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MORINGA LEAF SIMPLICIA ENCHANCED HEALTH STATUS AND IMMUNE RESPONSE OF TILAPIA AGAINST *Edwardsiella tarda* INFECTION

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ABSTRACT

Intensive fish aquaculture increases the risk of diseases like Edwardsiellosis caused by Edwardsiella tarda, which can lead to 30–50% mortality rates. While antibiotics have been used for disease control, excessive use can result in antibiotic residue and bacterial resistance. Moringa leaves (Moringa oleifera) are a promising herbal ingredient containing active compounds like glucomoringin, moringin, flavonoids, tannins, sapponins, alkaloids, and phenols, which can be used in aquaculture. This study aims to evaluate the effect of administering different doses of moringa leaf simplicia (MOLS) through feed for the prevention of E. tarda bacterial infection in tilapia. The research was conducted in two stages, namely in vitro and in vivo testing. The first stage was an in vitro test to determine the dose of moringa leaf extract that can inhibit the growth of E. tarda. The second stage was an in vivo test to evaluate the effect of feeding MOLS on growth performance, health status and immune response, and resistance of tilapia to E. tarda infection. In vivo tests were namely MOLS 12.5 g kg^{-1} (M12.5), 25 g kg^{-1} (M25), 50 g kg^{-1} (M50), positive control (K+) and negative control (K-). The test fish used were tilapia fish measuring 7.77 ± 0.06 g which were kept in 15 aquariums with density of 10 fish per aquarium for 30 days. Fish were challenged on the 31st day with E. tarda cell suspension 10^{6} CFU mL^{-1} which corresponds of the LD50 value-the bacterial dose that cause 50% mortality in the tested fish population and then the fish were maintained for 7 days. The results showed that increasing the addition of MOLS for 30 resulted in significant improvements (P<0.05) in growth of tilapia with control. The highest abundance of lactic acid bacteria was found in the M12.5 treatment, a significant difference (P<0.05) compared to the control treatment. MOLS also significantly improved the health status and immune response of tilapia (P<0.05), and suppressed the population of E. tarda in kidney and liver organs. Kidney histopathology showed normal to lightly damage in the MOLS treatment observed significantly lower (P<0.05) than the K+ treatment with lightly damage. Liver histopathology also showed normal to lightly damage in MOLS treatment observed significantly lower (P<0.05) than K+ with medium damage. M12.5 treatment showed the highest resistance significantly different (P<0.05) compared to K+ treatment. MOLS was able to improve the health status and immune response, as well as the resistance of tilapia to E. tarda infection with the best dose in feed with the addition of 12.5 g kg^{-1} MOLS.

KEYWORDS: *Edwardsiella tarda*; immunostimulant; moringa; simplicia; tilapia

INTRODUCTION

According to the FAO (2024), the main aquaculture production in Indonesia in 2021 was tilapia (*Oreochromis* sp.). Tilapia is widely cultivated across various regions and has developed well in Indonesia due to its high economic value (Suprayudi, 2013). Based on the performance report from the Ministry of Marine Affairs and Fisheries (KKP, 2024), tilapia production in 2023 reached 1.36 million tons, accounting for 65.82% of the target of 2.07 million tons. This indicates a need to increase production to meet the target. Tilapia holds great potential in aquaculture due to advantages such as relatively fast growth, suitability for high-density farming, ability to consume both natural and artificial feed, and high adaptability to a wide range of water quality conditions (Hendriana *et al.,* 2022).

A strategy to increase tilapia production in order to meet the target is through the development of intensive aquaculture systems. Intensive tilapia farming requires high stocking densities and increased

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feeding (Suprayudi *et al.,* 2017). However, intensive farming systems carry the risk of disease outbreaks in fish. Diseases in fish can hinder production, cause outbreaks, slow growth, prolong the rearing period, and ultimately lead to the death of the cultured fish. Fish diseases that commonly affect aquaculture commodities are often caused by bacteria, one of which is *Edwardsiella tarda*, the bacterium responsible for Edwardsiellosis. According to Narwiyani & Kurniasih (2011), factors influencing *E. tarda* infection include fish stress, particularly due to high stocking densities, poor water quality, and high organic matter content. Horizontal transmission of *E. tarda* occurs through contact between hosts or via the water.

So far, to minimize mortality caused by *E. tarda* infections, chemical substances (antibiotics) classified as safe have been used, with their application following government regulations (A'yunin *et al.,* 2020). The use of chemical treatments such as antibiotics has been restricted under the Ministry of Marine Affairs and Fisheries Regulation No. 1 of 2019 concerning the use of fish medication. Continuous use of chemical substances can lead to new problems, such as the accumulation of residues in fish tissues and the development of bacterial resistance (Maqsood *et al.,* 2009). Therefore, disease control using natural substances is needed, as they do not cause negative effects on consumers or the environment. Natural substances used as medicine without undergoing any processing are referred to as simplicia. One such natural material that can act as an immunostimulant is the moringa leaf (*Moringa oleifera*). Moringa leaves contain active compounds such as glucomoringin and moringin (Borgonovo *et al.,* 2020), as well as flavonoids, alkaloids, phenols, tannins (Ikalinus *et al.,* 2015; Riskianto *et al.,* 2021), and saponins (Arora *et al.,* 2013). Moringa leaves serve as an immunostimulant (Mansour *et al.,* 2018), and also have antimicrobial, anti-inflammatory (Borgonovo *et al.,* 2020), and antioxidant properties (Abadallah & Ali, 2018). Their abundant availability year-round, affordability, and ease of access make them a promising option for use.

The use of moringa leaves as an immunostimulant has been widely applied through various methods, including immersion, injection, and oral administration. The use of moringa leaf simplicia (MOLS) at a concentration of 15 mg/mL has been shown to enhance immune responses and control *Aeromonas hydrophila* infections in tilapia (Abd El-Gawad *et al.,* 2019); moringa leaf extract (MOLE) at 0.075 mg/mL injected into tilapia fingerlings improved non-specific immune responses against *A. hydrophila* (Subryana *et al.,* 2020); administration of MOLE at 0.0625 mg/mL helped control *Epistylis* sp. infestations in tilapia (Abdushamad *et al.,* 2022); extract concentrations of 25 mg/mL increased non-specific immune responses and resistance to *Vibrio alginolyticus* (Abidin *et al.,* 2022); and MOLS at 15 mg/mL improved nutrition and growth performance in *Pangasianodon hypophthalmus* (Windarti *et al.,* 2023). However, based on previous research, the use of MOLS to prevent *E. tarda* infections in tilapia has not yet been scientifically studied. Therefore, research is needed to evaluate the effectiveness of MOLS in preventing *E. tarda* infections in tilapia.

MATERIALS AND METHODS

This study consisted of two stages: in vitro and in vivo testing. The first stage involved in vitro tests to determine the effective dose of moringa leaf extract (MOLE) that can inhibit the growth of *E. tarda* bacteria. The second stage was an in vivo test to evaluate the effect of administering moringa leaf simplicia (MOLS) through feed on tilapia challenged with *E. tarda*. At the time this study was conducted, formal ethical approval from an animal ethics committee was not available. However, all experimental procedures were performed in accordance with standard animal welfare practices, ensuring the minimization of stress, pain, and discomfort to the experimental animals. The handling and maintenance of fish followed the principles of the 3Rs (Replacement, Reduction, and Refinement).

In Vitro Testing

Preparation of Moringa Leaf Extract

Moringa leaves were obtained from Blora, Central Java, Indonesia. The leaves were thoroughly washed and dried in an oven at 60°C. The dried leaves were then blended into a fine powder to produce simplicia. For inhibition zone and MIC testing, the MOLS was processed into an extract. The simplicia was macerated using 96% ethanol at a ratio of 1:4 (w/v) and shaken for 24 hours. The solution was filtered using Whatman No. 41 filter paper to obtain the filtrate and then remacerated twice with 96% ethanol. The filtrate was concentrated using a rotary vacuum evaporator at 40°C. The MOLE was stored at 4°C (Abidin *et al.,* 2022).

Preparation of *E. tarda* Bacteria

The bacterial isolate was cultured on brain heart infusion agar (BHIA) in petri dishes and incubated for 24–48 hours. The *E. tarda* bacteria were made resistant to the antibiotic rifampicin. The bacterial colonies that grew were inoculated into brain heart infusion broth (BHIB) and incubated on a shaker at 1400

rpm for 24 hours. The culture was then diluted using the serial dilution method.

Experimental Design In Vitro

The first phase of the study involved an in vitro test to determine the effective dose of MOLS that could inhibit the growth of *E. tarda* through inhibition zone and minimum inhibitory concentration (MIC) assays. The in vitro test was conducted using eight treatments with three replications each, with dosages based on the study by (Abidin *et al.,* 2022).

The treatments in this study consisted of several groups. The PBS control group used a medium containing phosphate-buffered saline (PBS). The Antibiotic control group used a medium supplemented with the antibiotic oxytetracycline (OTC) at a concentration of 50 ppm. The *E. tarda* control group was exposed to a medium containing *E. tarda*. Several experimental groups were treated with different concentrations of MOLE : M1.25 with 1.25 mg/mL, M2.5 with 2.5 mg/mL, M5 with 5 mg/mL, M10 with 10 mg/mL, and M20 with 20 mg/mL. These treatments aimed to evaluate the effectiveness of MOLE as an alternative treatment against *E. tarda* infection. The concentrations of 1.25–20 mg/mL of *M. oleifera* leaf extract (MOLE) used in this study were determined based on preliminary in vitro antibacterial assays against *E. tarda*, including inhibition zone and MIC tests. The selected dose range reflects a gradient from minimally effective to maximum tolerable concentrations suitable for dietary administration.

Inhibition Zone Test of *E. tarda* and MIC Test

The antibacterial activity of MOLE was tested using the Kirby-Bauer method. BHIA media was divided into several sections and labeled according to the respective treatments. *E. tarda* bacteria were inoculated onto the media using a sterile cotton swab, evenly spread across the agar surface. The MOLE was diluted using 50% dimethyl sulfoxide (DMSO) at 1 mL, with extract concentrations based to 1.25, 2.5, 5, 10, and 20 mg/mL. The positive control used oxytetracycline, the negative control used PBS, and the *E. tarda* control contained only the bacteria, with each treatment performed in triplicate. Sterile paper discs were soaked in the extract solution until fully saturated, then placed on the surface of the BHIA medium. The media were then incubated for 24–48 hours at 28°C. Clear zones (inhibition zones) formed around the discs were measured using a caliper with an accuracy of 0.05 mm.

MIC test was conducted using the broth dilution method based on Unegbu *et al.* (2020). Each falcon tube was filled with 5 mL of BHIB and treated with MOLE at concentrations of 1.25, 2.5, 5, 10, and 20 mg/mL. The positive control used oxytetracycline, the negative control used PBS, and the *E. tarda* control contained only the bacteria without any treatment. All treatments, except for the negative control, were inoculated with 5 µL of *E. tarda* bacterial suspension and shaken for 24 hours. The contents were then centrifuged at 5000 rpm for 5 minutes to separate the pellet and supernatant. The pellet was rinsed twice with PBS. The results of the dilution were measured using a spectrophotometer at a wavelength of 630 nm.

In Vivo Testing

Experimental Design In Vivo

The second phase of the study involved an in vivo test aimed at evaluating the effects of MOLS through feed on growth performance, health status, immune response, and resistance of ilapia to *E. tarda* infection. The in vivo experimental design consisted of five treatments with three replications each. The doses used in the in vivo test were based on the results of the in vitro test, with the extract doses converted into simplicia form using a 10% yield rate (Kusuma & Aprileili, 2022). This yield was calculated from the weight of the extract obtained (7.5 g) divided by the weight of the original simplicia (75 g), then multiplied by 100%.

The treatments consisted of five groups. The K– group received regular feed and was injected with PBS on day 31. The K+ group also received regular feed but was injected with *E. tarda*. The M12.5, M25, and M50 groups were fed diets containing 12.5, 25, and 50 g/kg of MOLS, respectively, and were all injected with *E. tarda* on day 31 to assess the effects of MOLS on disease resistance.

The preparation of the containers involved using 15 glass aquariums measuring 60 × 30 × 30 cm were used in the study. Each aquarium was first washed with soap and rinsed with clean water. They were then disinfected with 30 ppm chlorine for 24 hours while being aerated strongly. After disinfection, the aquariums were neutralized using 60 ppm sodium thiosulfate and aerated until the chlorine odor disappeared. The water was then drained, and the aquariums were dried before being filled with clean water that had been filtered for 24 hours using bioballs and dacron to a depth of 20 cm.

Test Feed Preparation

The test feed used commercial pelleted feed containing 30-35% protein. Mixing of MOLS into the feed was done by the repelleting method. Commercial fish

feed is pulverized into flour, then commercial feed is added to each MOLS according to the treatment, namely 12.5 g kg^{-1}, 25 g kg^{-1}, 50 g kg^{-1}. Then stirred using a mixer until homogeneous. Water was added, then 2% commercial binder. After the mixing process, the material was molded using a pellet molding machine. The pellet drying process is carried out using an oven at 60°C for 2 hours and then the feed is stored in an airtight container at room temperature. The diet composition of the four diets on Table 1.

Table 1. Formulation of experimental diets (g per 500 g)

Ingredient	Experimental feed tested diets			
	M0	M12.5	M25	M50
Moringa leaf simplicia	0	6.25	12.5	25
Commercial diet	490	483.75	477.5	465
Binder	10	10	10	10
Total	500	500	500	500

Meaurement of Water Quality

Water quality during the rearing period was checked periodically, every 3 days after syphoning in the morning for 30 days. Parameters measured in situ include temperature, pH, and dissolved oxygen (DO), while ex situ parameters include ammonia, nitrite, and nitrite. The measurement results of temperature, pH, DO showed that they were in accordance with the water quality standards based on SNI 7550:2009, namely temperature (27–29°C), pH (7.30–7.98), dissolved oxygen (DO) (7.7–9.4 mg L^{-1}). While the measurement results of nitrite (0.19–0.42 mg L^{-1}) are in accordance with the standard standards of Ebeling *et al.* (2006), nitrate (1.38–1.63 mg L^{-1}) in accordance with (PP No. 22 of 2021), and total ammonia nitrogen (TAN) (0.23–0.48 mg L^{-1}) according to Caldini *et al.,* 2015). All water quality parameters during rearing were within the water quality standards for tilapia aquaculture.

Observed Parameters

Growth Performance

Growth performance parameters were observed during the MOLS feeding period, namely the beginning of fish stocking (H0) and the end of the rearing period (H30).

Fish survival rate was observed from the beginning to the end of rearing, with calculations using the following formula (Dawood *et al.,* 2015):

$$SR\ (\%) = \frac{\text{fish harvested}}{\text{fish stocked}} \times 100$$

The average daily growth rate of fish was observed at the end of rearing, with calculations using the following formula (Steffens, 1989):

$$ADG = \frac{\text{final mean weight (g)} - \text{initial mean weight(g)}}{\text{rearing time (days)}}$$

The feed conversion ratio was observed at the end of maintenance, with the calculation using the following formula (Putra *et al.,* 2019):

$$FCR = \frac{\text{total feed given}}{\text{fish weight gain}}$$

Total feed intake is the amount of feed consumed by fish during maintenance. The amount of feed intake is calculated every day from the beginning to the end of maintenance, with the calculation using the following formula (Gabriel, 2019):

FI (g) = amount of initial feed (g)-amount of final feed (g)

Bacterial Count

Calculation of total LAB was carried out with total LAB that grew on Man Rogosa and Sharpe Agar (MRSA) media (Fardiaz, 1993). Preparation of MRSA by weighing MRSA as much as 67.13 g dissolved in 1000 mL of distilled water, then the MRSA solution was sterilized with an autoclave at 121°C for 15 minutes. Furthermore, the media was left until lukewarm, then aseptically the media was poured into Petri dishes and left until solid. Total LAB counting began with 0.1 g of crushed intestinal organ samples, then put into a tube containing 0.9 mL of sterile PBS, then homogenized. A total of 0.1 mL of sample was diluted into 0.9 mL of sterile PBS and serial dilutions were performed. The results of the dilution were spread as much as 50 ìL of each MRSA media. The media containing the culture was incubated for 24 hours at 37°C, then the total abundance of bacteria was calculated using the Total Plate Count (TPC) method with the formula (Fardiaz, 1993):

$$\text{LAB Count (CFU mL}^{-1}) = \frac{\text{number of colonies}}{\text{volume inoculated (mL) x dilution factor}}$$

Calculation of bacterial population using the TPC method (Barus *et al.,* 2017). The target organs of fish that will calculate the population density of *E. tarda*

bacteria are liver and kidney (Indriasari *et al.,* 2020). Organs were weighed as much as 0.1 g and then crushed, then serial dilutions were made using 0.9 mL of sterile PBS solution. Each dilution, the eppendorf tube was vortexed first before being inserted into the next media diluent to make it homogeneous. The results of the dilution were spread as much as 50 ìL on BHIA media. Incubation was carried out at 37ºC for 24 hours. Calculation of the number of colonies that grow with the formula (Madigan *et al.,* 2012):

$$\sum \text{Bacteria}\left(\text{CFU mL}^{-1}\right) = \text{number of colonies x } \frac{1}{\text{dilution factor}} \text{x } \frac{1}{\text{sample(mL)}}$$

Health Status and Response Immune

The parameters of health status and immune response were observed five times, namely, at the beginning of fish stocking into the aquarium (H0), at the end of the rearing period (H30), after the challenge test (H+1, H+3, and H+7).

Total erythrocytes were counted using the method of Blaxhall & Daisley (1973). Blood samples were sucked using an erythrocyte Thoma pipette containing a red stirring bulb to a scale of 0.5, then Hayem's solution was added to a scale of 101. After that, both ends of the pipette were closed using fingers and then homogenized by forming a figure 8 for 3-5 minutes until the blood was mixed. A total of 1-2 drops of blood are discarded, then the sample is put into a microtube or can be directly observed using a haemacytometer under a microscope with a magnification of 250 X, using the following formula:

$$\text{Hc (\%)} = \frac{\text{length of volume of red blood cells that settle}}{\text{length of total blod volume in the tube}} \times 100$$

Haemoglobin levels were observed using the Wedemeyer & Yasutake (1977) method, i.e. Sahli tubes were filled using 0.1 N HCl to scale 10 (red scale). Blood was sucked using a Sahli pipette as much as 0.02 mL, then put into a Sahli tube and allowed to stand for 3 minutes. After that, the Sahli tube was placed between 2 standard color tubes. Distilled water was added and stirred slowly using a stirring rod until the same color as the standard color. Haemoglobin levels were expressed in G% according to the yellow scale recorded on the Sahli tube.

Haematocrit levels (Hc) were observed and calculated using the method of Anderson (1993). Blood samples were sucked using a haematocrit tube slowly up to ¾ of the tube. The lower end of the haematocrit tube was closed using crystoseal to block the blood from escaping. Next, the blood-filled tube was centrifuged at 3500 rpm for 15 minutes. The hematocrit level can be calculated using the following formula:

Total leukocytes were counted using the method of Blaxhall & Daisley (1973). Blood samples were sucked using a Thoma leukocyte pipette containing a white stirring bulb to scale 0.5, then Turk's solution was added to scale 11. After that, both ends of the pipette were closed using a finger and then homogenized in a figure 8 shape for 3-5 minutes until the blood was mixed. A total of 1-2 drops of blood are discarded, then the sample is put into a microtube or can be directly observed using a haemacytometer under a microscope with a magnification of 250x, with the following formula:

$$\sum \text{Leukocytes (sel mm}^{-3}) = \sum \text{cells counted} \times \frac{1}{\text{vol. big box}} \times \text{dilution factor}$$

A total of 50 µl of fish blood samples and 50 µl of *Staphylococcus aureus* bacterial suspension were taken and then put into an Eppendorf tube and mixed and incubated at 28°C for 20 minutes. After that, a mixture containing bacterial suspension and blood was taken as much as 5 µl to make thin section preparations. The preparations were fixed using methanol solution for 5 minutes, followed by immersion using giemsa solution for 10 minutes and dried. Phagocytic activity was observed using a microscope and calculated using the formula of Anderson & Siwicki, (1993):

$$\text{Percentage of cells that necrosis (\%)} = \frac{\text{number of necrosis cells}}{\text{total number of cells}} \times 100$$

The respiratory burst value was calculated using the method proposed by Divyagnaneswari *et al.* (2007). Blood samples were put into a microplate as much as 50 µL and then incubated at 37°C for one hour. Blood was removed and rinsed using 100 µL PBS for three replicates. 0.2% nitroblue tetrazolium (NBT) solution of 50 µL was added to the microplate

and incubated again at 37°C for one hour. The 0.2% NBT solution was discarded and fixed using 100 µL of 100% methanol for 10 minutes, then the 100% methanol solution was discarded and rinsed again using 100 µL of 30% methanol for three replicates, with a 3-minute interval, then dried. The last process is to add 60 µL of potassium hydroxide (KOH) 2N and 70 µL of dimethyl sulphoxide (DMSO). Reading of the results can be done using a microplate reader at a wavelength of 630 nm.

Histopathology in fish was observed in the kidney and liver. Stages in making preparations are target organs fixed using Neutral Buffer Formaline (BNF) solution, dehydration, clearing, embedding, making tissue blocks and tissue staining using hematoxylin and eosin (Maulani *et al.,* 2017), followed by observation under a microscope. Histopathological analysis was performed qualitatively by calculating the percentage of necrosis in the observed organs with five different field of view using the method of Mustaqien *et al.* (2008) with the following formula:

Calculation of the amount of necrosis is followed by scoring the histopathology tissue that refers to the research of Wolf *et al.* (2015). The scoring of histopathology organs is presented in Table 2.

Data Analysis

Data were processed using Microsoft Excel 2019 and analyzed with SPSS 26 software. Prior to analysis, normality was assessed using the Shapiro-Wilk and homogeneity of variance was evaluated using Levene's tests, both at a significance level of ($P < 0.05$). After normality and homogeneity test ($P < 0.05$), statistical analysis was performed using 1 Way-ANOVA with a 95% confidence interval. If significant differences ($P < 0.05$) were detected among treatments, post-hoc test was performed using the Duncan test.

Table 2. Histopathology organ scoring

Necrosis	Score	Damage level
$P < 20\%$	0	Ordinary
$20\% \leq P < 40\%$	1	Lightly damage
$40\% \leq P < 60\%$	2	Medium damage
$60\% \leq P < 80\%$	3	Heavily damage
$P \geq 80\%$	4	Very heavily damage

RESULTS AND DISCUSSION

Inhibition and MIC Test

Inhibition zone test results show that all concentrations of moringa leaf extract (MOLE) have antibacterial activity against *E. tarda*, indicated by the formation of an inhibition zone in each treatment (Table 3). The zone of inhibition increased as the concentration of the extract increased, from 1.016 cm at 1.25 mg/mL to 2.086 cm at 20 mg/mL. Treatment with antibiotics (positive control) produced the largest zone of inhibition (3.058 cm), which was significantly higher than all other treatments ($P < 0.05$). Meanwhile, the PBS control and *E. tarda* control showed no inhibition zone, indicating the absence of antibacterial activity in both treatments.

Table 3. Inhibition zone and MIC test of moringa leaf extract on the growth of *E. tarda* bacteria

Treatments	Inhibition zone diameter (cm)	MIC test (Optical Density OD_{630})
PBS control	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Antibiotic control	3.058 ± 0.050^{f}	0.540 ± 0.036^{b}
E. tarda control	0.00 ± 0.00^{a}	2.378 ± 0.211^{g}
M1.25	1.016 ± 0.018^{b}	$2.117 \pm 0,151^{f}$
M2.5	1.234 ± 0.116^{bc}	1.931 ± 0.051^{ef}
M5	1.258 ± 0.073^{c}	1.785 ± 0.134^{e}
M10	1.792 ± 0.084^{d}	1.490 ± 0.087^{d}
M20	2.086 ± 0.201^{e}	1.272 ± 0.105^{c}

Different superscripts letters following (mean ± standard deviation); n = 3 in the same column indicate significant differences ($P < 0.05$). PBS control (negative control), Antibiotic control (positive control), *E. tarda* control (control *E. tarda*, M1.25, 2.5, 5, 10, 20 (moringa leaf extract 1.25, 2.5, 5, 10, 20 mg mL^{-1})

In contrast, the minimum inhibitory concentration (MIC) test results showed a different trend. At concentrations of 1.25–5 mg/mL, the reduction in bacterial growth was not significant to each other, with OD values remaining high (around 2.117 to 1.785), indicating weak inhibitory activity. Inhibitory effectiveness was only significant at higher concentrations of 10 and 20 mg/mL, with OD of 1.490 and 1.272 respectively, although still not as effective as antibiotics (OD 0.540). Thus, moringa extract showed significant antibacterial potential in the zone of inhibition test, but its effectiveness in the MIC test showed that high concentrations are needed to significantly inhibit bacterial growth.

The results of the inhibition zone test showed that all concentrations of MOLE formed a clear zone, as an indicator of inhibition of *E. tarda* growth. The higher the concentration of MOLE used shows the greater the inhibition zone formed, meaning that the bacterial activity of MOLE increases with increasing concentration of extract used. This is supported by MIC testing which also shows that the higher the concentration of moringa leaf extract used, it will be indicated by a decrease in the growth of *E. tarda* bacteria. The inhibition zone formed shows that moringa leaves have antibacterial activity, for example phenol, which works by inhibiting bacterial growth through inactivation of proteins in the cell membrane, which later binds to proteins in the bacterial cell wall structure through the formation of hydrogen bonds (Novita, 2016). The hydrogen bonds cause damage to cell wall proteins and cytoplasmic membranes, resulting in macromolecular and ionic imbalances in the cell (Hariati *et al.,* 2018). In addition, according to Dwicahyani *et al.* (2018), saponins found in moringa have the ability to affect bacterial cell wall tension by binding to lipopolysaccharides. This process increases the permeability of the cell wall while reducing its surface tension. Once inside the bacterial cell, the saponins disrupt the cell's metabolism. In addition, tannins also exhibit antibacterial properties by activating bacterial metabolic enzymes and inhibiting protein transport in the inner layer of the cell (Sapara *et al.,* 2016). The antibacterial mechanism of this combination of compounds works synergistically, thus increasing the effectiveness against bacteria (Rempe *et al.,* 2017).

Growth Performance

The growth performance of tilapia with feed containing MOLS for 30 days is presented in Table 4. Overall growth performance (FWB, ADG, FCR, SR) in the MOLS treatment (M12.5) showed no significant difference ($P>0.05$) compared to M0. However, there was a significant difference ($P<0.05$) in the M25 and M50 treatments which tended to decrease.

Giving MOLS 12.5 g kg^{-1} on the parameters of FBW, ADG, and FCR there was no significant difference ($P>0.05$) compared to the control (M0). This is in accordance with the research of Abd El-Gawad *et al.* (2019) showed that there was no significant difference ($P>0.05$) in moringa leaves included in tilapia feed at levels of 1.5% and 5% compared to the control on the additional weight of fish reared for 60 days. In addition, the provision of MOLS 50 g kg^{-1} was significantly lower ($P<0.05$) compared to the control. The low amount of FI results in low final weight produced. Research by El-Kassas *et al.* (2020) showed that the addition of moringa leaf flour (5% and 10%) reduced the amount of FI by tilapia compared to the control treatment. In addition, it is also in line with the research of Mapanao *et al.* (2021) there is a reduction in growth performance (weight gain, daily growth rate, specific growth rate in tilapia with feed treatment with the addition of moringa leaves more than 15%.

Table 4. Growth performance, FCR, FI and SR (mean ± standard deviation (SD), n=3) of tilapia fed with experimental feed for 30 days

Parameters	Experimental diets containing different levels of moringa leaf simplicia (g kg^{-1})			
	M0	M12.5	M25	M50
IBW(g)	7.77 ± 0.05	7.78 ± 0.06	7.74 ± 0.05	7.83 ± 0.06
FBW(g)	19.35 ± 0.89^{c}	18.05 ± 0.80^{c}	16.04 ± 0.60^{b}	11.57 ± 0.54^{a}
ADG (g/day)	0.39 ± 0.03^{c}	0.34 ± 0.03^{c}	0.28 ± 0.02^{b}	0.12 ± 0.02^{a}
FCR	1.20 ± 0.07^{a}	1.20 ± 0.09^{a}	1.34 ± 0.08^{ab}	1.47 ± 0.21^{b}
FI (g)	138.53 ± 2.16^{d}	123.17 ± 1.98^{c}	110.54 ± 0.54^{b}	53.90 ± 1.46^{a}
SR (%)	90 ± 0.00^{a}	96.67 ± 5.77^{a}	93.33 ± 5.77^{a}	90 ± 0.00^{a}

Different superscript letters following in the same row indicate significant differences ($P< 0.05$). IBW (Initial body weight), FBW (Final body weight), ADG (Average daily growth), FCR (Feed conversion ratio), FI (Feed intake), SR (Survival rate).

Reduced growth performance in fish given high doses of MOLS is thought to be caused by antinutrient content, compounds that can interfere with absorption and reduce nutrient utilization (Auwal *et al.,* 2019). Tannins and saponins are thought to exceed the normal limits that fish can accept, resulting in disruption of several physiological functions of treated fish (Mansour *et al.,* 2018). Therefore, there is a reduction in growth performance in fish. According to Richter *et al.* (2003), the addition of moringa leaf meal with a concentration of 10%, 20%, and 30%, followed by an increase in antinutrients such as tannins and saponins showed a significant reduction in growth performance such as the amount of feed consumption, specific growth, final weight of individuals. Similarly, Yuangsoi *et al.* (2014) reported that excessive tannins and saponins in moringa could negatively impact fish growth. Madalla *et al.* (2013) explained that high saponin levels reduce feed palatability, lowering feed intake. Furthermore, saponins can alter cell membrane structure and function by increasing mucosal permeability, thereby impairing nutrient absorption and transport (Mapanao *et al.,* 2021).

Health Status and Immune Response

Feeding tilapia with the MOLS for 30 days is proven to improve health status and immune response include total erythrocytes (TE), haemoglobin (Hb), haematocrit (Hc), total leukocytes (TL), phagocyte activity (AF), and respiratory burst (Rb) as presented in Table 5. The TE of tilapia at H0 was $1.69 \pm 0.06 \times 10^6$ cells mm^{-3}, then experienced the highest increase during 30 days of maintenance in the M12.5 treatment at $2.73 \pm 0.10 \times 10^6$ cells mm^{-3} which was significantly different ($P < 0.05$) with all treatments. The Hb of tilapia at H0 was 6.33 ± 0.76 G%, then experienced the highest increase on the 30th day of maintenance, namely M12.5 at 9.27 ± 0.46 G%, and M25 at 8.90 ± 0.10 G% and significantly different ($P < 0.05$) from all treatments. The Hc of tilapia at H0 was 17.13 ± 0.12%, then experienced the highest increase during 30 days of maintenance in the M12.5 treatment at 24.76 ± 0.32% and significantly different ($P < 0.05$) from K- at 21.88 ± 0.83%. The TL of tilapia at H0 was $2.33 \pm 0.12 \times 10^4$ cells mm^{-3}, then after 30 days of maintenance experienced the highest increase in the treatment of M12.5 at $2.92 \pm 0.07 \times 10^4$ cells mm^{-3} and M25 at $2.81 \pm 0.10 \times 10^4$ cells mm^{-3} which was significantly different ($P < 0.05$). The AF of tilapia at H0 was 40.54 ± 0.13%, then experienced the highest increase during maintenance in the M12.5 treatment of 47.34 ± 1.67% which was significantly different ($P < 0.05$) from the M50 treatment of 42.43 ± 0.88%.

After 30 days of MOLS administration, tilapia exhibited an increase in total erythrocyte (TE) count, remaining within the normal range for teleost fish (1.00–3.00 × 10v cells/mm^3; Royan *et al.,* 2014). This suggests that MOLS may stimulate erythropoiesis, potentially through bioactive compounds that enhance erythropoietin (EPO) production (Nurhayati *et al.,* 2023; Yanuartono *et al.,* 2019). Associated increases in hemoglobin (Hb) and hematocrit (Hc) were also observed, reflecting improved oxygen transport and

Table 5. Total erythrocytes (TE), haemoglobin (Hb), haematocrit (Hc), total leukocytes (TL), phagocytic activity (AF), and respiratory burst (Rb) of tilapia fed with experimental feed

Parameters	Times	Treatments				
		K-	K+	M12.5	M25	M50
Total erythrocyctes ($\times 10^6$ cells/mm^3)	H0	1.69 ± 0.06^a	1.69 ± 0.06^a	1.69 ± 0.06^a	1.69 ± 0.06^a	1.69 ± 0.06^a
	H30	2.31 ± 0.09^b	2.31 ± 0.09^b	2.73 ± 0.10^d	2.48 ± 0.08^c	2.09 ± 0.08^a
Haemoglobin (g%)	H0	6.33 ± 0.76^a	6.33 ± 0.76^a	6.33 ± 0.76^a	6.33 ± 0.76^a	6.33 ± 0.76^a
	H30	8.03 ± 0.25^b	8.03 ± 0.25^b	9.27 ± 0.46^c	8.90 ± 0.10^c	7.10 ± 0.26^a
Haematocrit (%)	H0	17.13 ± 0.12^a	17.13 ± 0.12^a	17.13 ± 0.12^a	17.13 ± 0.12^a	17.13 ± 0.12^a
	H30	21.88 ± 0.83^b	21.88 ± 0.83^b	24.76 ± 0.32^c	23.38 ± 1.22^{bc}	19.35 ± 1.07^a
Total leukocytes ($\times 10^4$ cells/mm^3)	H0	2.33 ± 0.12^a	2.33 ± 0.12^a	2.33 ± 0.12^a	2.33 ± 0.12^a	2.33 ± 0.12^a
	H30	2.44 ± 0.20^a	2.44 ± 0.20^a	2.92 ± 0.07^b	2.81 ± 0.10^b	2.63 ± 0.25^{ab}
Phagocyctic activity (%)	H0	40.54 ± 0.13^a	40.54 ± 0.13^a	40.54 ± 0.13^a	40.54 ± 0.13^a	40.54 ± 0.13^a
	H30	45.14 ± 0.81^b	45.14 ± 0.81^b	47.34 ± 1.67^c	42.84 ± 0.49^a	42.43 ± 0.88^a
Respiratory burst (OD 630 nm)	H0	0.10 ± 0.00^a	0.10 ± 0.00^a	0.10 ± 0.00^a	0.10 ± 0.00^a	0.10 ± 0.00^a
	H30	0.21 ± 0.01^a	0.21 ± 0.01^a	0.25 ± 0.01^a	0.23 ± 0.03^a	0.22 ± 0.01^a

Different superscripts letters following (mean ± standard deviation) in the same row indicate significant differences ($P < 0.05$). K- (negative control), K+ (positive control), M12.5, M25, M50 (moringa leaf simplicia 12.5, 25, 50 g kg^{-1} feed)

blood volume (Bintang *et al.,* 2024; Fitriany & Saputri, 2018). Additionally, total leukocyte (TL) counts increased but remained within the normal physiological range (Elbahnaswy & Elshopakey, 2020; Dotta *et al.,* 2014), with TL positively correlated with phagocytic activity (AF) and respiratory burst (RB), indicating enhanced immune function.

MOLS feeding for 30 days improved tilapia health and immune response after *E. tarda* challenge (Table 6). After being challenged with *E. tarda*, the health status and immune response of tilapia fluctuated. After the *E. tarda* challenge, TE, Hb, and Hc levels in tilapia decreased. However, by day 7, the M12.5 treatment showed the highest and significantly different ($P<0.05$) values: TE at 2.64±0.08/ ×/ 10v cells/mm³, Hb at 8.63±0.51/ g%, and Hc at 22.61±1.22%, indicating better physiological responses compared to other treatments.

TL, AF and RB values after the challenge test with *E. tarda* increased. The highest TL value after the challenge test occurred on day 1 of the M12.5 treatment at 3.59±0.25 × 10^4 cells mm^{-3} M25 at 3.41±0.18 × 10^4 cells mm^{-3} and significantly different ($P<0.05$) from the K- and K+ treatments. The AF value of tilapia fish increased, with the highest value obtained on the 1st day after the challenge test in the M12.5 treatment of 64.00±1.38% which was significantly different ($P<0.05$) from the K+, K- and M50 treatments. The highest RB value on the 3rd day after the challenge test in the M12.5 treatment was 0.81±0.02 and significantly different ($P<0.05$) from all treatments.

Table 6. Total erythrocytes (TE), haemoglobin (Hb), haematocrit (Hc), total leukocytes (TL), phagocytic activity (AF), and respiratory burst (Rb) of tilapia fed with experimental feed after the challenge test using *E. tarda*

Parameter	Times	Treatments				
		K-	K+	M12.5	M25	M50
Total erythrocyctes (× 10^6 cells/mm³)	H+1	2.28±0.07^{b}	1.77±0.19^{a}	2.55±0.12^{c}	2.21±0.10^{b}	1.90±0.11^{a}
	H+3	2.31±0.02^{b}	1.84±0.06^{a}	2.60±0.08^{c}	2.32±0.07^{b}	1.92±0.10^{a}
	H+7	2.40±0.09^{b}	2.07±0.12^{a}	2.64±0.08^{c}	2.38±0.02^{b}	2.07±0.13^{a}
Haemoglobin (g%)	H+1	8.03±0.06^{c}	5.93±0.23^{a}	8.10±0.36^{c}	7.47±0.31^{b}	5.80±0.35^{a}
	H+3	7.73±0.31^{bc}	6.10±0.36^{a}	8.27±0.64^{c}	7.23±0.25^{b}	5.73±0.61^{a}
	H+7	7.70±0.26^{b}	7.10±0.36^{b}	8.63±0.51^{c}	7.33±0.31^{b}	5.87±0.42^{a}
Haematocrit (%)	H+1	21.87±0.59^{c}	17.00±1.00^{a}	20.83±1.04^{c}	18.94±0.55^{b}	17.31±0.77^{a}
	H+3	21.67±0.29^{c}	17.96±0.72^{ab}	22.17±1.04^{c}	19.13±0.32^{b}	17.36±0.97^{a}
	H+7	22.07±0.93^{c}	18.04±1.04^{ab}	22.61±1.22^{c}	19.54±0.23^{b}	17.75±0.69^{a}
Total leukocytes (× 10^4 cells/mm³)	H+1	2.48±0.20^{a}	3.07±0.12^{b}	3.59±0.25^{d}	3.41±0.18^{cd}	3.21±0.10^{bc}
	H+3	2.51±0.08^{a}	2.93±0.10^{b}	3.42±0.28^{c}	3.22±0.04^{bc}	2.88±0.27^{b}
	H+7	2.61±0.10^{a}	2.83±0.04^{b}	3.21±0.13^{c}	2.92±0.09^{b}	2.86±0.17^{b}
Phagocytic activity (%)	H+1	44.94±1.42^{a}	60.30±0.66^{c}	64.00±1.38^{d}	62.25±1.75^{cd}	56.77±1.57^{b}
	H+3	45.40±4.38^{a}	57.80±0.49^{bc}	60.03±0.50^{c}	57.77±0.67^{bc}	55.40±2.26^{b}
	H+7	45.11±0.85^{a}	50.67±1.15^{b}	54.34±1.16^{c}	49.95±0.92^{b}	46.61±1.99^{a}
Respiratory burst (OD 630 nm)	H+1	0.21±0.01^{a}	0.48±0.01^{b}	0.77±0.01^{e}	0.66±0.03^{d}	0.61±0.01^{c}
	H+3	0.21±0.01^{a}	0.48±0.02^{b}	0.81±0.02^{d}	0.71±0.02^{c}	0.69±0.01^{c}
	H+7	0.22±0.01^{a}	0.47±0.03^{b}	0.62±0.05^{c}	0.53±0.04^{b}	0.51±0.05^{b}

Different superscript letters following in the same row indicate significant differences ($P<0.05$). K- (negative control), K+ (positive control), M12.5, M25, M50 (moringa leaf simplicia 12.5, 25, 50 g kg^{-1} feed)

After the H+1 challenge test, there was a decrease in TE, Hc, Hb levels in tilapia due to infection with *E. tarda* bacteria. This happens because *E. tarda* is a Gram-negative bacterium that produces 2 exotoxins in the form of hemoxilin and dermatoxin (Rodrigues *et al.,* 2019). Hemoxilin is able to lyse red blood cells so that blood and liberate haemoglobin which causes erythrocytes to decrease. The decrease in erythrocytes reduces the oxygen-carrying capacity in the blood which ultimately has a negative impact on fish health. Dermatoxins function to damage skin tissue and also cellulose membranes that will look like wounds or lesions in infected fish (Rozi *et al.,* 2022). After H+3, there is an effect of the addition of MOLS which is indicated by an increase in TE followed by an increase in Hb and Hc levels. According to Mohammadian *et al.* (2019), that the increase in TE determines the mechanism of fish resistance against

pathogens, affects fish health, and increases blood immune function. Increased Hb levels indicate a post-infection healing process that is influenced by the active compounds of MOLS. Moringa leaves contain flavonoids that have antioxidant properties. These antioxidants protect red blood cells from oxidative damage that can cause the destruction of red blood cells (hemolysis). The oxidation process can damage red blood cell membranes and reduce their lifespan, but the antioxidant compounds in moringa help maintain the stability and sustainability of red blood cells in the body. This also causes Hc and Hb levels to increase after the challenge test.

TL, AF, and RB values in infected fish are positively correlated. When fish are infected, the immune system will increase leukocyte production to fight the pathogen, with leukocytes playing a role in stimulating antibody formation and recognizing antigens (Syawal *et al.,* 2021). Immune cells such as macrophages and dendritic cells recognize the pathogen and release cytokines and chemokines that attract more leukocytes to the site of infection. AF increases when phagocytic cells such as neutrophils and macrophages engulf and destroy pathogens. The increase in AF is influenced by immunostimulants that activate receptors on phagocytic cells to digest bacteria (Elala & Ragaa, 2015). The phagocytosis process forms phagosomes that destroy microorganisms, and releases cytokines to increase immune activity (Biller & Takahash, 2018). This is in accordance with El-Kassas *et al.* (2022), who showed an increase in AF fed moringa leaf powder in tilapia. Furthermore, RB involves the production of reactive oxygen species (ROS) by neutrophils and macrophages to kill phagocytosed pathogens. ROS serve as a non-specific defense and antioxidant response to eliminate pathogens after phagocytosis (Harikrishnan *et al.,* 2018).

This is in accordance with the results of the study which showed a positive correlation between TL and both AF and RB parameters. Post-infection, TL, AF, and RB of MOLS treatment were higher than the control treatment ($P<0.05$) to strengthen the immune system against pathogens. This is in line with Mansour *et al.* (2018), as feeding with moringa leaves increases the RB value in *Sparatus aurata* fish. The increase is related to the active compounds contained in MOLS. Flavonoids can stimulate an increase in immune responses and an increase in lymphokine proliferation by T cells that stimulate phagocytic cells (Martinez *et al.,* 2019).

Bacterial Count

The highest abundance of lactic acid bacteria was found in the M12.5 treatment at 6.01 ± 0.05 log CFU g^{-1} which was significantly different ($P<0.05$) from the K and M50 treatments. However, it was not significantly different ($P>0.05$) with the M25 treatment. The process of nutrient absorption in the intestine can be supported by gut microflora that improve digestion. Lactic acid bacteria (LAB) residing in the gut play a role in fermenting carbohydrates to produce lactic acid, which not only improves fish immunity but also fights pathogenic bacteria and supports the digestive process (Ringo *et al,.* 2018). The addition of MOLS at a concentration of 12.5 g kg^{-1} was shown to increase the abundance of lactic acid bacteria (LAB) compared to the control. Anudeep & Radha (2018), mentioned that *M. oleifera* also contains oligosaccharides (fructooligosaccharides and malto-oligosaccharides), which can stimulate the growth of LAB organisms (Mensah *et al.,* 2012). Furthermore, this increase in beneficial gut microflora correlates with the elevated non-specific immune parameters observed in the M12.5 group, including total leukocytes (TL), phagocytic activity (AF), and respiratory burst (RB). The synergistic effect between gut microbiota modulation and immune activation suggests that MOLS improves tilapia health through both microbiological and immunological pathways.

The total population of *E. tarda* bacteria in the target organs of tilapia is presented in Table 7. The population of *E. tarda* bacteria at H+1 to H+7 after the challenge test showed a decrease. In the K- treatment, no *E. tarda* was detected in either the kidney or liver, indicating a healthy fish condition without exposure to pathogens. In contrast, in K+, a very high population of *E. tarda* was found, amounting to 7.86 ± 0.03 log CFU/g in the kidney and 8.04 ± 0.07 log CFU/g in the liver on the first day after challenge (H+1PC), and slightly decreased to 7.70 ± 0.00 and 7.86 ± 0.02 log CFU/g on the seventh day (H+7PC). The addition of feed containing MOLS was able to significantly reduce the population of *E. tarda* ($P<0.05$) compared to K+. Treatment M12.5 showed the highest effectiveness, with the number of bacteria in the kidney decreasing to 7.31 ± 0.02 log CFU/g at H+1PC and 7.12 ± 0.01 log CFU/g at H+7PC, while in the liver it was recorded at 7.77 ± 0.01 and 7.55 ± 0.02 log CFU/g respectively. Interestingly, the M25 and M50 treatments did not provide a greater reduction effect, and even tended to be less effective than M12.5. Overall, the population of *E. tarda* in the liver tended to be higher than the kidney in all treatment groups, indicating that the liver is an organ that is more susceptible to bacterial growth.

The *E. tarda* population was significantly lower in the kidney and liver organs with the addition of MOLS were less than the K+ treatment. MOLS is proven to

be able to suppress the growth of *E. tarda* bacteria. This is in accordance with El-Sheekh *et al.,* (2019), also argue that moringa leaf extract has significant antibacterial activity against various types of gram-positive and gram-negative bacteria. Giving natural immunostimulants can reduce the total plate count of bacteria in fish through an increase in the immune system and antibacterial activity (Harikrishnan *et al.,* 2018). Alkaloids contained in MOLS work by inhibiting enzymes that play a role in the DNA replication process. Inhibition of DNA replication functions so that bacteria cannot divide themselves so that growth is inhibited. In addition, alkaloids can also interfere with the formation of cross-bridges of the components that make up peptidoglycan, so that the cell wall layer is not completely formed and causes cell death (Riyadi *et al.,* 2021).

Table 7. Total population (mean ± SD, n = 3) of *E. tarda* bacteria in the kidney and liver of tilapia of H + 1PC = day one post challenge; H + 7PC = day seven post challenge

Target Organ	Treatment	Population of *E. tarda* (log CFU/g)	
		H + 1PC	H + 7PC
Kidney	K-	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	K +	7.86 ± 0.03^{d}	$7.70 \pm 0,00^{d}$
	M12.5	7.31 ± 0.02^{b}	$7.12 \pm 0,01^{b}$
	M25	7.35 ± 0.03^{b}	$7.15 \pm 0,04^{b}$
	M50	7.43 ± 0.02^{c}	$7.30 \pm 0,07^{c}$
Liver	K-	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	K +	8.04 ± 0.07^{d}	7.86 ± 0.02^{d}
	M12,5	7.77 ± 0.01^{b}	7.55 ± 0.02^{b}
	M25	7.82 ± 0.06^{b}	7.56 ± 0.06^{b}
	M50	7.94 ± 0.02^{c}	7.77 ± 0.02^{c}

Different superscript letters following in the same collumn indicate significant differences ($P < 0.05$). K- (negative control), K + (positive control), M12.5, M25, M50 (moringa leaf simplicia 12.5, 25, 50 g kg^{-1} feed)

Histopathology

Histopathological observations of tilapia kidney tissue on day 7 post-challenge with *E. tarda* (Figure 1) showed pathological changes, including liquefactive necrosis, characterized by damage to renal tubular structures and cell lysis. Damage was also seen in glomerular and tubular cells, characterized by loss of cell nuclei and irregular structures. Treatments M12.5,

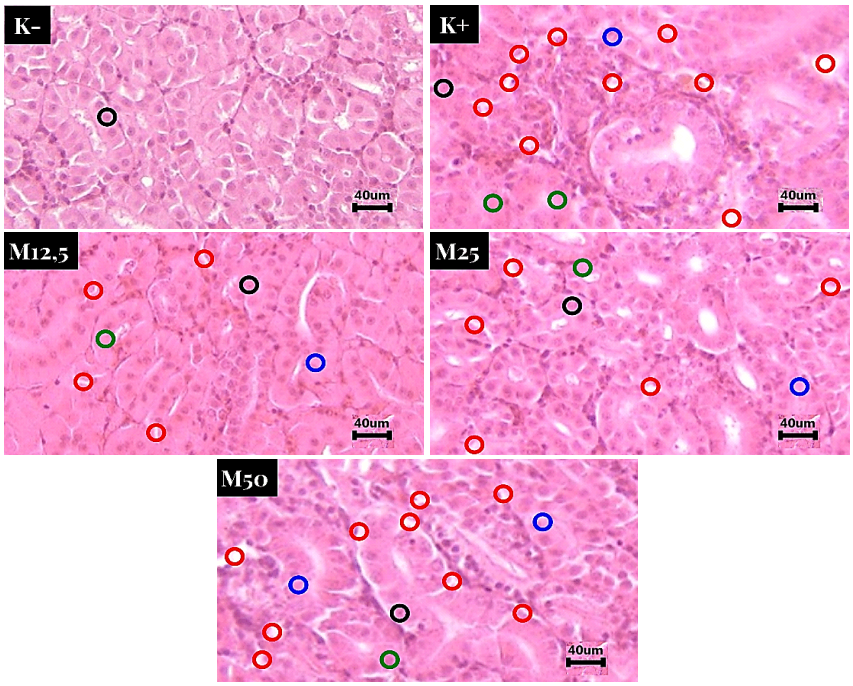

Figure 1. Histopathology of tilapia kidney on day 7 post-challenge with *E. tarda*. (✿) necrosis, (✿) normal cell, (✿) glomerular, (✿) tubular cells.

M25, and M50 showed significantly lower levels of kidney damage compared to the positive control group (K+) ($P<0.05$).

Histopathological examination of tilapia kidney tissue post-*E. tarda* challenge showed necrosis, as detailed in Table 8. *E. tarda* infection causes damage to the kidney and liver organs of tilapia. The damage occurs because these bacteria produce toxins that damage body cells, such as hemosilin and dermatoxin (Rozi *et al.,* 2022). This infection can trigger inflammation that disrupts the function of these organs. In the kidney, bacteria can cause damage to the glomerulus and tubules, leading to a decrease in filtering function as seen from the loss of cell nuclei and irregular size. The results of histopathology of kidney organs showed that the K+ treatment had lightly damage of 34.89% which was significantly different from the percentage of damage to the simplicia treatment which ranged from 13.86-25.21%. This is supported by El-Sheekh *et al.* (2019), which states that moringa leaf extract has the potential to repair damaged tissue due to antioxidant and anti-inflammatory contents such as flavonoids, tannins, and alkaloids so that it can prevent excessive damage to tilapia kidney organs after being infected with *E. tarda*.

Histopathological observation of tilapia liver tissue was conducted on day 7 post-challenge with *E. tarda* (data not displayed). Each treatment showed the presence of normal cells and necrotizing cells. The level of damage to tilapia liver tissue in treatments M12.5, M25, and M50 was lighter than the K+ treatment ($P<0.05$).

The histopathological analysis of liver tissue revealed necrosis caused by *E. tarda* infection, as shown in Table 9. Inflammation caused by infection can damage hepatocyte cells, disrupting detoxification and metabolic processes. This damage is further exacerbated if the infection is not treated immediately, which can lead to organ failure. The addition of MOLS shows that it can minimize organ damage caused by *E. tarda* infection. Damage is seen from the amount of necrosis that occurs in the liver. The results of histopathology of the liver showed that the K+ treatment had moderate damage of 45.65% which was significantly different from the percentage of damage in the MOLS treatment which ranged from 14.59-30.44%. This study is in accordance with previous research, feeding with herbal ingredients can reduce damage to fish internal organ tissue through reducing oxidative stress and inflammation (Harikrishnan *et al.,* 2018).

Table 8. Histopathological score of tilapia kidney tissue after challenge with *E. tarda*

Sample	Necrosis(%)	Score	Damage level
K-	9.87 ± 1.63	0	Ordinary
K+	34.89 ± 2.53	1	Lightly damage
M12.5	13.86 ± 2.52	0	Ordinary
M25	18.97 ± 2.35	0	Ordinary
M50	25.21 ± 2.85	1	Lightly damage

K- (negative control), K+ (positive control), M12.5, M25, M50 (moringa leaf simplicia 12.5, 25, 50 g kg^{-1} feed)

Table 9. Histopathology score of tilapia liver tissue after challenge with *E. tarda*

Sample	Necrosis(%)	Score	Damage level
K-	9.98 ± 2.36	0	Ordinary
K+	45.65 ± 2.58	2	Medium damage
M12.5	14.59 ± 2.12	0	Ordinary
M25	17.88 ± 2.73	0	Ordinary
M50	30.44 ± 2.25	1	Lightly damage

K- (negative control), K+ (positive control), M12.5, M25, M50 (moringa leaf simplicia 12.5, 25, 50 g kg^{-1} feed)

The survival rate (SR) of tilapia was observed before and after the challenge test. The SR before and after the challenge with *E. tarda* is shown in Figure 2. Before the challenge, SR was 100% for all treatments. After the challenge, SR fluctuated, except for treatment K- (100%). The highest SR was observed in treatment M12.5 (86.67 ± 5.77%), significantly different ($P<0.05$) from treatment M50 (76.67 ± 5.77%) and K+ (53.33 ± 5.77%).

Based on the results of the study, the addition of MOLS to tilapia infected with *E. tarda* showed significant results in SR. The K+ treatment which was given *E. tarda* bacterial infection without the addition of MOLS showed the lowest resistance of 53.33 ± 5.77%. Giving 12.5 g kg^{-1} of MOLS showed the highest SR of 86.67 ± 5.77% allegedly caused by active compounds in MOLS. Suteja *et al.,* (2016) stated that

flavonoids as antibacterials work by inhibiting DNA gyrase, inhibiting energy metabolism and bacterial growth, causing higher resistance to *E. tarda*. In addition, moringin can insert into bacterial DNA base pairs and inhibit the replication process. Moringin also stimulates oxidative stress, disrupting energy metabolism, especially carbohydrate metabolism, leading to disruption of the utilization of catabolic products for cell repair. As a result, bacteria are unable to maintain their survival and eventually die (Wen *et al.,* 2022).

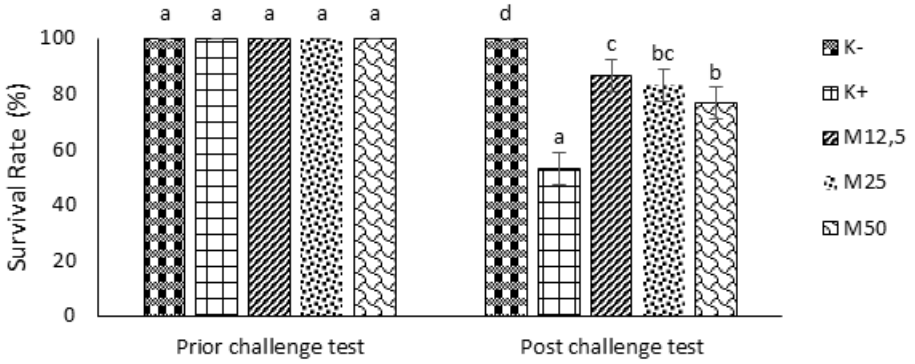

Figure 2. Survival rate of tilapia before and after challenge with *E. tarda*. Different superscripts letters following (mean ± standard deviation; n=3) indicate significant differences (P< 0.05). K- (negative control), K+(positive control), M12.5, M25, M50 (moringa leaf simplicia 12.5, 25, 50 g kg-1 feed).

CONCLUSION

Moringa leaf simplicia (MOLS) was able to improve the health status and immune response, as well as the resistance of tilapia to *E. tarda* infection with the optimal dose being 12.5 g kg^{-1} MOLS.

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