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Effectiveness of multiherbal leaf extract for the treatment of *Oreochromis niloticus* infected with pathogens

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ABSTRACT

The many herbal plants in Indonesia that contain antimicrobial substances such as *Calotropis gigantea*, *Moringa oleifera* and *Cassia alata* L. leaves are attractive and have the potential to be used to prevent and treat bacterial diseases in fish. This study aims to determine the effectiveness of administering a combination of multi-herbal ingredients, namely *M. oleivera, C. gigantea* and *C. alata* L. leaves in the treatment of *O. niloticus* tilapia infected with the pathogenic bacteria *S. agalactiae*. The experimental design used a completely randomized design with 6 treatment levels and 3 replications, namely treatment A (Fish that were not infected with *S. agalactiae*), B (Fish infected with *S. agalactiae*), C (Fish infected with *S. agalactiae* + soaked in thistle leaf extract 800 ppm+ *Moringa* leaf extract 1000 ppm)), D (Fish infected with *S. agalactiae* + soaked in *C. gigantea* leaf extract 800 ppm + *C. allata* leaf extract 10 ppm), E (Fish infected with *S. agalactiae* +soaked in *Moringa* leaf extract 1000 ppm + *C. allata* leaf extract 10 ppm) and F (*Moringa* leaf extract 1000 ppm+*C. gigantea* leaf extract 800 ppm + *C. allata* leaf extract 10 ppm). The results showed that the combination of *M. oleivera, C. gigantea* and *C. alata* L. extracts had a significant effect ($P<0.05$) on the survival rate of *O. niloticus*. Treatment (E), combination of *M. oleivera* leaf extract 1000 ppm + *C. alata* L leaf extract 10 ppm, showed the fastest healing of the body and the highest survival rate of tilapia, namely 80%.

Introduction

The cultivation commodity of freshwater tilapia (*Oreochromis niloticus*) is a fish that has important economic value and is easy to maintain. However, in cultivating these fish there are still several obstacles, including often being attacked by Streptococcosis. Streptococcosis disease in tilapia is caused by the pathogenic bacteria *Streptococcus agalactiae* (Gardenia *et al.* 2011; Suhermanto *et al.*, 2019). Nafiqoh *et al.* (2021) mentioned that the most common disease attacking tilapia farming activities is the bacteria *S. agalactiae*. Improving the immune system of farmed fish is an effective way to overcome this problem.

Improving the immune system can be done by immunostimulants. Immunostimulants are natural ingredients in the form of chemicals or drugs that can increase non-specific immune responses. Maryani and Rosdiana (2020) said that developing body resistance with environmentally friendly immunostimulants is the right choice, namely medicinal plants or phytopharmaceuticals. This natural ingredient acts as an antioxidant, but also increases the body's resistance to environmental aggressors (Chandra *et al.*, 2023; Akbar & Hasan, 2024).

Research related to the use of herbal ingredients from various plants containing secondary metabolites as antimicrobial substances has been very developed both nationally and globally. The large number of herbal plants in Indonesia that contain antimicrobial substances such as *Calotropis gigantea* leaves, *Moringa oleifera* leaves and *Cassia alata* L. leaves

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are attractive and have the potential to be used to prevent and treat bacterial diseases in fish (Arisa *et al.*, 2024). So far, the application of herbal ingredients as antimicrobials in fish infected with pathogens has been studied for each type of herbal, even though some of these herbal ingredients contain the same antimicrobial substances.

Research related to the combination of herbal ingredients or the use of multi-herbs has been carried out by Pratama *et al.* (2017), namely the use of noni fruit, papaya and turmeric by combining these ingredients is known to produce an effective and efficient formula in inhibiting *Aeromonas hydrophila*. Several other herbal ingredients that were tested on fish and shrimp were reported to also act as antimicrobials, including *Moringa oleivera*, *Calotropis gigantea* and *Cassia alata* L. (Arisa *et al.* 2021a; Arisa *et al.* 2021b; Arisa *et al.*, 2022). However, combinations related to the use of herbal ingredients *M. oleivera, C. gigantea* and *C. alata* L. have not been reported. Therefore, researchers will study the effectiveness of multiherbal leaf extracts for treating fish infected with pathogenic bacteria. The aim of this study was to determine the effectiveness of administering a combination of multi-herbal ingredients, namely *M. oleivera, C. gigantea* and *C. alata* L. leaves in the treatment of *O. niloticus* tilapia infected with the pathogenic bacteria *S. agalactiae.*

Materials and Methods

Location and Time

The research was conduted was from July to August 2023, at the Fish Hatchery and Breeding Laboratory, Faculty of Marine and Fisheries, Faculty of Veterinary Medicine, Syiah Kuala University and FKIP Chemistry Laboratory and Clinical Laboratory,, Banda Aceh.

The experimental design used a completely randomized design with 6 treatment levels and 3 replications, namely treatment A (Fish that are not infected with *S. agalactiae*), treatment B (Fish infected with *S. agalactiae*), treatment C (Fish infected with *S. agalactiae* + soaked in thistle leaf extract 800 ppm+ Moringa leaf extract 1000 ppm)), treatment D (Fish infected with *S. agalactiae* + soaked in *C. gigantea* leaf extract 800 ppm + *C. allata* leaf extract 10 ppm), treatment E (Fish infected with *S. agalactiae* + soaked in Moringa leaf extract 1000 ppm + *C. allata* leaf extract 10 ppm) and treatment F (Moringa leaf extract 1000 ppm + *C. gigantea* leaf extract 800 ppm + *C. allata* leaf extract 10 ppm). The test herbal ingredients were extracted referring to research by Arisa *et al.* (2024).

Test Fish

This research used 180 test fish measuring 7-8 cm in length. The test fish were put into the rearing container as many as 10 fish/container. The injection dose of the pathogen *S. agalactiae* in the test fish was 10^{-7} CFU ml^{-1} (Suhermanto *et al.,* 2020). Before infecting the pathogenic bacteria *S. agalactiae*, tilapia fry were first fasted for 24 hours. Sick fish after S. agalactiae infection will show clinical symptoms in the form of macroscopic symptoms including: swimming patterns, changes in the anatomy of external organs such as protruding eyes, hemorrhage, and peeling scales. The test fish were fed 3 times a day, namely at 07.00 AM, 12.00 AM and 17.00 PM.

Taking fish blood samples

Blood samples are taken using a syringe in the caudalis vein which is located just ventral to the vertebral bone. The blood that has been taken is then put into a tube and mixed thoroughly with anticoagulant in the form of EDTA. Blood samples were taken after infection on days 0, 1, 3, 5, 7 and 14. Each blood taken from each treatment was put into a microtube for testing and the blood image was seen (Dianti *et al.*, 2013).

Observed parameters

The research parameters measured were observations of clinical symptoms of sick fish, blood picture, survival and water quality of cultivation media. Observing clinical symptoms of sick fish means observing the behavior of the fish and changes that occur in the fish's body. Next, observing the blood picture that is measured is leukocytes, erythrocytes, hematocrit and hemoglobin.

a. The calculation of the number of leukocytes refers to Blaxhall and Daisley (1973), namely sucking a blood sample using a pipette containing a white stirrer to a scale of 0.5 ml, then adding a scale 11 turk solution, swinging it in the shape of a number 8 for 5 minutes until mixed evenly. Then throw away the first drop and then drop it into the hemacytometer and cover it with a cover glass, then observe it under a microscope. calculations were carried out on a small box hemacytometer.

$$\sum Leukocyte = Leukocyte\ x\ 50\ cell/mm^3 \quad (1)$$

b. Hematocrit calculation of hematocrit levels (He) was measured according to Anderson and Siwicki (1993). Determination of He levels is

carried out by: placing the blood sample into a microhematocrit tube up to approximately ¾ of the tube, then covering it with crytoseal to a depth of 1 mm. After that, centrifuged at a speed of 5000 rpm for 5 minutes. After that, the length of the blood that settles (a) and the total length of the blood volume contained in the tube (b) are measured. The levels are expressed as % volume of blood cell solids.

$$He\ (\%) = \left(\frac{a}{b}\right) x100\% \quad (2)$$

c. Hemoglobin (Hb) levels were measured using the Sahli method with a salinometer (Wedemeyer and Yasutake, 1977). First, the blood is sucked with a sahli pipette to a scale of 20 mm3 or a scale of 0.2 ml, then the tip of the pipette is cleaned with tissue paper. After that, the blood in the pipette was transferred into an Hb-meter tube filled with HCL 0.1 N scale 10 (red), stirred and left for 3-5 minutes. Add distilled water until the color of blood and HCl becomes the color of the standard solution on the Hb meter. Then read the scale, namely by looking at the surface of the liquid and checking it with a sahli tube scale which is visible on the gr % line scale (yellow) which means the amount of hemoglobin in grams per 100 ml of blood.

d. Survival rate

$$SR = \frac{Nt}{No} x100\%$$

Note: SR = Fish survival (%); Nt = Number of live fish at the end of rearing (fish), No = Number of fish at the beginning of rearing (fish).

Data Analysis

The survival rate parameters of tilapia were analyzed using the ANOVA test. If they have an effect, they will be tested further. then the results of observations of clinical symptom parameters of sick fish and hematology (leukocytes, hematocrit, hemoglobin) are presented descriptively.

Result

Clinical symptoms of tilapia that have been infected with *S. agalactiae* bacteria show that the fish has decreased appetite, dark body color, peeling scales, swimming at the bottom of the container, thin tail fins, hemorrhage on the skin, eyes and corneas are not bright (pale) and there are protrusions (lateral exophthalmi) (Figure 1). However, after being soaked with multi-herbal extracts, the fish experienced healing, namely the fish's body color returned to normal, the hemorrhage on the skin began to improve/heal, the tail fin grew as before/normal and the eye color returned to normal and there were no more eye protrusions (Figure 2).

Figure 1. Sick fish infected with S. agalactiae bacteria, a) eye protrusion; b) hemorrhage; c) flaky fins

Figure 2. Fish that have experienced recovery after soaking in multi-herbal ingredients

The results of observations of the blood picture parameters of healthy, sick tilapia and those treated with multi-herbal extracts changed during the study (Table 1-5).

Table 1. Description of tilapia leukocyte values

Treatment	Leukocyte Value x 10^3 (sel mm^{-3})					
	D0	D1	D3	D5	D7	D14
A	146,2	146,2	146,2	146,2	146,2	146,2
B	146,2	283,8	279,1	273,3	272,3	270
C	146,2	283,8	248,7	246,2	240,6	239,4
D	146,2	283,8	218,9	218,1	215,5	196,5
E	146,2	283,8	157,5	154,5	152,8	143,5
F	146,2	283,8	177,7	176,20	174,8	173,3

Table 2. Description of tilapia hamatocrit values

Treatment	Hematocrit Value (%)					
	D0	D1	D3	D5	D7	D14
A	21	21	21	21	21	21
B	21	13	15	14	16	17
C	21	13	16	15	17	18
D	21	13	16	17	19	20
E	21	13	17	18	20	26
F	21	13	15	16	18	19

Table 3. Description of tilapia hemoglobin values

Treatment	Hemoglobin Value (g/dL)					
	D0	D1	D3	D5	D7	D14
A	5	5	5	5	5	5
B	5	3,3	4	3,8	3,2	4
C	5	3,3	4,9	4,8	4,9	3,9

D	5	3,3	4,8	4,3	4,7	5
E	5	3,3	3,7	4,2	4,9	5,2
F	5	3,3	4,5	3,9	4,5	4,9

Table 4. Survival rate of tilapia during rearing

Treatment	Survival rate (%)
A	70,00 ± 10,00^{b}
B	36,66 ± 5,773^{a}
C	63,33 ± 5,773^{b}
D	73,33 ± 5,773^{b}
E	80,00 ± 10,00^{b}
F	65,00±0,00^{b}

Table 5. Data on water quality parameters during the research

Treatment	Suhu (^{0}C)	pH	DO (mg L^{-1})
A	28-29	6,5-7,5	5,1-5,5
B	28-29	6,7-7,3	5,2-5,6
C	29-30	7,1-8,2	5,1- 6,1
D	28-30	7,1-8,1	5,5- 6,3
E	28-30	7,1-8,3	6,1- 6,9
F	28-30	7,1-8,4	6,1- 6,8

Discussion

The results of observations on tilapia that had not been infected with Streptococcus agalactiae bacteria showed normal symptoms such as high appetite, active swimming, bright body color and scales that did not peel. According to Arie (2007) states that in general the characteristics of healthy tilapia are active movement, high appetite, bright skin color, scales that do not peel off and clear eye color. Meanwhile, after the tilapia fish were infected with bacteria, tests showed symptoms such as decreased appetite, dark body color, peeling scales, swimming at the bottom of the container, thin tail fins, hemorrhage on the skin, eyes and corneas were pale and appeared like lumps. According to Suhermanto *et al.* (2020), the occurrence of clinical symptoms in tilapia as a result of S. agalactiae infection may not occur partially, but there is a positive correlation in all organs in the fish's body. In general, the clinical symptoms shown by fish infected with S. agalactiae are eye abnormalities (exophthalmia, purulence, opacity), loss of balance in swimming (whirling disease), decreased appetite, red spots, and darker body color (Hardi, 2011; Dwinanti *et al.*, 2014; Lestari *et al.*, 2024). Test fish that have been infected and show a diseased condition are then soaked in *Moringa oleivera, Calotropis gigantea* and *Cassia alata* L. leaf extract for 24 hours. The application of multi-herbal extracts through a soaking system can simplify the treatment process, especially for small tilapia fish on a large scale. After immersion in multi-herbal extracts in tilapia fish showed changes in morphology and behavior. Changes in behavior are marked by the fish's response to food continuing to improve and the way the tilapia swims also looks more normal every day. The recovery of sick fish was clearly visible on the 7th day after immersion in the multi-herbal extract, marked by the peeling scales and tail fins which had previously been shriveled and their appetite starting to grow back. Based on observations during the research, the fastest fish recovery was in treatment E (*M. oleivera* leaf extract 1000 ppm + *C. alata* L leaf extract 10 ppm). Meanwhile, treatments C, D, F experienced healing on the 9th day after soaking and treatment B experienced a very long healing, namely on the 13th day, this was because treatment B was infected with *S. agalactiae* but was not given multi-herbal extract, so healing long time and also causes high mortality in tilapia fish.

Hematological profiles can provide valuable information regarding the internal health condition of aquatic organisms (Khalil *et al.*, 2023). Leukocytes are the most active unit of the body's defense system and circulate in the blood circulation in various types. Leukocytes in tilapia are part of the body's non-specific defense system. A decrease in the number of leukocytes is called leukopenia while an increase in the number of leukocytes is called leukocytosis. The leukocytes produced will be high if there is a viral infection in the tilapia's body and the fish's body is trying to fight it (Azhar, 2013). The number of fish leukocytes during the study ranged from 146.2 - 283.8 x 10^{3} cells mm-1. The number of leukocytes increased significantly after *S. agalactiae* bacterial infection and decreased again several hours later. The significant increase in the number of leukocytes after infection is thought to be a form of the body's resistance to pathogens that enter the body, resulting in the production of leukocyte cells in large numbers. According to Sugito *et al.* (2014), leukocytes will decrease if the fish is under stress. Leukocytes will increase when fish are infected as a form of the body's immune response against microorganisms. Healthy fish have lower white blood cells than sick fish (Utami, 2009).

Normal hematocrit levels in teleost fish range from 20-30% (Bond, 1979). Based on the results of observations from days 1 to 14, the hematocrit parameter value for post-infection tilapia ranged from 13-26%. While the fish is still in the healing process, the hematocrit value also increases. Royan *et al.* (2014) stated that this increase was caused by the fish starting to adapt to the new environment, so that the fish started to eat slowly like normal activities, so that there was a supply of nutrients in the body.

Based on the analysis of variance (ANOVA) test, immersion in multi-herbal extracts had a significant effect ($P<0.05$) on the survival of tilapia fish. Tilapia

survival ranges between 36% - 80%. The lowest survival percentage was found in the treatment that was not soaked in *M. oleivera, C. gigantea* and *C. alata* leaf extract, namely in treatment B with a value of 36%. Meanwhile, treatments that were soaked in multi-herbal extracts had a very high survival rate, namely 63-80%. This is because the addition of multi-herbal leaf extract can help improve the fish's immune system so that the survival rate is higher. According to Savitri *et al.* (2018) *M. oleifera* leaves contain antibacterial compounds in the form of suckedonin, triterpenoids and tannins which have a working mechanism to damage bacterial cell membranes.

The quality of water in the rearing media plays a very important role in the survival of fish, including temperature, acidity (pH) and dissolved oxygen, DO (Dissolved oxygen). Water quality measurements during tilapia fish rearing were measured twice at the beginning of the research and at the end of the research. When measuring DO during maintenance at 5.1-5.5 mg/l, this amount is still in the normal category and can be tolerated by tilapia, this is in accordance with the opinion of Putra *et al.* (2016) the dissolved oxygen content that can be tolerated by tilapia is 4-6 mg/l. Meanwhile, the results of pH measurements during maintenance ranged from 6.5-7.5, this value is still in the normal category and can be tolerated by tilapia. This is in accordance with the statement from Fitriadi *et al.* (2014) who explained that the pH value ranges from 7-9. Furthermore, water quality, namely temperature, based on the maintenance period is around 28-30°C, the temperature is still said to be normal and can be tolerated by tilapia, this is in accordance with the statement from (Fitriadi *et al.*, 2014), the optimal temperature is in the range 23-30°C.

Conclusion

The conclusion that can be drawn from this research is that the combination of *Moringa oleivera*, *Calotropis gigantea* and *Cassia alata* L. extracts has a significant effect (P<0.05) on the survival rate of tilapia (*O. niloticus*). The occurrence of a decrease in the number of leukocytes, an increase in the number of hemoglobin and hematocrit shows that the use of multi-herbal extracts can be used as an anti-bacterial to cure tilapia infected with S. agalactiae bacteria. Treatment E, namely a combination of *M. oleivera* leaf extract 1000 ppm + *C. alata* L leaf extract 10 ppm, showed the fastest healing of the body and the highest survival rate for tilapia, namely 80%.

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