each type of hemocyte cell emulates in the immune system of the shrimp. Hyaline cells increased in treatment C on the 21st day were thought to be due to increased phagocytic activity against pathogens. Hauton (2012) states that hyaline cells play a role in phagocytosis while granular cells contribute to the development of melanin, secrete antimicrobial peptides and are also involved in cytotoxic reactions. The mechanism of the different roles and changes in the different hemocyte cells is explained as follows. The hyaline hemocytes activate defense with the coagulation process in encountering a pathogen attack, a vital mechanism that protects the shrimp from fluid loss, as well as from the capture and immobilization of invasive microbes. Then, the granular hemocytes secrete protective enzymes that destroy the microbes by phagocytosis and/or encapsulation processes before being killed by other granulocytes. Once the microbes are encapsulated or phagocytised, the melanization process, which is also driven by the granular hemocytes, leaves them inert and prepares them for expulsion via the cuticular excretion or during the next molting period.

The results of this research demonstrated the survival of shrimp, hemolymph profiles and the bacteria inhibition were preferred when added with 7500 ppm of black cumin seed crude extract as supplemented in feed. The research found the use of black cumin extract at a proper quantity enriched in feed that may be worthwhile to aquaculture to raise vannamei shrimp survival to overcome vibriosis. More studies are required regarding the cost-effective methods of extraction for commercial applications of *N. sativa* extract and the possibility application of lower concentrations for administration long term.

Acknowledgements

Thanks are due to the Laboratory of Fishery and Marine Science, Halu Oleo University staff for their technical support. The authors also thank to Laboratory of Fish Health, Bogor Agricultural University for sharing isolate of bacteria.

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