Effect of herbal leaf extract on *Oreochromis niloticus* immunity infected with *Streptococcus agalactiae*

Iko Imelda Arisa[1,2,3*], *Julianda* Fatma[1], *Nurfadillah* Nurfadillah[1,2], *Cut* Nuzlia[1] and *Ulfa* Rahmi[1]

[1]Departement of Aquaculture, Marine and Fisheries Faculty, Universitas Syiah Kuala, Banda Aceh, Aceh, 23111Indonesia
[2]Research Center for Marine and Fisheries, Universitas Syiah Kuala, Banda Aceh, Aceh, 23111Indonesia
[3]Animal Histology and Fish Nutrition Laboratory, Marine and Fisheries Faculty, Universitas Syiah Kuala, Banda Aceh, Aceh, 23111 Indonesia

Abstract. Streptococcosis disease caused by the pathogen *S. agalactiae* is a serious problem in freshwater fish farming. This research aims to use multiherbal ingredients to stimulate the improvement of the fish's immune system in fighting infection by the pathogenic bacteria *S. agalactiae*. The research was carried out at the FKIP Chemistry Laboratory, the Fish Hatchery and Breeding Laboratory of the FKP, and the Laboratory of the Faculty of Medicine, Syiah Kuala University. The test fish used were tilapia measuring 7-8 cm length. The research method was carried out experimentally using a Completely Randomized Design consisting of 5 treatments and 3 replications, namely treatment A (negative control), B (positive control), C (addition of *C. gigantea*), D (addition of *M. oleivera*), E (addition of *C. alata* L). The test result data is analyzed using Analysis of Variance. The results of research on immersion in multiherbal extracts in fish infected with *S. agalactiae* showed an increase in the immune response as seen in blood parameters (leukocytes, hemoglobin, hematocrit) and a higher survival rate compared to those without immersion in the extract. The highest survival rate was obtained in the treatment of 10 ppm of *C. alata* L leaves extract at 83.33%.

1 Introduction

Tilapia (*Oreochromis niloticus*) has high economic value [1], and is an important commodity in the freshwater fish business in Indonesia to meet animal consumption needs. Commercial cultivation of tilapia can trigger stress, which can increase the fish's susceptibility to disease [2]. According to Nasution *et al*. [3], one of the obstacles in the cultivation business, especially tilapia, is the disease Streptococcosis, which is caused by bacterial infection.

* Corresponding author: ikoimeldaarisa@usk.ac.id

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Furthermore, Heckman *et al*., [4] mentioned that *Streptococcus iniae* is a bacterial pathogen that has reappeared in freshwater and marine aquaculture the worldwide.

Diseases caused by pathogenic bacteria will reduce the value of tilapia production because the survival rate of the fish will be low. Treatment of the disease that has been applied includes giving antibiotics. The existence of resistance to various antibiotics shows that Streptococcosis is no longer effective with various types of antibiotics and also that the use of antibiotics has been banned for treating diseases in farmed fish [5]. Therefore, alternative medicine is needed using natural plants as herbal medicine, including *Moringa oleivera* leaves, *Calotropis gigantea* leaves, and *Cassia alata* L. leaves. This natural plant is known to contain antibacterial compounds to treat fish and shrimp infected with bacterial diseases [6, 7, 8].

Previous research has classified herbal ingredients as rich in substances immune enhancer or immunostimulan, where the immunostimulant is herbs can occur by the mechanism of modulating non-specific immune responses and is currently quite widely used to control diseases in fish. Traditionally, herbs and their extracts have been proven effective as performance enhancers for the immuune system and are also recommended as good alternative immunostimulants in aquaculture. According to Muahiddah & Diamahesa [9], the use of natural ingredients for immunostimulants can increase the non-specific immunity of fish toward off disease attacks and environmentally friendly. This research aims to use multiherbal ingredients to stimulate the improvement of the fish's immune system in fighting infection by the pathogenic bacteria *S. agalactiae.*

2 Material and Methods

This study used a completely randomized design (CRD) with three repetitions and five treatments as its experimental setup. The following can be used to express how extract administration is combined for each treatment:
P1 = Fish free of *S. agalactiae* infection
P2 = Fish with *S. agalactiae* infection
P3 = Fish soaked in 800 ppm of *C. gigantea* leaves extract after being infected with *S. agalactiae*
P4 = Fish soaked in 1000 ppm of *M. oleivera* leaves extract after being infected with *S. agalactiae*
P5 = Fish treated with *C. alata* L leaves extract (10 ppm) after being infected with *S. agalactiae*

2.1 Preparation of Extracts

2.1.1 Cassia alata L leaves extract

Cassia alata leaves were washed with clean water and dried in the sun until the leaves were easily crushed. The dried leaves are ground into flour. A total of ±500 g of *C. alata* leaves powder was soaked in 3 L 96% PA absolute ethanol for 24 hours. The results of the immersion are filtered (Whattman No.41), the results are extracted using a rotary evaporator, then the extract is stored at -5 °C until it is used for testing.

2.1.2 Moringa aloivera leaves extract

Moringa aloivera leaves are dried at room temperature for approximately 4 days with supervision. The dry sample (simplisia) was cut into pieces and then ground using a blender until it became simplicia powder. Simplicia powder was weighed 130 g and put into an Erlenmenyer. Then soak (maceration) with 2 liters of ethanol solution and soak for 24 hours. After 24 hours, the solution was filtered using a filter and evaporated using an evaporator [6].

2.1.3 Calotropis gigantea leaves extract

Calotropis gigantea are dried at room temperature for approximately 4 days under supervision. The dried sample (simplisia) is cut into pieces then ground using a blender until it becomes simplicia powder. 500 g of simplicia powder was weighed and put into a container. Then it was soaked (maceration) with 4 liters of 95% absolute ethanol solution and soaked for 24 hours. After 24 hours, the solution was filtered using a filter and evaporated using an evaporator. Next, the resulting extract is weighed for specific gravity and stored in a freezer until used for testing. According to the level of treatment [10].

2.2 Application of multi-herbal leaves to tilapia infected with the pathogen *S. Agalactiae*

The maintenance container used in the experiment was a 12 L jar filled with 10 L of water. The number of container prepared was 15 units and each was filled with 10 fish/container. The container used is a clean or sterile bucket. The test tilapia fish measuring 7-8 cm. The feed used during maintenance is commercial feed with a protein content of 30%. Tilapia fish are fed three times a day at 07.00, 12.00 and 17.00 WIB as much as 5% of body weight.

Before applying the multiherbal extract, healthy fish were first taken that had been adapted to the rearing container for one week, then the fish were injected with the S. agalactiae pathogen at 105 CFU/ml, which is the LD50 dose [11]. After injection of *S. agalactiae*, the fish were left for 2 hours to observe for clinical symptoms of Streptococcosis. After that, soak it with multiherbal extracts in a container according to the dosage for each treatment for ± 15 minutes. Next, the fish were transferred to a rearing test container and then kept for 14 days and observations were made regarding clinical symptoms, blood picture, survival rate and weight growth of the fish. Control water quality by siphoning 10% of the total water volume of each container every day.

2.3 Observed parameters

Parameter measurements in the study included observing clinical symptoms of sick fish, blood picture, survival rate and growth of tilapia fish. Clinical symptoms of sick fish are observed by observing the fish's behaviour and changes that occur in the fish's body. The fish blood features observed include leukocytes, erythrocytes, hematocrit and haemoglobin. Blood parameter measurements were carried out 4 times, namely on days 0, 1, 3 and 5 after the challenge test/pathogenic bacterial infection

a. Total leukocyte counts were calculated according to Blaxhall and Daisley [12]. The calculation is done by: the blood sample is sucked with a pipette containing a white stirrer to a scale of 0.5 ml, then turk's solution is added to a scale of 11, shaken or swung in a number 8 shape for 5 minutes to mix homogeneously. The first drop was discarded,

then dropped into a hemacytometer and covered with a cover glass, observed under a microscope. calculations are carried out on a small box hemacytometer.

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

b. Hematocrit (He) levels were measured according to Anderson and Siwicki [13]. The He level is determined by: the blood sample is put into a microhematocrit tube until approximately ¾ of the tube, then the end is plugged with crytoseal to a depth of 1 mm. After that, it was 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. He levels are expressed as % volume of blood cell solids and are calculated by

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

c. Hemoglobin (Hb) levels used the Sahli method with a salinometer [14]. First, the blood is sucked with a sahli pipette to a scale of 20 mm^3 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 is transferred to an Hb-meter tube filled with 0.1 N HCL to a scale of 10 (red), stir and leave for 3-5 minutes. Add distilled water until the color of the blood and HCl is the color of the standard solution in the Hb meter. Then read the scale, namely by looking at the surface of the liquid and checking it with the Sahli tube scale which is seen on the gr% line scale (yellow) which means the amount of hemoglobin in grams per 100 ml of blood.

d. Survival rate

$$SR\ = \frac{Nt}{No}\ x100\% \quad (3)$$

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).

2.4 Data analysis

Data from testing results of multiherbal extracts for each treatment for tilapia survival rate parameters were analyzed using the ANOVA test, if it had an effect it would be tested further. Furthermore, the results of observations of clinical symptom parameters of sick fish and hematology (leukocytes, hematokrit, hemoglobin) are presented descriptively.

3 Results and Discussion

3.1 Clinical symtoms

The results of observations on tilapia that had not been infected with *S. agalactiae* bacteria showed symptoms of high appetite, active swimming, bright body color and scales that did not peel. According to Arie [15] 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 bacterial infection, tilapia 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 pale corneas. A longer time of S. agalactiae bacterial

infection in the blood will affect clincal symptoms and changes in the profile and composition of tilapia blood cells [16]. Clinical symptoms of fish infected with *S. agalactiae* bacteria and after soaking with multiherbal extracts (*C. gigantea* leaves extract, *C. alata L* and *M. oleivera*) are presented in Table 1. After soaking with multiherbal leaf extract, it is known that the fish's condition recovers slowly. Herbal planys have properties that enhance the host's immunity and act as antibacterial and antiviral agents [17].

Table 1. Clinical symptoms before, after bacterial infection and after immersion in multi-herbal leaf extract

No	Characteristics of Fish	Picture
1	Characteristics of healthy fish (before bacterial infection and extract soaking): - the movement is active - high appetite - bright skin color - scales do not peel off - clear eye color	
2.	Characteristics of sick fish (after bacterial infection and before extract soaking) : - Scales peel off - Thin caudal fin - The eyes and corneas are pale - Hemorrhage of the skin - Protruding eyes	 
3.	Characteristics of fish (after extract soaking) - The fish's body color returns to normal/bright - Hemarogy on the skin began to improve - The fish's fins begin to grow normally - The eyes have started to returned to normal	

3.2 Tilapia blood parameters

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 fish's body and the fish's body tries to fight it [18,19,20].

Changes in the number of leukocytes in *O. niloticus* blood observed in the negative control treatment showed that the leukocyte value was 146.2×10^3 cells mm^{-3} and the leukocyte value in the positive control treatment infected with *S. agalactiae* bacteria was 283.8 -273.3 $\times10^3$ cells mm^{-3}. In the P1 treatment, the leukocyte value was obtained at (146.2×10^3 cells mm^{-3}) and P2 (283.8×10^3 cells mm^{-3}) showed that the leukocyte value increased, this was due to infection with the *S. agalactiae* bacteria and stress in the fish. The increase in leukocytes is the fish's response to increase the body's defense against bacterial attacks. Leukocytes have various functions related to the removal of foreign objects (including pathogenic microorganisms). The increase in leukocytes in the body is caused by

leukocytes acting as the body's defense which quickly reacts to the penetration of antigens into the fish's body. please follow the writing format Description of tilapia leukocyte values during the study is in Table 2.

Table 2. Description of tilapia leukocyte values

Treatment	Leukocyte (x 10^3 cells mm^{-3})			
	D0	**D1**	**D3**	**D5**
P1	146.2	146.2	146.2	146.2
P2	146.2	283.8	279.1	273.3
P3	146.2	269.3	248.7	246.2
P4	146.2	269.3	218.9	189.3
P5	146.2	269.3	160.1	159.5

Hemoglobin (Hb) is a metalloprotein in blood plasma which functions as a transporter of oxygen from the gills which is pumped by the heart to all cells and organs of the body. In the infection treatment and after soaking in herbal extracts, the hemoglobin value at the start of the study was 6 g $100ml^{-1}$ then decreased after being injected with the pathogen *Streptococcus agalactiae*, on day 1 the hemoglobin value was 3.3 g 100 ml^{-1} then gradually increased until the 5^{th} day with a value of 3-4,8 g $100ml^{-1}$.

Table 3. Hemoglobin

Treatment	Hemoglobin Value (g $100ml^{-1}$)			
	D0	**D1**	**D3**	**D5**
P1	6	6	6	6
P2	6	3.3	3	3.3
P3	6	3.3	4.9	3
P4	6	3.3	4.6	4.8
P5	6	3.3	4.6	4.2

The results of hematocrit observations on day 0 did not cause symptoms because the fish were still in normal condition with a value of 21%. Normal hematocrit levels in teleost fish range from 20-30% [21]. Day 1 after bacterial infection in tilapia which had been carried out for 24 hours showed clinical symptoms such as reduced appetite, swimming at the bottom of the container, thin tail fins, hemorrhoids on the skin and eyes and pale corneas as indicated by a decreased hematocrit value with value of 13%, after the fish causes these symptoms, it is then given extracts from multi-herb leaves in different doses, after which it is soaked for 24 hours. The results of observations on days 3 and 5 showed that the fish were still in the healing process because the extract was still reacting.

Table 4. Hematocrit

Treatment	Hematocrit Value (%)			
	D0	**D1**	**D3**	**D5**
P1	21	21	21	21
P2	21	13	15	14
P3	21	13	15	14
P4	21	13	16	17
P5	21	13	6	18

3.3 Survival rate

Based on the results of ANOVA analysis of research data, the effect of giving multiherbal extracts to tilapia infected with the *S. agalactiae* pathogen showed that giving this extract had a significant effect (P<0.05) on survival (Table 5).

Table 5. Survival rate tilapia fish for 14 days of rearing

Treatment	Survival Rate (%)
P1	70.00 ± 10.00^{c}
P2	36.67 ± 5.77^{b}
P3	0^{a}
P4	80 ± 5.77^{c}
P5	83.33 ± 10.00^{c}

In treatment P3 an average value of 0.00% was obtained, in which case all the fish in the rearing container died. This is caused by soaking a little longer, when soaking is too long, the substances contained can turn into poison which causes the digestive system to be disturbed. In treatment P5 was the best treatment with an average value of 83.33%. Meanwhile, the P2 treatment (positive control) showed a decrease in the viability of *O. niloticus* fish by 36.67% due to infection by the pathogen *S. agalactiae* and no treatment was carried out (Table 5). Haq and Prasetio [22] stated that one of the causes of low fish survival could be parasitic, bacterial or viral infections. And the fish immune system plays an important role ini fish survival when fighting pathogenic bacteria during infection [23, 24].

4 Conclusion

The use of herbal ingredients containing active antimicrobial compounds can suppress the development of disease in tilapia caused by the pathogenic bacteria *Streptococcus agalactiae*. Based on research results, the use of *C. alata* L leaf extract at a dose of 10 ppm had the highest survival rate for tilapia with a value of 83.33%. The description of the blood parameters (leukocytes, hemoglobin and hematocrit) of tilapia fish soaked with multiherbal extract shows that the fish's immune response is better than infected fish without soaking in the extract.

Acknowledgment

The author would like to thank the Research and Community Service Institute of Syiah Kuala University for financial support for this research and all parties who have assisted in the process this research.

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