The Effectiveness of Cherry (*Muntingia calabura L*) leaves extract on gourami (*Osphronemus goramy*) seeds infected with the bacteria *Aeromonas hydrophila*

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Abstract. Gourami (*Osphronemus goramy*), a high-value fish, faces challenges in growth and survival due to bacterial infections, particularly *Aeromonas hydrophila*. This study analyzed the effects of cherry (*Muntingia calabura*) leaves extract at different doses on gourami infected with *A. hydrophila*. A completely randomized design (CRD) with five treatments and three repetitions was used: Treatment A (negative control), Treatment B (positive control), and Treatments C, D, and E (fish challenged with *A. hydrophila* and immersed in 50 ppm, 70 ppm, and 90 ppm of cherry leaves extract, respectively). Results showed that cherry leaves extract significantly improved fish survival and health. Treatment C (50 ppm) yielded the best outcomes, with 76.66% survival, leucocyte counts of 165.3 x 10^3 cells/mm^3, and hemoglobin levels of 8 g/dL. It also reduced mucus production, healed ulcers, and stabilized blood parameters. Thus, 50 ppm cherry leaves extract was the most effective dose for enhancing gourami health and survival against *A. hydrophila* infections.

1 Introduction

Gourami (*Osphronemus goramy*) is one of the main freshwater fish in Indonesia and has been cultivated for commercial purposes [1]. However, this has experienced several obstacles in the cultivation of gourami, especially regarding fish health management. Reports from [2] indicated that disease outbreaks have occurred in several production centers where gourami is cultivated. One of them is a bacterial disease caused by infection of *Aeromonas hydrophila*. An outbreak of *A. Hydrophila* has been associated with resulting in red spots appearing on the fish and causing damage to the skin, gills and internal organs. This is a pathogenic bacterium that causes the disease Motile Aeromonas epticemia (MAS), especially for freshwater fish species in tropical waters such as carp, nile tilapia, and gourami [3, 4].

Treatment by giving antibiotics has often been done, but this treatment can cause bacteria to become resistant and increase residues in the fish, which has a negative impact on consumers and the environment [5]. Therefore, it is necessary to find alternative medicinal ingredients from natural ingredients that is relatively safe for both fish and the environment

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to treat disease-causing bacterial infections. One of the natural plants that has the potential to act as an immunostimulant is the cherry plant (*Muntingia calabura*). The contents of cherry leaves (*M. calabura*) have antioxidant potential which can be attributed to the high content of phenolic compounds [6].

Cherry leaves contain flavonoids, saponins and tannins which have antibacterial and anti-inflammatory properties [7]. Flavonoids from several plants can have antibacterial properties, where flavonoids can release energy transduction into the cytoplasmic membrane of bacteria and inhibit bacterial motility [8]. Saponin can increase the permeability of the lipid bilayer to regulate antibody access to the cytoplasmic surface of cells for transmembrane protein synthesis. Saponin has also been shown to have a strong effect in inducing platelets in the blood [9]. One of the other compounds in cherry leaves is tannin, this tannin compound has an effect natural antibacterial by inhibiting cell membrane transport enzymes to bacteria [10].

The previous study of [11, 12] has been done about the use of cherry leaves extract as a treatment on Nile tilapia (*Oreochromis niloticus*) and Siamese catfish (*Pangasianodon hypophthalmus*). The best treatment for Nile tilapia was found at a dose of 60 ppm, achieving a survival rate of 81.67%, while for Siamese catfish, the optimal dose was 3,600 ppm, resulting in a survival rate of 86.67% for the test fish. Thus, cherry leaves extract is effective in treating fish fry infected with *A. hydrophila.* The previous study depicted that cherry leaves extract has strong potential for development in fish disease treatment. However, the use of cherry leaves extract on gourami has not yet been tested. Subsequently, the main objective of the present study was to experimentally assess the effectiveness of cherry (*Muntingia calabura L*) leaves extract on gourami seeds (*Osphronemus goramy*) infected with the bacteria *A. hydrophila.*

2 Material and methods

2.1 Experimental design

This study used a Completely Randomized Design (CRD) with five treatments and three replications. The treatment involved soaking gourami seeds in cherry leaves extract at varying concentrations based on the research by [11]. The specific treatments were as follows: treatment A (negative control, without cherry leaves extract and not challenged with *A. hydrophila* bacteria), treatment B (positive control, without cherry leaves extract and challenged with *A. hydrophila* bacteria, treatment C (challenged with *A. hydrophila* bacteria and immersion into 50 ppm cherry leaves extract), treatment D (challenged with *A. hydrophila* bacteria and immersion into 70 ppm cherry leaves extract), and treatment E (challenged with *A. hydrophila* bacteria and immersion into 90 ppm cherry leaves extract).

2.2 Cherry leaves extract preparation

Cherry leaves which have been used in this study was the 4-10th leaves from the top of each stalk which are still green and fresh [12]. The leaves were washed first with running water and then dried. Then the leaves were cut into small pieces and blended until they become powder. The powdered leaves were soaked and then macerated using 96% ethanol for 3 days and stirred periodically, then filtered using a funnel and filter paper to separate the filtrate from the dregs. After the maceration process, it was then evaporated with a Rotary Vacuum Evaporator at a temperature of 40°C to obtain a thick extract that is free from solvents [13].

2.3 Fish rearing

There were 150 fish reared into 25L water buckets with a length of fish 9-10 cm and age range of 120-160 days. Before conducting research, the containers were cleaned to avoid contamination. Then, gourami seeds were first acclimatized for 20 minutes in a container for adapting to the new environment. The adaptation process in the rearing container was carried out for a week before pathogen infection. The procedure perfomed in this study followed by Universitas Syiah Kuala guideline (Code No. 958/2015) on the animal care and use in research.

2.4 Bacterial infection on gourami fish

The gourami seeds that will be infected with *A. hydrophila* bacteria were healthy fish seeds with a fish size of 9-10 cm which were adapted for 24 hours before being infected with the bacteria. *A. hydrophila* bacteria were obtained from the Laboratory of the Faculty of Agriculture, with a bacterial density of 10^8 CFU/ml for 24 hours. Infection was carried out using the immersion method with 1 mL/1000 mL of water, and during infection the fish were not given food [12]. Fish showed symptoms resulting from infection with *A. hydrophila* bacteria, such as red spots on their backs, reduced appetite, and the presence of ulcers [14].

2.5 Treatment with cherry leaves extract

Treatment was carried out by adding cherry leaves extract into a jar containing water and infected fish at different doses according to the treatment. The time used for soaking was 24 hours after the fish showed clinical symptoms post-infection [15]. After the soaking process was complete, the water was changed using clean water and the fish were observed to see the effect of the soaking results on the condition of the infected fish.

2.6 Blood collection from fish

The fish were first anesthetized with clove oil at a dose of 0.05 mL/L until the fish fainted. Before blood collection, the 1 mL syringe and ependorf tube were moistened with 10% EDTA to prevent blood clotting. Blood collection was carried out in the caudalis vein, then the blood in the syringe was put into an Eppendorf tube to be used to observe total leucocytes, haemoglobin and lymphocytes. Blood sampling from test fish was carried out 3 times, namely on day 1 after infection, day 7 after soaking in cherry leaves extract, and day 14 after soaking in cherry leaves extract.

2.7 Research parameters

2.1.1 Survival rates

Survival rates (SR) were calculated according to the following equation [16]:

$$SR\ (\%) = N_t/N_0 \times 100 \quad (1)$$

where N_t is the final number of fish (ind) and N_0 is the initial number of fish (ind)

2.1.2 Observation of haemoglobin

The procedure for calculating haemoglobin levels was carried out using the Sahli method. First, the blood sample was sucked using a Sahli pipette to a scale of 20 mm^3 or on a scale of 0.2 ml. Then cleaned the pipette tip with tissue paper. Then, the blood in the pipette was transferred into an Hb-meter tube filled with 0.1 N HCl to a scale of 10 (red). Next, the blood was then stirred with a stirring rod for 3 to 5 minutes. After that, distilled water was added to the tube until the color of the blood becomes the color of the standard solution in the Hb-meter. Haemoglobin levels were expressed in g/dL.

2.1.3 Leucocyte count

The calculation of the number of leucocytes was done according to [17], first the blood sample was sucked with a pipette containing a white agglomerate to a scale of 0.5. Then, Turk's solution was added to a scale of 10 and the pipette was swung in a figure 8 (the same as stirring to count the number of red blood cells) for 3-5 minutes so that the blood mixes evenly. After that, the first two drops of blood solution from the pipette were discarded, then drip the solution into the haemocytometer, after which it was covered with a cover glass. The liquid will fill the counting chamber capillary. The total number of white blood cells or leucocytes was counted using a microscope with a magnification of 10 times. The total number of leucocytes was calculated by counting the cells contained in 4 small boxes, and the number was calculated according to the formula:

$$Leucocyte = \frac{w1+w2+w3+w4}{N} \times P \quad (2)$$

Information:
Leucocyte: Calculation of the number of leucocyte (103 cells/mm3)
w: Number of cells
N: Number of counting rooms (4)
P: diluent (20 x 10)

2.8 Data analysis

Data obtained from testing blood imaging parameters were analyzed using descriptive methods. Then, survival data were analyzed by Analysis of Variance (ANOVA) and Duncan's further test.

3 Result and discussion

O. goramy before infection showed normal symptoms in each treatment. On the contrary, generally it showed several symtomps after being infected by *A. Hydrophila* such as ulcers appear, red spots appear, skin color fades, and the scales peeled off (Fig. 1). Moreover, there is a decrease in fish appetite and the appearance of excessive mucus on the surface of the fish's body. This indicated that the body's defense system has been activated to fight against the *A. hydrophila* bacteria. In accordance to [14] and [18], fish infected with *A. hydrophila* bacteria will causes several clinical symptoms including fading skin color, excess mucus production, the appearance of red spots or wounds on the surface of the fish's body.

After immersion into cherry leaves extract (day 14), it can be seen some changes such as reduced mucus and fish swim actively in each treatment particularly in treatment C. However, other treatments also effective in treating gourami seeds.

Fig. 1. Ulcers, excessive mucus, peeled-off scales.

After immersion into cherry leaves extract (day 14), it can be seen some changes such as reduced mucus, the ulcers disappear, the scales are back to normal, and fish swim actively in each treatment particularly in treatment C. However, other treatments also effective in treating gourami seeds (Fig. 2).

Fig. 2. The ulcers disappear and the scales are back to normal.

3.1 Blood profile

3.1.1 Leucocyte

Observing the number of leucocytes before treatment with cherry leaves extract, the leucocyte value in healthy fish and fish infected with *A. hydrophila* bacteria was 156.8×10^3 cells/mm^3 and 287.2×10^3 cells/mm^3, respectively (Table 1). The increase in leucocyte values occurred due to *A. hydrophila* bacterial infection and the increase in leucocytes was a response from the fish to defend the body from bacterial attack. This statement is in accordance with [19] which states that leucocytes have various functions, which are closely related to eliminating foreign objects (including pathogenic microorganisms), and the supporting statement is according to [20], the increase in leucocytes in the body is because leucocytes function for defence body, which reacts quickly to the entry of antigen into the fish's body.

After soaking fish on cherry leaves extract at day 7, the leucocyte value began to decrease in treatment C with a value of 237.7×10^3 cells/mm^3, treatment D with a value of 255.4×10^3 cells/mm^3, and treatment E with a value of 272.6×10^3 cells/mm^3. Meanwhile, leucocyte values continued to increase in treatment B with a value of 345.8×10^3 cells/mm^3. This is caused by an *A. hydrophila* bacterial infection without treatment using cherry leaves extract. The high number of leucocytes in the positive control treatment is because leucocytes are an important part of the immune system which plays a role in producing antibodies that can fight viruses, bacteria and fungi. When fish are sick and when an infection occurs, leucocytes will move towards the site of infection to provide rapid defence against infectious genes [21]. The number of normal fish leucocyte cells ranges from 20,000-150,000 cells/mm^3 [22].

Table 1. Leucocyte value before and after immersion into cherry leaves extract.

Treatments	Leucocyte ($\times 10^3$ sel/mm^3)			
	H0	H1	H7	H14
A (negative control)	156.8	156.8	160.3	158.5
B (positive control)	156.8	287.2	345.8	339
C (50 ppm)	156.8	287.2	237.7	165.3
D (70 ppm)	156.8	287.2	255.4	178.1
E (90 ppm)	156.8	287.2	272.6	193.5

Information:
H0: before infection with *A. hydrophila*
H1: After infection with *A. hydrophila*
H7: After immersion into cherry leaves extract
H14: recovery period

On day 14, the leucocyte decreased in each treatment: treatment C with a value of 165.3×10^3 cells/mm^3, treatment D with a value of 178.1×10^3 cells/mm^3, and treatment E with a value of 193.5×10^3 cells/mm^3. This decrease was influenced by immersion in cherry leaves extract, which aids the immune system in combating bacterial infections. The bactericidal properties of flavonoid, saponin, and tannin compounds in cherry leaves extract effectively kill pathogenic bacteria, reducing the need for leucocytes to be produced in large numbers [23]. Moreover, the flavonoid, tannin, and saponin content found in kersen leaves (*Muntingia calabura*) serves as an antibacterial agent. Its mechanism of action involves denaturing cellular proteins in the bacterial cell wall structure, leading to cell lysis by increasing cell membrane permeability, denaturing proteins, and inhibiting the synthesis of bacterial nucleic acid components [24]. The mechanism of action of active compounds, particularly flavonoids, in stimulating the immune system is by accelerating leucocyte activation, enabling the phagocytosis of foreign objects to occur rapidly [25]. Consequently, the leucocyte value will return to normal, as observed in treatments involving immersion using cherry leaves extract.

The decrease in leucocyte on day 14 in this study align with the study of [26], where the leucocyte counts in Sangkuriang catfish infected with *A. hydrophila* decreased to 107×10^3

cells/mm³ on day 14. According to [11], excessive administration of cherry leaves extract at high doses can turn some active compounds into toxins for test fish. This is based on the toxicity test by [11], which showed that the LC50 test results indicated that concentrations above 100 ppm of cherry leaves extract caused 50% mortality in test fish fingerlings within 24 hours. Active compounds such as saponin can become toxic at certain levels. If fish cannot tolerate high doses of saponin, this compound will become toxic and harmful to fish. [27] stated that saponin is a toxic compound for fish and exhibits haemolytic activity that can damage red blood cells and impair the fish's respiratory process. Furthermore, based on the leucocyte count calculations in this study, the best value was obtained in treatment C at 165.3 $\times 10^3$ cells/mm³, while the lowest value was observed in treatment B at 339 $\times 10^3$ cells/mm³.

3.1.2 Haemoglobin

Haemoglobin (Hb) is a metalloprotein in blood plasma that functions to transport oxygen from the gills, pumped by the heart, to all cells and organs of the body. The Hb value of gourami seeds were 8 g/dL which was under normal conditions. In gourami seeds infected with *A. hydrophila* bacteria, the Hb value decreased to 2 g/dL. This decrease was caused by the *A. hydrophila* infection which attacked the fish's body and interferes with the function of haemoglobin. According to [28], the decrease in haemoglobin levels is due to bacteria attacking the host's body, thereby hindering the haemoglobin's function and resulting in the fish experiencing haemolysis in the blood.

After immersion on day 7, the Hb levels in treatment C (50 ppm) were 6 g/dL, in treatment D (70 ppm) were 5 g/dL, and in treatment E (90 ppm) were 3 g/dL. On day 14, the Hb value in treatment C was 8 g/dL, in treatment D was 7 g/dL, and in treatment E was 5 g/dL. Based on the haemoglobin concentration, the best value was obtained in treatment C at 8 g/dL, while the lowest value was observed in treatment B at 3 g/dL (Table 2).

Table 2. Haemoglobin value before and after immersion into cherry leaves extract.

Treatments	Haemoglobin (g/dL)			
	H0	**H1**	**H7**	**H14**
A (negative control)	8	8	8	8
B (positive control)	8	2	4	3
C (50 ppm)	8	2	6	8
D (70 ppm)	8	2	5	7
E (90 ppm)	8	2	3	5

3.2 Survival rates

Based on the current study, the addition of cherry leaves extract influenced the survival rate of the fish. ANOVA test showed use of cherry leaves extract on gourami seeds infected with *A. hydrophila* have a significant effect ($P<0,05$) on survival rate of gourami seeds. The best survival rate was obtained in treatment C (50 ppm) with an average value of 76%. The increase in survival in treatment C was influenced by the antibacterial activity contained in the cherry leaves extract, which could inhibit the growth of *A. hydrophila*, thus reducing the infection level and allowing the fish to gradually recover. Cherry leaves extract contains flavonoids, tannins, and saponins, which serve as antibacterial agents [29]. In addition to its antibacterial function, saponin can also stimulate the immune system [7]. The active flavonoid compounds can form complexes with proteins through hydrogen bonds, thereby damaging bacterial cell membranes. Meanwhile, the lowest survival rate was observed in treatment B (positive control), with an average of 40%, due to the absence of treatment with cherry leaves extract after infection with *A. hydrophila*. This statement is supported by [30], who stated that one of the causes of low fish survival could be parasitic, bacterial, or viral infections.

Table 3. Survival rate of gourami seeds at the end of experiment.

Treatments	**Survival rates (%)**
A (negative control)	$56.66{\pm}11.54^{bc}$
B (positive control)	$40.00{\pm}0.00^{a}$
C (50 ppm)	$76.66{\pm}5.77^{d}$
D (70 ppm)	$66.66{\pm}5.77^{cd}$
E (90 ppm)	$50.00{\pm}0.00^{ab}$

Note: The average values in the same column with different superscripts indicate that they are significantly different and the values with the same superscript in the column indicate that they are not significantly different.

3.3 Water quality parameters

Table 4. Water quality parameters.

Perlakuan	**Awal**			**Akhir**		
	pH	**DO (mg/L)**	**Suhu (°C)**	**pH**	**DO (mg/L)**	**Suhu (°C)**
A	7.78	6.0	29.3	7.65	7.1	30
B	7.67	6.5	30.1	7.52	7.0	29.4
C	7.63	7.3	29.1	7.78	6.2	29.2
D	7.65	6.6	30.3	7.64	7.0	30.3
E	7.68	6.1	29.8	7.89	6.4	30.1

Water is a very important component in fish rearing media and must meet the required quality and quantity standards. Water quality plays a crucial role in the survival of fish. The observed water quality parameters aim to provide information that the media used for rearing gourami fry is in a controlled condition. The temperature measurements during this study ranged from 29.3°C to 30°C, the DO (Dissolved Oxygen) measurements ranged from 6 to 7 mg/L, and the pH measurements ranged from 7.6 to 7.7. These measurement ranges are still considered suitable for gourami rearing, where the ideal temperature is 29°C-30°C [31], the DO range is 4-9 mg/L, and the pH range is 7-8 [32].

4 Conclusion

In summary, immersion in cherry leaves extract (*Muntingia calabura*) has an effect on treating *Osphronemus goramy* seeds infected with *Aeromonas hydrophila*, with the best dose being in treatment C (50 ppm), resulting in a survival rate of 76%.

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