Effectiveness of fermentation of *Avicennia marina* leaf extract on *Oreochromis niloticus* (Linnaeus, 1758) contaminated with pathogenic bacteria *Aeromonas hydrophila*

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Abstract. This study investigated the effectiveness of *Avicennia marina* (Forssk.) Vierh. mangrove leaf extract in overcoming *Aeromonas Hydrophila* (Chester, 1901) Stanier, 1943 bacterial infection in Nile tilapia (*Oreochromis niloticus* (Linnaeus, 1758)). The study was conducted from March to July 2023 using a completely randomized design with four treatments (control, 11%, 12%, and 13% extract) and three replicates. *Avicennia marina* leaf extract was obtained through fermentation for 14 days and applied through the feed. Parameters observed included hematological profile (total erythrocytes, hemoglobin, hematocrit, total leukocytes, and leukocrit) and fish survival rate. The result showed that *Avicennia marina* extract significantly improved hematological profiles and survival rate ($p<0.05$), with the 12% dose providing the highest erythrocyte count (1.87×10^{12}/L), increased post-challenge leukocyte count (24.3×10^{9}/L), and a 91% survival rate. In contrast, the control group showed 0% survival after the challenge. The presence of bioactive compounds such as phenolics, saponins, and terpenoids in the extract is likely to have enhanced the fish's immune response and resistance to bacterial infection. These results suggest that *Avicennia marina* leaf extract, especially at a concentration of 12%, is a promising natural agent for improving fish health and survival against *Aeromonas Hydrophila* infection.

Key Words: antibiotic, *Avicennia marina* leaf, bioactive compound, mangrove, pathogen.

Introduction. Due to its high economic value and good adaptability, Nile tilapia farming has become one of the fastest-growing aquaculture sectors in Indonesia and globally (FAO 2020). However, the intensification of Nile tilapia farming often faces challenges in bacterial disease outbreaks, especially those caused by *A. hydrophila* (Ofek et al 2023). *A. hydrophila* infection can cause Motile Aeromonas Septicemia (MAS) disease, characterized by skin lesions, fin necrosis, and high mortality rates, resulting in significant economic losses for fish farmers (Zhao et al 2019).

Tilapia-intensive aquaculture practices also face challenges related to high stocking densities, suboptimal water quality, and environmental stress that can increase fish susceptibility to pathogen infection, especially *A. hydrophila* (Shen et al 2018). This condition is further exacerbated by climate change, which causes fluctuations in water temperature and other water quality parameters, which can increase pathogenic bacteria's virulence (Li et al 2020). The use of antibiotics to combat *A. hydrophila* infections in aquaculture has raised significant concerns regarding antibiotic resistance and negative impacts on the aquatic environment. Therefore, an urgent need to develop effective, sustainable, and environmentally friendly disease control strategies.

Utilizing mangrove leaf extracts as natural antibacterial and immunostimulating agents offers a promising solution. Mangrove ecosystems, widespread in tropical and subtropical coastal regions, serve as coastal protection, biodiversity habitats, and valuable phytochemical resources (Friess et al 2019). Recent studies have shown that mangrove species contain bioactive compounds, such as flavonoids, tannins, and terpenoids, with significant pharmacological potential (Abaho et al 2022). Effectiveness of mangrove leaf extracts in improving growth, immune response, and disease resistance in Nile tilapia. For example, the administration of *A. marina* extract in feed increases phagocytic activity and the production of reactive oxygen species (ROS), which are important defense mechanisms in the fish immune system (Abdel-Tawwab et al 2021).

The use of mangrove extracts through the feed is practical and allows for the efficient uptake of bioactive compounds through the fish's digestive system (Soudant et al 2022). However, extract standardization, dose optimization, and evaluation of long-term impacts on aquatic ecosystems still require further research (Zhu 2020). Therefore, the authors are interested in researching the effectiveness of mangrove leaf extract in tackling *A. hydrophila* infection in Nile tilapia.

This study looked for an alternative natural antibiotic using fermented *A. marina* leaf extract contaminated with the pathogenic bacteria *A. hydrophila*. Fermentation of *A. marina* leaf extract is thought to produce bioactive compounds as an alternative to natural antibiotics. The study aimed to analyze the effect of fermentation on the survival of Nile tilapia (*O. niloticus*) infected with the pathogenic bacteria *A. hydrophila*.

Material and Method

Time and place. This research was carried out in March- July 2023, sampling the leaves of mangrove *A. marina* from the mangrove forest ecosystem of Pangkalan Sesai Village, Dumai City, Riau Province, Indonesia. Fermentation analysis of *A. marina* leaf extracts, bioactive compound tests, and hematology tests of Nile tilapia infected with pathogenic bacteria *A. hydrophila* were conducted at the Marine Microbiology Laboratory, Faculty of Fisheries and Marine Sciences, Universitas Riau, Indonesia.

Methods. The method used in this research is an experimental method that applies a complete randomized design (CRD) with four treatments and three replicates. The treatments were T0 (without the addition of *A. marina* extract), T1 (with the addition of *A. marina* extract to feed at a dose of 11%), T2 (12% dose), and T3 (13% dose). Each treatment was challenged with *A. hydrophila* bacteria.

Mangrove extract preparation. Experiments were conducted in a one-liter laboratory scale extractor. The extractor was equipped with an agitator to provide intense mixing during extraction, a condenser for recondensing solvent vapors due to elevated temperature, and a water bath fitted with a thermostat to ensure constant temperature. Extraction was carried out for 360 min, and during the process, samples of fermented *A. marina* leaf (5 mL each) were drawn and then evaporated at 45ºC for yield analysis purposes. After extraction, the mixture was separated sequentially from the residual solid using Whatman 41 filter paper, followed by centrifugation at 6000 RPM for 15 min and filtration using Whatman 42. The solid-free mixture was evaporated using a Rotary Vacuum Evaporator (Buchii, Switzerland) at 50ºC with gradual depressurization from 30 kPa to 7 kPa (Patra et al 2022).

The fermentation technique of *A. marina* mangrove leaf extract was taken as much as 3 kg, and then 750 g of brown sugar was added. Furthermore, 6 L of clean water was added (1:0.25:2). After that, all the ingredients were blended until smooth and put into the fermentation container and then tightly closed. Then, the fermentation process was carried out for 14 days. Before use, *A. marina* extract was subjected to phytochemical analysis, including bioactive compounds from fermented *A. marina* leaf extract, such as alkaloid, steroid, phenolic, flavonoid, saponin, and terpenoid compounds.

Preparation of Avicennia marina extract feed. The pellets used were Hi-Pro-vite 781-2, a commercial fish feed produced by PT Central Pangan Pertiwi Animal Feedmill Co. Ltd, Karawang, West Java, Indonesia. The composition of this fish pellet is 31-33% protein, 4-6% fat, 3-5% moisture, and 9-10% water content. Then, the pellets were added with *A. marina* extract by spray method with doses (11%, 12%, and 13%). After that, the pellets were dried before being given to the fish.

Fish acclimatization and rearing. The container used in fish rearing is a black container with a capacity of 100 L and filled with 80 L of brackish water (5 ppt salinity). Each container was filled with a stocking density of 20 fish. Nile tilapia used were obtained from farmers in Pekanbaru City, Indonesia, with a length of 11.00±1.00 cm and a weight of 14.00±1.00 g. The fish were reared for 10 days and fed with brackish water (salinity 5 ppt). Each rearing container is also treated as a recirculation system, aiming to maintain optimal water quality for fish. Fish rearing was performed for 10 days by feeding with *A. marina* leaf extract. Feeding was done twice, at 09:00 AM and 03:00 PM, as much as 5% of body weight (Effendi et al 2025).

Blood sampling. During the study, blood samplings were conducted 3 times: before the treatment, on the 10th day after treatment, and on the 14th day after being infected with *A. hydrophila*. The fish were sedated using clove oil (0.01 mL L^{-1} water) to reduce stress. As the fish lost balance and became unresponsive, the blood was withdrawn from the vena caudalis using a 1 mL syringe wet with 10% EDTA. The blood was then kept in an Eppendorf tube and was ready for further hematological analysis, such as the number of erythrocytes, leucocytes, hemoglobin, hematocrit, and leucocyte following Blaxhall & Daisley in (Witeska et al 2022).

Data analysis. Data on hematology parameters (the total number of erythrocytes, hematocrit, hemoglobin, and total number of leukocytes) and survival were tabulated and analyzed using SPSS version 24. If necessary, data were analyzed using a One Way ANOVA and continued by a Student Newman Keuls (SNK) test.

Results and Discussion

Fermentation test of Avicennia marina leaf extract. Based on *A. marina* leaf samples, the fermentation process of *A. marina* leaf extract is carried out to identify the content of bioactive compounds thought to have potential as antibacterial ingredients to overcome the attack of pathogenic bacteria *A. hydrophila* in mariculture. Bioactive compounds are essential (carotenoids, omega-3) and non-essential (vitamins, polyphenols) found in nature. Screening analysis of bioactive compounds from fermented *A. marina* leaf extract can be seen in Table 1.

Table 1

Screening of *Avicennia marina* mangrove leaf extract

No.	*Mangrove leaf*	
	Bioactive compounds	*Avicennia marina*
1	Alkaloid	-
2	Steroid	-
3	Phenolic	+
4	Flavonoid	-
5	Saponin	+
6	Terpenoid	+

Description: +: positive (identified); -: negative (not identified).

Table 1 shows that the test results of bioactive compounds in the fermentation of *A. marina* leaf extract contain antibacterial ingredients. The test results of bioactive compounds in the extra fermentation of *A. marina* leaves can be identified as phenolic compounds, saponins, and terpenoids, while alkaloids, steroids, and flavonoid compounds are not identified.

The plant's ecological adaptability and pharmacological potential influence the bioactive compounds, such as phenolics, saponins, and terpenoids. According to Annas et al (2023), *A. marina* leaves contain high concentrations of phenolic compounds, which are critical for their antioxidant properties. Saponins are another class of compounds found in *A. marina* leaves. They are known for their surfactant properties, which can disrupt pathogen cell membranes (Rozirwan et al 2022). Terpenoid compounds are part of the plant's secondary metabolites that help it adapt to the harsh coastal environment where mangrove forests thrive (Wu et al 2023). The phytochemical profile of *A. marina* can be influenced by external conditions such as pollution and habitat, which can affect the bioactive potential of the plant (Rozirwan et al 2022).

***Hematological test of Nile tilapia*.** To see the condition of Nile tilapia contaminated with pathogenic bacteria *A. hydrophila*, hematological tests were carried out, namely total number of erythrocytes, hemoglobin levels, and hematocrit of Nile tilapia that had been given artificial feed containing fermented *A. marina* leaf extract in the early stages (0 days), then Nile tilapia was kept in a maintenance bath for 10 days.

Furthermore, fermentation of *A. marina* leaf extract was carried out on experiment feed, which could maintain the hematological values of Nile tilapia under normal conditions for 14 days (post-challenge test). The results of a hematologic examination of Nile tilapia can be seen in Table 2.

Table 2

Hematological mean values and standard deviation of parameter

Parameters	Treatment			
	T0	*T1*	*T2*	*T3*
		Day 10		
RBC ($x10^{12}$/L)	1.43 ± 0.03^a	1.56 ± 0.02^b	1.69 ± 0.02^c	1.66 ± 0.02^c
HCT (%)	28.33 ± 0.58^a	29.00 ± 1.00^a	3100 ± 1.00^b	29.33 ± 0.58^a
Hb (g dL^{-1})	7.27 ± 0.12^a	7.73 ± 0.12^b	8.07 ± 0.12^c	7.87 ± 0.12^{bc}
WBC ($x10^9$ L)	19.10 ± 0.20^a	19.70 ± 0.10^b	20.70 ± 0.30^c	19.90 ± 0.10^b
LEU (%)	1.67±0.58	1.67±0.58	1.67±0.58	1.67±0.58
		Post challenge test		
RBC ($x10^{12}$/L)	0.00 ± 0.00^a	1.76 ± 0.05^b	1.87 ± 0.02^c	1.81 ± 0.02^b
HCT (%)	0.00 ± 0.00^a	30.67 ± 0.58^b	31.67 ± 0.58^c	30.33 ± 0.58^b
Hb (g dL^{-1})	0.00 ± 0.00^a	8.07 ± 0.12^b	8.40 ± 0.20^c	8.33 ± 0.12^c
WBC ($x10^9$ L)	0.00 ± 0.00^a	22.4 ± 0.40^b	24.3 ± 0.20^d	23.2 ± 0.40^c
LEU (%)	0.00 ± 0.00^a	2.00 ± 1.00^b	1.67 ± 0.58^b	1.67 ± 0.58^b

Description: superscript on the same line indicates an influence between treats; ($P<0.05$); P0 (control), P1 (dose 11%), P2 (dose 12%), P3 (dose 13%); RBC: Red Blood Concentration; HCT: Hematocrit; Hb: Hemoglobin; LEU: Leukocrite.

Table 2 shows that the hematological condition of Nile tilapia after being fed with feed containing different concentrations of fermented mangrove leaf extract showed an effect between treatments ($p<0.05$) and was in the normal range. Giving mangrove leaf fermentation concentration T2 (12% dose) showed the highest increase in erythrocyte cell number on day 10 and post-challenge compared to the treatment of T0 (control), T1 (11% dose), and T3 (13% dose). The total number of erythrocytes of Nile tilapia given fermented mangrove leaf extract ranged from 1.69-1.87x1012/L, hematocrit ranged from 31.00-31.67%, hemoglobin ranged from 8.07-8.40 g dL^{-1} and were still in the normal range. According to Gazali et al (2023), normal tilapia erythrocyte total ranged from 1.34-2.11 $x10^{12}$/L, hematocrit 26.17-33.19%, and hemoglobin 6.26-11.2 g dL^{-1}. The increased erythrocyte cell number of Nile tilapia is thought to be due to bioactive compounds in mangrove leaf extract fermentation, namely phenolics, saponins, and terpenoids. These bioactive compounds can increase the activity of hematopoietic (head kidney, pronephros) to produce blood.

In the control, post-challenge test, hematological measurements could not be taken because there was 100% mortality. This indicates that feeding without adding mangrove leaf extract cannot increase fish immunity *to A. hydrophila* bacterial infection. *A. hydrophila* bacterial infection attacks the blood-producing organs and can reduce erythrocyte function, lowering red blood cell counts and hemoglobin levels. According to (Yacoub et al 2021), Hemoglobin concentration and hematocrit values are often reduced in fish affected by bacterial infections. This reduction is attributed to the damage and functional impairment of erythrocytes caused by bacterial toxins.

Fish erythrocytes are usually ellipsoidal and nucleated, varying in size and age between species. The number of erythrocytes is influenced by activity level, water temperature, and oxygen availability. Hemoglobin and hematocrit concentrations are directly correlated with erythrocyte counts, as they are the main components of these cells responsible for oxygen transport (Witeska et al 2020). In addition, environmental stressors and bacterial infections can cause a decrease in hemoglobin and hematocrit values that can cause anemia in fish (Prahardika & Styawan 2022).

The total leukocytes and leukocrite of Nile tilapia, after being given feed containing mangrove leaf extract for 10 days, amounted to 19.10-20.70x10^9/L. Post-challenge tests on fish fed with mangrove extract increased to 22.40-24.30x10^9/L, while in the control treatment (T0), this could not be observed because mortality reached 100%. This indicates that feeding with mangrove leaf extract can increase the immune response of tilapia so that fish can maintain their survival, ranging from 80-91% (Figure 2). An ANOVA test shows that feeding with the addition of mangrove leaf extract *A. marina* influences the total number of leukocytes and leukocrit in Nile tilapia ($p<0.05$). The addition of as much as 12% gives the best results on the value of total leukocytes and leukocrit in Nile tilapia.

During maintenance, the total leukocytes of saline tilapia are still within the normal range. According to Lestari (2021), normal Nile tilapia leukocytes are >18.9x10^9 /L. The increase in the number of leukocytes is thought to be caused by the content of secondary metabolite compounds from *A. marina* extracts, such as phenolics, saponins, and terpenoids. An increase in leukocyte count after the administration of mangrove extracts generally indicates a positive immune response to pathogen exposure or environmental stress, where leukocytes, particularly lymphocytes, play an important role in maintaining homeostasis and fighting infection. Conversely, a decrease in leukocytes may indicate excessive physiological stress or toxic effects from a dose of extract that is too high. Laith et al (2020) hybrid Nile tilapia showed that *Excoecaria agallocha* leaf extract significantly increased the white blood cell count when administered at a 50 mg/kg concentration. This suggests that the extract contains agents that stimulate leukocyte production, enhance fish's immune response, and protect fish from oxidative stress, thus increasing resistance to environmental challenges (Muahiddah & Salim 2023). Generally, leukocyte counts are influenced by feed nutrition, environment, and fish health status.

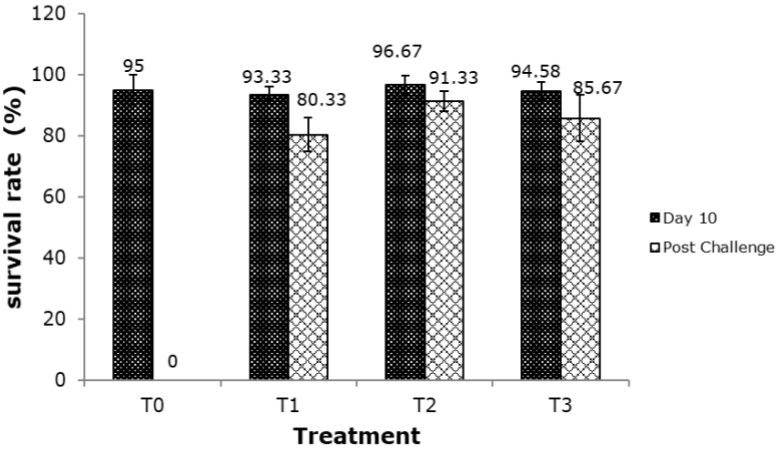

Figure 3. Survival rate of Nile tilapia.

Total leukocytes increased in Nile tilapia after post-challenge treatment due to the entry of pathogenic bacteria *A. hydrophila* into the body of fish so that it experiences stress. This causes the total level of leukocytes in the body of Nile tilapia to increase. This is thought to be related to the fermentation of mangrove leaf extract, increasing the immune response of the Nile tilapia body so that total leukocytes increase. According to Kane et al (2016) and Soltani et al (2019), an increase in total leukocytes can be considered a good indicator of the immune response of fish, indicating an increase in immune cells.

The results of calculating the survival rate in Nile tilapia after post-challenge test treatment were the highest SR of 91.33%. The results of the observation of the survival rate after the post-challenge tests are P0 (control) treatment, and survival is 0%. This shows that Nile tilapia that are fed without fermented mangrove leaf extract experience death due to attacks by pathogenic bacteria *A. hydrophila*.

Conclusions. The study shows that the addition of *A. marina* leaf extract to the experimental feed of Nile tilapia significantly enhances immune responses and survival rates (91%) against *A. hydrophila* infection, with a 12% dose optimally improving hematological parameters such as total number of erythrocytes, hemoglobin, hematocrit, and total number of leukocytes. These results highlight the potential of *A. marina* as a natural, sustainable alternative to synthetic antibiotics. By promoting fish health and reducing reliance on chemicals, this approach supports the global shift toward eco-friendly and resilient aquaculture practices and offers a promising solution to enhance productivity, food security, and the long-term viability of fish farming.

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