Jurnal Akuakultur Indonesia 21 (2), 161–177 (2022)

Original article DOI: 10.19027/jai.21.2.161-177

Utilizing of black cumin *Nigella sativa* flour to increase the immunity system of tilapia *Oreochromis niloticus* against *Aeromonas hydrophila* bacteria attack

Penggunaan tepung jintan hitam *Nigella sativa* untuk meningkatkan sistem ketahanan tubuh ikan nila *Oreochromis niloticus* terhadap serangan bakteri *Aeromonas hydrophila*

Norma Rizki Silviana[1*], Rosidah[1], Wahyuniar Pamungkas[1], Roffi Grandiosa[1]

[1]Faculty of Fisheries and Marine Science, Universitas Padjadjaran Jalan Raya Bandung - Sumedang km 21 Jatinangor 45363 Sumedang West Java, Indonesia
*Corresponding author: normarizki420@gmail.com

ABSTRACT

This study aims to determine dose of black cumin flour in fish feed diets which can improve the disease resistance of tilapia against the attack of *A. hydrophila*. This study was used tilapia juvenile with a size of 7 – 8 cm. This study was conducted from March – May 2021 and the experimental method is completely randomized design (CRD) with 5 treatments and 3 replications. The treatment used were addition of black cumin flour into fish feed with doses 0 (A), 20 (B), 35 (C), 50 (D), and 65 (E) g/Kg feed. The treatments fed by supplemental diets contained black cumin flour was conducted for 14 days, then a challenge test was carried out with the infection of *A. hydrophila* bacteria by immersion for14 days. *A. hydrophila* bacteria used to infect fish had a density of 108 CFU/mL. The Parameters used were total leukocyte, hematocrit, total leukocyte differential, clinical symptoms, survival rate and water quality. The result showed that black cumin flour was effective to increase the immunity system of tilapia. The dose of 50 g/Kg feed was effective showed increase the highest percentage increase in white blood cells of 64.56%, compared to treatments B, C, and E which showed an increase in white blood cells of 47.40%, 48.82% and 1.2%, respectively. Treatment A decreased the percentage of white blood cells by 19.52%. The 50 g dose of black cumin showed the highest percentage value of hematocrit and leukocyte differential in tilapia fry, respectively 34%, lymphocytes 84.3%, monocytes 6%, and neutrophils 9.7% with the fastest recovery period (4 days) and after the challenge test resulted in the highest survival rate of 95%.

Keywords: *Aeromonas hydrophila,* tilapia juvenile, black cumin, immune system

ABSTRAK

Penelitian ini bertujuan untuk menentukan dosis tepung jintan hitam dalam formula pakan ikan yang dapat meningkatkan ketahanan tubuh ikan nila terhadap serangan bakteri *A. hydrophila*. Penelitian ini menggunakan benih ikan nila dengan ukuran 7-8 cm. Penelitian ini dilaksanakan pada bulan Maret – Mei 2021 dan metode eksperimen yang digunakan adalah model Rancangan Acak Lengkap dengan lima perlakuan dan tiga ulangan. Perlakuan yang digunakan adalah penambahan tepung jintan hitam pada pakan dengan dosis 0 (A), 20 (B), 35 (C) 50 (D) dan 65 (E) g/Kg pakan. Pemeliharaan ikan uji yang diberikan perlakuan yang mengandung tepung jintan hitam dilakukan selama 14 hari, setelah itu dilanjutkan uji tantang menggunakan bakteri *A. hydrophila* dengan metode immersi selama 14 hari. Bakteri *A. hydrophila* yang digunakan untuk menginfeksi ikan memiliki kepadatan 108 CFU/mL. Parameter yang digunakan yaitu leukosit, hematokrit, differensial leukosit, gejala klinis, kelangsungan hidup dan kualitas air. Hasil penelitian memperlihatkan tepung jintan hitam dapat meningkatkan ketahanan tubuh ikan nila. Dosis jintan hitam 50 g per 1 kg pakan paling efektif, terlihat dari persentase peningkatan sel darah putih tertinggi sebesar 64.56%, dibandingkan perlakuan B, C, dan E yang masing masing menunjukan peningkatan sel darah putih sebesar 47.40%, 48.82% dan 1.2%. Perlakuan A mengalami penurunan persentase sel darah putih sebesar 19.52%. Dosis jintan hitam 50 g menunjukkan nilai persentase hematokrit dan differensial leukosit yang paling tinggi pada benih ikan nila, masing-masing sebesar 34%, limfosit 84.3%, monosit 6%, dan neutrofil 9.7% dengan masa pemulihan tercepat (4 hari) dan setelah di uji tantang menghasilkan kelangsungan hidup tertinggi yaitu sebesar 95%.

Kata kunci: *Aeromonas hydrophila*, benih ikan nila, jintan hitam, sistem imun

INTRODUCTION

Based on data from the Directorate General of KKP (2019) Tilapia (*Oreochromis niloticus*) production from 2015 to 2019 experienced an average increase of 9.20%. The presence of disease attack in a major factor in achieving maximum success in cultivation activities. As according to FAO (2020) fish disease is a problem often faced in aquaculture activities, it is projected to increase 32% between 2018 to 2030.

One of disease that often attack tilapia is red spot disease or Motile Aeromonas Septicemia (MAS) caused by *Aeromonas hydrophila* (Ashari *et al.,* 2014). According to Lukistyowati and Kurniasih (2012), disease caused by *A. hydrophila* bacteria can cause fish mortality to reach 80-100%. The right action to overcome this disease is through preventive measures, including proper feeding in quality and quantity, water quality control and present of immunostimulant. The use of immunostimulants for disease prevention from natural ingredients is relatively safer than synthetic chemicals (Cheng *et al*., 2014). One of the natural ingredients that can be used for immunostimulants is black cumin.

Black Cumin (*Nigella sativa*) known as Habbatussauda is efficacious as an antioxidant, anticancer, anticholesterol, antifungal, antibacterial, and also as an immonomodulator (Asniyah, 2009). Some studies prove that black cumin as an immunomodulator can play a role as the resilience of the fish body against disease. As research conducted by Valenta *et al.* (2018) showed that cork fish fed with 3.5% black cumin for 14 days were effective in preventing *A. hydrophila.* The result of research conduced by Sa'adah *et al.* (2015) showed that the addition of black cumin was effective for echance the immune system of tilapia. The dose 3.5% of black cumin into feed can inhibit and prevent the development of *S. agalactiae* infection in tilapia with a survival rate of tilapia of 90%, hematocrit level of 30%, value of lower feed conversion and higher absolute weight and length growth after infection.

MATERIALS AND METHODS

Materials in this study include 320 tilapia fry (size 7-8 cm), black cumin flour 1 kg, isolate *Aeromonas hydrophila* bacteria with a density 108 CFU/ml, commercial feed in the form of floating pellets, Carboxymethy cellulose (CMC), turk solution, giemsa solution, and immersion oil. Tools in this study include 15 aquariums size (36 x 22 x 26) cm^3, 2 fiber tubs, aerator, heater, rake, thermometer, pH meter, DO meter, heparin tube, haemocytometer, scalpel, microscope, hand counter, hematocrite capillary pipe, centrifuge, critoseal, ose needle, bunsen, petri dish, hot plate, autoclave, incubator, cuvet, test tube, and spectrophotometer.

Reseach method

This study used an experimental method of completely randomized design model with 5 treatments and 3 replicates, the treatment used was black cumin flour mixed into feed with different dose of 0 g, 20 g, 35 g, 50 g, and 65 g.

Research Procedure

Fish Acclimatization

Aquariums that had been filled with water were aerated for 24 hours. A total of 20 fish with a size of 7-8 cm were placed in each aquarium for acclimatization for 7 days. As fish are poikilotherm animals, the water temperature will have an impact on their metabolism (Stankeviciute *et al*., 2018). After the process of transporting fish to new waters, fish also need time to habituate to changes in water quality, feed, routines and other external factors. So, even when the animals seem to be in good health. Fish acclimatization aims to adjust the condition of fish to the new water conditions, proper acclimatization reduces the complications of fish disease before the study was conducted (Brønstad, 2022).

Feed Composition

1 kg of commercial feed was mixed with black cumin according to the predetermined concentrations of 20, 35, 50, and 65 g/kg. Carboxymethyl cellulose (CMC) was added to the feed according to the specified formulation as a binder. Tilapia that have been acclimated are then gived feed treatment containing black cumin according to the treatment. Feeding was carried out for 14 days (Valenta *et al.,* 2018). The frequency of feeding was carried out three times a day at 08.00 am, 11.00 am, and 16.00 pm (Rosidah *et al.,* 2019).

Preparation of *Aeromonas hydrophila* Bacterial Isolate

The preparation process for fure isolates of bacteria is Nutrient Agar (NA) dissolved with distilled water in an erlenmeyer, then heated on a hot plate. Nutrient agar solution was poured into a petri dish. isolation of *A. hydrophila* bacteria on nutrient agar media was carried out in laminar air flow. Pure colonies that grew on nutrient agar media were liquid cultured on nutrient broth media. The liquid culture results were put into a cuvvete and then measured the density using a spectrophotometer with a wevelenght of 540nm.

Challenge Test

The challenge test was conducted on the 15th day after feed treatment with *A. hydrophila* bacteria. The challenge test was carried out using the immersion method with a bacterial solution (10^8 CFU/mL) of 20 mL into each aquarium. Then observations were made for 14 days. During the challenge test, fish were fed without treatment. In this study the determination of LD_{50} of *A. hydrophila* in tilapia based on the results of previous studies. Thanh *et al.* (2021) that the lethal dose of bacterial infection *A. hydrophyla* by immersion ranged from 10^7-10^8 CFU mL-1.

Observation Parameters

Leukocytes

The leukocyte count was observed three times, before treatment, after treatment, and after the challenge test with A. hydrophila bacteria. The white blood cell procedure was described according to Blaxhall and Daisley (1973): The microscope was prepared with a magnification of 100 times, then the Neubauer Improved Counting Chamber was placed under the microscope and observed until small boxes were seen where the white blood cell counts were visible. The test fish were taken from the aquarium and the base of the tail was slashed using a scalpel. Take fish blood and then add Turk's solution to a dilution of 10. The suction rubber is released from the pipette and both ends of the pipette are pressed with the thumb so that the liquid does not come out, then it is moved in a circular direction for 3 minutes to make it even. The counting chamber is dripped with liquid blood through the Haemocytometer trench, let stand for a while then covered with Cover Glass. Counting of white blood cells is carried out under a microscope with the help of a Hand Counter.

Leukocyte counts were performed using the formula (Simmons, 1980):

$$\text{SDP per ml of blood} = \frac{\text{NI} \times \text{Multiplier factor}}{0.4}$$

Description:

Ni = Leukocyte count

Multiplier factor = Dilution number (10 times)

0.4 = Total volume of blood in the box

Hematocrit

Hematocrit percentage is the volume of erythrocytes in fish blood. Internal hematocrit levels were measured by the method (Anderson & Siwicki, 1993). The hematocrit procedure is: Entering the fish blood sample into the microhematocrit tube by capillary until it is filled 80%. The end of the tube is plugged with Critoseal or wax. Centrifuged for 15 minutes at a speed of 1.200 rpm. Measurement of hematocrit levels is done by comparing the high volume of red blood cell solids with the volume of whole blood in the sample tube.

Hematocrit counts were performed using the formula (Anderson & Siwicki, 1993):

$$\text{Hematocrit (\%)} = \frac{\text{H1}}{\text{H2}} \times 100$$

Description:

H1 = High volume of red blood cells

H2 = High volume of whole blood cell volume

Leukocyte differential

The procedure for calculating the differential leukocyte according to Amlacher (1970): Hold the object glass with the index and thumb. Drop a little fish blood on a clean object glass on the right. Place another object glass to the left of the drop of blood at an angle of 30 degrees. Pull the object glass to the right until it touches the blood. After the blood has spread along the edge of the second object glass, push the second object glass to the left while maintaining an angle of 30 degrees so that the blood slide is thin enough so that it is easy to observe. After that the review is aired. To facilitate observation, the blood was stained with Giemsa dye.

Giemsa blood staining procedure. The freshly smeared blood in the object glass is air-dried (air fixation), then fixation in methanol solution for 5 minutes. Soak the smear preparation in diluted Giemsa solution (1:20) for 15 minutes. Then, rinse with distilled water and air dry. Drop the immersion oil. The finished preparations were then placed under a microscope and observed with a magnification of 400 times. The percentage of leukocyte cells was calculated by observing

10 fields of view and each type of differential leukocyte counted was grouped according to its type.

Calculation of the differential number of leukocyte including lymphocytes, monocytes, neutrophils according to Amlacher (1970):

$$\% \text{ Lymphocytes} = \frac{L}{100} \times 100$$

$$\% \text{ Monocytes} = \frac{M}{100} \times 100$$

$$\% \text{ Neutrophils} = \frac{N}{100} \times 100$$

Macroscopic Clinical Symptoms

Observation of clinical symptoms include morphological changes characterized by damage to the tilapia body in the form of distended abdomen (dropsy), thin fins, ulcers, red spots (hemorrhagic), pop eyes (exophthalmia), fish movement response to shock and response to food.

Survival Rate

Survival rate calculations can be calculated using the formula (Effendie, 2002) :

$$SR = \frac{Nt}{No} \times 100$$

Description:

SR = Survival rate (%)

Nt = Number of fish juvenile alive at the end of rearing (fish)

No = Number of fish juvenile alive at the beginning of rearing (fish)

Water Quality

Water quality observation were observed every day to keep the fish in a optimal state, supporting data were taken at the beginning and end of the study. Measurement of water quality parameters includes temperature, pH, DO (dissolved oxygen).

Data Analysis

Data on the result of leukocytes, hematocrit, and survival rate obtained were analyzed using ANOVA with a confidence level of 95%, if there is a significant difference continued with the Duncan test (Gasperz 1991). Differential leukocytes, clinical symptoms, and water quality were analyzed desriptively.

RESULT

White Blood Cells (Leukocytes)

Based on the observation of the test fish after being treated with black cumin flour, leukocyte levels increased. This shows that blck cumin can increase leukocyte levels as an indocator of an increase in the body. The highest increase occurated in treatment D (50 g) with a total leukocyte value of 12.59×10^4 cells/mm^3 (Figure 1) and a percentage increase of 64.56% (Table 1).

The ANOVA test result show that the provision of black cumin has an effect on the leukocyte levels of tilapia fry. Duncan test result showed that treatments A and E were significantly different from treatments B, C, and D. Treatments C and D were not significantly different. Treatments A and E resulted in leukocyte counts of about 6.39×10^4 cells/mm^3 dan 7.53×10^4 cells/mm^3, respectively while treatments B and C were 11.07×10^4 cells/mm^3 dan 11.35×10^4 cells/mm^3.

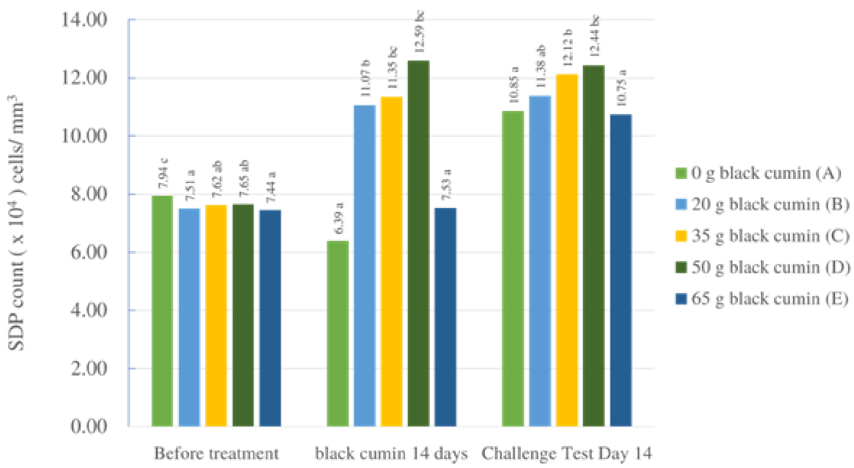

Figure 1. Tilapia White Blood Cell Count (Leukocytes) During Research.

Results on day 14 after the challenge test, treatments A and E showed a high increase in leukocytes, treatment B had a slight increase, while treatments C and D decreased (Figure 1, Table 1). Duncan test results showed that treatments A and E were not significantly different, but significantly different from treatments B, C and D. Despite the high leukocyte levels, treatment D experienced a decrease in leukocyte levels compared to before the challenge test with a percentage decrease of 1.20% (Table 1).

Hematocrit

Based on observations, tilapia fry after being treated with black cumin, all treatments experienced an increase in the amount of hematocrit, except for treatment C which experienced a slight decrease (Figure 2). While treatment A (control) did not experience an increase or decrease.

Duncan's test results showed that treatments A, B, D and E were significantly different from treatment C and produced the lowest hematocrit levels of 27%. The highest hematocrit level was obtained in treatment B, which amounted to 37%.

Post-challenge tilapia hematocrit levels showed different percentages for each treatment. The ANOVA test results showed that each treatment did not show a significant difference. Treatment D produced the highest hematocrit level of 37%.

Leukocyte Differential

Lymphocytes

The number of tilapia lymphocytes before being treated with black cumin flour ranged from 78.0-81.0%, after being treated it increased to 82.7 - 84.3%. The highest increase occurred in treatment D while the untreated ones decreased from 78.0 to 76.3% (Figure 3). After the challenge test, the number of lymphocytes in all treatments

Table 1. Percentage increase/decrease of leukocytes after treatment and after challenge test.

Treatment	Average Number Leukocytes (cells/mm^3) Before Treatment	After Treatment	After Challenge Test	Increase/Decrease Leukocytes (%) After Treatment	After Challenge Test
A (0 g)	79408	63908	108542	-19.52%	69.84%
B (20 g)	75075	110658	113792	47.40%	2.83%
C (35 g)	76233	113450	121217	48.82%	6.85%
D (50 g)	76492	125875	124358	64.56%	-1.20%
E (65 g)	74433	75333	107450	1.21%	42.63%

Note: (-) indicates a decrease in the number of leukocytes.

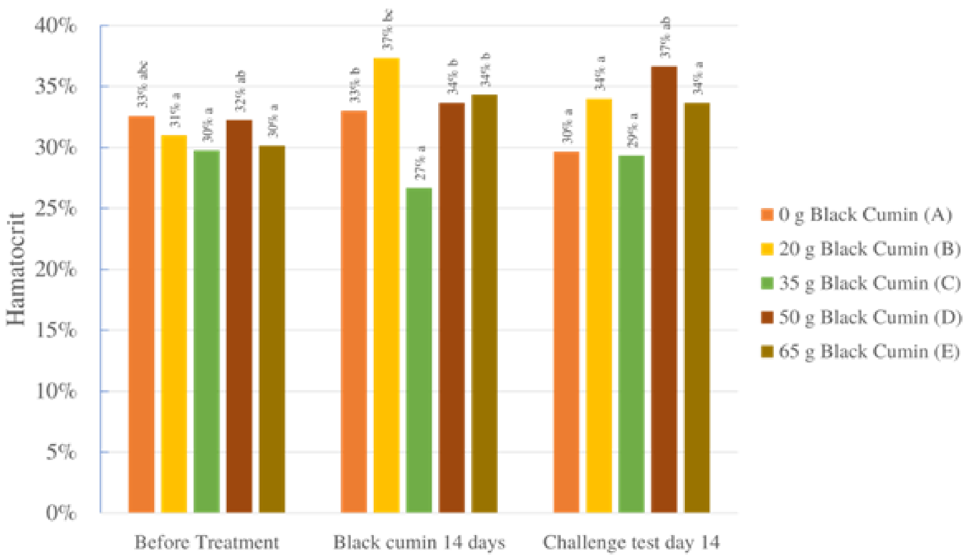

Figure 2. Hematocrit (%) levels during the research.

decreased. The highest increase in the number of lymphocytes occurred in treatment D and the lowest decrease was in treatment A (Figure 3).

Monocytes

The number of tilapia monocytes before being treated with black cumin flour ranged from 6.0-8.0%. After being treated, it increased to 6.0 - 10.7%. On the 14th day after the challenge test, the percentage of monocytes increased in each treatment, the value ranged from 10.7-16.3% (Figure 4).

Neutrophils

The number of neutrophils before being treated with black cumin flour ranged from 12.0 - 14.0%. After being treated, it decreased the value ranged from 7.7% - 13.0%. While after the challenge test, the percentage of neutrophils increased in each treatment to 13.0-14.7%. Treatment D produced a higher neutrophil value of 14.7% (Figure 5).

Macroscopic Clinical Symptoms

Observation of macroscopic clinical symptoms such as changes in body surface damage, response to shock and feed.

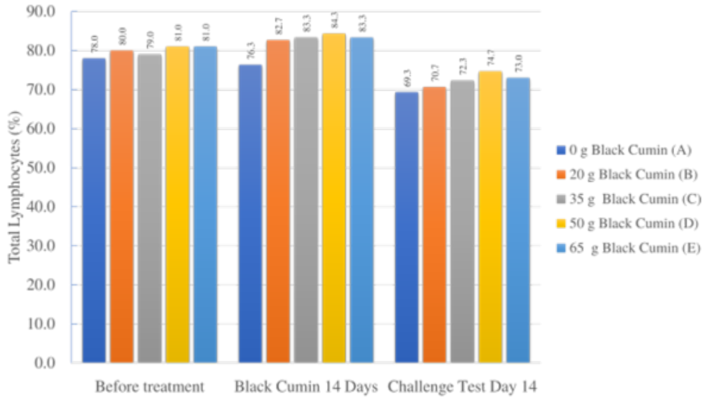

Figure 3. Lymphocyte count during the reseach.

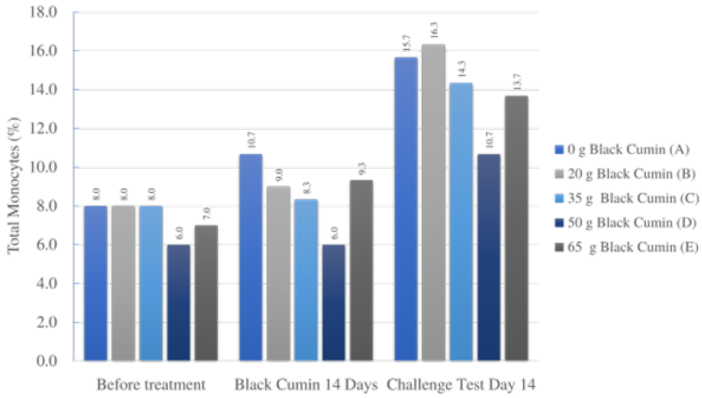

Figure 4. Monocyte count during the research.

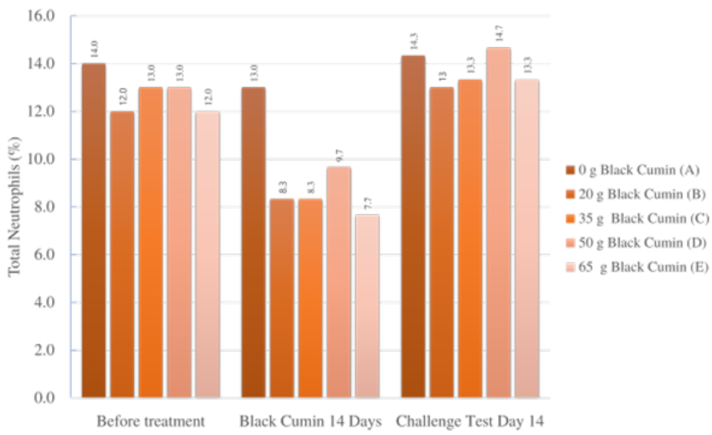

Figure 5. Neutrophils count during the research.

Body Surface Damage

Based on observations, in treatment A, body damage was seen on the 6th day, namely red spots (haemorrhagic) (Figure 6d) and even then it did not occur in all test fish. Fish in treatments B and E began to show red spots at the 24th hour or one day after infection, treatment C red spots were seen on day 3 and treatment D red spots were seen on day 4. Starting on day 4, the test fish in treatments A and B showed clinical symptoms of red spots and ulcers (Figure 6c), while in treatment B the test fish began to experience dropsy on day 6, while in treatment A dropsy began to appear on day 7. Other treatments until the end of observation did not experience dropsy. The test fish in treatments C and D started to appear on the 6th day. Clinical symptoms in the form of pop eye (Figure 6e) began to appear on day 6, namely in treatment A test fish, in treatment B appeared on day 8, treatment C and E appeared on days 9 and 10. Until day 14 (end of observation) test fish in treatment D only experienced clinical symptoms in the form of red spots and thin fins in the mild category and did not occur in all test fish (15% of test fish) (Table 2).

Based on Table 2, body damage in the form of haemorrhage occurred in treatment A on day 1, and on day 2 experienced thin fins, while the test fish in treatment E experienced haemorrhage on day 2. Treatment C, D, and E experienced hemoraghi

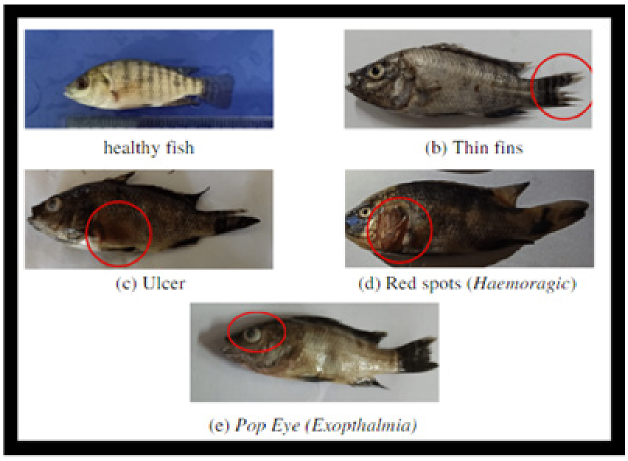

Figure 6. Body surface damage of tilapia infected with *Aeromonas hydrophila* bacteria.

Table 2. Body surface damage for 14 days.

		Days													
Treatment	Replicates	1	2	3	4	5	6	7	8	9	10	11	12	13	14
A (0 g)	1	-	d	d	c	bc	bcd	bde	bde	ce	ce	-	-	-	-
	2	-	d	b	bc	bc	bcd	abe	abe	cd	cd	-	-	-	-
	3	d	b	b	b	bd	bde	bd	abde	bcde	bcd	-	-	-	-
B (20 g)	1	-	d	d	c	c	acd	acd	acd	acde	b	b	de	be	b
	2	-	d	c	bc	bc	bcd	bcd	bcd	bcd	bce	bce	bce	-	-
	3	-	-	d	bd	d	cd	cd	bce	bce	bce	bce	bce	bce	-
C (35 g)	1	-	-	d	d	d	bd	bd	abd	abde	be	be	be	-	-
	2	-	-	-	d	d	d	d	bcd	bd	bd	be	bd	b	b
	3	-	-	-	d	d	d	d	d	d	d	-	-	-	-
D (50 g)	1	-	-	-	d	d	d	d	bd	bd	b	-	-	-	-
	2	-	-	-	d	d	bd	bd	d	d	b	-	-	-	-
	3	-	-	-	d	d	bd	d	d	d	bd	-	-	-	-
E (65 g)	1	-	-	-	d	d	d	d	cd	cd	de	-	-	-	-
	2	-	d	d	d	d	d	d	bd	bcd	-	-	-	-	-
	3	-	-	-	d	d	bd	bd	bd	bd	-	-	-	-	-

Description: (a) Dropsy (distended abdomen), (b) Thin fins, (c) Ulcer/Wound, (d) Haemorrhagic (Red Spot), (e) Exopthalmia (Pop eye), (-) no body damage.

on day 4 (Figure 6c) while treatments A and B experienced ulcers. Treatment A experienced dropsy, thin fins, ulcers, haemorrhagic and exopthalmia from day 5 to day 10. But in contrast to treatments B and C experienced dropsy, thin fins, ulcers, haemorrhagic and exopthalmia until day 14. Treatments D and E only experienced thin fins, ulcers, haemorrhagic and exopthalmia until day 10.

Fish Movement Response

Observations after the challenge test showed diverse responses in each treatment (Table 3).

Based on Table 3, on day 1 fish in all treatments gave a good / normal response to movement, then day 3 to day 8 test fish in treatments A, B, and E showed a low movement response, but the test fish that survived until the end of the observation showed a normal movement response. Test fish in treatment C (most / 93%) and treatment D from the beginning to the end of the observation showed no change in response to movement, all of them showed a normal response (Table 3). Day 3 to day 8 fish in treatment E showed the lowest movement response or no response, while day 6 treatment C began to show a fairly low response to motion.

Feed Response

The observation of feed response showed different responses in each treatment (Table 4).

Based on Table 4, fish in treatments A, B, and E on days 1 and 2 showed a low to moderate response. Test fish in treatment C showed a low response on days 7 and 8. Test fish in treatment D from the beginning of observation until the end of observation there was no change in feed response. This shows the clinical symptoms that occur in fish in treatment D in the mild category.

Survival Rate

The results of observations in all treatments showed varying percentages. Survival values ranged from 66% to 95% (Figure 7).

The highest percentage increase in survival was obtained in treatments D and E at 95% and 93% respectively, treatment C at 87%, while treatment B at 66% was the lowest survival value. The low survival rate in treatment B is thought to be due to infection with *A. hydrophila* bacteria so that fish experience many deaths after bacterial infection. The results of the ANOVA test showed that the provision of black cumin affects the percentage of survival rate in tilapia. The Duncan test results showed that treatments A and B were not significantly different from treatment C. Treatment C was not significantly different from treatment D and E, but treatment D (50g) showed the highest survival value, which 93%.

Table 3. Fish Movement Response.

		Days													
Treatment	Replicates	1	2	3	4	5	6	7	8	9	10	11	12	13	14
A (0 g)	1	++	++	++	++	+	+	+	+	++	++	++	++	++	++
	2	++	++	+	+	+	+	+	+	++	++	++	++	++	++
	3	++	++	++	++	+	+	+	+	++	++	++	++	++	++
B (20 g)	1	++	++	++	++	++	++	++	++	++	++	++	++	++	++
	2	++	++	+	+	+	+	+	+	++	++	++	++	++	++
	3	++	++	++	+	+	+	+	+	++	++	++	++	++	++
C (35 g)	1	++	++	++	++	++	+	++	++	++	++	++	++	++	++
	2	++	++	++	++	++	+	++	++	++	++	++	++	++	++
	3	++	++	++	++	++	+	++	++	++	++	++	++	++	++
D (50 g)	1	++	++	++	++	++	++	++	++	++	++	++	++	++	++
	2	++	++	++	++	++	++	++	++	++	++	++	++	++	++
	3	++	++	++	++	++	++	++	++	++	++	++	++	++	++
E (65 g)	1	++	++	++	++	++	++	++	++	++	++	++	++	++	++
	2	++	++	++	++	++	++	++	++	++	++	++	++	++	++
	3	++	++	+	+	+	+	+	+	++	++	++	++	++	++

Description: (-) No response, (+) Passive/ low startle response, (++) Active/ high motion response (normal).

Table 4. Feed Response.

		Days													
Treatment	Replicates	1	2	3	4	5	6	7	8	9	10	11	12	13	14
A (0 g)	1	++	+	++	+++	+++	+++	+++	+	+++	+++	+++	+++	+++	+++
	2	+	+	++	+++	+++	+++	+++	+	+++	+++	+++	+++	+++	+++
	3	++	+++	++	+++	+++	+++	+++	+	+++	+++	+++	+++	+++	+++
B (20 g)	1	++	+++	+++	+++	+++	+++	+++	+	+++	+++	+++	+++	+++	+++
	2	+++	+++	+++	+++	+++	+++	+++	+	+++	+++	+++	+++	+++	+++
	3	+	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++
C (35 g)	1	+++	++	+++	+++	+++	+++	+	+	+++	+++	+++	+++	+++	+++
	2	+++	+++	+++	+++	+++	+++	++	+	+++	+++	+++	+++	+++	+++
	3	+++	+++	+++	+++	+++	+++	++	+	+++	+++	+++	+++	+++	+++
D (50 g)	1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	3	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
E (65 g)	1	++	+	+++	+++	+++	+++	+++	+	+++	+++	+++	+++	+++	+++
	2	++	+++	+++	+++	+++	+++	+++	+	+++	+++	+++	+++	+++	+++
	3	+++	+++	+++	+++	+++	+++	+++	+	+++	+++	+++	+++	+++	+++

Description: (+) Low feed response, (++) Medium feed response, (+++) High feed response (normal).

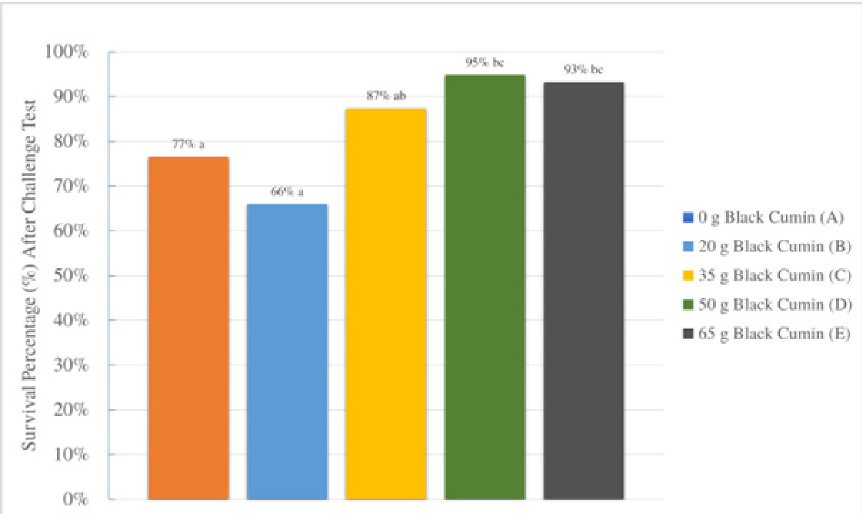

Figure 7. Survival after challenge test.

Table 5. Water quality value.

	Temperature (°c)		pH		DO (mg/l)	
Treatment	Start of Research	End of Research	Start of Research	End of Research	Start of Research	End of Research
A	30-32	30-31	7.18-7.55	6.11-7.34	6.6-6.7	4.5-5.6
B	30-32	29-31	7.3-7.5	6.4-6.7	6.5-6.8	4.3-4.6
C	31-33.5	30	7.13-7.9	6.7-7.8	6.6-6.9	4.7-4.9
D	30-32	30	7.5-7.9	6.95-7.12	6.6-6.8	5-5.1
E	30-32	30	7.28-7.6	6.7-7	6.5-6.9	4.8-5.2
Optimal (SNI: 7550 - 2009)	25-32 (SNI)		6.5-8.5 (SNI)		>3 (SNI)	

Water Quality

During the rearing period until the challenge test, water quality measurements included temperature, pH, and DO (dissolved oxygen) in the aquarium media of each treatment (Table 5).

Based on Table 5 the water quality used for tilapia rearing during the research period is in controlled and normal conditions according to Indonesian National Standards.

DISCUSSION

Leukocyte

Leukocytes or white blood cells are very important as an indicator of fish health response and see directly the condition of the immune system against disease attacks (Yousefi *et al*., 2021). Based on the results obtained, the test tilapia that has been given treatment using black cumin shows an increase in white blood cells (leukocytes). This shows that black cumin can increase the immunity system of tilapia, indicated by an increase in leukocyte levels. Ramadhana *et al.* (2019) black cumin can increase white blood cells, because the bioactive compounds contained in black cumin are thymoquinon, alkaloids and tannins. Immunostimulant treatment will stimulate the productivity of the immune system and inhibit the growth of pathogenic bacteria that cause disease (inactivation of bacterial attack), and can play a role in increasing the phagocytic activity of leukocytes gradually (Monalisa *et al.,* 2018). The dose of 50 g black cumin flour produces the highest increase in leukocyte levels, this is because the active compound can work optimally to increase leukocyte levels, because at a dose of 65 g there is a decrease in leukocyte levels due to the high content of the active compound. As according to Ahmed (2018) active content compounds contained in black cumin when used in high doses cause toxic effects and paralyze (muscle tension).

At day 14, treatment A and E showed a significant increase in leukocyte levels, indicating that the fish were in a state of disease. A significant increase in leukocytes indicates that the fish is infected by a pathogen, the infected body will cause inflammation that induces the body to produce leukocytes as an immune response (Devi *et al.*, 2019). The test fish in treatments C and D showed decreased leukocyte levels, indicating that the fish were heading towards a healthy condition. This is shown by mild clinical symptoms and a normal response to shock and feed. As according to Arindita *et al.* (2014) the decrease in total fish leukocytes after infection is due to the response given being able to stabilize the fish so that the fish does not show a significant increase in leukocyte cells due to body adaptation. The main characteristic of the non-specific immune response is characterized by the movement of Leukocyte cells that directly enter the infected body tissue, this is because white blood cells have the ability to penetrate the capillary wall without associating strictly with certain organs or tissues, so that these white blood cells can work independently like a single cell organism in counteracting pathogenic attacks that enter the body and then form an immunity (Rojo *et al*., 2018).

Hematocrit

Hematocrit is the percentage of the number of red blood cells in the blood whole blood volume unit (Sahfitri *et al*., 2021). Hematocrit level aims to determine the effect of the use of immunostimulants and can be used to determine the condition of fish after being given immunostimulants (Putra *et al*., 2015). The average hematocrit of fish ranges from 27.3% - 37.8% (Hardi *et al.,* 2011). Based on the results after the treatment of black cumin, treatment B and E shows an increase in hematocrit levels. According to Astuti *et al.* (2017) that the increase in percentage of hematocrit, is due to the influence of immunostimulants. Bioactive compounds contained in black cumin (alkaloids, triterpenoids, steroids, saponins, phenolics and flavonoids) that function as immunostimulant, antibacterial and antioxidant (El-Hack *et al.,* 2021). The mechanism of antioxidant activity in the body is to prevent the increase of free radicals (reactive oxygen species) and protect early damage to erythrocyte cells (Naiel *et al*., 2020). According to Ahmadifar *et al.* (2019) bioactive can also stimulate the body to activate several antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD) which can inhibit hemolysis when lipid peroxidation occurs in erythrocyte cell membranes (oxidative stress), as a result of blood hemoglobin levels increase, and also cause the number of red blood cells to increase. While in treatment C and D showed a decrease in the number of hematocrit, which may be caused by stress when the fish is taken for blood sampling. Witeska *et al.* (2022) stress considerably affects hematological parameters and may disturb the results of analyses, stress in fish may be caused by various factors, such as drastic changes in environmental conditions, and also due to blood sampling that takes too long.

The average percentage of fish hematocrit in all treatments at the beginning of the challenge test showed a decrease, this could be due to bacterial attacks that secrete toxins resulting in hemolysis, at the same time it can also occur in order to stabilize the number of blood cells when leukocyte production increases to form immunity against bacterial infections. Fish attacked by pathogenic bacteria *A. hydrophila* will cause symptoms of stress, injury and damage to internal organs resulting in hemolysis anemia and making red blood cell values decrease (anemia) (Prasetio *et al.,* 2018). Treatment C and D after the challenge test compared with other treatments showed an increase in hematocrit, it can be an indicator of fish become healthy again gradually. Trilia *et al.* (2014) an increase in the number of red blood cells will be an effector for a faster increase in specific immune responses (antibodies) in sufficient quantity to relieve bacterial infection. The number of red blood cells also is an effector in improving non-specific immunity (innate immune system), increasing the number of erythrocytes will accelerate the transport of oxygen, nutrients and other compounds to regenerate damaged cells during inflammation (Anderson *et al*., 2018).

Leukocyte Differential

Lymphocytes

Lymphocyte cells have the highest presentation compared to other cell types (Novita *et al.,* 2020). The increase in lymphocyte values occurred in treatments given black cumin (B, C, D, and E). Treatment D showed the highest lymphocyte values. The increase in the number of lymphocytes indicates that black cumin has and impact as an immunostimulant can increase the proliferation of lymphocyte cells, which has an impact on increasing immunity system (Hidayati *et al*., 2019). Immunostimulant mechanism in increasing the number of lymphocytes by stimulating macrophages to produce interleukins, then producing more lymphocytes and plasma cells (antibodies) when attacked by bacterial pathogenic agents (Ojueromi *et al*., 2022).

The number of lymphocytes tilapia decreased in all treatments after the challenge test. Low lymphocytes level indicate that the fish is sick or infected with a certain disease (Gabaudan & Verlhac, 2006). Leukocytes has decreased due to fish infected with bacteria *A. hydrophyla* will cause inflammation and hyperemia (Dong *et al*., 2017). According to Tang *et al*. (2020) hyperemia is a condition of accumulation blood cells in a particular tissue or organ due to inflammation, which will then cause narrowing of the capillary blood vessels. Inflammation also causes the movement of lymphocyte cells to the infected tissue, then the lymphocyte cells will recognize certain antigens to produce antibodies, some of the lymphocyte cells will carry out phagocytic activity on bacteria and then lysis on themselves (rupture), then send a signal for the body to produce differentiation of other leukocyte cells (monocytes and neutrophils) to help destroy pathogens that enter the body (Aliko *et al*., 2018).

Monocytes

Monocyte cells are one of the largest types of white blood cells, their function is to eat dead cells and attack pathogens that enter by phagocytosis (Rustikawati, 2012). Yuliana *et al*. (2021) also stated that monocytes can differentiate into macrophages, and generally there are many in areas affected by infection or inflammation. Based on the results of all treatments given black cumin, resulting in a significant increase in the number of monocytes, it can be due to the effect of immunostimulants in black cumin which increases the number of monocytes. according to Abarike *et al*. (2019) immunostimulant is able to stimulate the activity of hematopoietic precursor to increase the production of monocyte cells.

Based on the results after the challenge test, treatment D showed the lowest increase in monocytes after being infected by pathogens, meaning the low virulence of bacteria *A. hydrophila* or it could be due to the content of black cumin as an immunostimulant and antibacterial. Al-Ali *et al.* (2008) that in the friction oil contained in black cumin isolate thymohydroquinone which has potential as an antibacterial. The highest percentage of monocytes after infection, indicating the severity of virulence in fish, means that more bacteria or pathogens enter the body (Lattanzi *et al*., 2016). Waliani *et al.* (2015) also stated that the increase in the number of monocytes as macrophages is an immune response of fish that depends on the level of disease symptoms during infection, then it will decrease when the fish starts to get better or healthy again.

Neutrophils

Neutrophils are one type of white blood cells (granulocytes) that are abundant in the body, neutrophils attach to the walls of blood vessels that serve to block bacterial infections that try to enter the blood through wounds and produce superoxide compounds used to degrade pathogenic bacteria (Saffari *et al*., 2018). Increased of lymphocytes causes a decrease in the production of differentiation of other types of white blood cells because the energy and components of the cell constituents at that time are more focused on multiplying lymphocytes than other leukocyte cells (Kurniawan *et al.,* 2013). The value of neutrophils decreased due to the absence of bacterial activity because according to the main function of neutrophils is to destroy foreign material through phagocytic processes (Yuliana *et al.,* 2021). Hartika *et al.* (2014) also stated that a low neutrophil presentation indicates the absence of microorganism attack so that neutrophils have not been produced by the fish body.

Based on the results after the challenge test, Treatment D produced a higher neutrophil value of 14.7%, and the control treatment amounted to 14.3% The increase in the number of neutrophils can indicate that there is neutrophil attack activity on antigens. Because neutrophils are the first leukocyte cells that respond when there is an attack of bacteria that cause inflammation in the body (Selders *et al.,* 2017). In accordance with the statement Suhermanto *et al.* (2013) that neutrophils will increase during infection due to the influence of external chemical stimuli by immunostimulants. Neutrophils work as a non-specific body defense through the process of phagocytosis, namely chemotaxis, where phagocyte derivative cells will migrate to infected areas, ingestion of foreign substances (antigens), and destruction of antigens by lysozyme enzymes in phagolysosomes, so that without stimulation from foreign substances in the form of bacteria, viruses and pathogens neutrophils will not increase their cell activity (Yengkhom *et al.,* 2018).

Macroscopic Clinical Symptoms

Body Surface Damage

Based on the results after the challenge test, Treatment D shows better on the immunity system compared to other treatments, this is due to the effect of a dose of 50 g of black cumin which can increase the number of leukocytes so that the formation of fish immunity system to *Aeromonas hydrophila* bacterial attack is characterized by less severe body damage with the fastest fish recovery period (4 days). The body surface damage and recovery period of each test fish treatment differed. Rodrigues *et al*. (2019) stated that each individual (fish) has a different immunity system to the severity of the disease, it can be influenced by bacterial pathogenicity factors, fish age, sex, nutritional status and stress levels to the environment.

The characteristics of fish attacked by *Aeromonas hydrophila* on tilapias are bleeding on the chest, severe dropsy, hemorrhagic skin, ulcerations, exophthalmic and showed darkened skin (Abdel-Latif *et al*., 2022). Treatments A and B showed increasingly severe wounds on some of the test fish that continued, so that they eventually died. Treatment A experienced the most severe body damage due to the absence of body defenses provided, seen from the number of leukocytes that did not increase so that the immunity system and survival of fish was low. Hyperpigmentation or darkened skin can be caused due to changes in the physiology of fish that experience stress due to bacterial infection, and disruption of the hormonal system resulting in changes in skin color (Gómez *et al*., 2021). While haemorrhagic or red spots can be due to the nature of *Aeromonas hydrophila* bacteria that make tissue inflammation and some organs of the fish are damaged and bleeding occurs (Basri *et al*., 2020). furthermore, when the disease becomes more systemic, it can cause swelling in the eyes of fish (exopthalmia) and bacteria can attack the walls of the digestive tract and vascular system of fish (Laith *et al*., 2017).

Motion Response

Based on the results of the challenge test on the 3rd day after infection, until the 8th day showed a slow swimming response, it could be due to damage to some organs of swimming fish or fish are stressed after infection with *A. hydrophila* disease. In clinical case reports of fish infected with *Aeromonas* spp., it was reported that fish may present anorexia and abnormal swimming, sometimes described as swimming near the water surface (Gallani *et al.,* 2020). In some fish there is also characterized by fish that only move to the surface to take food and oxygen, and fast in overcoming the shock response. this is in accordance with the statement Jasmanindar *et al.* (2020) fish stricken with disease *A. hydrophila* generally shows symptoms of abnormal motion, slow swimming, damaged fins, ulcers or ulcers characterized by injuries to the skin and muscles. *A. hydrophila* infection also shows symptoms of injury to some swimming organs of fish (fish fins) and inflammation of the muscles of the fish. If the virulence gets worse (the severity of the disease) then it is possible for the fish to become impaired ability to move or passive response (Dong *et al*., 2017).

Based on the results after the challenge test, treatment D is the best in preventing disruption of swimming response compared to other treatments, it is due to the addition of immunostimulants from black cumin, able to prevent symptoms of aeromonas disease. Awad and Awaad (2017) reported that immunostimulants can help trigger healing from disease attack and reduce the impact of clinical symptoms of bacterial or viral infection attacks, immunostimulants are also known to stimulate anti-inflammatory responses, so that injured fish body tissues can be more quickly restored.

Feed Response

Based on the results of the challenge test after infection with *A. hydrophila* bacteria, showed changes in feeding behavior in all treatments, become passive or less response to feed, it can be due to inflammation and damage to some organs of fish infected with the disease. Abasubong *et al.* (2018) reported that disease attacks in fish can cause fish to be injured in several digestive organs in the body, this affects the physiological condition of the fish and makes the fish have no appetite for feed. Prasetio *et al*. (2018) also reported the change in water quality and the injection of bacteria in test fish can affect the physiological condition of fish in responding to feed.

Treatment D with a dose of 50 g of black cumin shows the best feed response, this is indicated by a high feed response in fish on day 1, as research conducted by Dontriska *et al.* (2014) black cumin has an bioactive compounds in the form of nigelline which serves to increase fish appetite when compared to other treatments that show low feed response. The use of black cumin in feed is able to improve the immune system of fish, because it has bioactive such as alkaloids, flavonoids and terpenoids as immunostimulants and appetite-stimulants, thereby stimulating fish recovery from disease to be more efficient (Bektaş *et al*., 2019).

Survival Rate

Based on the results of the challenge test,the addition of black cumin at doses of 35g, 50g and 65g showed a good effect on the immune system of fish against disease attacks due to immunostimulant effects. In accordance with the statement of Maryani and Rosdiana (2020) that the optimal point of fish in increasing fish immunity is due to secondary metabolite compounds to fight bacterial attack *A. hydrophila* is characterized on the result of the presentation of the highest white blood cell count among other treatments. Black Cumin has an antibacterial, anti-inflammatory and immunostimulating effect, Additionally, exhibited potent antibacterial activity against bacterial disease, thus, enabled the fish to resist the infection and enhancing the fish survival rate (Mahboub *et al*., 2022).

Jafarzadeh *et al.* (2021) also reported that bioactive ingredients (flavonoids, phenolic compounds) in feed, can function as immunomodulators that stimulate the activation of macrophages to produce interleukin, then interleukin will accelerate the maturation of lymphocyte cells to differentiate into B cells and T cells, both types of lymphocyte cells will then form an antibody response, and increase the percentage of survival of fish when infected with the disease However, the addition of high doses that exceed the optimal dose becomes less effective. Herbal plants if not used in the right dosage have negative effects in the form of toxins and allergies that can interfere with the health of the body (Yilmaz *et al.,* 2011).

Water Quality

Temperature, pH, and DO in all treatments showed optimal results, according to Khairumman and Amri (2013) that tilapia fish can live at a temperature of 14 - 38°C. the pH value for tilapia rearing is 6 - 8.5 (Kordi 2010). Optimal tilapia growth requires a minimum oxygen content of 3 mg / L (Kordi 2010). studies have shown that the use of Black Cumin does not negatively affect water quality. Fish can grow at optimal water quality, in this condition the metabolism increases so that the fish's appetite is also good. if the water quality is sufficient standard, then the growth of fish can be more optimal. However, if the water used in aquaculture has been contaminated or has a quality that does not meet the requirements for fish farming, then the fish will be attacked by diseases or parasites that live in the water (Abdel-Tawwab *et al*., 2019).

CONCLUSION

Based on the research, it can be concluded that black cumin flour at a dose of 50 g is most effective in increasing the immunity system of tilapia, producing the highest increase in leukocyte levels (64.46%), hematocrit levels 34%, lymphocytes 84.3%, monocytes 6%, neutrophils 9.7% and after being challenged with *Aeromonas hydrophila* bacteria experienced the fastest recovery period (4 days) and high survival 95%.

REFERENCE

Abarike ED, Kuebutornye FKA, Jian J, Tang J, Lu Y, Cai J. 2019. Influences of immunostimulants on phagocytes in cultured fish: a mini review. Reviews in Aquaculture 11: 1219–1227.

Abasubong KP, Liu WB, Zhang DD, Yuan XY, Xia SL, Xu C, Li XF. 2018. Fishmeal replacement by rice protein concentrate with xylooligosaccharides supplement benefits the growth performance, antioxidant capability and immune responses against *Aeromonas hydrophila* in blunt snout bream (*Megalobrama amblycephala*). Fish & Shellfish Immunology 78: 177–186.

Abdel-Latif HMR, Shukry M, Abd-Elaziz RA. 2022. Clinico-pathological findings and expression profile of inflammatory cytokines, apoptosis, and oxidative stress-related genes draw mechanistic insights in Nile tilapia reared under ammonia-N exposure and *Aeromonas hydrophila* challenge. Fish & Shellfish Immunology.

Abdel-Tawwab M, Monier MN, Hoseinifar SH, Faggio C. 2019. Fish response to hypoxia stress: growth, physiological, and immunological biomarkers. Fish Physiology and Biochemistry 45: 997–1013.

Ahmadifar E, Moghadam MS, Dawood MAO, Hoseinifar SH. 2019. Lactobacillus fermentum and/or ferulic acid improved the immune responses, antioxidative defence and resistance against *Aeromonas hydrophila* in common carp (*Cyprinus carpio*) fingerlings. Fish & Shellfish Immunology 94: 916–923.

Ahmed MEA. 2018. Study of Physicochemical Properties of *Nigella sativa* Oil Extracted by Mechanical Pressing and by n-Hexane Solvent (Doctoral dissertation, Sudan University of Science and Technology).

Al-Ali, Abdul AA, Mohammad AR, Nisar AS. 2008. Oral and Intraperitoneal LD 50 of Thymoquinone, An Active Principle of *Nigella sativa*, in Mice and Rats. Journal Ayub Medical College Abbottabad 20: 25–27.

Aliko V, Qirjo M, Sula E, Morina V, Faggio C. 2018. Antioxidant defense system, immune response and erythron profile modulation in gold fish, *Carassius auratus*, after acute manganese treatment. Fish & Shellfish Immunology 76: 101–109.

Amlacher E. 1970. Textbook of fish disease. New York: Neptune. 302 page.Anderson DP, Siwicki AK. 1993. Basic Hematology and Serology for Fish Health Programs. Paper presented in second symposium on diseases in Asian Aquaculture. Aquatic Animal Health and the Environment. Phuket, Thailand: 185–202.

Anderson HL, Brodsky IE, Mangalmurti NS. 2018. The evolving erythrocyte: red blood cells as modulators of innate immunity. The Journal of Immunology 201: 1343–1351.

Arindita C, Sarjito, Prayitno SB. 2014. The Effect of *Aloe vera* Powder on Food toward Survival rate and Blood Profile of *Cyprinus carpio* Infected by *Aeromonas hydrophila*. Journal of Aquaculture Management and Technology 3: 66–75.

Ashari Chairanitansyah, Tumbol, Reiny A, Kolopita EF. 2014. Bacterial Disease Diagnose on Nile Tilapia (*Oreochromis niloticus*) Culture in Fixed Net Cage in Lake Tondano. Aquaculture 2: 24–30.

Asniyah. 2009. Antimicrobial effect of Black Cumin Oil (*Nigella sativa*) on the growth of *Escherichia coli* in Vitro. Journal Of Biomedicine 1: 25–29.

Astuti APK, Hastuti S, Haditomo AHC. 2017. The Effect of Extracts Curcuma in the Diets as Immunostimulant for Java Barb (*Puntius javanicus*) with Bacterial Challenge Test. Journal of Aquaculture Management and Technology 6: 10–19.

Awad E, Awaad A. 2017. Role of medicinal plants on growth performance and immune status in fish. Fish & Shellfish Immunology 67: 40–54.

Basri L, Nor RM, Salleh A, Yasin IS, Saad MZ, Abd. Rahaman NY, Barkham T, Amal MNA. 2020. Co-infections of tilapia lake virus, *Aeromonas hydrophila* and Streptococcus agalactiae in farmed red hybrid tilapia. Animals 10: 2141.

Bektaş ZH, Savaşer S, Akçimen U, Ceylan M, Yener O, Bulut C. 2019. Using of black cumin seed powder (*Nigella sativa*) as immunostimulant and growth promoter in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Turkish Journal of Fisheries and Aquatic Sciences 19: 987–999.

Blaxhall PC, Daisley KW. 1973. Routine Haematological Methods for Use With Fish Blood. Journal Fish Biology 5: 557–581.

Brønstad A. 2022. Good Anesthesia Practice for Fish and Other Aquatics. Biology 11: 1355.

Cheng G, Hao H, Xie S, Wang X, Dai M, Huang L, Yuan Z. 2014. Antibiotic alternatives: The substitution of antibiotics in animal husbandry. Frontiers Microbiology 5: 1–15.

Devi G, Harikrishnan R, Paray BA, Al-Sadoon MK, Hoseinifar SH, Balasundaram C. 2019. Effects of aloe-emodin on innate immunity, antioxidant and immune cytokines mechanisms in the head kidney leucocytes of *Labeo rohita* against *Aphanomyces invadans*. Fish & Shellfish Immunology 87: 669–678.

Directorate General of aquaculture Ministry of Marine Affairs and Fisheries. Work Report. 2019

Dong HT, Techatanakitarnan C, Jindakittikul P, Thaiprayoon A, Taengphu S, Charoensapsri W, Khunrae P, Rattanarojpong T, Senapin S. 2017. *Aeromonas jandaei* and *Aeromonas veronii* caused disease and mortality in Nile tilapia, *Oreochromis niloticus* (L.). Journal of Fish Diseases 40: 1395–1403.

Dontriska, Sasanti AD, Yulisman. 2014. Effectiveness of Black Cumin (*Nigella sativa*) Flour for Prevent Infection *Aeromonas hydrophila* Infection on Catfish. Journal Of Indonesian Swamp Aquaculture 2: 188–201. Effendie MI. 2002. Fisheries Biology. Bogor: Yayasan Pustaka Utama.Yogyakarta. 163 page.

El-Hack A, Mohamed E, Abdelnour SA, Khafaga AF, Taha AE, Abdel-Latif HMR. 2021. *Nigella sativa* Seeds and Its Derivatives in Fish Feed. In Black cumin (*Nigella sativa*) seeds: Chemistry, Technology, Functionality, and Applications pp. 297–315 Springer.

FAO. 2020. The State of World Fisheries and Aquaculture. Food Agric 165.

Gabaudan J, Verlhac V. 2006. Critical review of the requirements of ascorbic acid in cold and cool water fishes (salmonid, percids, plecoglossids and flatfishes). Ascorbic Acid in aquatic organisms: Status and perspectives, 33p.

Gallani SU, Valladão GMR, Assane IM, de Oliveira Alves L, Kotzent S, Hashimoto DT, Pilarski F. 2020. Motile Aeromonas septicemia in tambaqui *Colossoma macropomum*: Pathogenicity, lethality and new insights for control and disinfection in aquaculture. Microbial Pathogenesis 149: 104512.Gasperz V. 1991. Experiment Design Method. Bandung, C.V. Amico.

Gómez de Anda FR, Vega-Sánchez V, Reyes-Rodríguez NE, Martínez-Juárez VM, Ángeles-Hernández JC, Acosta-Rodríguez I, Campos-Montiel RG, Zepeda-Velázquez AP. 2021. The Nasal Epithelium as a Route of Infection and Clinical Signs Changes, in Rainbow Trout (*Oncorhynchus mykiss*) Fingerlings Infected with Aeromonas spp. Applied Sciences 11: 9159.

Hardi EH, Sukenda, Harris E, Lusiastuti AM. 2011. Characterization and Phatogenity of ß and Non Haemolytic *Streptococcus agalactiae* in Cultured Nila Tilapia. Veterinary Journal 12: 152–164.

Hartika R, Mustahal, Putra AN. 2014. Tilapia Blood Parameters with The Addition of Different Dose of Prebiotics in Feed. Fishreries and Marine Journal 4: 259–267.

Hidayati T, Akrom A, Indrayanti I, Sagiran S. 2019. Black cumin seed oil increase leucocyte and CD4Thelper number in sprague-dawley rats induced with dimethylbenzanthracene. International Journal of Public Health Science 8: 238–245.

Jafarzadeh A, Nemati M, Jafarzadeh S, Chauhan P, Saha B. 2021. The immunomodulatory potentials of interleukin-27 in airway allergies. Scandinavian Journal of Immunology 93: e12959.

Jasmanindar Y, Salosso, Dahoklory. 2020. Immunostimulant *Gracilaria verrucosa* in catfish farming *Clarias* sp. Aquatic Journal 3: 67–72.

Khairuman H, Amri K. 2013. Tilapia Fish Farming. Jakarta, AgroMedia.

Kordi KMGH. 2010. Tilapia Farming in Tarpaulin Pond. Yogyakarta, Lily Publisher.

Kurniawan, Prayitno SB, Sarjito, Lusiastuti AM. 2013. The Effect of soursop leaf extract (*Annona muricata* L) on blood profile and survival of Sangkuriang Catfish (*Clarias gariepinus* Var. Sangkuriang) infected with *Aeromonas hydrophila* bacteria. Journal of Aquaculture Management and Technology 2: 50–62.

Laith AA, Ambak MA, Hassan M, Sheriff SM, Nadirah M, Draman AS, Wahab W, Ibrahim WNW, Aznan AS, Jabar A. 2017. Molecular identification and histopathological study of natural Streptococcus agalactiae infection in hybrid tilapia (*Oreochromis niloticus*). Veterinary World 10: 101.

Lattanzi S, Cagnetti C, Provinciali L, Silvestrini M. 2016. Neutrophil-to-lymphocyte ratio predicts the outcome of acute intracerebral hemorrhage. Stroke 47: 1654–1657.

Lukistyowati I, Kurniasih. 2012. Detection of Aerolysin Gen from *Aeromonas hydrophila* in Common Carp Fed with Garlic Extract. Veterinary Journal 13: 43–50.

Mahboub HH, Elsheshtawy HM, Sheraiba NI, Fahmy EM, Mohamed EAA, Abdelnaeim NS, Mohamed DI, Ismail TA, Ahmed SAA. 2022. Dietary black cumin (*Nigella sativa*) improved hemato-biochemical, oxidative stress, gene expression, and immunological response of Nile tilapia (*Oreochromis niloticus)* infected by *Burkholderia cepacia*. Aquaculture Reports 22: 100943.

Maryani, Rosdiana. 2020. The Role of Immunostimulan Yellow Roots *Arcangelisia flava* Merr in Image System Immunication Description Common Carp (*Cyprinus Carpio* L). Journal of Indonesian Swamp Aquaculture 8: 22–36.

Monalisa SS, Rozik M, Pratasik SB. 2018. Effectivity of Arcangelisia flava as immunostimulant to prevent streptococcosis on Nile tilapia, *Oreochromis niloticus*. Aquaculture, Aquarium, Conservation & Legislation 11: 1834–1843.

Naiel MAE, Shehata AM, Negm, SS, Abd El-Hack ME, Amer MS, Khafaga AF, Bin-Jumah M, Allam AA. 2020. The new aspects of using some safe feed additives on alleviated imidacloprid toxicity in farmed fish: A review. Reviews in Aquaculture 12: 2250–2267.

Novita, Setyowati DN, Astriana BH. 2020. Profile of Blood of Seabass Infected by *Vibrio* sp. with The Addition of *Aloe vera* Extract. Fisheries Journal 10: 55–69.

Ojueromi OO, Oboh G, Ademosun AO. 2022. Black Seed (*Nigella sativa*): A Favourable Alternative Therapy for Inflammatory and Immune System Disorders. Inflammopharmacology 1–21.

Prasetio E, Rachimi, Hermawansyah M. 2018. Use of *Aloe vera* in Feed As Immunostimulant to Hematology of Biawan Fish (*Helostoma teminckii*) Tested at The Test With Bacteria. Ruaya Jounal 6: 60–73.

Putra GP, Mulyana, Mumpuni FS. 2015. The Effect of Extracts of Tumeric (*Curcuma xanthorrhiza* Roxb) Against The Mortality Rate and Blood Picture of Nilem (*Osteochilus hasselti*) Fry That Had Been Challenged by *Aeromonas hydrophila* Bacteria. Mina Science Journal 1: 6–78.

Ramadhana AW, Avifah RN, Agustono, Al Arif MA, Lamid M . 2019. Effect Addition of Black cumin (*Nigella sativa*) Flour in Commercial Feed to Survival Rate, Specific Growth Rate, Feed Consumption Level, Feed Convertion Ratio, and Feed Efficiency of Mozambique Tilapia *Oreochromis mossambicus*).

Rodrigues MV, Dias MFF, Francisco CJ, David GS, da Silva RJ, Junior JPA. 2019. *Aeromonas hydrophila* in Nile tilapia (*Oreochromis niloticus*) from Brazilian aquaculture: a public health problem. Emergent Life Sciences Research 5: 48–55.

Rojo-Cebreros AH, Ibarra-Castro L, Martínez-Brown JM. 2018. Immunostimulation and trained immunity in marine fish larvae. Fish & Shellfish Immunology 80: 15–21.

Rosidah, Buwono ID, Lili W, Suryadi IB, Triandika AR. 2019. The resistance of sangkuriang catfish (*Clarias gariepinus* Burchell 1822) against *Aeromonas hydrophila* bacteria given moringa leaf extracts (*Moringa oleifera* L.) through the feed. Indonesian Journal of Ichthyology 19: 97–113.

Rustikawati I. 2012. The effectiveness of *Sargassum* sp. extract to leukocyte differentiation of tilapia (*Oreochromis niloticus*) infected by *Streptococcus iniae*. Journal of Aquatics 3.

Sa'adah R, Sasanti AD, Taqwa FH. 2015. Application of black cumin (*Nigella sativa*) powder to prevent infection of *streptococcus agalactiae* on nile tilapia (*Oreochromis niloticus*). Journal of Indonesian Swamp Aquaculture 3: 58–69.

Saffari S, Keyvanshokooh S, Zakeri M, Johari SA, Pasha-Zanoosi H, Mozanzadeh MT. 2018. Effects of dietary organic, inorganic, and nanoparticulate selenium sources on growth, hemato-immunological, and serum biochemical parameters of common carp (*Cyprinus carpio*). Fish Physiology and Biochemistry 44: 1087–1097.

Sahfitri I, Wulandari, Aminatul Zahra. 2021. Blood Profile of White Snapper *Lates calacalifer* Feed Containing *Gracilaria* sp. Intek Aquaculture 5: 59–70.

Selders GS, Fetz AE, Radic MZ, Bowlin GL.

2017. An overview of the role of neutrophils in innate immunity, inflammation and host-biomaterial integration. Regenerative Biomaterials 4: 55–68.

Simmons A. 1980. Techical Hemarology. 3rd Edition. J.B. Lippicot Company. Philadelphia. Toronto.

Stankevičiūtė M, Sauliutė G, Makaras T, Markuckas A, Virbickas T, Baršienė J. 2018. Responses of biomarkers in Atlantic salmon (*Salmo salar*) following exposure to environmentally relevant concentrations of complex metal mixture (Zn, Cu, Ni, Cr, Pb, Cd). Part II. Ecotoxicology 27: 1069–1086.

Suhermanto A, Andayani S, Maftuch. 2013. Effect of total phenol of sand sea cucumber (*Holothuria scabra*) on non-specific immune response of carp (*Cyprinus carpio*). Journal of Sustainable Earth 13: 225–233.

Tang Y, Zeng W, Wang Y, Wang Q, Yin J, Li Y, Wang C, Bergmann SM, Gao C, Hu H. 2020. Comparison of the blood parameters and histopathology between grass carp infected with a virulent and avirulent isolates of genotype II grass carp reovirus. Microbial Pathogenesis 139: 103859.

Thanh Dien L, Linh NV, Sangpo P, Senapin S, St-Hilaire S, Rodkhum C, Dong HT. 2021. Ozone nanobubble treatments improve survivability of Nile tilapia (*Oreochromis niloticus*) challenged with a pathogenic multi-drug-resistant *Aeromonas hydrophila*. Journal of Fish Diseases 44: 1435–1447.

Trilia NAO, Setyawan A, Adiputra YT, Wardiyanto. 2014. Immunogenicity Combination Vaccine Inactivated Whole Cell *Aeromonas salmonicida* and Black Cumin (*Nigella sativa*) in Carp (*Cyprinus carpio*). Journal of Aquaculture Engineering and Technology 2: 250–257.

Valenta Mery, Sasanti DA, Yulisman. 2018. Different Time Duration of Feed Contain Black Cumin on Survival and Growth of Snakehead Challenged by *Aeromonas hydrophila.* Journal of Indonesian Swamp Aquaculture 6: 2303–2960.

Waliani RF, Gunanti M, Laksmi S. 2015. Effect Of Extract Sargassum sp. with Methanol Solvent in Feed on Erythrocythes and Differential Leucocythes of African Catfish (*Clarias gariepinus*). Jurnal Ilmiah Perikanan Dan Kelautan 7: 213–218.

Witeska M, Kondera E, Ługowska K, Bojarski B. 2022. Hematological methods in fish–Not only for beginners. Aquaculture 547: 737498.

Yengkhom O, Shalini KS, Subramani PA, Michael RD. 2018. Non-specific immunity and disease resistance are enhanced by the polysaccharide fraction of a marine chlorophycean macroalga in *Oreochromis niloticus* (Linnaeus, 1758). Journal of Applied Ichthyology 34: 556–567.

Yilmaz S, Ergun S, Turk N. 2011. Effects of Cumin-suplemented Diets on Growth and Disease (*Streptococcus iniae*) Resistance of Tilapia (*Oreochromis mossambicus*). The Israeli Journal of Aquaculture 64: 768–773.

Yousefi M, Ghafarifarsani H, Hoseinifar SH, Rashidian G, Van Doan H. 2021. Effects of dietary marjoram, *Origanum majorana* extract on growth performance, hematological, antioxidant, humoral and mucosal immune responses, and resistance of common carp, *Cyprinus carpio* against *Aeromonas hydrophila*. Fish & Shellfish Immunology 108: 127–133.

Yuliana A, Wulandari R, Zahra A. 2021. Provision of *Sargassum* sp. Extract Through Commercial Feed of Hematocrit Value and Leukocyte Differential in Pomfret *Trachinotus blochii*. Intek Aquaculture 5: 36–49.