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The Immunity Performance of Nile Tilapia (*Oreochromis niloticus*) after the Exposure of Botanical Insecticide with Azadirachtin Active Ingredient

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

This study aims to determine the exposure effect of insecticide with the azadirachtin active ingredient on the immunity of nile tilapia. This research was conducted in March and August for the preliminary test and September-October 2022 for the main test at Ciparanje Inland Aquaculture Hatchery, Padjadjaran University. This study used the experimental method of Complete Randomized Design with five treatments and three repeats. The nile tilapia used is 6-7 cm. The

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treatment used is exposure to insecticide with different doses, namely A (0 ppm), B (26.25 ppm), C (52.5 ppm), D (78.75 ppm), E (105 ppm). Parameters observed included red blood cell count, white blood cell count, feed response, shock response, and macroscopic symptoms up to day 14. Red blood cell and white blood cell data were analyzed in a quantitative descriptive manner. Data on feed response, shock response, and macroscopic symptoms were carried out in a qualitative descriptive manner. Water quality data were analyzed in a quantitative descriptive manner. The results showed that exposure of insecticide with azadirachtin active ingredient interfered fish health by increasing white blood cell count, lowering red blood cell count, disrupting feed response and shock response. The higher the concentration of insecticide given, the more it affects the immunity of tilapia. Treatment without the addition of insecticides is the best treatment in this study.

Keywords: Azadirachtin; immunity; insecticide; nile tilapia; oreochromis niloticus.

1. INTRODUCTION

Efforts to increase agricultural output continue to be made to meet the increasing food needs of the community. However, there are several problems, one of which is pest attack. This causes pesticides to be widely used in the agricultural and plantation sectors to overcome these pest attacks. Pest attacks can damage agricultural products and cause losses to farmers. Pest attacks can cause crop failure and agricultural losses of up to 50% [1].

Insecticide is one type of pesticide with chemical or botanical active ingredients used to control or eradicate insect type disturbing organisms [2]. At this time, botanical insecticides are more widely used than chemical insecticides. This is due to the use of chemical insecticide in the long term can produce residues and have a negative impact on the environment so that farmers use botanical insecticide as an alternative to overcome plant pests [3]. In addition, botanical insecticides are safer for the environment because they are made from natural ingredients.

Neem plants are one of the plants whose secondary metabolites can be used as botanical insecticide [4]. Leaf or seed extracts from neem plants contain *azadirachtin* compounds. *Azadirachtin* compounds have an influence on insect pests, such as inhibitors of feeding activity, inhibitors of skin development and turnover, rejection of ejection, and can cause death in insects [5] .

Insecticides used in agriculture can pollute aquaculture waters. This is because the location of aquaculture is generally close to agricultural locations, there are even fishery activities located in rice fields (mina padi). The flow of water from agricultural areas will be distributed to water bodies with lower locations such as aquaculture ponds so that insecticides used in agricultural activities can be carried away by the flow of water. Insecticides that enter aquaculture water bodies can be a threat to aquatic biota, especially fish [6].

Waters that are polluted by the use of insecticides can be identified using a toxicity test. Toxicity tests can be performed to determine the level of toxicity of insecticides at certain concentrations. One of the aquatic biota that can be used to carry out toxicity tests is fish. Fish is one of the animals that can be used as a bioindicator of pollution levels, water quality, and changes in the aquatic environment. One type of fish that can act as a bioindicator is nile tilapia (*Oreochromis niloticus*). Tilapia is one of the aquatic biota recommended by USEPA (United States Environmental Protection Agency) as a toxicology test animal. This is because nile tilapia has been widely cultivated, its distribution is quite wide, has a good ability to tolerate adverse environmental conditions, and is easy to maintain in the laboratory so it is good for use as a bioindicator [7].

The purpose of this study was to determine the exposure effect of insecticide with *azadirachtin* active ingredient on nile tilapia immunity so that it can be used as a reference and information on tolerable concentrations and long-term effects after exposure of botanical insecticide with *azadirachtin* active ingredient on nile tilapia immunity.

2. METHODOLOGY

2.1 Time and Place

This research was carried out at the Ciparanje Inland Aquaculture Hatchery, Faculty of Fisheries and Marine Science, Padjadjaran University in March and August 2022 for Preliminary Tests.

Meanwhile, the main test was carried out from September to October 2022.

2.2 Tools and Materials

The tools used in this study were an aquarium measuring 50 x 29.5 x 35.5 cm^3, a concrete tub 2 x 1 x 0.5 m^3, blowers and aeration equipment, heaters, cup clips, millimeter blocks, digital scale, siphon device (water hoses), syringe, mercury thermometer, DO meter, pH meter, stopwatch, Thoma pipet, haemocytometer, cover glass, binocular microscope, scissor, labels, camera.
While the ingredients used, namely nile tilapia nirwana measuring 6-7 cm, insecticide with *azadirachtin* active ingredient, feed, potassium permanganate (PK), Ethylene Diamine Tetra-Acetic Acid (EDTA) solution, Hayem solution, Turk's solution.

2.3 Research Methods

This study used a randomized design complete with five treatments and three repeats. The treatment given is:

Treatment A	= Control (Without addition of insecticide)
Treatment B	= Addition of insecticide 25% LC50 (26,25 ppm)
Treatment C	= Addition of insecticide 50% LC50 (52,5 ppm)
Treatment D	= Addition of insecticide 75% LC50 (78,75 ppm)
Treatment E	= Addition of insecticide LC50 (105 ppm)

2.4 Research Procedure

2.4.1 Research preparation

The preparations carried out include the preparation of containers, the preparation of insecticides, and the acclimatization of fish. Preparation of containers is carried out by washing tools to be used such as aquariums, aeration hoses, aeration stones using Potassium Permanganate (PK), then the tools are dried. Next, the aquarium is laid out according to a predetermined layout and labeled. The aquarium was filled with 30 L of water in preliminary tests. While in the main test as much as 40 L. Aquarium is equipped with a heater and aeration device.

The insecticide used is prepared by being taken using a syringe according to a predetermined concentration. The syringe is inserted into the insecticide to be used and the plunger part is gently pulled so that the insecticide is sucked into the syringe. Then prepared cup clip as a container to store insecticide to be used. Then, the plunger on the syringe is gently pressed into the cup clip container, then the cup clip container is tightly closed.

The nile tilapia that will be used are acclimatized for 3 days to ensure the condition of the fish is healthy. During acclimatization, fish are fed as much as 3% of the biomass. After acclimatization, the fish are introduced into the aquarium and observations are made. The preparatory stage of this research was carried out in the preliminary test and the main test.

2.4.2 Preliminary test

The treatment in the preliminary test refers to the USEPA recommendation, which is 0; 10; 100; and 1000 ppm. The test was carried out with 4 treatments and 2 repetitions. Tilapia used as many as 15 heads/aquarium. The aquarium used 8 pieces and the aquarium filled with water as much as 30 L. However, because the lower threshold and upper threshold ranges in the treatment were too large, additional treatment was carried out by reducing and enlarging the treatment concentration between 10 ppm and 1000 ppm. Additional concentrations are used, namely 50 ppm, 75 ppm, 100 ppm, 125 ppm, 150 ppm, 175 ppm, 250 ppm, and 500 ppm. Observations were made for 96 hours by observing the death of fish every 24 hours. The results obtained were analyzed for regression using SPSS 2021 software. The results that have been obtained are concentrations that are thought to be deadly to test fish as much as 50%. After 96 hours of observation, an LC_{50} value of 105 ppm was obtained.

2.4.3 Main test

The treatments to be used in the main test, namely 5 treatments and 3 repeats. Tilapia used as many as 20 heads/aquarium. The aquarium used as many as 15 pieces and filled with water as much as 40 L. The concentration to be used in each treatment refers to the LC_{50} value that has been obtained in the preliminary test. The treatment to be used, namely control (without the addition of insecticide), 25% LC50 (26.25 ppm), 50% LC50 (52.5 ppm), 75% LC50 (78.75 ppm), and 100% LC50 (105 ppm). During the observations, the fish were fed as much as 3% of the biomass. Water quality maintenance is carried out by replacing water by water hoses as much as 10% every three days. The observations were conducted for 14 days by observing red blood cell count, white blood cell count, feed response, shock response, and macroscopic symptoms, and water quality.

2.4.4 Observation parameters

2.4.4.1 Total Red Blood Cells (Eritrosit)

Observation of the number of red blood cells was carried out on days 0, 3, 7, 10, and 14. According to [8], the calculation of the total of red blood cells is as follows:

$$\text{RBC} = \frac{A1 + A2 + A3 + A4 + A5}{5} \times 25 \times 10 \times 200$$

Description :

RBC = Red Blood Cells;
A1,A2,A3,A4,A5 = Observed box sample;
5 = Total of sample boxes;
25 = The entire total of red blood cells boxes;
10 = Box thickness (mm);
200 = Dilution (mm^3)

2.4.4.2 Total White Blood Cells (Leukosit)

Observation of the total of white blood cells was carried out on days 0, 3, 7, 10, and 14. According to [8], the calculation of the total of white blood cells is as follows:

$$\text{WBC} = \frac{\mathbf{A1 + A2 + A3 + A4}}{\mathbf{4}} \text{ X } 16 \text{ X } 10 \text{ X } 20$$

Description:

WBC = White Blood Cells;
A1,A2, A3, A4 = Observed box sample;
4 = Total of sample boxes;
16 = The entire total of red blood cells boxes;
10 = Box thickness (mm);
20 = Dilution (mm3)

2.4.4.3 Feed Response

The observation of feed response refers to the research of [9] and carried out until the 14th day. Observation of feed response is calculated through the response time of fish when eating the given feed. The response rate of fish feed is as follows:

- (+++) = Fish respond to feed under 30 seconds
- (++) = Fish respond to feed 31 – 90 seconds
- (+) = Fish respond to feed 91 – 180 seconds
- (-) = Fish don't respond to feed

2.4.4.4 Shock Response

The observation of shock response refers to the research of [9] and carried out until the 14th day. The observation of the shock response is calculated through the number of active fish after the shock is given. The shock response rate of fish is as follows:

- (+++) = >80% of fish respond to shock
- (++) = 60 – 80% of fish respond to shock
- (+) = 30 – 60% of fish respond to shock
- (-) = Fish don't respond to shock

2.4.4.5 Macroscopic symptoms

Observation of macroscopic symptoms was carried out after insecticide treatment by observing body damage to the outside, such as torning fins, fading body color, bleeding, and distended abdomen until day 14.

2.4.4.6 Water Quality

Water quality parameters observed include temperature, acidity (pH), and dissolved oxygen. Observations on water quality were made on days 0, 3, 7, 10, and 14.

2.5 Data Analysis

Red blood cell and white blood cell count data were analyzed in a quantitative descriptive manner. Data on feed response, shock response, and macroscopic symptoms were carried out in a qualitative descriptive manner. Water quality data is analyzed descriptively quantitatively and compared with Indonesian National standard.

3. RESULTS AND DISCUSSION

3.1 White Blood Cell Count

White blood cells are blood cells that function as non-specific defenses in the immune system of fish. Changes in white blood cell count are indicators of fish in poor condition [10]. The following is a graph of the results of observing the number of white blood cells.

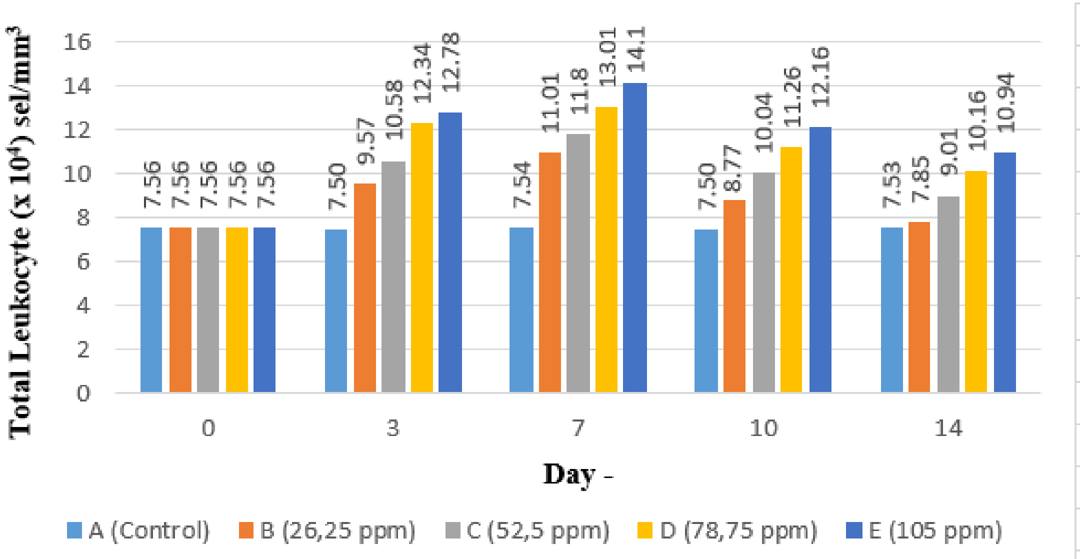

Fig. 1. Graph of total Leukocyte

An increase in the number of white blood cells in treatment given insecticide exposure compared to control treatment is a sign that fish health is being disrupted. The highest increase in white blood cell count is found on day 7 and gradually decreases on day 10. An increase in the number of white blood cells is a form of defense response from the tilapia body against foreign substances that enter the body [11]. This is supported by a statement from [12] which states that white blood cells have an important role in the body's defense mechanism, consisting of granulocytes to overcome bacterial infections, lymphocytes as antibody-producers, and monocytes as phagocytes to restore injured tissue. Exposure to insecticide causes disruption of body tissues and stress to tilapia so that the body responds by increasing the number of white blood cells as a form of the body's defense system [13].

This is supported by the condition of the fish in the research which showed symptoms of stress, such as increased operculum and mouth openings, fish tended to go limp or stay still on the surface of the water. This is in accordance with the research by [13] and [14] which showed that there was an increase of the white blood cells in stressed fish. In addition, the observations showed that the number of white blood cells would increase along with the increasing concentration of the insecticide given. This is in accordance with the research of [13] which showed that there was an increase in the number of white blood cells in nile tilapia along with the increasing concentration of the insecticide given. A decrease in the number of white blood cells indicates that fish are beginning to adapt to their living environment and is a phase of recovery in fish. This is supported by an improved feed response and shock response from day 10. According to [15], the number of normal white blood cells in tilapia ranges from 20,000 – 150,000 cells/mm^3 so that the number of white blood cells in tilapia during the study was relatively normal.

3.2 Red Blood Cell Count

Red blood cells function to bind oxygen which will be used as energy through the process of catabolism [16]. The following is a graph of the results of observing the number of red blood cells.

The lowest decrease in red blood cell count was found on day 7 and gradually improved on day 10. As the concentration of insecticide applied to the treatment increases, the number of red blood cells decreases. This is because the active substance of the insecticide enters the body of the fish and causes damage to red blood cells. This is in accordance with the research of [17], which shows that increasing pesticide concentrations can increase damage to tilapia red blood cells so that the number of red blood cells decreases.

A decrease in the number of red blood cells indicates anemia in tilapia. Anemia occurs due to damage to red blood cells and reduced release of red blood cells in the blood circulation. Anemia that occurs in fish is related to impaired iron absorption (Fe), where iron plays an important role in the synthesis of hemoglobin in the blood [18]. A decrease in the number of red blood cells causes an obstruction of the supply of nutrients to cells, tissues, and organs. This leads to a decrease in body activity and stunted growth in fish [7]. According to [15], the number of normal red blood cells in tilapia ranges from 20,000 – 3,000,000 cells/mm^3 so that the number of red blood cells in tilapia during the study was normal.

3.3 Feed Response

Fish health can be known through the response of fish to feed. Fish with healthy conditions will have a good feed response [19]. The following is the results of observations of feed response.

A decreased feed response is caused by exposure to insecticides and causes stress in fish. Stressful conditions experienced by fish cause behavioral changes, such as loss of appetite and rejection of feed [20]. This is in line with [21], who states that stress experienced by fish can cause disruption of physiological conditions characterized by loss of appetite of fish. This is supported by the condition of the amount of feed left when feeding. When associated with the results of observing the number of red blood cells, the low number of red blood cells causes a decrease in fish body activity. This causes the fish to become weak, fall silent at the bottom or hang below the surface of the water, and decreased appetite [22].

3.4 Shock Response

Observation of the shock response is carried out by knocking on the walls of the treatment aquarium. This is done to determine the response or reflex of fish to stimuli given from outside [19]. If the fish is in a healthy condition, the fish will immediately move when given a surprise. The following is the result of observing the shock response.

Based on the observations, treatment A and B on days 1 to day 14 did not decrease shock response. Meanwhile, in treatment C, D, and E experienced a decrease in shock response. In treatment C, the response to shock decreased until day 6. Meanwhile, the shock response in treatment D was low until day 5 and treatment E was low until day 6. The decreased shock response is thought to be due to stress experienced by fish due to exposure to insecticides and decreased metabolism so that fish become weak and respond less to the shock given. This is supported by the research of [6] which stated that exposure of insecticide disrupted the fish's respiratory system so that fish lacked oxygen and became weak which resulted in a decreased response to stimuli in the form of shock.

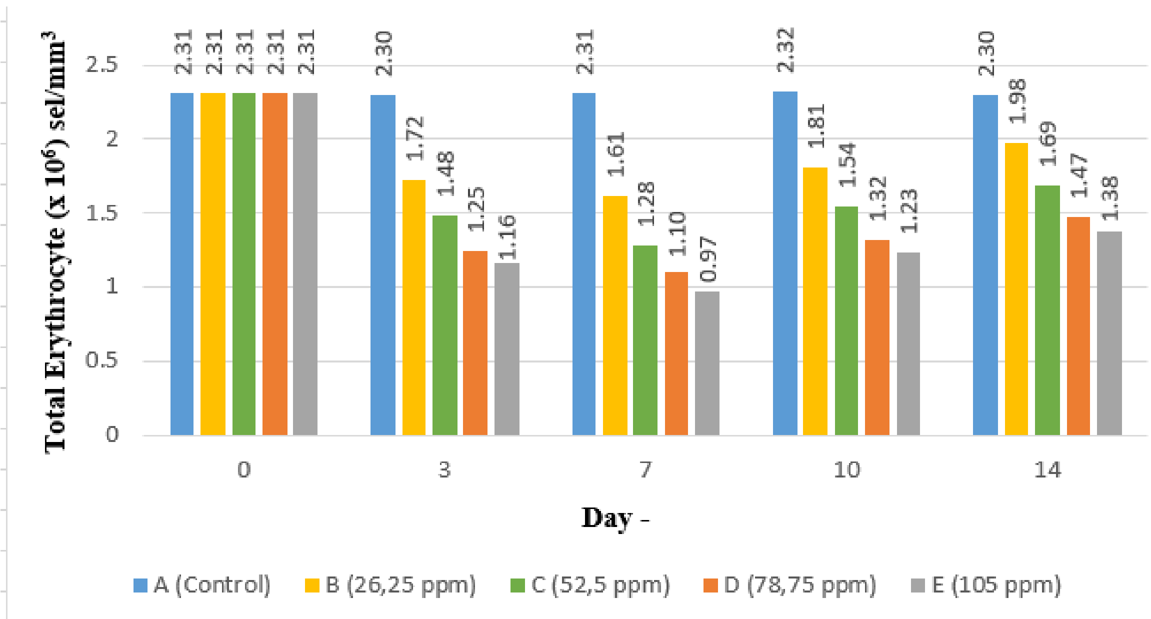

Fig. 2. Graph of total erythrocyte

Table 1. Feed Response

Treatment	Repetition	Day to-													
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
A	1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	3	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
B	1	++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	2	++	++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	3	++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
C	1	++	++	++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++
	2	++	++	++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++
	3	++	++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
D	1	+	+	+	+	+	+	+	++	++	++	++	++	++	+++
	2	+	+	+	+	+	+	+	++	++	++	++	++	+++	+++
	3	+	+	+	+	+	+	+	++	++	++	++	+++	+++	+++
E	1	+	+	+	+	+	+	+	+	+	++	++	++	++	+++
	2	+	+	+	+	+	+	+	+	+	++	++	++	+++	+++
	3	+	+	+	+	+	+	+	+	+	++	++	++	++	+++

Description: (+++) fish respond to feed under 30 seconds, (++) fish respond to feed 31-90 seconds, (+) fish respond to feed 91-180 seconds, (-) fish don't repond to feed

Table 2. Shock response

Treatment	Repetition	Day to-													
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
A	1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	3	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
B	1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	3	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
C	1	++	++	++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++
	2	++	++	++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++
	3	++	++	++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++
D	1	+	+	+	+	+	++	++	++	++	++	++	++	+++	+++
	2	++	+	+	+	+	++	++	++	++	++	++	++	+++	+++
	3	+	+	+	+	+	++	++	++	++	++	++	+++	+++	+++
E	1	+	+	+	+	+	+	++	++	++	++	++	++	+++	+++
	2	+	+	++	+	+	++	++	++	++	++	++	++	+++	+++
	3	+	+	+	+	+	++	++	++	++	++	++	++	+++	+++

Description: (+++)>80% of fish respond to shock, (++) 60-80% of fish respond to shock, (+) 30-60% of fish respond to shock, (-) Fish don't respond to shock

3.5 Macroscopic Symptoms

Macroscopic symptoms are observed from day 1 to day 14 by observing damage to the outside of the body of the fish. Observations were made after insecticide exposure was given at each treatment. The following are the results of observations of macroscopic symptoms.

Based on observations, the macroscopic symptoms that appear are excess mucus, faded body color, peeling scales, and torn fins. Exposure to insecticides causes fish to produce more mucus on their bodies to protect the exposed body from damage. The same was expressed by [23] which states that fish will produce mucus in their bodies as a form of defense due to exposure to pesticides which are pollutants in the fish's environment. However, this causes the supply of oxygen to the fish's body to decrease so that the fish's body weakens and causes the body color to fade. This is in line with [6], which states that exposure to insecticides can cause fish body color to fade and torn fin in fish due to weakening of the fish body due to insecticide exposure.

3.6 Water Quality

Water quality observed during the study included temperature, pH (acidity degree), and dissolved oxygen. The following are observations of water quality.

Based on the results of water quality observations (temperature, pH, dissolved oxygen) that have been carried out show that the value of water quality during the study was within the optimal range for tilapia according to [24]. In addition, exposure to insecticide provided does not affect the water quality of maintenance media because water quality is controlled according to the demands of a complete randomized design method that is not allowed for other factors other than the treatment given (exposure of botanical insecticide with *azadirachtin* active ingredient).

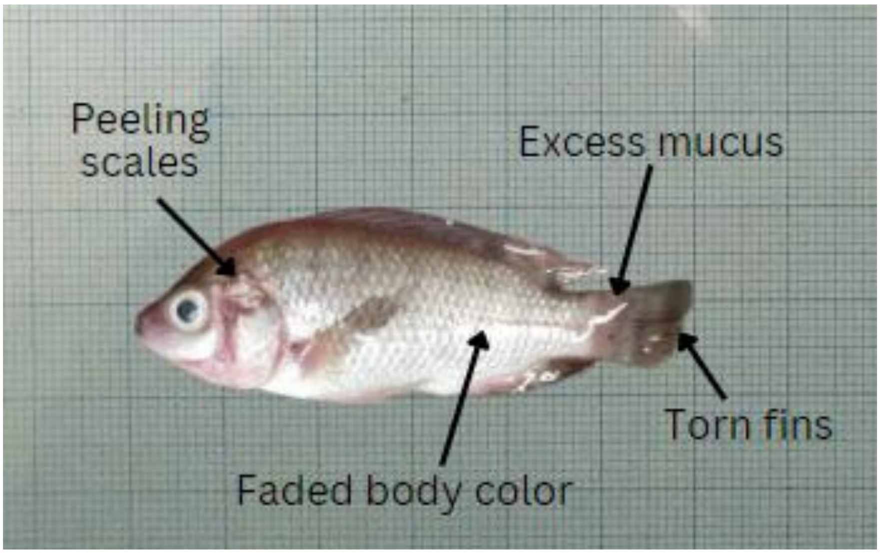

Fig. 3. Macroscopic symptoms

Table 3. Water Quality

Treatment	Day 3, 7, 10, dan 14			Day 21 dan ke-28		
	Temperature (°C)	pH	DO (mg/L)	Temperature (°C)	pH	DO (mg/L)
A	29,8 - 30,7	6,7 - 6,9	5,6 - 6,1	29,8 - 30,9	6,8 - 6,9	5,7 - 6,1
B	29,9 - 30,7	6,6 - 6,8	5,4 - 5,8	30,1 - 30,9	6,6 - 6,8	5,5 - 5,9
C	29,9 - 31	6,7 - 6,9	5,7 - 6,1	29,9 - 30,5	6,6 - 6,7	5,6 - 6,1
D	29,7 - 30,9	6,5 - 6,9	5,6 - 5,9	30,1 - 31	6,5 - 6,8	5,7 - 5,9
E	30,1 - 31	6,6 - 6,8	5,5 - 5,8	30,4 - 31	6,5 - 6,8	5,6 - 5,9
Optimal [24]	25 -32	6,5 - 8,5	≥ 5	25 -32	6,5 - 8,5	≥ 5

4. CONCLUSION

Based on the results of research that has been done, exposure to insecticides affects the health of tilapia, the higher the exposure to insecticides given, the health of tilapia decreases. The results showed that exposure of insecticide with *azadirachtin* active ingredient disrupted the health of nile tilapia by increasing the white blood cell count, decreasing the red blood cell count, disrupting feed response and shock response, and causing several macroscopic symptoms in the nile tilapia body likes excess mucus, faded body color, peeling scales, and torn fins. The peak of disruption of fish health on the 7th day and after that the health condition of the fish gradually improves. Treatment without insecticide is the best treatment in this study.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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