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The Performance of L-Theanine as an Immunosimulant to Reduce Stress Levels in Motivant Fish (*Helostoma temminkii*)

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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

Farmed fish that are stressed are susceptible to various diseases that will affect their health, thereby reducing their reproductive abilities. Immunostimulants are substances that can enhance or stimulate the fish's immune system by interacting directly with cells that activate the immune system. The purpose of this study was to analyze the performance of L-theanine as an immunostimulant to reduce stress levels in monkfish. This research was conducted at the Wet Laboratory of the Faculty of Fisheries and Maritime Affairs, Lambung Mangkurat University, South Kalimantan Province. The treatment that will be given is as follows, namely Treatment A with feed

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added at a dose of L-Theanine 0 mg/kg. Treatment B feed was added with a dose of L-Theanine 300 mg/Kg. Treatment C was supplemented with a dose of L-Theanine 600 mg/Kg. Treatment D was supplemented with a dose of L-Theanine 900 mg/kg. The observed haematological conditions were hematocrit, erythrocytes and blood glucose. The addition of feed with a dose of L-Theanine 300 mg/kg was able to maintain immunostimulants to reduce stress levels in animal fish according to hematological measurements in the form of hematocrit, erythrocytes and blood glucose.

Keywords: Biawan fish; L- theanin; immunistulants; stress.

1. INTRODUCTION

Biawan fish (*Helostoma temminkii*) is a native Indonesian fish found in several rivers in Sumatra and Kalimantan. Biawan fish live in rivers, tributaries and inundation areas from upstream to downstream, even in river estuaries which are hilly and forested along the sides. This fish has high economic value, has prospects for developing aquaculture with great opportunities, the selling price is quite expensive, is an important commodity in the freshwater fish business but this fish is still rarely cultivated to date [1].

Stress is defined as the effect of any environmental changes or encouragement to achieve homeostasis or changes in stability reaching normal limits. Biawan fish are very sensitive to any changes both externally and internally, this condition causes them to be susceptible to stress [2]. Stressed animal fish are easily infected by several diseases that will affect their health, thereby reducing reproductive performance (eg growth, yield, survival and feed efficiency). Immunostimulants are substances that can enhance or stimulate the immune system of fish by interacting directly with cells that activate the immune system [3]. The stress response of fish can be divided into primary, secondary and tertiary phases. The primary effect on stress is an endocrine response that stimulates metabolic and osmotic adaptation (secondary phase) [4]. Under stressful conditions, fish will develop a new homeostasis by changing their metabolism. This response to stress is controlled by the endocrine system through the release of cortisol and catecholamine hormones from chromaffin cells and stimulation from the hypothalamus-pituitary- interrenal (HPI) as the main axis in the release of corticosteroids into the blood circulation. The mechanism of action of immunostimulants in stimulating the immune system is by increasing the activity of phagocytic cells [5].

The L-theanine compound is known as one of the neutron conductors found in the brain. This compound is absorbed in the intestinal brush border membrane because it is fat soluble, while it is transported to the brain via the preferred transport system of leucine from the blood lining the brain. theanin also increases GABA levels in the brain which brings a feeling of comfort [6]. Direct administration of L-theanine into the striatum of the brain by microinjection also causes a significant increase in DA release in a dose-dependent manner. As mentioned that the release of DA, one of the neurotransmitters, has a major effect on human emotions, these results suggest that L-theanine can affect metabolism and/or release of several neurotransmitters in the brain [7].

Irsadi [8] the L-Theanine content in green tea can reduce stress levels in fish. L-Theanine can be a progressive muscle relaxant to reduce anxiety levels. This condition is the basis that giving β-glucan can manage stress and increase immunity. L Theanin as an immunostimulant can be used as an alternative in aquaculture during the rematuration process of broodfish. There is a need for more studies regarding the use of L Theanin as an immunostimulant to enhance the non-specific immune system in fish which is very necessary in an effort to reduce stress levels in fish which can inhibit the rematuration process [9]. L-theanine has been studied for its effects on fish stress response and immune function. Stress can negatively impact the immune system of fish, making them more susceptible to diseases. L-theanine, when administered to fish, has shown potential in reducing stress and enhancing immune function.

Research conducted on various fish species, such as rainbow trout and goldfish, has demonstrated that L-theanine supplementation can improve fish's resistance to stressors, such as handling, transportation, or changes in environmental conditions. It has been observed to decrease cortisol levels (a stress hormone) and increase the activity of antioxidant enzymes, which help protect the fish's cells from oxidative stress.

Furthermore, studies have indicated that L-theanine may positively influence the immune response in fish. It has been shown to enhance the production of immune-related molecules, such as cytokines and immunoglobulins, which are crucial for immune defense . However , it's important to note that the research on L-theanine as an immunosimulant and stress reducer in fish is still limited, and more studies are needed to fully understand its effects and potential applications. Additionally, the effectiveness of L-theanine may vary depending on the fish species and the specific stressors they encounter. The aim of the study was to analyze the performance of L-theanine as an immunostimulant to reduce stress levels in monkfish.

2. MATERIALS AND METHODS

2.1 Location and Time of Research

This research was conducted at the Wet Laboratory of the Faculty of Fisheries and Maritime Affairs, Lambung Mangkurat University, South Kalimantan Province. The Biawan fish to be used in this study came from the Bincau Freshwater Fish Hatchery, South Kalimantan. The required number of broodstock is 60 female broodstock, body weight from 40 grams to 100 grams. Maintenance of brood fish during the 15 day study by providing artificial feed which was given pellet feed which was added with L – Theanin 3 times a day morning at 07:00, noon at 12:00 and evening 17:00 with a normal dose of 5% of the total weight of the fish .

2.2 Experimental Designs

According to previously research , the use of L-Theanine to increase fish immunostimulants is at a dose of 200 mg / kg. Therefore the treatments given is as follows, namely:

- Treatment A was feed added with a dose of L- Theanine 0 mg / kg
- Treatment B feed added with a dose of L-Theanin 300 mg/Kg
- Treatment C feed added with a dose of L-Theanine 600 mg/Kg
- Treatment D feed added with a dose of L-Theanine 900 mg/kg

Hematologic analysis of fish using a Hematology Analyzer at the Banjarbaru City Veterinary Center, South Kalimantan

2.3 Data Analysis

The parameter data observed in this study were analyzed descriptively and the results were presented in graphs and tables. Covariance analysis (ANCOVA) was applied to examine differences between treatments in terms of growth patterns. One-way ANOVA test was used to ascertain whether there was a difference in the length-weight measurement of snakehead fish reared in indoor containers between the four treatments. If there is, then the test (HSD) is applied. All tests were analyzed at the 0.05 significance level using the Microsoft excel.

3. RESULTS AND DISCUSSION

Analysis of the performance of L-theanine as an immunostimulant to reduce stress levels in monkfish was carried out by haematological testing. Hematology is a branch of medical science that studies blood, blood-forming organs and their diseases. Hematology studies disorders, diagnosis, treatment, recovery and prevention of diseases that attack blood and its components [10]. The hematological conditions observed in this study were the hematocrit, erythrocytes and blood glucose of the biawan fish.

3.1 Hematocrit

Hematocrit is the percentage of the volume of erythrocytes in fish blood or the ratio between the volume of blood cells and blood plasma. The hematocrit can provide an indication of the health of the fish and help determine the occurrence of abnormalities due to the use of immunostimulants. The hematocrit value can be calculated by the number of red blood cells contained in fish blood [11]. The results of the calculation of the hematocrit of the monkfish during the study can be seen in the following Fig. 1.

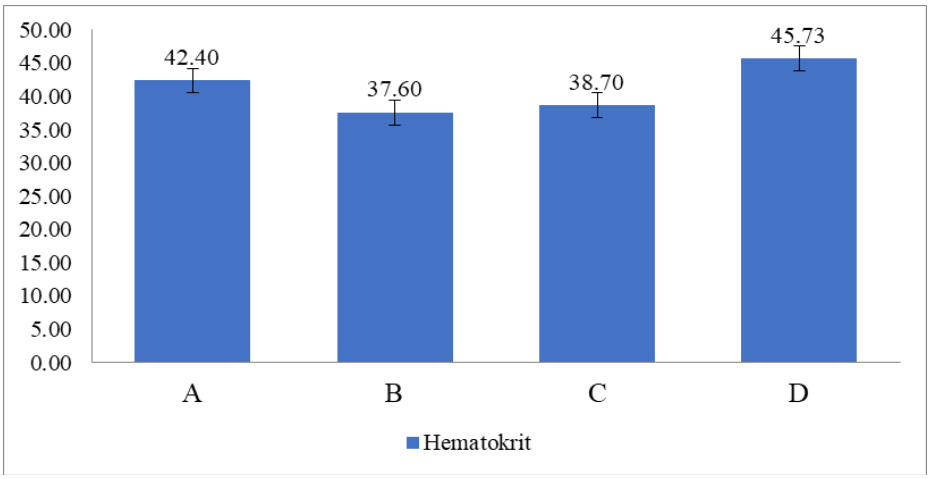

Fig. 1. Hematocrit value of biawan fish

Based on Fig. 1 it can be seen that the percentage of hematocrit for each treatment was different. But overall, the hematocrit value of the Bawan fish is still within normal limits. The value of hematocrit analysis of monkfish that was given treatment with feed substitution with a dose of L-Theanine, namely A as a control (42.40%), Treatment B (37.60%), Treatment C (38.70%) and Treatment D (45.73) %). The results of the normality test for the hematocrit of the Biawan fish obtained a Li Max value of 0.91 < Li Table 5% 0.242, so it can be concluded that the data is normally distributed. The results of the Barlett variance homogeneity test obtained the value X2 count -7.284 <X2 table 5.991, which means homogeneous. The results of the ANOVA analysis of diversity showed that the calculated F value was 1.900 < F table 5%, namely 4.07, which meant that there was no difference between treatments.

The highest hematocrit value during the study was in treatment D (45.73%). This condition is due to the influence of L- Theanin contained in the feed. According to Abdullah [6] in Faizah [12] fresh water fish are said to be healthy if their hematocrit levels range from 22-60%. A hematocrit concentration of <22% indicates that the fish is anemic and the fish may be sick or stressed. Giving L-Theanine to feed can help increase the immunity of the animal fish.

Hematocrit shows the percentage of solids (red blood cell levels, etc.) to the amount of blood fluids. The higher the hematocrit percentage, the thicker the blood concentration. This happens because there is seepage (leakage) of fluid out of the blood vessels while the amount of solids remains, so the blood becomes thicker. A decrease in hematocrit occurs when the body experiences acute blood loss, sudden blood loss, for example during anemia, leukemia, chronic kidney failure, malnutrition, deficiency of vitamins B and C. An increase in hematocrit above normal occurs in dehydration, severe diarrhea, eclampsia, the effects of surgery, burns and others.

Hematocrit is the ratio of the number of red blood cells to the total volume of blood calculated as a percentage. If your hematocrit is known to be 20%, it means that there are 20 milliliters of red blood cells per 100 milliliters. The description of the blood of an organism can be used to determine the health condition being experienced by fish, one of which is seen from the percentage of hematocrit which has an important role as a defense against bacterial attacks that enter the fish's body. When fish are stressed, the hematocrit value will tend to decrease. Stress in fish also causes physiological deviations and hormonal imbalances, causing blood components to also change.

Based on various studies that changes in hematocrit values will have a negative impact because they affect the viscosity (thickness) of blood, high or low hematocrit causes an increase and vice versa will slow blood flow in the capillaries and speed up the work of the heart. Sodium and potassium ions present in body fluids, both extracellular fluids (blood) and intracellular fluids (cytoplasmic fluids) are able to influence the work of epinephrine to suppress excessive spleen contractions, so that the contractions that occur in the erythrocytes become stable while maintaining the hematocrit value in the blood at normal range.

The results of the analysis provide an illustration that at each sample point the Hematocrit levels varied in each treatment group. With an average value of initial treatment = 41.5%, Treatment A = 42.4% Treatment B = 29.3% Treatment C = 38.7% Treatment D = 45.7% which is the spread in each observation group above the minimum recommended level. Hematocrit can be influenced by season, food and hormonal factors. According to Fange (1992), in tolerant fish, hematocrit levels are generally relatively constant between 20-40%. The normal value of tilapia hematocrit ranges from 27-37% [13]. Tilapia hematocrit values range from 28.00 – 35.13%. Thus the Hct value of tilapia is classified as healthy.

Calculation of the percentage of hematocrit is one of the approaches used to be able to recognize the fish's body when stressed. Hematocrit is the percentage of the volume of red blood cells (erythrocytes) in fish blood. If the hematocrit value is less than 25%, it indicates anemia (Kuswardani, 2007). The mechanism for changing the percentage of hematocrit during stress starts from receiving information on the causes of stress factors by the receptor organs. Furthermore, the information is conveyed to the hypothalamus part of the brain through the nervous system. The hypothalamus instructs the chromaffin cells to secrete catecholamines via sympathetic nerve fibers. The presence of these catecholamines will activate lipopolysaccharide which attacks blood components whose function can reduce the hematocrit in fish. The results of examination of the hematocrit can be used as an indicator to determine the health condition of fish, a hematocrit value of less than 25% indicates anemia. A low hematocrit can also indicate contamination, lack of food for fish, low protein feed, vitamin deficiency, stress or infection [14].

Stress in fish also causes physiological deviations and hormonal imbalances, causing blood components to also change. Changes in blood picture and blood chemistry, both qualitatively and quantitatively, can determine the state of health. A decrease in the hematocrit value indicates the uncomfortable condition of an organism and causes anemia (Nirmala et al., 2012). A hematocrit value below 30% indicates a deficiency of erythrocytes. The number of red blood cells, hematocrit, and hemoglobin decreased with increasing concentration of mercury in the rearing medium. The decrease in hematocrit and hemoglobin levels in fish blood due to mercury is affected by contamination, absorption and accumulation of mercury which can cause anemia in fish.

A decrease in the value of the hematorit can occur if, at the time of stress, the fish cannot maintain homestatic conditions where at the General Adaptation Syndrome (GAS) stage a defense reaction occurs against the stressor which causes changes in heart rate, changes in respiration, and blood supply capacity. When a defense reaction occurs, the body will provide a primary response in the form of increased secretion of corticosteroids and catecholamines.

The measured hematocrit value is directly related to the number of erythrocytes and hemoglobin levels. When fish experience stress, the contraction of the spleen will decrease so that the circulation of red blood cells becomes weak, so that the hematocrit value decreases. The occurrence of stress on fish will cause an increase in heart rate, blood pressure, increase in blood glucose and release of cortisol. At times of stress, cortisol suppresses the immune system, causing lymphocytes to increase and increase cortisol secretion can also cause a decrease in monocytes and basophils. In the body of fish, monocytes play a role in phagocytosis of foreign bodies. These cells have the ability to kill various types of pathogenic agents, including bacteria and worm larvae.

Stress consists of 3 components, namely stressor, process and response. The term stress does not only refer to the source of stress but the interrelationships between the three. A stressor is an event, situation, or object that causes stress and causes a stress reaction as a result. There are four kinds of stress factors, namely chemical stressors, which can be in the form of water quality, pollution, the presence of nitrogen compounds and metabolic waste substances. Biological stressors, can be density, parasites, microbes, fungi and bacteria. Physical stressors, in the form of sound, and light intensity. Procedural stressors include handling, transportation and disease management (Linder et al., 2013).

3.2 Erythrocytes

Erythrocytes are also known as red blood cells. The main role of erythrocytes is to transport oxygen from the lungs throughout the body of the fish. The results of the analysis of the monkfish's erythrocytes during the study period can be seen in the following Fig. 2.

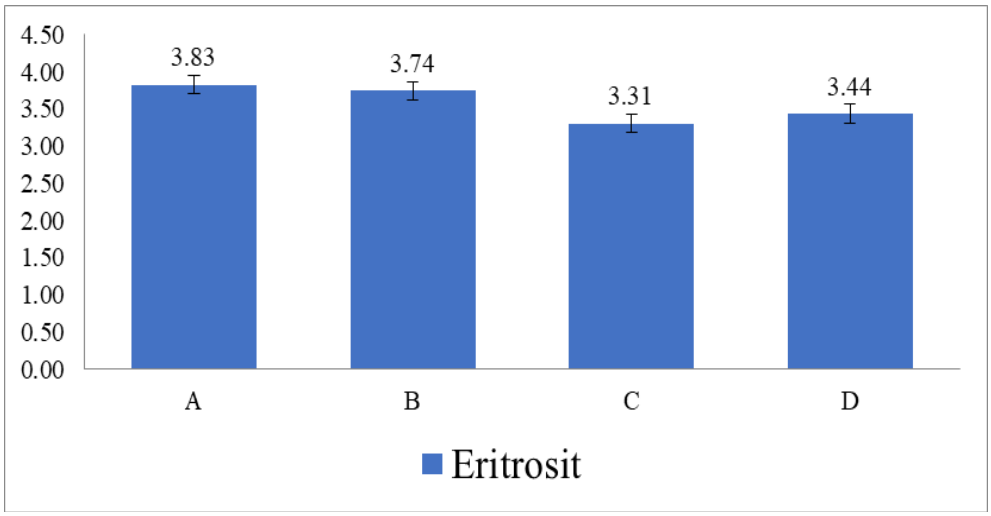

Fig. 2. Erythrocyte value of biawan fish (x 10 ^{6} / µL)

Based on Fig. 2, it can be seen that the percentage of erythrocytes in each treatment was different. But overall, the value of the red blood cells is still within normal limits. The value of the erythrocyte analysis of animal fish treated with feed substitution with a dose of L-Theanine, namely A as a control (3.83 x10 6 $^{/}$ µL), Treatment B (3.67 x10 ^{6}/µL), Treatment C (3.71 x10 ^{6}/µL) and Treatment D (3.18 x10 ^{6}/µL). The results of the normality test for the liliefors erythrocytes of the Biawan fish obtained a Li Max value of 0.438 < Li Table 5% 0.242, so it can be concluded that the data is normally distributed. The results of the Barlett variance homogeneity test obtained the value X2 count 1.685 <X2 table 5.991, which means homogeneous. The results of the ANOVA analysis of diversity showed that the calculated F value was 0.541 < F table 5%, namely 4.07, which meant that there was no difference between treatments.

The highest erythrocyte value during the study was given the animal feed containing L-Theanine, namely in treatment A (3.83 x 10 ^{6} / µL). In teleost fish, the normal number of normal red blood cells or erythrocytes ranges from 1.05 x 10 ^{6} – 3.0 x 10 ^{6} cells/mm ^{3} (Royan *et al.,* 2014). The value of the erythrocytes in this study ranged from 3.17-3.83 x10 ^{6} /µL. This amount is still within the reasonable limits of normal fish erythrocytes. This condition means that the addition of L-Theanine in the feed has a positive impact on the red blood cells' erythrocytes.

According to several studies, if there is an increase in red blood cells, it indicates the effort of the fish's body homeostasis in an effort to increase hemoglobin to bind oxygen. Fish that live in low-oxygen water will experience hematopoiesis so that their erythrocytes increase as an effort to adjust to the addition of oxygen.

Hidayah (2019) the value of erythrocytes given to the feed means that the addition of the plant extract has a positive impact on the erythrocytes of the Biawan fish. Erythrocytes ranged from 2.07-3.05 x10 ^{6} / µL. The red *blood cell count* (RBC) is the number of erythrocytes per cubic millimeter or microliter. Robert [15] as with hematocrit, low erythrocyte levels indicate anemia. Meanwhile, high levels indicate that fish are under stress [16].

The shape and small size of erythrocytes is an adaptive value for oxygen and carbon dioxide, namely as a carrier that can quickly spread throughout the network. The normal range for the number of fish erythrocytes in general is 20,000-3,000,000 cells/mm³, thus the number of fish erythrocytes studied is normal with a healthy category (Oktavia, 2011), animals that actively move will have a lot of erythrocytes because they will consume a lot of oxygen, Erythrocytes function as oxygen transport in the blood. The number of erythrocytes varies depending on age, sex, hormones and environment [17].

3.3 Blood Glucose

Blood glucose is glucose found in the blood which is formed from carbohydrates in food and stored as glycogen in the liver and skeletal muscles (Joyce, 2007). Blood glucose is a picture of the stress response as a result of the

release of the hormone cortisol in the hypothalamus through the bloodstream to the liver to remodel glycogen into glucose, so that blood glucose increases. When a fish encounters a stressful situation, such as a predator threat or environmental disturbance, the hypothalamus in the brain is activated. This triggers the release of corticotropin-releasing hormone (CRH) from the hypothalamus. Then CRH stimulates the anterior pituitary gland to release adrenocorticotropic hormone (ACTH) into the bloodstream. ACTH, in turn, stimulates the adrenal glands to release cortisol (also known as the stress hormone). Blood glucose is the main source of energy and an important element to support the metabolism of fish cells, especially brain cells. The results of the blood glucose analysis of the monkfish during the study period can be seen in the following Fig. 3.

Based on Fig. 3, it can be seen that the percentage of blood glucose in the monk fish was different for each treatment. But overall the blood glucose value of the monkfish is still within normal limits. Value of blood glucose analysis of animal fish treated with feed substitution with a dose of L-Theanine, namely A as a control (48.67 mg/dL), Treatment B (46.00 mg/dL), Treatment C (44.00 mg/dL) and Treatment D (65.33 mg/dl). The results of the normality test for the blood glucose lilies of monkfish obtained a Li Max value of 0.303 < Li Table 5% 0.242, so it can be concluded that the data is normally distributed. The results of the Barlett variance homogeneity test obtained the value X2 count - 9.422 < X2 table 5.991, which means homogeneous. Analysis results ANOVA diversity shows calculated F value 1,370 < F table 5%, which is 4,066 which means No There is difference between treatment. Glucose value blood without addition of L- Theanine tend more tall compared to the stockfish given Biawan fish feed containing L - Theanine.

Based on results analysis addition of L- Theanine with doses of 300mg/Kg and 600mg/Kg provide good results _ For push glucose blood of biawan fish. Generally, fish blood glucose levels that are considered normal range from 40-90 mg/dL. If the blood glucose state of the fish is not normal, it will interfere with the life of the fish and can even cause death. Giving L-Theanine to feed can help maintain and increase the immunity of the cattle fish.

The content of L-Theanine has been shown to be able to reduce blood glucose levels in Biawan fish that experience stress due to environmental changes during transportation. From the test results it can be concluded that treatment B with L-Theanine substitution 300 mg/L is the best treatment. This is based on the efficiency of the ingredients and the results which show normal levels in fish and the stability of the measurement results. Normal fish blood glucose levels contain 40-90 mg/dL, the blood glucose content is almost the same as blood glucose in humans, namely 70-110 mg/dl (Rahardjo et al., 2011).

According to the study Most of the glucose production is mediated by the action of cortisol which stimulates hepatic gluconeogenesis and also stops the peripheral uptake of sugar. The presence of blood glucose is determined by diet, time of end of meal, status of liver glycogen stores, developmental stage and season.

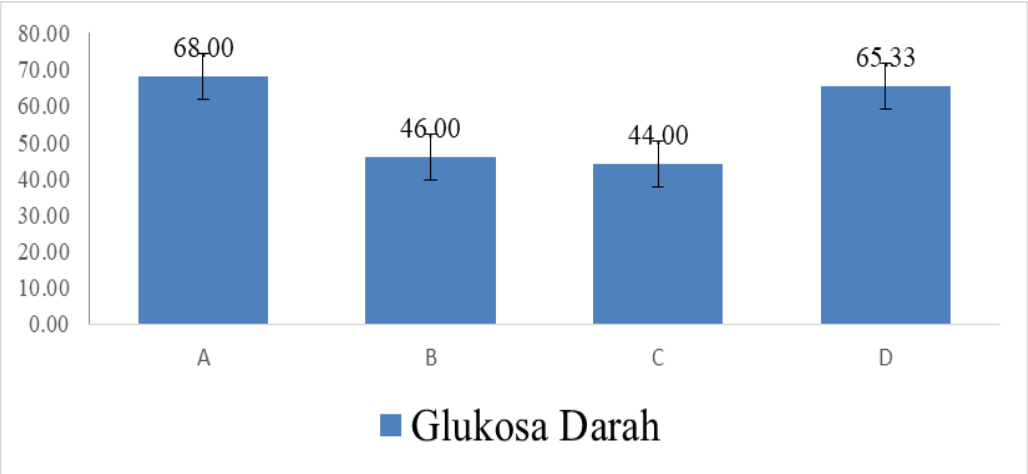

Fig. 3. Biawan fish blood glucose value (mg/dL)

High blood glucose levels stimulate the thyroid gland and increase the production of thyroxine. High thyroxine can trigger lymphocytopenia (low lymphocytes) in the blood. Then the sympathetic nervous system overreacts, which causes lymphatic contraction, increasing the respiratory rate and blood pressure. Stress in fish is defined as a number of physiological responses that occur when fish are trying to maintain homeostasis. When a fish experiences stress, it responds by developing a new homeostatic state by changing its metabolism.

Stress can increase blood glucose levels. Physically, stress can be seen from the behavior of fish, such as less aggressive movements, decreased fish appetite, and fish body color becomes dark. Changes in fish blood glucose can also be an indication of stress in fish caused by external factors in the aquatic environment such as drastic environmental changes, domestic and industrial waste pollution. Changes in environmental conditions will cause a high demand for blood glucose supply [18,19].

4. CONCLUSION

Addition feed with dose of L- Theanine 300 mg/kg is able maintain immunostimulant For push stress levels in animal fish in accordance with measurement hematology form hematocrit , erythrocytes and glucose blood.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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