The effect of *Moringa oleifera* leaf extract in feed as an immunostimulant for *Aeromonas hydro*phila bacterial infections

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Abstract. This study investigates the impact of adding *Moringa oleifera* leaf extract to feed as an immunostimulant for tilapia infected with *Aeromonas hydrophila*. The experiment followed a Completely Randomized Design (CRD) with six treatments and three replications. The findings revealed that incorporating Moringa leaf extract significantly influenced survival rates ($P<0.05$). The highest survival rate (83.33%) was observed with a dose of 150 mg kg-1. In conclusion, doses of 100-150 mg kg-1 of Moringa leaf extract produced the best results, including reduced clinical symptoms, survival rates (70-83.33%), leukocyte count (173-223 x 10^3 cells mm^{-3}), hematocrit (27-28%), and hemoglobin levels (6.6-7 g dL^{-1}), indicating its potential as an effective immunostimulant for tilapia.

1 Introduction

Nile Tilapia (*Oreochromis niloticus*) is one of the key commodities developed to support national food security, economic stability, and community welfare. Indonesia, China, and Egypt are among the main global producers of tilapia [1, 2]. One common issue in aquaculture is disease outbreaks that can reduce the quality and value of the fish. Diseases in tilapia are often caused by pathogenic bacteria such as *Aeromonas hydrophila*. This bacterial infection can cause stress in fish and weaken their immune system [3]. [4] reported that *Aeromonas hydrophila* is an opportunistic Gram-negative bacterium that can cause rapid fish mortality, reaching 80-100%. One preventive measure in aquaculture is to utilize natural materials or medicinal plants as sources of immunostimulants.

Immunostimulants are substances, either natural or synthetic, that enhance and strengthen the immune response against pathogens, improve the body's resistance, and reduce the risk of infection. Using immunostimulants in aquaculture is an attractive alternative to decreasing reliance on antibiotics and increasing the sustainability of aquaculture production. They boost

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innate immune responses, thereby improving fish immunity against diseases and lowering fish mortality due to infections. Additionally, immunostimulants promote growth and accelerate wound healing [5]. They are often derived from bacteria, brown algae, red algae, terrestrial fungi, and other materials with potential immunostimulant properties. Their use has been widely proven to prevent diseases in fish. The immunostimulant effects of glucan, chitin, lactoferrin, and levamisole have been reported to protect fish and shrimp from disease outbreaks [6].

Imunostimulan dapat digunakan secara efektif dengan memasukkannya ke dalam pakan ikan. Praktik ini bertujuan untuk meningkatkan pertumbuhan ikan, meningkatkan pemanfaatan pakan, dan mencegah wabah penyakit dalam akuakultur [7]. Imunostimulan bekerja langsung pada sel sistem imun, sehingga membuatnya lebih aktif. Namun, imunostimulan sintetis harganya cukup mahal. Oleh karena itu, perlu dicari sumber imunostimulan alternatif yang hemat biaya dan mudah digunakan, seperti ekstrak daun kelor [8].

Moringa leaves (*Moringa oleifera*) are known as one of the natural materials that can function as immunostimulants. The chemical composition of moringa leaves includes various vitamins (A, C, E, K, B1, B2, B3, B6), flavonoids, alkaloids, saponins, tannins, and terpenoids [9, 10]. Flavonoids function as antioxidants that can stop free radical chain reactions, while saponins act as immunostimulant agents [11]. Previous research has shown that the white blood cell count in catfish (*Clarias* sp.) increased after being given moringa leaf extract in feed for 21 days, although the count remained within the normal range (77,400–91,467 cells mm^{-3}). Additionally, soaking vannamei shrimp (*Litopenaeus vannamei*) larvae infected with Vibrio harveyi in a moringa leaf extract solution at a concentration of 1000 ppm inhibited bacterial growth and increased the survival rate of the shrimp larvae [12]. However, to date, the use of moringa leaf extract in feed as an immunostimulant for tilapia (*Oreochromis niloticus*) infected with *Aeromonas hydrophila* has not been reported, making this research necessary.

2 Material and methods

The fish used were 180 fish measuring 8-10 cm with a stocking density of 1 fish L^{-1} [13]. The water volume was filled with 10 liters per treatment unit and aeration was installed in each container. Each plastic tank contains 10 tilapia fish. This study employed a completely randomized design (CRD) with three replicates and six treatments in its experimental setup. The treatment combinations were as follows:

- A = 0 mg kg^{-1} feed of *M. oleifera* leaf extract, without *A. hydrophila* infection
- B = 0 mg kg^{-1} feed of *M. oleifera* leaf extract, with *A. hydrophila* infection
- C = 100 mg kg^{-1} feed 0f *M. oleifera* leaf extract, with *A. hydrophila* infection
- D = 125 mg kg^{-1} feed of *M. oleifera* leaf extract, with *A. hydrophila* infection
- E= 150 mg kg^{-1} feed of *M. oleifera* leaf extract, with *A. hydrophila* infection
- F = 175 mg kg^{-1} feed of *M. oleifera* leaf extract, with *A. hydrophila* infection

2.1 Preparation of *Moringa aloivera* leaves extract

Moringa oleifera leaves are dried at room temperature for about four days under supervision. The dried sample (simplicia) is then cut into pieces and ground using a blender to produce simplicia powder. 130 g of this powder is then weighed and placed into an Erlenmeyer flask. It is then soaked (macerated) with 2 liters of ethanol solution and left to soak for 24 hours. After this period, the solution is filtered and the filtrate is evaporated using an evaporator [12].

2. 2. Mixing the test feed with moringa leaf extract

The feed used in this research was commercial pellet feed. The test feed was added with Moringa leaf extract at different doses for each treatment. Mixing Moringa leaf extract and FF999 pellet feed with 35% protein content using the coating method. The feed is mixed with egg white until evenly mixed to bind the extract to the feed. Next, mix the extract according to the dose which has been diluted with distilled water evenly into the feed which has been weighed according to the fish weight requirements per week. Air dry the food for 15 minutes, store the dried food in a jar and then store it in a dry place.

2. 3. Maintenance fish and feeding

The test fish were raised for 28 days, with feeding provided three times daily at 08:00 AM, 11:00 AM, and 04:00 PM, at a rate of 5% of the fish's body biomass. For the first 14 days, the fish were given feed mixed with Moringa leaf extract. On the 15th day, the fish were infected with *A. hydrophila* bacteria, and feeding with the mixed feed continued for another 14 days post-infection. The procedures in this study adhered to the Universitas Syiah Kuala guidelines (Code No. 958/2015) for animal care and use in research.

2. 4. Infection of *A. hydrophila*

Aeromonas hydrophila bacterial infection was carried out on the 15th day with a density of 10^8 CFU mL^{-1}. The challenge test was carried out by infecting the fish with *A. hydrophila* bacteria by soaking for 24 hours in a bacterial solution. Put 10 ml into each container with a bacterial requirement of 1 ml 1 L^{-1} of water. After the fish were challenged, clinical symptoms and survival were observed until day 14. Blood sampling was carried out on the day H-0 (Before treatment, H-14 (After treatment), H-1, H-7 and H-14 after infection then observed leukocytes, hemoglobin and hematocrit.

2. 3. Observed parameters

The study's parameter measurements encompassed the observation of clinical symptoms in sick fish, blood parameters, survival rates, and water quality. Clinical symptoms were assessed by monitoring the fish's behavior and any physical changes. Blood parameters observed included leukocytes, hematocrit, and hemoglobin levels. These blood measurements were taken at three intervals: on days 1, 7, and 14 following the challenge test/pathogenic bacterial infection.

a. The total leukocyte count is determined following the method described by Blaxhall and Daisley [14]. This procedure involves drawing a blood sample into a pipette containing a white blood cell diluent up to the 0.5 ml mark. Turk's solution is then added to bring the volume to scale 11. The mixture is then shaken or swirled in a figure-eight motion for 5 minutes to ensure thorough mixing. The first drop of the mixture is discarded, and the remainder is placed on a hemocytometer, covered with a coverslip, and examined under a microscope. The leukocytes are counted in the small squares of the hemocytometer.

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

b. The hematocrit (He) level is measured following the method described by Anderson

and Siwicki [15]. To determine He, a blood sample is placed into a microhematocrit tube, filling it to about ¾ of its volume. The end of the tube is then sealed with a 1 mm layer of crytoseal. The tube is centrifuged at 5000 rpm for 5 minutes. Subsequently, the length of the settled blood (a) and the total length of the blood volume in the tube (b) are measured. The hematocrit level is expressed as the percentage of the solid cell blood volume and is calculated by comparing these two measurements.

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

c. The hemoglobin (Hb) level is measured using the Sahli method with a salinometer [16]. The procedure involves drawing blood into a Sahli pipette until it reaches the 20 mm^3 mark or 0.2 ml, and then cleaning the pipette tip with tissue. The blood is then transferred into an Hb-meter tube containing 0.1 N HCl, filling it to the 10 scale (red), mixed, and allowed to sit for 3-5 minutes. Afterward, aquades is added until the color of the blood and HCl solution matches the standard solution color on the Hb-meter. Finally, the measurement is read by observing the liquid surface and noting the hemoglobin level in grams per 100 ml of blood using the Sahli tube scale (yellow).

d. Survival rate

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

Note: SR represents the fish survival rate (%); Nt = denotes the number of live fish at the end of rearing, No indicates the number of fish at the beginning of rearing.

2. 4. Data analysis

The data from the testing of herbal extracts for each treatment on tilapia survival rate parameters and hematology parameters (leukocytes, hematocrit, hemoglobin) were analyzed using the ANOVA test. If a significant effect was found, further tests were conducted. Additionally, the results of clinical symptom observations in sick fish is presented descriptively.

3. Results and discussion

3.1 Clinical symtoms

Observation of clinical symptoms in Nile tilapia after infection with *Aeromonas hydrophila* bacteria showed morphological changes, including wounds on the body, reddening of the fish, a ragged tail, peeling of the dorsal fin and scales, decreased appetite, reduced responsiveness, fish swimming at the bottom and some at the surface of the water, and some fish swimming erratically. According to [17], the presence of red spots around the abdomen, slightly protruding eyes, and damage to the tips of the fins indicates that the fish are infected with Motile Aeromonas Septicemia (MAS) caused by *A. hydrophila* infection. After being given moringa leaf extract, the fish's condition improved, as indicated by increased appetite and the regrowth of fins and tail. The slowest recovery occurred in treatments C and D, on the 8th day post-infection, while the fastest recovery occurred in treatment E, on the 7^{th} day.

3.2 Tilapia blood parameters

The observation of white blood cells in Nile tilapia was conducted to assess changes in leukocyte levels before treatment with feed supplemented with moringa leaf extract, after the treatment, after the challenge test, and on the final day of maintenance. White blood cells in fish serve as a non-specific defense mechanism. Each treatment resulted in a different increase in the number of white blood cells, both before treatment, after treatment, and after the challenge test. An overview of the leukocyte value of tilapia during the study is presented in Table 1.

Table 1. Nile tilapia leukocyte value during the study.

Treatment	Leukocyte Value (10^3 sel mm^{-3})				
	Before Infection		After Infection		
	H-0	H-14	H-1	H-7	H-14
A	110.4	116.633± 2.09^{a}	123.136± 2.60^{a}	125.633± 1.44^{a}	133.517±3.29^{a}
B	110.4	115.30± 0.90^{a}	268.30± 0.98^{f}	287.267± 1.32^{f}	288.827±1.65^{e}
C	110.4	365.56± 1.00^{bc}	193.30± 3.01^{d}	175.133± 2.20^{d}	155.587±1.99^{c}
D	110.4	139.681 ±1.49^{c}	185.133± 1.73^{c}	167.067± 2.24^{c}	147.267±1.57^{a}
E	110.4	146.348 ±2.04^{d}	173.016± 2.41^{b}	156.90± 1.40^{b}	142.167±1.62^{b}
F	110.4	132.25± 2.41^{b}	223.133± 2.34^{e}	215.60± 2.12^{e}	201.176±2.62^{d}

Based on the data presented in Table 1, on day 0 (before treatment), the leukocyte count was recorded at 110.40×10^3 cells mm^{-3}. On day 14, after administering moringa leaf extract through feed to tilapia, there was an increase in the white blood cell count compared to treatments A and B. According to [18], the phytochemical content of moringa leaves (*Moringa oleifera* Lam) includes gallic tannins, steroids, triterpenoids, flavonoids, saponins, anthraquinones, catechol tannins, alkaloids, and reducing sugars. Bioactive compounds in plants that function as immunostimulants include alkaloids, flavonoids, phenols, terpenoids, and steroids [19]. [20] stated that flavonoids have antiseptic and antimicrobial activities, and they can disrupt bacterial energy metabolism, thus hindering bacterial growth.

The results of the blood test in Table 1 on day 1 after the challenge test showed the following leukocyte counts: in treatment A, the leukocyte count was 123.136×10^3 cells mm^{-3}, which is still within the normal range; in treatment C, it was 193.30×10^3 cells mm^{-3}; in treatment D, it was 185.1×10^3 cells mm^{-3}; in treatment E, it was 173.016×10^3 cells mm^{-3}; and in treatment F, it was 223.133×10^3 cells mm^{-3}. These results indicate an increase in the total white blood cells due to the *A. hydrophila* infection, as the fish produced white blood cells to defend against the bacterial attack. [21] also stated that when fish are infected, the number of white blood cells increases to protect the body as a defense against bacterial infections. In treatment B, there was a significant increase in the number of white blood cells after the challenge test, with the highest leukocyte count among all treatments, reaching 268.3×10^3 cells mm^{-3}. This indicates that the Nile tilapia infected with *Aeromonas hydrophila* showed more severe clinical symptoms compared to the other treatments. According to [22], when the leukocyte count in fish is low, the fish becomes more susceptible to pathogenic bacterial infections. The normal leukocyte count in Nile tilapia is between 20,000 - 150,000 cells mm^{-3}. [23] Futher stated that he active immunostimulant compounds found in jengkol peel extract, such as flavonoids, alkaloids, saponins, tannins, glycosides, and steroids, function as immunostimulants. These compounds can stimulate the immune system in juvenile gourami to enhance their defense against *Aeromonas hydrophila* infections by inducing the proliferation of white blood cells.

Other blood parameters, such as hemoglobin, on day 0 (before treatment) were measured at 6 g dL^{-1}. On day 14 (after the administration of the immunostimulant moringa leaf extract), the hemoglobin levels ranged from 6 to 7 g dL^{-1}, which is considered normal. The addition

of moringa leaf extract at various doses in the feed is believed to maintain the hemoglobin levels of Nile tilapia within the normal range. Hemoglobin levels within the normal range indicate that there is sufficient oxygen bound in the blood, suggesting that the fish are healthy. On day 1 post-infection with *Aeromonas hydrophila* bacteria, hemoglobin levels decreased in treatments B (+), C (100 mg kg^{-1} feed), D (125 mg kg^{-1} feed), E (150 mg kg^{-1} feed), and F (175 mg kg^{-1} feed), with values ranging from 3.8 to 4.5 g dL^{-1}. [24] stated that a decrease in blood hemoglobin is associated with a reduction in the number of red blood cells, which is likely caused by hemolysis within the blood. Hemolysis occurs due to the destruction of red blood cells caused by bacterial toxins, known as hemolysins, which break down and destroy hemoglobin in the blood. An overview of the hemoglobin value of tilapia during the study is presented in Table 2.

Table 2. Nila tilapia hemoglobin value during the study

Treatment	Hemoglobin value (g dL^{-1})				
	Before Infection		After Infection		
	H-0	H-14	H-1	H-7	H-14
A	6	6.5± 0,86	6.6± 0.52^{c}	6.8±0.76^{c}	7.0± 1.00^{b}
B	6	6.3± 1,52	3.1± 0.28^{a}	3.3± 0.57^{a}	4.3±0.57^{a}
C	6	6.6 ± 1,15	3.6± 0.57^{ab}	4.3 ±0.57^{ab}	6.5± 0.50^{b}
D	6	6.6± 0,57	3.8± 0.76^{ab}	4.5± 0.50^{b}	6.8± 0.76^{b}
E	6	7.0 ± 1,00	4.5± 0.50^{b}	4.8 ± 0.28^{b}	7.0± 1.00^{b}
F	6	6.4± 0,40	3.5± 0.50^{ab}	3.5±0.50^{a}	5.3±0.57^{a}

On day 7, the hemoglobin level in treatment C increased to 4.3 g dL^{-1}, and continued to rise to 6.5 g dL^{-1} by day 14 post-infection. Treatment D also showed an increase on day 7 post-infection with a value of 4.5 g dL^{-1}, which continued to rise to 6.8 g dL^{-1} by day 14. Treatment E exhibited an increase in hemoglobin levels from day 7 to day 14 post-infection, reaching 7.0 g dL^{-1}. Treatment F showed a slight increase on day 14 post-infection with a value of 5.3 g dL^{-1}. [25] stated that the increase in hemoglobin levels is due to the activity of flavonoids and tannins, which act as antioxidants, protecting hemoglobin from oxidation. Moringa leaves contain a relatively high amount of iron (Fe), which helps in the formation of red blood cells, thus increasing hemoglobin levels in the blood. In the B (positive control) treatment, hemoglobin levels on days 7 and 14 post-infection remained below normal because no moringa leaf extract was added to the feed.

Based on the research results, the hematocrit level on day 0 (before treatment) was 26%. On day 14, after the administration of moringa leaf extract as an immunostimulant, there was a slight increase, but the level remained within the normal range. However, on day 1 post-infection, the hematocrit level decreased, with the lowest value observed in the B treatment at 14%. The hematocrit values for treatments C, D, E, and F were 16.3%, 16%, 18%, and 14.3%, respectively. This decrease occurred because the fish were infected with *Aeromonas hydrophila*. On day 7 post-infection, the hematocrit levels began to increase, with values of 21.3% for C, 20% for D, 22.3% for E, and 17.0% for F. By day 14 post-infection, the hematocrit levels continued to rise, reaching normal levels ranging from 25.6% to 27.6%. The increase in hematocrit levels indicates that the fish are in a healthy state. This also proves that the administration of moringa leaf extract can help enhance the fish's immune system. The hematocrit values throughout the study are presented in Table 3.

Table 3. Nile tilapia hematocrit value during the study.

Treatment	Hematokrit value (%)				
	Before Infection		After Infection		
	H-0	H-14	H-1	H-7	H-14
A	26	26.0± 1.00	26.6± 0,57^{c}	28.0±1,00^{d}	26.0± 1,00^{b}
B	26	26.3± 1.52	14.0± 1.00^{a}	16.6± 1.52^{a}	18.3±1.52^{a}
C	26	27.3 ± 0.57	16.3± 1.55^{ab}	21.3 ±1.52^{bc}	25.6± 1.15^{b}
D	26	28.3 ±1.15	16.0 ± 2.00^{ab}	20.0± 2.00^{abc}	26.6± 1.15^{b}
E	26	28.6 ± 1.52	18.0± 1.00^{b}	22.3 ± 2.08^{c}	27.6± 0.57^{b}
F	26	26.6± 0.57	14.3± 0.57^{a}	17.0±1.00^{ab}	17.6±1.52^{a}

3.3 Survival rate

Over the 28-day observation period, feeding fish with different doses of moringa leaf extract as an immunostimulant and infecting them with *A. hydrophila* bacteria had a significant impact on the survival rate of Nile tilapia. Treatment E, with a dose of 150 mg kg^{-1} of feed, showed the highest survival rate, averaging 83.33%. In contrast, treatment B (positive control) had the lowest survival rate, averaging 53.33%. The lower survival rate in treatment B was attributed to the absence of moringa leaf extract in the feed as an immunostimulant, both before and after the *A. hydrophila* infection, resulting in a weaker immune system compared to the other treatments.

Table 4. Survival rate of tilapia during study.

Treatment	Survival Rate (%)
A	73.33± 5.77^{bc}
B	53.33± 5.77^{a}
C	70.00± 10.00^{b}
D	76.67± 5.77^{bc}
E	83.33 ± 5.77^{c}
F	56.67± 5.77

Water quality is pivotal to the success of aquatic animal husbandry, influencing the growth rate and survival of Nile tilapia. To ensure optimal conditions, water quality parameters such as temperature, pH, and dissolved oxygen (DO) were measured weekly throughout the study. The recorded values were as follows: temperature ranged from 27-29°C, DO levels from 5.3-6.5 mg L^{-1}, and pH from 7.4-8.5. The ideal temperature for the growth of Nile tilapia is between 25-33°C [26], and the optimal pH range is 7-9 [27].

4. Conclusion

Incorporating moringa leaf extract into feed can boost the immunity of tilapia. A dose of 150 mg kg^{-1} has demonstrated significant enhancement of the tilapia immune system, particularly in combating *Aeromonas hydrophila* infections, with a survival rate of 83.33%. Utilizing moringa leaf extract as a herbal component in fish feed presents an effective alternative to antibiotics for managing fish diseases.

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