Effect Of Giving Crude Extract Of Stink Bean Pod (Parkia Speciosa) On The Hematology Of Nile Tilapia (Oreochromis Niloticus) Infected With Pseudomonas Aeruginosa Bacteria

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Abstract: Regular intake of antibiotics can cause pathogenic resistance, pollute the environment, and threaten the consumers health. A natural material that can be utilized is stink bean pod (P. speciosa). This study was aimed to perceive the effect of crude extract of stink bean pod (P. speciosa) on the hematology of Nile tilapia (O. niloticus) infected with P. aeruginosa bacteria. The method employed in the study was experimental method, while the research design was Completely Randomized Design (CRD). The design of this study consisted of 4 treatments namely crude extract of stink bean pod (P. speciosa) at a dose of 10 ppm, 25 ppm, 40 ppm, and 55 ppm; and 2 controls namely positive control with 30 ppm Tetracycline antibiotics and negative controls with 3 replications. Blood samples were taken within12 hours, 24 hours, 36 hours, and 48 hours after infection. The observation results revealed that the lowest value of erythrocyte, hematocrit, and hemoglobin was within 24 hours after infection, while the highest value was within 48 hours after infection. In leukocyte and leukocyte differentials, the highest value was within 24-hour observation, while the lowest value was within 48-hour observation. Thus, giving crude extract of stink bean pod (P. speciosa) could affect the hematology of Nile tilapia (O. niloticus) infected with P. aeruginosa bacteria.

Index Terms: P. speciosa, P. aeruginosa, hematology, O. niloticus.

1 INTRODUCTION

Niletilapia (O. niloticus) aquaculture has developed rapidly in Asia, especially in Indonesia, but it still requires encouragement in order to become the mainstay of fisheries commodity, especially in aquaculture areas(1). The increase of Nile tilapia production can be seen from the statistical data that is quite significant in the last five years; the production rose 4.95 million metric tons (estimated value, $10.3 billion). Indonesia ranked third as the producer of cultured Nile tilapia in the world after China Indonesia, and Egypt being the three largest producers(2). There are some advantages of Nile tilapia such asable to adapt to environmental conditions with a wide range of salinity, easy to obtain, having specific flavor and firm texture, easy to be served, having less thorns, and inexpensive (3). Fish disease is a major obstacle in aquaculture businesses since it can cause several disadvantages such as decreased production, decreased water quality, and even total death. Fish disease can be caused by several types of pathogens such as viruses, parasites, fungi and bacteria, and several types of bacteria that commonly attack fresh water fish (4). One type of Pseudomonas bacteria that spread fast is P. aeruginosa. These bacteria have zoonotic properties that can transmit diseases from animals or fish to humans and vice versa (5). The use of antibiotics is indeed able to prevent disease. However, the overuse of antibiotics can cause pathogenic resistance, pollute the environment, and threaten the consumers health.

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Therefore, the use of natural material can be an alternative to cure diseases Nile in tilapia due to bacterial infections(6). A natural material that can be utilized as an alternative is stink bean pod (P. speciosa). Stink bean pod has some benefits such as having antioxidants, reducing stress, having anti-inflammatory property, and inhibiting bacteria because it has almost all-important chemical compounds such as tannins, terpenoids, thiazolidine-4-carbosilic acid, phenols, flavonoids, alkaloids, cyclic polysulfides, and djenkolik acid (7). Thus, it is necessary to explore the crude extract of stink bean pod as a material that can be used to overcome the problem of bacterial infection in fish. This study was aimed to determine the effect and ideal dose of crude extract of stink bean pod (P. speciosa) on the hematology of Nile tilapia (O. niloticus) infected with P. aeruginosa bacteria.

2 MATERIAL AND METHOD

2.1 Research Design

Completely Randomized Design (CRD) consisting of 4 treatments was employed in this study. The treatments were crude pod extract of stink bean (P. speciosa) at the dose of 10 ppm, 25 ppm, 40 ppm, 55 ppm; and there were 2 controls consisting of positive control with 30 ppm Tetracycline antibiotics and negative control with 3 replications. The blood samples were taken within 12 hours, 24 hours, 36 hours, and 48 hours after infection.

2.2 Crude Extraction of Stink Bean Pod

Stink bean pod powder was soaked (maceration) by dissolving 500 grams of simplicia powder with 2,500 mL of ethanol 96% in a glass jar. Then, it was closed by using plastic wrap and then kept for 72 hours (3 days). Next, the sample was filtered by using filter paper in order to obtain filtrate and waste. The filtrate was evaporated with a Rotary Evaporator to obtain the crude extract of stink bean pod. The extract from evaporation was put into a film bottle and wrapped with aluminum foil and stored in a refrigerator. The extract was phytochemically tested

to detect the compounds contained in the stink bean pod.

2.3 Invitro Test

This test was conducted to determine the ability to inhibit bacterial growth by giving P. speciosa extract. The inhibition zone was marked by the presence of a clear zone formed around the blank disk. The medium used was PSA. The clear zone diameter was measured by using calipers (mm).

2.4 Hematology Test

Blood Samples Collection

The fish blood samples were taken by using a 1 ml syringe. Before being used, the syringe was rinsed with Na citrate as an anti-coagulant. Then, the blood was drawn at the caudal peduncle with a 45° needle position and was pulled slowly until the blood entered the tube. The blood samples were stored in eppendorf tube.

Erythrocyte

At red blood cell (erythrocytes), blood samples mixed with Na citrate were taken by using thomaerythrocyte pipettes to a scale of 0.5. Then, the Hayem's solution was also taken until the scale showed the number 100. The pipettes were homogenized and the firth four drops were discarded. The next drop was dropped on the hemocytometer and covered with a glass cover. After that, it was observed under a microscope at 1000x magnification and the total erythrocyte was calculated. The erythrocyte calculation formula is as follows:

$$Total\ Erythrocyte = \sum N\ x\ 10^4 cell/mm^3$$

Note :N : Counted erythrocytes

Leukocyte

At red blood cell, blood samples mixed with Na citrate were taken by using thoma leukocyte pipettes to a scale of 0.5. Then, the Turk's solution was also taken until the scale showed the number 101. The pipettes were homogenized and the first four drops were discarded. The next drop was dropped on the hemocytometer and covered with a glass cover. After that, it was observed under a microscope at 1000x magnification and the total leukocyte was calculated. The leukocyte calculation formula is as follows:

$$Total\ \ Leukocyte = \sum N\ x\ 50\ cell/mm^3$$

Note :N : Counted leukocyte

Leukocyte Differential

The blood sample was dropped on the first object glass. The second object glass was placed 45° above the first object glass. Then, the object glass was slide in the opposite direction to form a thin layer of blood. Next, the specimen was left to dry, fixed with methanol for 5 minutes, rinsed with distilled water and dried again. After that, it was given 10% Giemsa's solution for 10 minutes, rinsed with distilled water, and dried again. Next, the specimen was observed under a microscope at 1000x magnification in order to count the lymphocyte, monocyte and neutrophil. The formula to count the percentage of leukocyte differential is as follows:

Note :

L : Total lymphocytes

M : Total monocytes

N : Total neutrophils

Hematocrit

Hematocrit level was measured by dipping one tip of a microhematocrit tube into an Eppendorf tube containing blood. Blood would flow in capillary motionfilling ¾ of the tube. The tip of the tube containing blood was clogged by using crystoceal and centrifuged by using a hemofuge at a speed of 5000 rpm. Next the length of the blood that settled (a) and the total length of blood volumein the tube (b) were measured (8). The formula

$$\%\ Lymphocyte = \frac{L}{100} x100\%$$

$$\%\ Monocyte = \frac{M}{100} x100\%$$

$$\%\ Neutrophil = \frac{N}{100} x100\%$$

for measuring hematocrit level is as follows:

$$Hematocrit = \frac{a}{b} x100\%$$

Note:

a : Length of the settled blood

b : Total length of blood in tube

Hemoglobin

The procedure for calculating hemoglobin level was based on the Sahli's method. First, the blood sample was sucked by using a Sahli pipette up to a scale of 20 mm^3 or on a scale of 0.2 ml. Then, the tip of the pipette was cleaned with a tissue. Next, the blood in the pipette was transferred into the Hb-meter tubefilled with 0.1 N HCl up to a scale of 2. After that, the blood was stirred with a stirring rod to make it homogeneous. Next, distilled water was added to the tube until the blood color turned into the color of standard solution in the Hb-meter. Hemoglobin level was expressed in G%.

Supporting Parameter

Supporting parameters in this study were observation of clinical symptoms, water quality and survival rate of fish during the study.

Data Analysis

The data obtained were then analyzed statistically by using analysis of variance or F test (ANOVA). It was performed to perceive the effect of the treatment (independent variable) on the response of measured parameter or F test. If the F test value shows significant difference or highly significant difference, then Least Significant Difference test (LSD) is performed to determine the difference between the two treatments.

3 RESULTS AND DISCUSSION

The Disk Test

On the invitro test using the disk test, it was found that each treatment of crude extract of the stink bean pod (P. speciosa) indicated different abilities to inhibit bacterial growth. In this study, The presence of clear zone indicated that the crude extract of stink bean pod (P. speciosa) was able to inhibit the growth of P. aeruginosa bacteria. From the above treatment, the highest resulted was treatment D with a dose of 55 ppm,

while the lowest resulted was treatment A with a dose of 10 ppm. Compound contained in the crude extracts of stink bean pod (P. speciosa) serves as antioxidant, antibacterial, antiviral, anti-inflammatory, anti-allergic, and anti-cancer (9). If more extract is given, the ability to inhibit the bacterial growth will be higher. It has been proven that giving crude extract of stink bean pod (P. speciosa) can inhibit the growth of P. aeruginosa bacteria.The average graph of the disk testare shown in Figure 1.

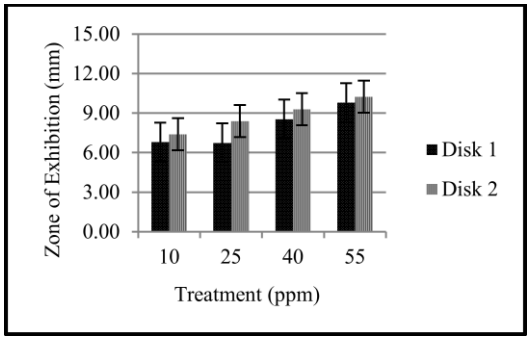

Fig. 1. The averagegraph of the disk test. Disk 1 and disk 2 were repeated treatments.

Blood Profile

Erythrocyte

In this study, The highest resulted were shown by treatment D with a dose of 55 ppm, while the lowest resulted was treatment A with a dose of 10 ppm. Based on the results, the total erythrocytes decreased at 12-hour and 24-hour observation. Decrease in total erythrocytes indicates that fish is in a state of stress and an indication of foreign material in the body (10). However, there was an increase in erythrocyte production at 48-hour observation with a range of 1.58 x 10^6 cell/mm^3 to 1.81 x 10^6 cells/mm^3 due to the reaction of crude extract of stink bean (P. speciosa) to P. aeruginosa bacteria.

The total red blood cells in fish affected by pathogens tend to decrease until the end of the observation (11). Erythrocyte has a function to supply oxygen to the fish's body. Giving crude extract of stink bean pod (P. speciosa) has been proven to increase the number of erythrocytes and inhibit the growth of P. aeruginosa bacteria (12). The average graph of erythrocyte are shown in Figure 2.

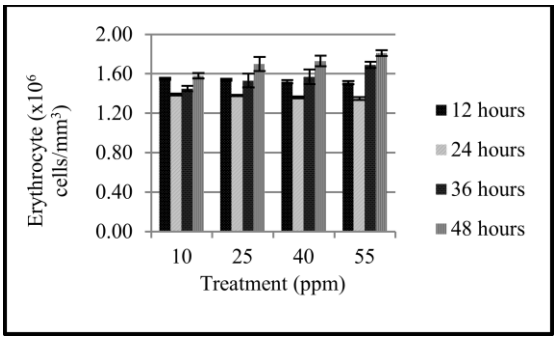

Fig. 2. The average graph of erythrocyte.

Leukocyte

Based on the data obtained, total leukocytes increased until the 24-hour observation, then it decreased after the 36-hour observation. The total leukocytes increased at 24-hour observation. Unhealthy fish will produce many leukocytes to phagocyte bacteria and synthesize antibodies(13). In this research, there was a decrease in leukocyte production at 48-hour observation with a range of 14.30 x 10^4 cells/$mm^3$to 16.45 x 10^4 cells/mm^3 due to the reaction of crude extract of stink bean (P. speciosa) to P. aeruginosa bacteria. The lowest resulted was treatment D with a dose of 55 ppm since it reached the normal limit. Meanwhile, the highest resulted was treatment A with a dose of 10 ppm due to the fact that leukocytes act as the body's defense system that prevents pathogens attack. More leukocytes will develop into the area of infection as a defense action of the body (13). An increase in the number of leukocytes is a reaction from fish resistance which also escalates. In addition, an increase in the total leukocytes of infected fish is presumably due to the nature of leukocytes that are active or move towards infected or damaged organs. If the infection has been treated, the total leukocytes will be the same as its initial number (10).The average graph of leukocyte are shown in Figure 3.

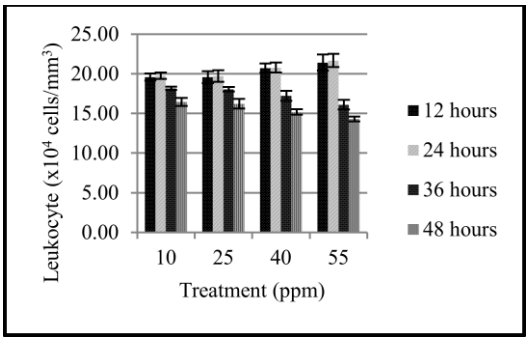

Fig. 3. The average graph of leukocyte.

Leukocyte Differential

Lymphocyte

Based on the results of this study, the total lymphocyte increased within 24-hour observation (14), the increase in lymphocytes produced by Nile tilapia has a significant role in increasing the immune response of Nile tilapia against disease and infection. In this research, there was a decrease in lymphocyte production within 48-hour observation with a range of 84.67% to 88.00% due to the reaction of crude extract of stink bean (P. speciosa) to P. aeruginosa bacteria. The lowest resulted was treatment D with a dose of 55 ppm, while the highest resulted was treatment A with a dose of 10 ppm. Lymphocytes have a role in producing antibodies and maintaining the fish body condition to stay healthy (15). It has been proven that giving crude extract of stink bean pod (P. speciosa) can decrease the total lymphocyte and inhibit the growth of P. aeruginosa bacteria.The average graph of lymphocyte areshown in Figure 4.

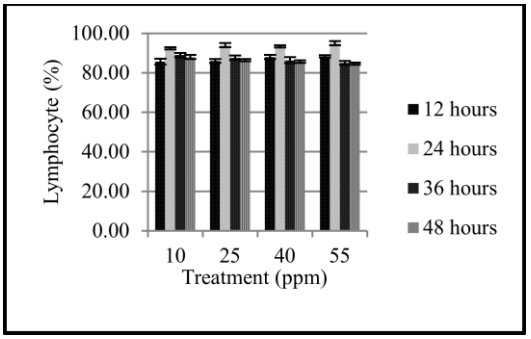

Fig. 4. The average graph of lymphocyte..

Monocyte

Based on the results of this study, Thus, it could be concluded that the total monocytes increased within 24-hour observation. There is an infection caused by a foreign material, the monocytes will transport quickly leaving the blood vessels to the infected area to do phagocytosis (16). In this research, there was a decrease in monocyte production within 36-hour and 48-hour observation with a range of 4.33% to 7.67% due to the reaction of crude extract of stink bean (P. speciosa) to P. aeruginosa bacteria. The lowest resulted was treatment D with a dose of 55 ppm since the total monocytes reached the normal limit. Meanwhile, the highest resulted was treatment A with a dose of 10 ppm due to the fact that monocytes act as the immune system that prevents pathogens attack. Explained that monocytes function as macrophage agents that phagocyte or swallow foreign material in the body (17). It has been proven that giving crude extract of stink bean pod (P. speciosa) can decrease excessive number of monocytes and inhibit the growth of P. aeruginosa bacteria.The average graph of monocyte areshown in Figure 5.

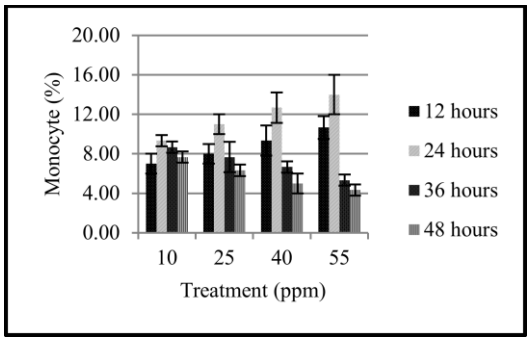

Fig. 5. The average graph of monocyte.

Neutrophil

Based on the results of this study, it could be concluded that neutrophils increased within 24-hour observation. An increase in total neutrophils is a result of the immune mechanism that works in response to an infection in the body (18). However, there was a decrease in neutrophil production within 36-hour and 48-hour observation with a range of 11.33% to 15.00% due to the reaction of crude extract of stink bean (P. speciosa) to P. aeruginosa bacteria. The lowest resulted was treatment D with a dose of 55 ppm since it reached the normal limit. Meanwhile, the highest resulted was treatment A at a dose of 10 ppm since neutrophils function to attack foreign materials. This is in accordance with Havixbeck et al.'s explanation (1988) which mentioned that the main function of neutrophils is destructing foreign materials through phagocytosis process. It has been proven that giving crude extract of stink bean pod (P. speciosa) can decrease excessive number of neutrophil and inhibit the growth of P. aeruginosa bacteria. The average graph of neutrophil areshown in Figure 6.

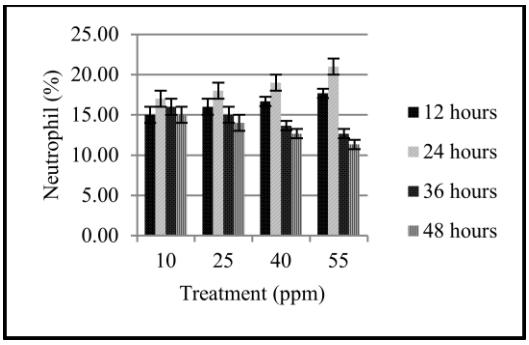

Fig 6. The average graph of neutrophil.

Hematocrit

Based on the results of this study, it could be concluded that the hematocrit level decreased within 12-hour and 24-hour observation. The decrease in the percentage of hematocrit level is caused by numerous infections (19). In this study, there was an increase in hematocrit level within 36-hour and 48-hour observation with a range of 30.67% to 35.00% due to the reaction of crude extract of stink bean (P. speciosa) toP. aeruginosa bacteria. The highest resulted was treatment D with a dose of 55 ppm since it reached the normal limit. Meanwhile, the lowest resulted was treatment A at a dose of 10 ppm due to erythrocyte damage caused by bacterial infection which led toa decrease in hematocrit level. Infection and stress can interfere the immune system by giving a negative impact on fish growth and affecting the blood composition including total erythrocytes and hematocrit level (20). It has been proven that giving crude extract of stink bean pod (P. speciosa) can increase hematocrit level and inhibit the growth of P. aeruginosa bacteria.The average graph of hematocrit are shown in Figure 7.

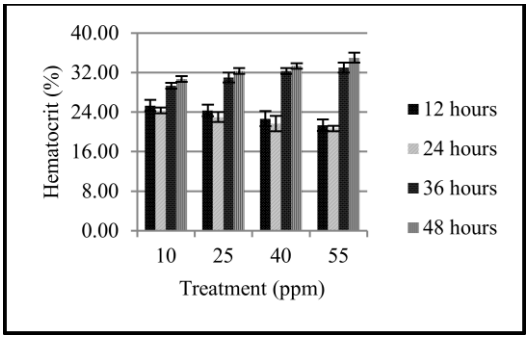

Fig. 7. The average graph of hematocrit

Hemoglobin

Based on the results of this study, it could be concluded that the hemoglobin level decreased within 12-hour and 24-hour observation. Hemoglobin level that is below the normal range indicates low level of protein in fish feed, deficient vitamin, and poor water quality or infected fish (21). In this study, there were an increase in hemoglobin level within 36-hour and 48-hour observation with a range of 5.80 G% to 6.70 G% due to the reaction of crude extract of stink bean (P. speciosa) to P. aeruginosa bacteria. The highest resulted was treatment D with a dose of 55 ppm since it reached the normal limit. Meanwhile, the lowest resulted was treatment A at a dose of 10 ppm due to the fact that hemoglobin is a content of red blood cell pigment that binds oxygen to produce energy. Hemoglobin determines the immunity level in fish because it possesses a close relation with the binding of oxygen by the blood (22). It has been proven that giving crude extract of stink bean pod (P. speciosa) can increase hemoglobin level and inhibit the growth of P. aeruginosa bacteria.The average graph of hemoglobin are shown in Figure 8.

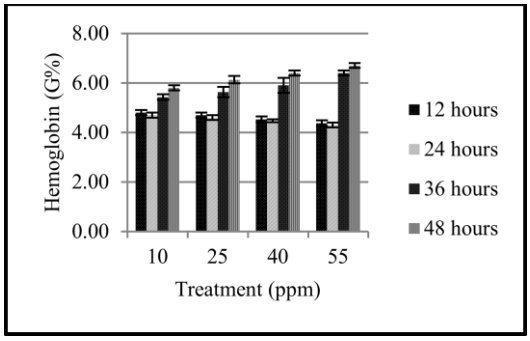

Fig.8. The average graph of hemoglobin

The Clinical Symptoms

The clinical symptoms of Nile tilapia (O. niloticus) before being infected with P. aeruginosa bacteria were bright and having clean body color, having complete organs, and able to swim normally. While the clinical symptoms of Nile tilapia (O. niloticus) after being infected with P. aeruginosa bacteria were indicated some changes including pale body color, partial peeled scales, protruding eyes, slowing operculum movements, passive swimming on the water surface, and lack of appetite. Lack of appetite in fish may be caused by stress as a result of the test treatment. The symptoms of fish infected with these bacteria are red lumps at the base of the pectoral fin, swollen abdomen, full body ulcers, internal bleeding, bleeding around the mouth, opercula, and necrosis in the spleen and kidney tissue (23). Other symptoms of Nile tilapia (O. niloticus) infected with P. aeruginosa bacteria are pale body color, protrusion of the eyeball (exophthalmia), flaky scales, flaky fins, severe wounds in infected areas, and weak swimming ability (24).

Survival Rate

It could be concluded that the survival rate of Nile tilapia increased due to the ability of crude extract of stink bean pod (P. speciosa) to inhibit the growth of P. aeruginosa bacteria and improve the survival rate of Nile tilapia. The highest resulted was treatment D with a dose of 55 ppm, while the lowest resulted was treatment A with a dose of 10 ppm. Biotic factors affecting survival rate are competitors, parasites, predation, fungi, population density, adaptability of animals and human handling. Meanwhile, abiotic factors affecting survival rate are physical and chemical nature of an aquatic environment (25).The average graph of survival rate areshown in Figure 9.

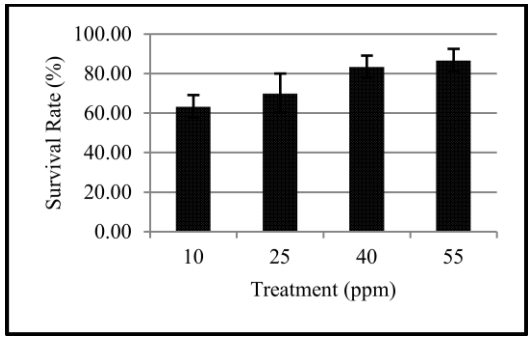

Fig.9. The average graph of survival rate.

Water Quality

Based on the observation of water quality, the data obtained included temperature, dissolved oxygen, and pH which were performed during 14days offish rearing period. The range of temperature measurement was 25.2-26.8^{o}C, range of Dissolved Oxygen (DO) was 5.5-8.4 mg/L, and range of pH level was 7.11-8.14. The results of water quality measurement were still in the normal range and could be tolerated by fish.

4 CONCLUSION

Based on the research that was carried out, it could be concluded that giving crude extract of stink bean pod (P. speciosa) affected variation the hematology of Nile tilapia (O. niloticus) infected with P. aeruginosa bacteria. Then, the ideal dose of crude extract was 55 ppm (treatment D).

5 ACKNOWLEDGMENT

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