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Effect of *Gracilaria verrucosa* Extract as an Immunostimulant on the Non-Specific Immune System of Striped Catfish (*Pangasius hypophthalmus*)

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ABSTRACT

One of the failures of freshwater fish farming is often caused by the attack of *A. hydrophila* bacteria. This bacterial attack can be prevented by administering immunostimulants derived from *G. verrucosa* extract. This study aims to determine the non-specific immune response of striped catfish to *Aeromonas hydrophila* infection following supplementation of *G. verrucosa* extract in feed. Five doses were administered in this study: 1 ml, 1.5 ml, and 2 ml per kg of feed, as well as a positive control and a negative control. Blood samples were collected every week after infection, and the parameters observed were total erythrocyte and leucocyte counts, as well as leucocyte differential counts. The results showed that *G. verrucosa* extract significantly increased blood cell counts. The highest dose was found to be the most effective in increasing the non-specific immune response of striped catfish against *A. hydrophila* infection.

Keywords: *Aeromonas hydrophila*, *Gracilaria verrucosa*, immunostimulant, *Pangasius hypophthalmus*

INTRODUCTION

The fisheries sector in Indonesia aims to increase the production of various aquatic commodities, including freshwater species. Striped catfish (*Pangasius hypophthalmus*) is one of the most popular freshwater fish in Indonesia and has become an economically significant fish with extensive cultivation in Southeast Asia (Gao *et al.*, 2021). However, its cultivation is not free from obstacles, such as the motile *Aeromonas septicemia* (MAS) disease caused by infection with *Aeromonas hydrophila.* MAS is an acute bacterial disease that can cause death in fish up to 100% (Semwal *et al.*, 2023). This disease can occur due to the weak immune system of fish caused by poor water quality and organic matter (Abdella *et al.*, 2024).

One of the efforts to control and prevent diseases in striped catfish is using immunostimulants to strengthen the fish's defense system. Immunostimulants are biological compounds that can enhance non-specific defence mechanisms by promoting the activity of phagocytic cells and improving their bactericidal properties (Farooqi and Qureshi, 2018). One source of immunostimulants is *Gracilaria verrucosa*, which can increase phagocytic activity and stimulate respiratory burst response due to its polysaccharide content (Zahra *et al.*, 2017).

This study aims to determine the effect of *G. verrucosa* administration as an immunostimulant on the immune system of striped catfish through total erythrocyte counts, leukocyte counts, and leukocyte

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differential counts following infection with *A. hydrophila* bacteria.

MATERIALS AND METHODS

Time and Place

This study was conducted from February to April 2023 at the Faculty of Health, Medicine, and Life Sciences Laboratory, Universitas Airlangga, Banyuwangi.

Experimental Design

The experiment was carried out at the Integrated Laboratory of the Faculty of Health, Medicine, and Natural Sciences, Airlangga University, Banyuwangi, from February to April 2023. A total of 200 healthy catfish were used and divided into 20 aquariums (10 fish/50 L). This study used a completely randomised design (CRD) with five treatments and four replications. The treatments included a negative or laboratory control, P1 (positive control), P2 (administration of *G. verrucosa* extract at 1 g/kg feed), P3 (administration of *G. verrucosa* extract at 1.5 g/kg feed), and P4 (administration of *G. verrucosa* extract at 2 g/kg feed.

Feeding was conducted twice a day at 08.00 and 16.00 with a percentage of feed amounting to 3% of the fish's body weight. Administration with *G. verrucosa* extract was carried out for up to 20 days, followed by a challenge test using *A. hydrophila*. Non-specific immune responses were observed for up to eight days after infection.

Preparation of *G. verrucosa* Extract

G. verucosa was sourced from traditional cultivation sites in Sidoarjo, East Java. The seaweed was rinsed with fresh water to eliminate residual salts and surface microorganisms and then air-dried in a shaded area to avoid direct sun exposure (Zahra *et al.*, 2017). Once dried, it was ground and sieved through a 60-mesh filter to obtain a fine powder. The powder was macerated in 96% ethanol at a 1:2 (w/v) ratio for 48 hours in a sealed glass container, stirring daily using a spatula. The resulting mixture was filtered to separate the filtrate from the residue, and the filtrate was concentrated using a rotary vacuum evaporator at 50 ^{o}C to obtain a thick ethanol extract of *G. verrucosa*.

Diet Preparation

The preparation of the test feed was adopted from Zahra *et al.* (2017). The thick extract of *G. verrucosa* was first dissolved in 1 ml of sterile distilled water. The test feed given during this study was an artificial feed containing 30% protein. The extract solution was sprayed evenly onto 500 grams of prepared commercial pellet feed. The feed was air-dried on a tray and stored in a sealed container.

Blood Collection

Fish blood sampling for antibody titer test was conducted before treatment (day 0), during treatment (days 7 and 14), after immunostimulant administration (day 21), and after infection with *A. hydrophila* (day 28). Fish samples taken represented 10% of the population for each replication. Fish blood sampling was done using a 1 ml syringe moistened with EDTA (Akram *et al.*, 2022). The blood samples were inserted into a microtube to calculate the blood profile, including total erythrocyte count, total leukocyte count, and leukocyte differential count (Belshe *et al.*, 2004). Antibody titer was determined to evaluate fish immunity following immunostimulant administration.

Challenge with *A. hydrophila*

After 20 days of feeding with *G. verrucosa*, all treatment groups except P0 or the negative control were challenged by immersing *A. hydrophila* at a density of 10^6 CFU/ml for 10 minutes. The fish were then returned to their

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respective aquariums, and behavioral changes in the fish were observed after infection.

Total Leukocyte Count

Leukocyte counts were determined using the method by Lugowska *et al.* (2017). Samples were processed with Turk's solution and observed under a microscope at 400x magnification. The total leukocyte count was calculated using the following formula:

$$\left(\frac{A}{N}\right) \text{x} \left(\frac{1}{V}\right) \text{x } fp$$

Where:
A = number of leukocyte cells counted
N = Number of hemocytometer squares observed
V = volume of each hemocytometer square observed
Fp = dilution factor

Total Erythrocyte Count

Erythrocyte counts were determined using the method by Witeska *et al.* (2022). Samples were observed using Hayem's solution under a microscope at 400x magnification. Counting was performed in five small hemocytometer squares. The total erythrocyte count was calculated using the following formula:

$$\left(\frac{A}{N}\right) \text{x} \left(\frac{1}{V}\right) \text{x } fp$$

Information:
A = number of leukocyte cells counted
N = number of hemocytometer squares observed
V = volume of each hemocytometer square observed
Fp = dilution factor

Leukocyte Differential Count

Leukocyte differentials were assessed by distinguishing cell types based on their morphology, following the method by Chernyavskikh *et al.* (2018). Blood smears were prepared and stained with Giemsa, then observed under a microscope at 1000× magnification to determine the percentages of monocytes, lymphocytes, and neutrophils. Counting was performed systematically, starting from the upper edge of the smear, moving downward, then shifting laterally, and repeating this until 100 cells were counted. The percentages of lymphocytes, neutrophils, and monocytes were calculated using the following formula:

$$\% \text{ lymphocytes} = 100\% \times \frac{l}{100}$$

$$\% \text{ monocytes} = 100\% \times \frac{M}{100}$$

$$\% \text{ neutrophils} = 100\% \times \frac{N}{100}$$

Data Analysis

The data obtained were analyzed using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test (DMRT) at a significance level of 5%. The analyses were performed using IBM SPSS Statistics version 24.

RESULTS AND DISCUSSIONS

Total Erythrocyte Count

Red blood cells contain hemoglobin, which binds oxygen (Seibel *et al.*, 2017). In fish, erythrocytes are nucleated cells with many cytoplasmic organelles (Martins *et al.*, 2021). The results of total erythrocyte counts are presented in **Table 1**. The total erythrocyte counts increased significantly after the administration of *G. verrucosa* extract, particularly in treatments P2, P3, and P4, compared with P0 and P1. P4 had a significantly different value ($p < 0.05$) compared to the control treatment without the administration of *G. verrucosa* extract during the study period, except at day 21, which was not significantly different from P0. In contrast,

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P2 during the study period had no significantly different value compared with P1 or P0 ($p > 0.05$). Erythrocyte counts can serve as indicators of fish health status, reflecting the ability of the immune system to defend against pathogenic bacteria (Stosik *et al.*, 2020). The results also showed that P1, or the positive control, showed a decrease in erythrocyte counts during the study period, with the lowest value recorded at 0.71×10^6 cells/mm^3. According to Alsaid *et al.* (2014), fish infected by the disease often experience a decrease in erythrocyte counts caused by impaired kidney and lymphatic function, which produce erythrocytes. *A. hydrophila* enters the kidneys through the bloodstream

Table 1. Total Erythrocyte Counts During the Study Period (mean ± SD)

Treatment	Total Erythrocyte Counts ($\times10^6$ cell/mm^3)				
	D0	D7	D14	D21	D28
P0	2.15 ± 0.07^{c}	2.36 ± 0.04^{b}	2.47 ± 0.01^{b}	2.53 ± 0.03^{a}	2.50 ± 0.02^{c}
P1	2.32 ± 0.01^{ab}	2.47 ± 0.02^{b}	2.58 ± 0.03^{b}	1.55 ± 0.57^{b}	0.71 ± 0.18^{d}
P2	2.20 ± 0.06^{bc}	2.47 ± 0.12^{b}	2.56 ± 0.05^{b}	2.86 ± 0.05^{a}	2.58 ± 0.05^{bc}
P3	2.34 ± 0.10^{a}	2.71 ± 0.01^{a}	2.75 ± 0.01^{a}	3.02 ± 0.12^{a}	2.77 ± 0.01^{ab}
P4	2.29 ± 0.01^{ab}	2.73 ± 0.04^{a}	$2,82 \pm 0.09^{a}$	2.99 ± 0.02^{a}	2.84 ± 0.09^{a}

Description: (P0) negative control; (P1) positive control; (P2) administration of 1 ml/l extract + *A. hydrophila* infection; (P3) administration of 1.5 ml/l extract + *A. hydrophila* infection; (P4) administration of 1 ml/l extract + *A. hydrophila* infection.

and infects the renal tubules, causing red blood cells to rupture. *A. hydrophila* secretes aerolysin toxins, which cause hemolysis and enterotoxicity, leading to red blood cell rupture and tissue damage, especially in the fins (Singh *et al.*, 2013).

In contrast, the treatments given feed containing *G. verrucosa* had higher erythrocyte counts compared with the control, P0, and P1 treatments. The total erythrocyte counts of the treatments supplemented with *G. verrucosa* extract ranged between 2.20-3.02 × 10^6 cells/mm^3. These values were higher than those of P0 and P1 without the supplementation of *G. verrucosa* extract, which ranged from 0.71-2.58 10^6 cells/mm^3. This is attributed to the phytochemical compounds of *G. verrucosa* extract, including alkaloids, flavonoids, and steroids (Hakim *et al.*, 2020). In addition, *G. verrucosa* contains iron and minerals such as potassium (Khaled *et al.*, 2014), which can increase red blood cell production. This finding is supported by Gupta (2014), who stated that iron is needed in erythrocyte production.

Total Leukocyte Count

Leukocytes play a role in defending organisms against foreign substances such as bacteria and viruses (Mokhtar *et al.*, 2023). The total leukocyte count was found to be fluctuating. The results of total leukocyte counts are presented in **Table 2**. The total leukocyte counts increased in P0, P1, P2, and P4 but decreased in P3 after seven days post-infection. The highest value in this study was found in P4 (5.99×10^4 cells/mm^3), which was not significantly different from P2 ($p > 0.05$) but significantly different from other treatments ($p < 0.05$). Furthermore, on day 14 the values increased again in P0, P1, and P2, with the highest value found in P2 (6.03×10^4 cells/mm^3), which was significantly different from P3 ($p < 0.05$). The increases in leukocyte counts in P2 and P3 were due to the flavonoid content of *G. verrucosa*, which can help activate leukocyte cells and increase their antibody-like functions (Kilani-Jaziri *et al.*, 2015).

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On days 21 and 28, the highest values were recorded in P0 (7.41 and 5.82 $\times 10^4$ cells/mm^3, respectively), which were significantly different ($p < 0.05$) from other treatments. The increase in total leukocyte counts reflected the immune system's attempt to prevent rapid bacterial infection by mobilizing leukocyte cells to the infected area (Nugrahani *et al.*, 2021).

The high number of leukocytes indicates the response of the fish's immune system aimed at enhancing disease resistance. According to Galagarza *et al.* (2017), the normal total leukocyte count in striped catfish ranges from 3-9 $\times$ 10^4 cells/mm^3. The fluctuations in the number of leukocytes in circulation indicate the role of leukocytes in fighting disease and pathogens.

Table 2. Total Leukocyte Counts During the Study Period (mean ± SD)

Treatment	Total Leukocyte Counts (x 10^4 cell/mm^3)				
	D0	D7	D14	D21	D28
P0	2.64 ± 0.12^{b}	3.87 ± 0.93^{bc}	4.84 ± 0.73^{a}	7.41 ± 0.61^{a}	5.82 ± 1.03^{a}
P1	3.65 ± 0.81^{b}	3.89 ± 0.43^{bc}	5.22 ± 0.87^{a}	6.08 ± 1.07^{ab}	2.01 ± 0.31^{d}
P2	3.44 ± 0.23^{b}	5.14 ± 0.71^{ab}	6.03 ± 0.04^{a}	4.55 ± 0.07^{bc}	3.58 ± 0.01^{cd}
P3	5.49 ± 0.33^{a}	3.35 ± 0.34^{c}	2.58 ± 0.59^{b}	4.31 ± 0.83^{bc}	5.39 ± 0.97^{ab}
P4	5.15 ± 0.07^{a}	5.99 ± 0.40^{a}	5.74 ± 0.04^{a}	3.02 ± 0.34^{c}	3.91 ± 0.35^{bc}

Description: (P0) negative control; (P1) positive control; (P2) administration of 1 ml/l extract + *A. hydrophila* infection; (P3) administration of 1.5 ml/l extract + *A. hydrophila* infection; (P4) administration of 1 ml/l extract + *A. hydrophila* infection

Leukocyte Differential Count

Leukocyte differentiation comprises lymphocytes, monocytes, and neutrophils (Barrera et al, 2023). Leukocyte differentiation in fish is a form of immune response possessed by the fish immune system (Mokhtar *et al.*, 2023). The research showed that P4 had the highest value and was significantly different ($p < 0.05$) compared to other treatments in all leukocyte differential parameters, namely lymphocytes, monocytes, and neutrophils (**Figure 1**). Total lymphocyte counts increased in all treatments after the administration of *G. verrucosa* extract and *A. hydrophila* infection. Total lymphocyte counts in the control treatment ranged from 43.6% to 79.05%, while those treated with *G. verrucosa* extract ranged from 74.95% to 92%. An increase in lymphocyte count is a sign of an enhanced immune system stimulated by immunostimulants (Gordan *et al.*, 2015; Pothiraj *et al.*, 2023).

Therefore, *G. verrucosa* extract fed to fish in P2, P3, and P4 stimulated an increase in lymphocytes, which strengthens the immune system of fish. According to Syaieba *et al.* (2019), the increase in total lymphocyte count is due to the flavonoid content in *G. verrucosa* extract, which triggers lymphocyte proliferation. Lymphocytes function in the immune system by recognizing antigens through specific receptors found on cell membranes. When an antigen enters the body, T lymphocytes cannot identify the antigen without specific receptors that enable T cells to stimulate B cells and release antibodies (Kavathas *et al.*, 2019).

The increase in total monocyte counts was also observed following the administration of *G. verrucosa* extract and bacterial infection. Monocytes can function as macrophages and can be found in areas of inflammation or infection in fish (Zhu & Su, 2022). The percentage of monocytes in P4 was the highest, reaching 26%. *G. verrucosa* extract stimulated

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increased monocyte counts in striped catfish, resulting in higher phagocytic activity in the *G. verrucosa* extract treatments compared to the control treatment in fighting *A. hydrophila* bacterial infection. The increase in monocyte counts is an indicator of an increased non-specific immune response stimulated by immunostimulants, as the most visible effect of immunostimulants is facilitating the function of phagocytic cells such as monocytes and neutrophils (Gordan *et al.*, 2015; Pothiraj *et al.*, 2023). Monocyte counts in fish increase with the onset of infection (Roberts, 2012).

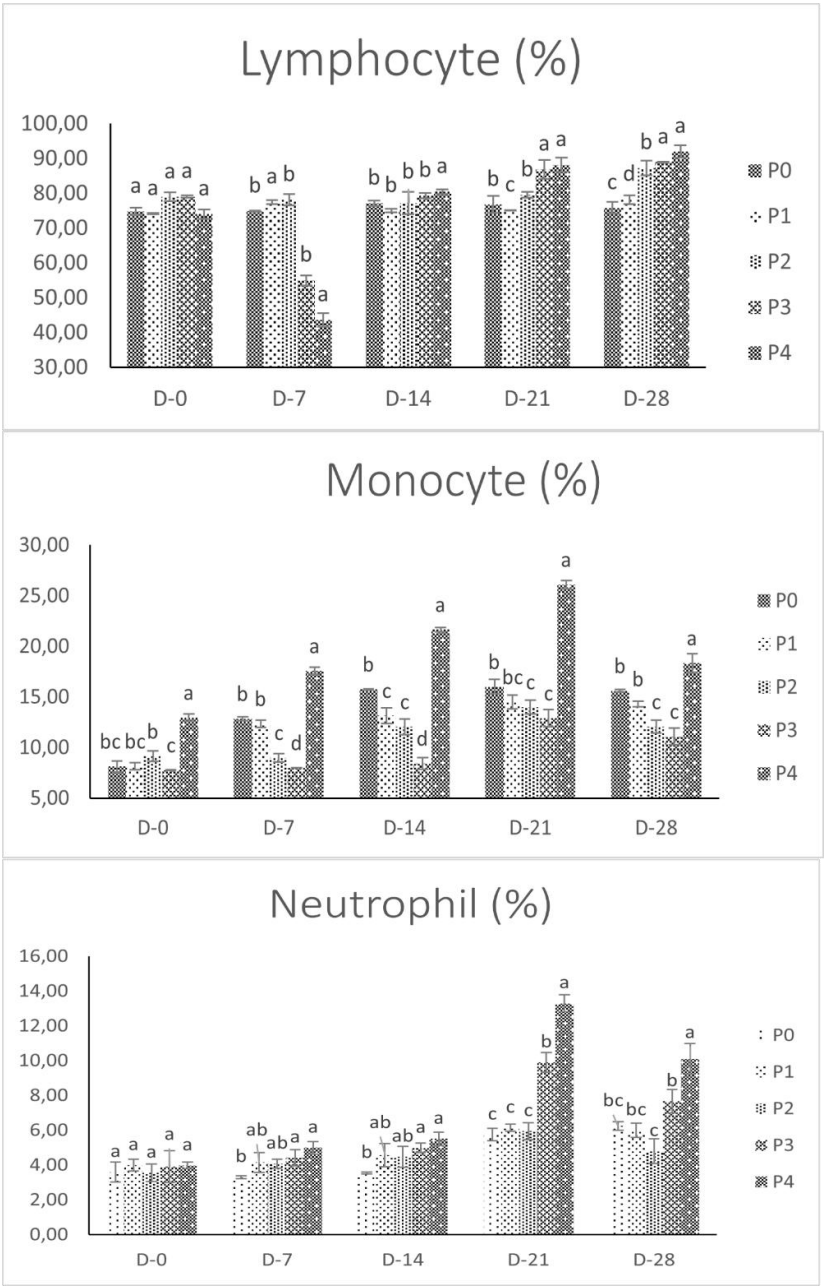

Figure 1. Percentage of leukocyte differential counts, including lymphocytes (top), monocytes (middle), and neutrophils (bottom), in *Pangasius hypopthalmus* treated with *G. verrucosa* extract for 28 days after *A. hydrophila* infection. Based on ANOVA and Duncan's tests, different letters on each bar indicate statistically significant differences ($p < 0.05$). Values are presented as mean ± standard deviation.

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Neutrophil counts were also the highest in P4, reaching 13.5% on day 21 and 10.12% on day 28 (**Figure 1**). However, these values were lower than the normal range of 15-18% (Sulieman and Habeeb, 2024). This condition may be related to increased lymphocyte counts, as the balance between neutrophils and lymphocytes is often regulated by physiological stress responses involving cortisol, which typically triggers neutrophilia and lymphopenia. Therefore, low neutrophil counts may reflect a phase of inflammation resolution or a shift toward adaptive immune dominance characterized by increased lymphocyte activity (Buonacera *et al.*, 2022). Neutrophils are leukocytes that also play a role in the body's defense mechanisms (Lee *et al.*, 2022). Harikrishnan *et al.* (2010) stated that neutrophils in the blood would increase when an infection occurs because neutrophils act as the body's first line of defense. Increased neutrophil counts are related to the process of phagocytosis.

Leukocyte differential counts comprise neutrophils, lymphocytes, and monocytes, which are interrelated in responding to pathogen infections in fish. Neutrophils are the first immune cells to react to pathogen attacks by phagocytosis and produce antimicrobial compounds such as nitric oxide and reactive oxygen species (ROS) (Rieger and Barreda, 2011). Subsequently, monocytes play a role in phagocytosis through phagolysosomes and can replace the function of neutrophils when their numbers decrease (Aliko *et al.*, 2018). Monocytes also function as a specific immune system activation by stimulating lymphocytes (Leirião *et al.*, 2012). Lymphocytes consist of B lymphocytes, which play a role in antibody production, and T lymphocytes, which play a role in recognizing and expressing infected cells (Aliko *et al.*, 2018). Lymphocyte activation occurs after phagocytosis, and activation is carried out by neutrophils and monocytes, thus forming an adaptive immune response in catfish.

CONCLUSION

This study demonstrates that the supplementation of *G. verrucosa* extract to fish feed can stimulate the non-specific immune response of striped catfish by increasing the number of erythrocytes, leukocytes, lymphocytes, monocytes, and neutrophils following infection with *A. hydrophila*. The higher concentration of the extract in fish feed was associated with the stronger immunity of the fish.

AUTHOR CONTRIBUTIONS

The contributions of each author are as follows: AHF drafted the manuscript and designed the tables and graphs. SAS performed data collection and drafted the initial manuscript. LS, HK, DSB, HHBM, and PRY formulated the conceptual ideas and revised the article.

CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

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