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Hematology and Health Status of *Pangasianodon hypophthalmus* Fed with *Moringa oleifera* Enriched Pellets and Infected with *Aeromonas hydrophila*

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ABSTRACT

Moringa oliefera leaves can be used to improve the health of fish in general. To understand the effectiveness of moringa leaf powder addition in enhancing the immunity of *Pangasionodon hypopthalmus* toward *Aeromonas hydrophila* attack, a study was conducted from June to September 2022. The fish were fed with the moringa powder enriched pellets and then were infected with *A. hydrophila*. There were four treatments applied, namely negative control (no moringa and no infection), positive control (no moringa, infection), T1 (5 g/kg, infection), T2 (10 g/kg, infection), and T3 (15 g/kg, infection). The fingerlings of *P. hypopthalmus* (3.5±0.5g body weight) were reared for 45 days (1 fish/4L water) and fed 3 times/day, 5% of body weight. *A. hydrophila* was infected through injection (0.1 mL of 10^8 CFU/mL) on the 31st day. The hematology of the fish was checked on the 31st day and on the 14th day after infection (or on the 45th day of the research). Results showed that after being treated with moringa for 30 days, the fish's hematology in all treatments showed almost no difference. However, the phagocytosis index was slightly higher in the moringa-treated fish, recording around 20.33% (in NC and PC) and 23.33-25.67% (in T1, T2, and T3, respectively). On the 14th day after the infection, the phagocytic Index increased to 26.67, 29.00, and 30.33% in T1, T2 and T3, respectively. No positive control fish survived by the end of the experiment, while 88.89 – 97.77% of moringa-treated fish survived, and the infection wound was completely cured. Data obtained indicate that the moringa addition in the fish feed pellets effectively improves the immunity of *P. hypopthalmus* toward *A. hydrophila* infection.

INTRODUCTION

Pangasianodon hypophthalmus is one of the favorite fishes in Riau, Indonesia. This fish may be sold fresh or smoked in the market, and many Riau cuisines use this type of fish as a basic ingredient. This fish is also rich in protein content and has relatively high economic value. This species is commonly cultured. It is carnivorous, able to tolerate environmental changes, and grows rapidly **(Chowdhury & Ro, 2020; Chowdhury *et al.*, 2020)**.

In the intensive culture of *P. hypophthalmus*, however, several common problems occur. High stocking densities, poor culture management, and inadequate water circulation may cause stress and increase the spread of infectious diseases in the culture ponds **(Hasan *et al.*, 2020)**. Motile Aeromonas Septicemia (MAS), caused by *Aeromonas hydrophila*, is one of the most common diseases affecting cultured fish in tropical areas. Fish suffering from this disease show organ and tissue damage, growth delays, and even mass mortality **(Zanowicz *et al.*, 2020; Yousefi *et al.*, 2021)**. In terms of aquaculture, the MAS disease outbreaks may cause great loss.

Antibiotics can be used to prevent and cure fish from the MAS disease attack. However, the use of antibiotics in the consumed fish causes many negative effects, as the antibiotic residue is present in the consumed fish meat **(Nguafack *et al.*, 2020)**. Antibiotic use also contributes to the occurrence of new strains of bacteria that are resistant to the antibiotic **(Thornber *et al.*, 2020)**. The use of natural antibiotic might be better as it is relatively safe and do not leave any residue in fish meat.

Another approach to reducing the risk of microbial infections is by improving fish health through natural sources such as *Moringa oleifera*, often referred to as a "superfood." **Raghoji (2021)** stated that *Moringa* spp. is a nutritious food containing various phytochemicals beneficial to health. **Abalaka *et al.* (2021)** reported that *Moringa* leaves contain bioactive compounds such as flavonoids, alkaloids, phenols, steroids, triterpenoids, saponins, tannins, and anthraquinones. The leaves, fruits, bark, and roots have been used medicinally for antitumor, anti-inflammatory, and antihypertensive purposes, and to reduce cholesterol, as antioxidants, antidiabetics, hepatoprotectives, antibacterials, and antifungals **(Abdel-Latif *et al.*, 2022)**.

Moringa leaves are also rich in vitamins A and C, calcium, potassium, iron **(Aborhyem *et al.*, 2016)**, and crude protein (251 g/kg). However, the plant also contains anti-nutritional compounds such as tannins, lectins, phytates, and oxalates **(Karthivashan *et al.*, 2015)**. Additionally, **Teclegeorgish *et al.* (2021)** noted that *Moringa* leaves contain phenolic compounds such as flavonoids and phenolic acids (e.g., quercetin and kaempferol) that act as antioxidants. They also possess antimicrobial properties against several bacteria, including *Klebsiella* spp., *Pseudomonas aeruginosa*, *Trichoderma* spp., *Aspergillus flavus*, *Bacillus cereus*, *Staphylococcus pneumoniae*, and *Escherichia coli*, as well as *A. hydrophila* **(Mbokane & Moyo 2018; Oladeji *et al.*, 2019)**.

It has been proven that adding *Moringa* leaf powder to fish feed pellets enhances growth and immunity. **Elabd *et al.* (2019)** found that adding 15g of *Moringa* per kilogram of feed improved the immunity and survival of *Oreochromis niloticus*, while 20g per kilogram boosted growth and hematological parameters in *Clarias* spp. **(Olusola & Olorunfemi 2017)**. To date, however, there is no information on the effectiveness of

Moringa leaf powder in promoting the growth and immunity of *P. hypophthalmus* infected with *A. hydrophila*. Since fish health status and metabolic changes can be assessed through hematological parameters **(Adi *et al.*, 2020)**, this study aimed to evaluate the hematological response of *P. hypophthalmus* fed with *M. oleifera*-enriched pellets when challenged with *A. hydrophila*.

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MATERIALS AND METHODS

This study was conducted in the Marine Biotechnology, Fisheries and Marine Faculty, Universitas Riau, Pekanbaru, Indonesia from June to September 2022. A CRD (complete random design) was applied. There were five treatments and three replications/ treatment. The treatments applied were as follows:

NC = Negative control (no moringa and no *A. hydrophila* infection)
PC = Positive control (no moringa and was infected with *A. hydrophila*)
T1 = Moringa powder addition (5g/kg feed) and was infected with *A. hydrophila*
T2 = Moringa powder addition (10g/kg feed) and was infected with *A. hydrophila*
T3 = Moringa powder addition (15g/kg feed) and was infected with *A. hydrophila*

The fish used in this study were fingerlings of *P. hypopthalmus*, around 3.5±0.5g body weight. In total, 300 fish were used in this study. Before the treatment, the fish were adapted for a week.

Moringa enriched fish feed pellets preparation

The fish were fed commercial fish feed pellets mixed with moringa leaves powder during the study. The moringa powder was made by sun drying of the moringa leaves, grinding them using a blender, followed by sieving. The moringa leaves were converted to powder, and the feed pellets were then crushed, formed into small granules (around 1 mm diameter), and sun-dried **(Windarti *et al.*, 2023)**.

Fish rearing

The fish were reared in 60 × 30 × 30 cm aquaria filled with 60L of freshwater, with a stocking density of one fish per 4L of water. The rearing period lasted for 30 days. Fish were fed three times a day—at 08:00 AM, 01:00 PM, and 06:00 PM. The total feed provided daily was equivalent to 5% of the fish's body weight **(Effendi *et al.*, 2025)**.

***Aeromonas hydrophila* infection**

After being fed with *Moringa*-enriched pellets for 30 days, the fish were infected with *A. hydrophila*. On the 32nd day, each fish was intramuscularly injected with 0.1mL of *A. hydrophila* suspension at a concentration of 10^8 CFU/mL, targeting the muscle area behind the head. Following the injection, the fish were reared and continuously fed with *Moringa*-enriched pellets for an additional 14 days. During this period, clinical signs of Motile Aeromonas Septicemia (MAS) were observed and monitored.

Blood sampling

Blood sampling was conducted at three different time points: prior to treatment (baseline), 30 days after treatment, and 14 days after infection with *A. hydrophila*. Fish were sedated using clove oil (0.01 mL/L of water) to minimize stress. Once the fish lost balance and became unresponsive, blood was drawn from the caudal vein using a 1mL syringe pre-coated with 10% EDTA. The collected blood was stored in Eppendorf tubes and prepared for hematological analysis, which included measurements of erythrocyte count, leukocyte count and differentiation, hemoglobin concentration, and hematocrit, following the method of Blaxhall & Daisley, as cited in **Witeska *et al.* (2022)**.

To determine the Phagocytic Index, blood was collected in hematocrit capillary tubes and centrifuged at 11,000 RPM for 3 minutes. After erythrocytes had settled, the capillary was cut to discard the erythrocyte portion. The remaining leukocyte layer and serum were transferred into a microtube and mixed with 50µL of a *Staphylococcus aureus* suspension (10^8 cells/mL). The mixture was homogenized and incubated for 20 minutes at room temperature. Subsequently, 5µL of the suspension was smeared on a glass slide and fixed with 95% methanol. Once dried, the slide was stained using a combination of Giemsa and Carbol Fuchsin, and examined under a binocular microscope. The Phagocytic Index was calculated as:

Phagocytic Index = (Number of macrophages containing engulfed bacteria ÷ Total number of macrophages counted)

Data analysis

Data on hematological parameters—including erythrocyte count, hematocrit, hemoglobin, total leukocyte count, leukocyte differentiation, phagocytic index, and survival rate—were tabulated and analyzed using SPSS version 24. One-way ANOVA was used to determine statistical differences among treatments. Where significant differences were found, a Student-Newman-Keuls (SNK) post hoc test was applied.

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RESULTS

Survival of *P. hypopthalmus*

The data obtained in this study indicate that the addition of *Moringa* leaf powder to fish feed pellets positively influenced fish immunity. Following injection with *A. hydrophila*, all fish exhibited clinical signs of Motile Aeromonas Septicemia (MAS).

Affected fish became frantic, and redness developed around the injection site, which later progressed into open wounds. Feed intake decreased significantly. By the fourth day post-infection, the wounds had worsened into large open ulcers (Fig. 1).

The positive control group (infected with *A. hydrophila* but not treated with *Moringa*) showed the poorest performance, with multiple mortalities observed. All fish in the positive control group died by the fifth day post-infection. In contrast, although some mortality was also observed among the *Moringa*-treated groups, the majority of fish survived. By the end of the experiment, survival rates in the *Moringa*-treated groups ranged from 88.9% to 97.77% (Fig. 2).

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Fig. 1. *P. hypopthalmus* with ulcer due to *A.hydrophila* infection

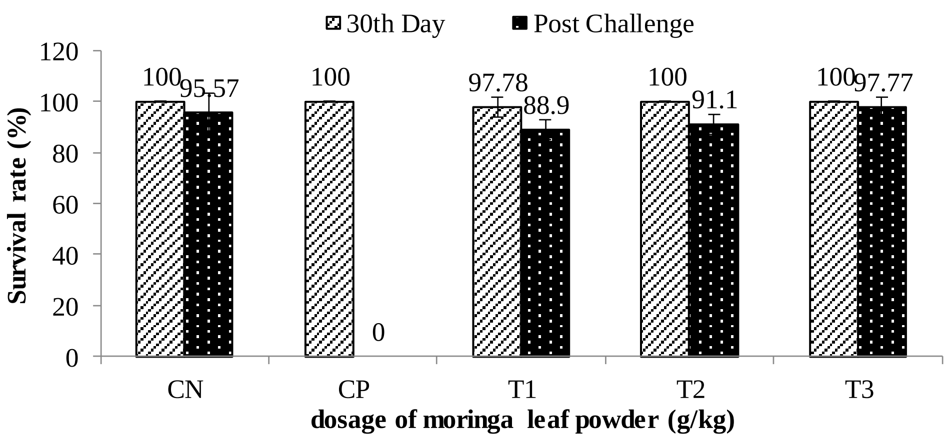

Fig. 2. The survival rate of *P. hypopthalmus* on the 30th day and the 14th day post-challenge with *A. hydrophila*

Although the *Moringa*-treated fish exhibited clinical signs of MAS disease and developed ulcers at the injection site, they were able to recover without further intervention. Around 6–7 days post-infection, the wounds began to heal, with the ulcerated areas gradually covered by a layer of transparent skin. By the 14th day after infection, the wounds were fully healed (Fig. 3).

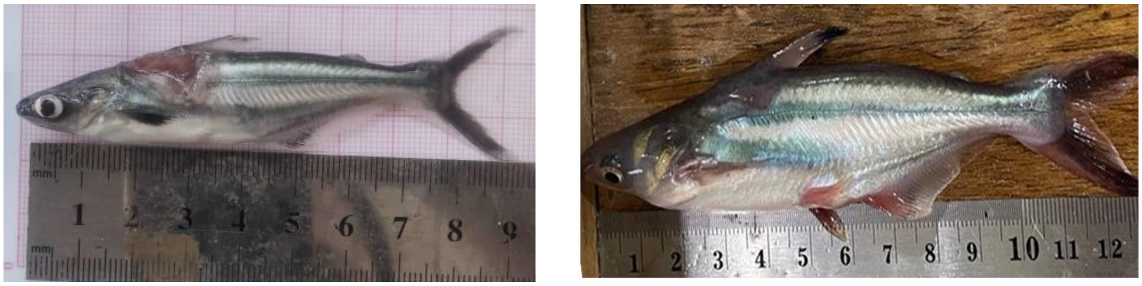

Fig. 3. The moringa-treated fish on the 14th day after the infection, and the wound was healed

Hematology of *P. hypopthalmus*

In this study, the addition of *Moringa* leaf powder to fish feed pellets significantly influenced the hematological parameters of the fish. Fish fed with *Moringa*-enriched pellets showed significantly higher erythrocyte counts, hematocrit levels, and hemoglobin concentrations ($P < 0.05$) compared to fish that received no *Moringa* in their diet.

The inclusion of 15g of *Moringa* powder per kilogram of feed pellets yielded the best results. After 30 days of feeding, the erythrocyte counts in the treated fish were higher than those in pre-treatment, negative control, and positive control groups, ranging from 1.93 to 2.10 × 10^6 cells/mm^3, compared to 1.82–1.84 × 10^6 cells/mm^3 in the control groups. In addition to elevated erythrocyte numbers, the hematocrit and hemoglobin levels in *Moringa*-treated fish were also significantly higher than those in both the pre-treatment group and the non-*Moringa* groups (Table 1). These hematological indicators suggest that the fish were in a healthy and normal physiological condition.

Following infection with *A. hydrophila*, the hematological condition of the *Moringa*-treated fish remained relatively stable or slightly improved. The treated fish exhibited significantly higher erythrocyte counts, hematocrit levels, and hemoglobin content compared to the negative control group. Among the treatments, fish in the T3 group showed the best hematological results, with the highest erythrocyte and hemoglobin values. However, hematological data from the positive control group (infected but not fed with *Moringa*) were unavailable, as all fish in this group died by the 4th to 5th day post-infection (Table 1).

Table 1. Erythrocyte of *P. hypopthalmus* during the research

Parameter	**Treatments**				
	NC	**PC**	**T1 (5 g/kg)**	**T2 (10 g/kg)**	**T3 (15 g/kg)**
Pre-treatment					
Total erythrocyte (x10^6 corpuscle/mm^3)	1.56±0.06	1.56±0.06	1.56±0.06	1.56±0.06	1.56±0.06
Hematocrit (%)	27.67±0.58	27.67±0.58	27.67±0.58	27.67±0.58	27.67±0.58
Hemoglobin (g/dL)	5.93±0.31	5.93±0.31	5.93±0.31	5.93±0.31	5.93±0.31
30th day after being treated with moringa					

Total erythrocyte (x10^6 corpuscle/mm^3)	1.84±0.01^a	1.82±0.02^a	1.93±0.01^b	2.06±0.03^c	2.10±0.01^d
Hematocrit (%)	30.33±0.58^a	30.67±0.58^a	32.33±0.58^b	32.67±0.58^b	33.33±0.58^b
Hemoglobin (g/dL)	6.60±0.20^a	6.53±0.1^a	7.13±0.11^b	7.47±0.11^c	7.53±0.11^c
14th day after being infected with *A. hydrophila*					
Total erythrocyte (x10^6 corpuscle/mm^3)	1.91±0.03^b	-	2.11±0.03^c	2.21±0.03^d	2.31±0.03^e
Hematocrit (%)	31.00±0.00^b	-	33.33±0.58^c	35.67±0.58^d	37.67±0.00^e
Hemoglobin (g/dL)	7.20±0.20^b	-	7.53±0.11^c	7.73±0.11^c	8.33±0.11^d

Note: Superscript on the same line indicates an influence between treats (P<0.05); Hematological data of the positive control fish were not available as all of the fish died in the 4th-5th days after the infection

Leucocyte differentiation and phagocytic activity

In general, the *Moringa*-supplemented diet had a positive effect on the leukocyte profile of the fish. On the 30th day of treatment, the total leukocyte and lymphocyte counts in *Moringa*-treated fish were higher than those observed in fish fed a non-*Moringa* diet (Table 2). Additionally, the phagocytic index of the negative control (NC) and positive control (PC) groups was significantly lower than that of the *Moringa*-treated groups (P< 0.05). Among the treatments, the T3 group (15 g *Moringa*/kg feed) showed the highest phagocytic index, which was significantly different from those of the T1 and T2 groups (Table 2).

On the 14th day post-infection with *A. hydrophila*, the T3 group continued to show the highest phagocytic index, recorded at 30.33 ± 0.58%. This result indicates a marked improvement in the fish’s immune response due to the *Moringa*-enriched diet.

Table 2. Leucocyte differentiation and phagocytic index of *P. hypopthalmus*

Parameter	**Treatments**				
	NC	**PC**	**T1 (5 g/kg)**	**T2 (10 g/kg)**	**T3 (15 g/kg)**
Pre-treatment					
Total Leucocyte (x10^4 cell/mm^3)	6.28±0.60	6.28±0.60	6.28±0.60	6.28±0.60	6.28±0.60
Lymphocyte (%)	75.67±0.58	75.67±0.58	75.67±0.58	75.67±0.58	75.67±0.58
Monocyte (%)	9.33±0.58	9.33±0.58	9.33±0.58	9.33±0.58	9.33±0.58
Neutrophil (%)	6.33±0.58	6.33±0.58	6.33±0.58	6.33±0.58	6.33±0.58
Thrombocyte (%)	8.67±0.58	8.67±0.58	8.67±0.58	8.67±0.58	8.67±0.58
Phagocytic index (%)	17.00±1.00	17.00±1.00	17.00±1.00	17.00±1.00	17.00±1.00
30th day after being treated with moringa					
Total Leucocyte (x10^4 cell/mm^3)	7.87±0.07^a	7.81±0.08^a	7.96±0.01^a	8.27±0.11^b	8.60±0.16^c
Lymphocyte (%)	80.33±0.58^a	80.00±1.00^a	82.33±0.58^b	82.67±0.58^{bc}	83.67±0.58^c
Monocyte (%)	7.67±0.58^b	7.67±0.58^b	6.33±0.58^a	6.67±0.58^{ab}	6.33±0.58^a
Neutrophil (%)	6.67±0.58^b	6.67±0.58^b	6.67±0.58^b	5.67±0.58^{ab}	5.33±0.58^a
Thrombocyte (%)	5.33±0.58	5.67±0.58	4.67±0.58	5.00±1.00	4.67±0.58
Phagocytic index (%)	20.33±0.58^a	20.33±0.58^a	23.33±0.58^b	24.33±0.58^b	25.67±0.58^c
14th day after being infected with *A. hydrophyla*					

Total Leucocyte ($x10^4$ cell)	8.26±0.12 ^{b}	-	9.27±0.07^{c}	9.56±0.06^{d}	9.79±0.09^{e}
Lymphocyte (%)	80.67±0.58^{b}	-	83.33±0.58^{c}	85.33±0.58^{d}	86.67±0.58^{e}
Monocyte (%)	7.67±0.58^{c}	-	5.00±1.00^{b}	4.67±0.58^{b}	4.33±0.58^{b}
Neutrophil (%)	6.33±0.58^{c}	-	4.67±0.58^{b}	4.67±0.58^{b}	4.33±0.58^{b}
Thrombocyte (%)	5.33±0.58^{c}	-	7.00±1.00^{b}	5.33±0.58^{b}	4.67±0.58^{b}
Phagocytic index (%)	21.33±0.58^{b}	-	26.67±0.58^{c}	29.00±1.00^{d}	30.33±0.58^{e}

Note: Superscript on the same line indicates an influence between treats ($p<0.05$); Hematological data of the positive control fish were not available as all of the fish died in the 4th-5th days after the infection

DISCUSSION

One of the major challenges in aquaculture is the occurrence of disease outbreaks caused by pathogenic bacteria. In Indonesia, Motile Aeromonas Septicemia (MAS), commonly caused by *Aeromonas hydrophila*, is a prevalent issue in cultured fish. While chemical antibiotics can be effective in treating infections, their use poses risks, including the accumulation of antibiotic residues in fish meat and the development of antibiotic-resistant bacterial strains. As a safer alternative, natural herbal antibiotics have gained attention due to their efficacy, safety, and lack of harmful residues.

Moringa oleifera, known as a "superfood" for humans, has shown multiple benefits when used in aquaculture. Previous studies have reported that *Moringa* promotes fish growth **(Adeniji *et al.*, 2019; Nadia *et al.*, 2021)**, enhances reproductive performance **(Gad *et al.*, 2019)**, exhibits antibacterial properties **(Korni *et al.*, 2020)**, improves both immunity and overall growth **(Emam *et al.*, 2021)**, and serves as an antioxidant source **(El-Kassas *et al.*, 2022)**.

In this study, supplementing fish feed with *Moringa* leaf powder significantly increased the survival rate of *Pangasianodon hypophthalmus* following infection with *A. hydrophila*. Prior to the challenge test, all fish across treatments were in good health, as indicated by their hematological profiles. However, post-infection, the positive control group (infected, no *Moringa* diet) rapidly deteriorated—ulcers deepened by the second day, appetite declined, activity dropped, and all fish died by the fifth day.

In contrast, *Moringa*-treated fish exhibited milder clinical signs. While ulcers did form and appetite decreased initially, signs of recovery appeared by the fourth or fifth days. Transparent tissue began covering the wounds, and by the 14th day, nearly all ulcers were fully healed (Fig. 3). The survival rate among *Moringa*-treated fish ranged from 88% to 97%, despite the use of no chemical antibiotics, indicating that recovery was due to enhanced immune response.

This enhanced immunity was evident in the hematological and immune parameters. On day 30, the total leukocyte count in *Moringa*-treated fish ranged from 79,600 to 86,000 cells/mL, increasing to 92,700–97,900 cells/mL by day 14 post-infection. These levels indicate a robust immune system. According to **Abbas *et al.* (2014)**, increased leukocyte numbers correlate with enhanced immunity. This immune improvement is likely linked to the bioactive compounds in *Moringa* leaves, such as tannins, flavonoids, phenols, saponins, alkaloids, and vitamins A, C, E, K, and B-complex **(Abalaka *et al.*, 2021)**. **Hai (2015)** emphasized that flavonoids and vitamin E act as immunomodulators, enhancing both innate and adaptive immune functions.

Furthermore, **Shahbazi and Bolhassani (2016)** noted that immunostimulants can enhance non-specific immunity by promoting phagocytic activity—critical for eliminating pathogens. Although saponins (also present in *Moringa*) may inhibit digestive enzymes when consumed in high amounts **(Del *et al.*, 2018)**, at the low dosages used in this study, they may act as immunostimulants **(Hashemi & Davoodi, 2012)**. The results here support the idea that small quantities of *Moringa* leaf powder enhance immunity without adverse effects.

Health status was also reflected in erythrocyte counts, hemoglobin levels, and hematocrit values, particularly in the T3 group (15g/ kg feed). These fish showed significant improvements in these parameters compared to other groups, indicating stimulation of hematopoietic organs such as the lymph and kidneys. **Fazio *et al.* (2015)** reported that increased erythrocyte and hematocrit levels signify enhanced erythropoiesis and oxygen-carrying capacity, thereby improving energy metabolism. Similar hematological improvements were previously reported in *Oreochromis niloticus* **(Sherif *et al.*, 2014)** and *Clarias gariepinus* **(Eyiwumi *et al.*, 2018)** fed with *Moringa*-supplemented diets.

A. hydrophila infection is known to reduce erythrocyte counts due to its septicemic nature **(Kumar *et al.*, 2016; Nurhalisa *et al.*, 2022)**. However, in this study, *Moringa*-treated fish maintained higher erythrocyte counts even after infection, indicating that hematopoiesis continued effectively **(Azlan *et al.*, 2021)**. This shows that *Moringa* supplementation supports the fish's ability to maintain healthy blood parameters during infection.

Leukocytes, particularly phagocytic cells such as monocytes and macrophages, are crucial for immune defense. **Esteban *et al.* (2015)** described phagocytosis as a key mechanism for pathogen clearance in teleost fish. In this study, the phagocytic index increased significantly in *Moringa*-treated fish, especially in the T3 group, suggesting that the diet enhanced innate immune responses and helped the fish resist *A. hydrophila* infection.

Recovery from MAS in this study was closely linked to immune defense. Fish in the T3 group showed increased lymphocyte counts, indicating an increase in T-cell production. **Windarti *et al.* (2023)** stated that increased lymphocyte levels reflect enhanced T-cell activity and lymphoid tissue regeneration. Similar findings were observed in other studies using *Moringa*-supplemented diets in the Nile tilapia and African catfish **(Sherif *et al.*, 2014; Eyiwumi *et al.*, 2018)**.

Several natural herbal immunostimulants have been used successfully in aquaculture. Diets enriched with herbal compounds are known to increase phagocytic activity **(Dhayanithi *et al.*, 2015)**. In this study, fish fed with *Moringa* had a significantly higher phagocytic index compared to controls, indicating stimulation of blood cell differentiation and macrophage proliferation. **Kurniawan *et al.* (2025)** also observed that immunostimulants in fish feed trigger blood cell proliferation and differentiation. On day 14 post-infection, the elevated phagocytic index in the *Moringa*-treated groups confirmed improved non-specific immune responses, supporting the fish's ability to combat bacterial infections.

CONCLUSION

The addition of 15g of *Moringa* leaf powder per kilogram of fish feed pellets enhanced the immune response of *Pangasianodon hypophthalmus* and effectively mitigated the fatal effects of *Aeromonas hydrophila* infection.

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