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The Effect of the Croton Leaf (*Codiaeum variegatum*) Against *Edwardsiella tarda* Infection in the Gourami Fish (*Osphronemus gouramy*)

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ABSTRACT

Gourami fish is a popular and economical fishery culture commodity in Indonesia. One of the bacterial diseases that is an obstacle in gourami fish culture is Edwardsiellosis, caused by the *Edwardsiella tarda* bacteria. The aim of this study was to examine the viability of croton leaf (*Codiaeum variegatum*) as a natural substitute for synthetic antibacterial in addressing *E. tarda* infection. This study used a two-phase approach, identification of antibacterial compounds from *C. variegatum* leaves and their effectiveness against *E. tarda*. The treatments were negative control (K-), positive control (K+), *C. variegatum* with doses of 500, 550, 600, 650, and 700mg/ L (seven treatments and three replication). The results indicated that *C. variegatum* primarily comprised antibacterial compounds of flavonoids, alkaloids, tannins, and terpenoids. The utilization of the raw extract from *C. variegatum* significantly influenced the infected gourami fish's hematology. It was found that 600mg/ L was the most efficient dose to overcome *E. tarda* infection in gourami fish, leading to significant improvements hematological parameter. Moreover, histopathological examination indicated enhancements in tissue profile, as evidence by lower congestion, necrosis, and degeneration score. Additionally, after being treated with 600mg/ L dose of *C. variegatum* extract, the gourami fish's survival rate reached 83%. These findings underscore the potential efficacy of *C. variegatum* as a promising treatment for managing gourami fish infection caused by *E. tarda*. Nonetheless, further investigation is warranted to evaluate the practical application of *C. variegatum* in gourami fish aquaculture.

INTRODUCTION

The Indonesian populace's interest in fish eating is rising concurrently with the enhancement of fisheries output value. Total aquaculture production in 2017 reached 16.87 million tons **(Andrian *et al*., 2024)**. Of the freshwater cultured fish production, the majority (75.71%) is contributed by fish commodities that have long been domesticated in Indonesia, such as gourami fish (*Osphronemus gouramy)* **(Nugroho, 2012)**. Gourami fish is a type of freshwater fish native to Indonesia which has economic value, nutrition, and affordable price **(Budiana & Rahardja, 2018)**. Gourami fish also has relatively stable high selling price (2.28 USD/kg) **(Al-Baiquni, 2019)**. According to **Saraswati *et al.* (2020)**, the high production of gourami fish cannot be separated from intensive culture which is currently widely implemented in Indonesia. If intensive culture is not managed well, the environmental balance will be disturbed, so that the fish become stressed and can trigger the development of disease. According to **Narwiyani and Kurniasih (2011)**, intensive culture is prone to causing fish stress, especially due to high stocking densities, poor water quality conditions and also high organic matter content.

One of the causes of infectious diseases is bacterial attacks. Bacterial disease is a process where bacterial organisms capable of causing disease enter the body or tissue and cause trauma or damage **(Rajme-Manzur *et al*., 2021)**. *Edwardsiella tarda* is one of the bacterial disease affecting gourami fish culture. *E. tarda* bacteria is a pathogen that can attack freshwater biota, including fish. The bacteria of *E. tarda* is the cause of Edwardsiellosis or Emphysematous Putrefactive Disease (EPD). Fish affected by Edwardsiellosis will have pale skin, excessive mucus, wounds, if scratched they will emit a foul odor and inflammation from the anus to the base of the tail. *E. tarda* infection causes a decrease in the productivity of gourami fish farming and results in losses for farmers. Transmission of *E. tarda* bacterial infection occurs horizontally, namely through contact between one host and another via water **(Tungkup *et al.*, 2021)**. According to **Narwiyani and Kuniasih (2011)**, Edwardsiellosis infection can cause the death of up to 80% of fish in nature.

Zafran *et al.* (2020) explained that the use of antibacterials has been widely used in aquaculture and is believed to be the most effective solution, such as the use of chloramphenicol and oxytetracycline which are used to inhibit the growth of *E. tarda*. In addition, according to **Rosidah and Afizia (2012)**, the use of antibiotics is quite effective in treating disease, but increases the proliferation of antibiotic-resistant pathogens. Another negative effect of using antibiotics is the accumulation which is dangerous for environmental health. Organisms in that environment can be exposed to and adapt to antibiotics, which may cause them to become resistant to those drugs, leading to behavioral changes and disrupting their reproduction.

Natural ingredients that can be used as antibacterial for *E. tarda* are crude extract of red ginger extract (*Zingiber officinale var. Rubrum*) **(Prastiti *et al.*, 2015)** and *Rhizophora* sp. leaves **(Syawal *et al.*, 2017)**. Another alternative medicinal ingredient that can be used to treat the bacterial disease *E. tarda* is the leaves of the croton plant (*Codiaeum variegatum*). Several studies have examined the potential antibacterial properties of *C. variegatum*; however, its implementation to treat fish disease is not reported **(Fauziyyah *et al*., 2021)**. It is crucial to initiate research and investigations focused on identifying active compounds within *C. variegatum* possessing antibacterial properties. Additionally, these studies should explore the viability of using these substances as possible medication candidate to treat gourami fish infection caused by *E. tarda*.

MATERIALS AND METHODS

1. Experimental design

The experimental fish specimens were submerged in a pure culture of *E. tarda* bacteria in order to infect them. Ten fish were cultured in 20 liters of water (30 x 30 x 30 centimeter tank; 27-30ºC. Fish mortality observations were conducted over a period of 96 hours **(Wulandari *et al.*, 2014)**. Both descriptive and experimental approaches were used in this study. While the descriptive method was used to give a thorough picture of the natural bioactive components found in *C. variegatum* and its effects of the gourami fish, the experimental approach was conducted to collect quantitative data. Several treatments used in this study were *C. variegatum* doses of 500, 550, 600, 650, and 700mg/ L (each replicated three times, with ten fish per replication), a positive control (K+), and a negative control (K-). These doses were chosen based on earlier *in vitro* studies by **Fauziyyah *et al.* (2021)**.

2. Fish and bacteria used in this study

The gourami fish (size 7–10cm) samples were provided by fish farms in Tulungagung, East Java, Indonesia. A pure culture of *E. tarda* was procured from BUSKIPM Jakarta and grown using Tryptic Soy Broth (TSB) as the growth medium in order to test for antibacterial activity.

Using the procedure described by **Murwantoko *et al.* (2013)**, the LD50 (Lethal Dose 50) test was performed to ascertain the concentration and amount of time needed for the bacterium to kill 50% of the experimental fish. The LD_{50} test involved using various concentrations of pure *E. tarda* bacteria, including 10^9 cells/ml, 10^8 cells/ml, 10^7 cells/ml, 10^6 cells/ml, and 10^5 cells/ml. The density of *E. tarda* utilized in the study was determined to be 2.53 x 10^7 cells/mL based on the LD_{50} results. Fish were put into treatment containers for this test, with 10 fish per container for stocking density.

A disc diffusion test was used in the antibacterial activity assessment to determine the effect of the crude extract of *C. variegatum* leaves inhibited the growth of *E. tarda*. According to **Ekklesia *et al.* (2020)**, the diameter of the clear zone was calculated by deducting the diameter of the paper disc (6mm) from the clear zone area findings in order to estimate the influence of the extract and to assess the efficacy of the diffusion method.

3. Extraction of *C. variegatum*

The *C. variegatum* leaves utilized in this study were sourced from the Materia Medica Herbal Laboratory in Batu, Indonesia, and subjected to extraction using ethanol as the maceration solvent. To confirm the presence of active compounds in the extract, several tests were conducted, including phytochemical screening and FTIR (Fourier-transform infrared spectroscopy) **(Patle *et al.*, 2020)**. The primary aim of phytochemical screening was to identify active compounds in the plant extract, such as saponins, tannins, flavonoids, alkaloids, and terpenoids, within the crude extract of *C. variegatum* leaves.

Additionally, the extract underwent LC_{50} (Lethal Concentration) testing to determine the concentration that could induce 50% mortality and establish a safe dosage that did not result in mortality among experimental fish **(Harbawi, 2014)**. In this test, fish were placed into treatment containers at a density of 10 fish per container, and the aquariums were filled with the extract according to the designated treatment. Observations were made on fish behavior, mortality rate, and time elapsed. The test was repeated if mortality in the control treatment exceeded 10%.

4. Histopathology analysis

Gourami fish histological investigations were performed in order to ascertain the properties of liver tissue. The staining technique employed Haematoxylin Eosin that had been fixed with 10% formalin and calculations were performed to evaluate the effect of *C. variegatum* extract on edwardsiellosis **(Maftuch *et al.*, 2015)**. Histopathological examination of fish can provide an overview of tissue changes in fish infected with disease to detect the presence of infective pathogenic components through micro-anatomical observations of abnormal changes at the tissue level.

5. Blood analysis

Hematological observations were carried out after the bacterial infection process and maintenance for 7 days by taking blood from gourami fish after being infected with *E. tarda* bacteria right at the linea lateralis of the fish using a 1ml syringe which had previously been given anticoagulant in it. From fish blood taken, erythrocytes, hemoglobin, hematocrit, leukocytes and leukocyte differential were counted **(Arlanda *et al.*, 2018)**. Using a 1ml syringe filled with 0.5µL of EDTA as an anticoagulant, blood was drawn from the gourami fish's lateral line for the observations. This test's goal was to evaluate the condition of the infected gourami fish after *C. variegatum* extract was administered. 3 iterations of blood analysis were performed, and the results were monitored prior to, during, and after therapy.

6. Survival rate analysis

To evaluate the efficacy of *C. variegatum* in treating Edwardsiellosis, the survival rate was determined using the formula provided by **Kasnir *et al.* (2023)**. The assessment of survival rate extended over a period of 7 days, with 3 replications for each treatment group and a sample of 10 fish in each replication.

$$SR = \frac{Nt}{No}\ x\ 100\%$$

Notes:

SR : Survival rate (%)

Nt : The number of fish (individual) at a given time point t

No : The initial individual fish count at the beginning of the experiment

7. Data analysis

The Statistical Package for the Social Sciences (SPSS) 25.0 was used to analyze the data in this study using a completely randomized design (CRD).

RESULTS

1. Active compound present in *C. variegatum*

Table 1. FTIR spectrophotometer wavelength absorption data from *C. variegatum* extract

FTIR wavelength (cm^{-1})	Functional groups	Group
3451.323	O-H stretching	Terpenoid
2925.981	C-H aliphatic	Alkaloid
2857.214	C-H aliphatic	Alkaloid
1739.613	C=O	Tannin
1455.579	C-H aliphatic	Alkaloid
1378.940	C-H	Flavonoid
1166.680	C-N	Alkaloid
1087.236	C-N	Alkaloid
839.255	C-H	Flavonoid
568.710	C-H bending	Tannin
3451.323	O-H stretching	Terpenoid
2925.981	C-H aliphatic	Alkaloid
2857.214	C-H aliphatic	Alkaloid
1739.613	C=O	Tannin

The result of FTIR test revealed the presence of several active compounds in the crude extract of *C. variegatum* (Table 1). Four compounds, namely flavonoids, alkaloids, terpenoids, and tannins, were detected in this study.

2. Histopathology analysis

The result of hispatology in this study is shown in Fig. (1). There were no indications of cellular damage in the liver tissue from the healthy fish. This contrasts with infected fish, where congestion is apparent. Interestingly, treatment of *C. variegatum* with 600mg/ L showed better result compared with other infected fish. The histopathological scoring test result is exhibited in Fig. (2). The treatment exhibiting the lowest average score for abnormal histopathological findings was observed at a concentration of 600mg/ L, resulting in scores of 1.8 for degeneration, 0.93 for congestion, and 1.13 for necrosis. This suggested that the best dosage of *C. variegatum* for gourami fish to overcome *E. tarda* infection was 600mg/ L. Remarkably, congestion increased significantly in the treatments of 650 and 700mg/ L, after damage scored decreased at the concentration of 500 and 600mg/ L.

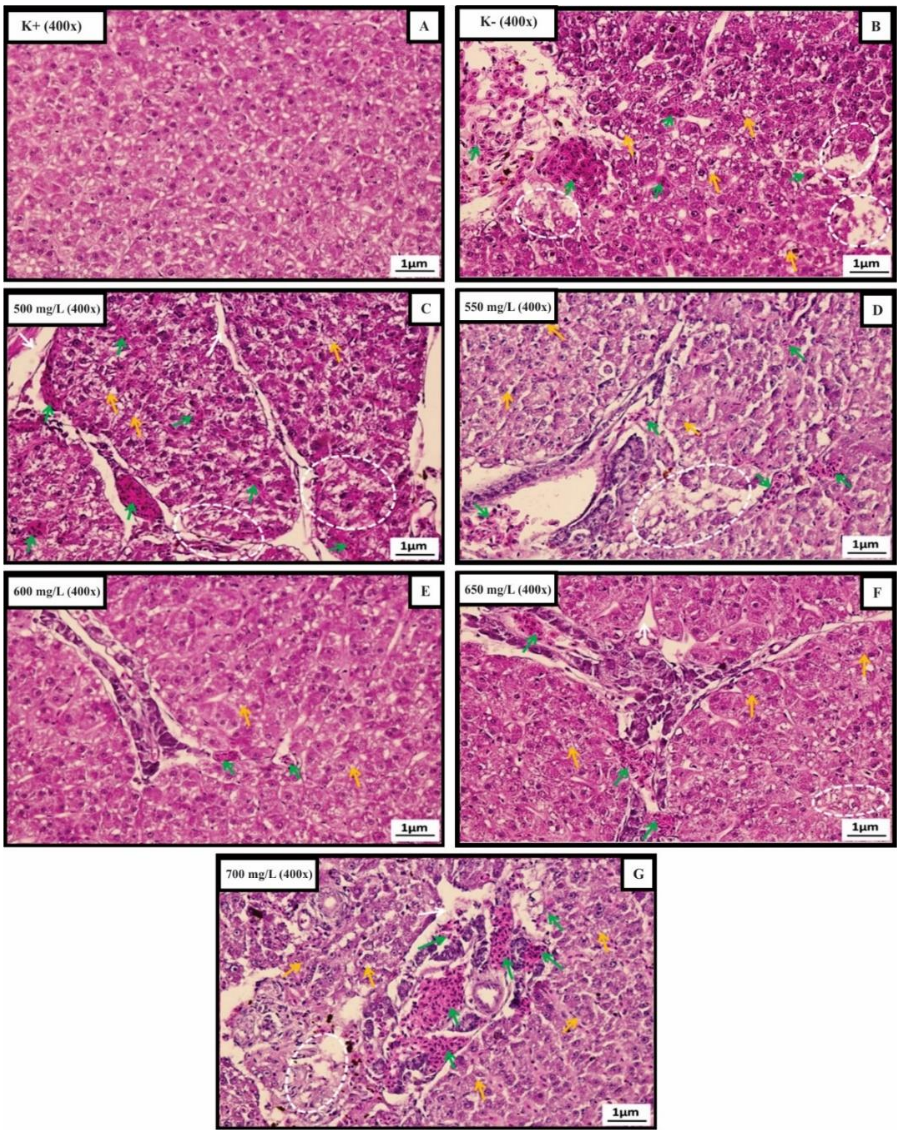

Fig. 1. Liver histopathological overview of gourami fish / 400x. **(A)** Normal (non-infected) /K+, **(B)** Infected fish (not-treated) / K-, **(C)** Treated with 500 mg/L. **(D)** Treated with 550 mg/L. **(E)** Treated with 600 mg/L. **(F)** Treated with 650 mg/L. **(G)** Treated with 700 mg/L, (Yellow Arrow: Degeneration; Green Arrow: Congestion; White Circle: Necrotic Area)

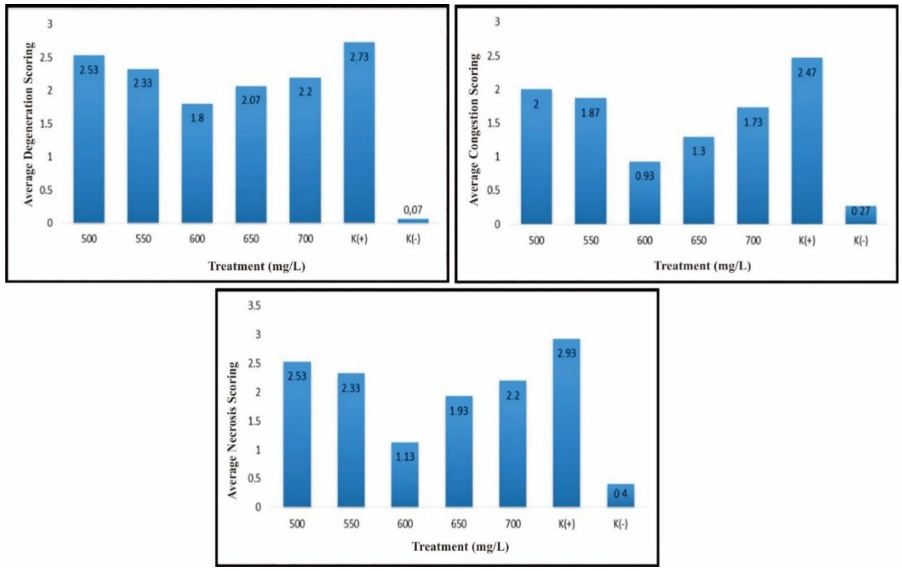

Fig. 2. Histopathological scoring test results showing: degeneration (Upper, Left), congestion (Upper, Right), and liver necrosis of gourami fish (Bottom). K+: Normal (non-infected), K- : Infected fish (not-treated)

3. Hematology test

The results of hematology test (hematocrit, hemoglobin, and erythrocyte level) are shown in Fig. (3). The dose of 600mg/ L achieved the ideal hematocrit value (29.67%). According to regression analysis, the extract of *C. variegatum* improved the hematocrit levels by 79%. Likewise, the treatment of 600mg/ L resulted in a notable rise in hemoglobin levels, which reached 4.5g%. It is indicated that *C. variegatum* can improve the hemoglobin level by 81%. The treatment of 600mg/ L also showed the largest rise in erythrocyte values, reaching 3.20 x 10^6 cells/mm^3. Erythrocyte counts, which had dropped to 1.8 x 10^6 cells/mm^3 after infection, were significantly increased by the crude extract of *C. variegatum*, with a 93% impact. According to these results, the extract concentration of 600mg/ L was the most successful in raising blood parameters in gourami fish after *E. tarda* infection. On the other hand, at dosages of 650 and 700mg/ L, hematocrit, hemoglobin, and erythrocyte levels decreased.

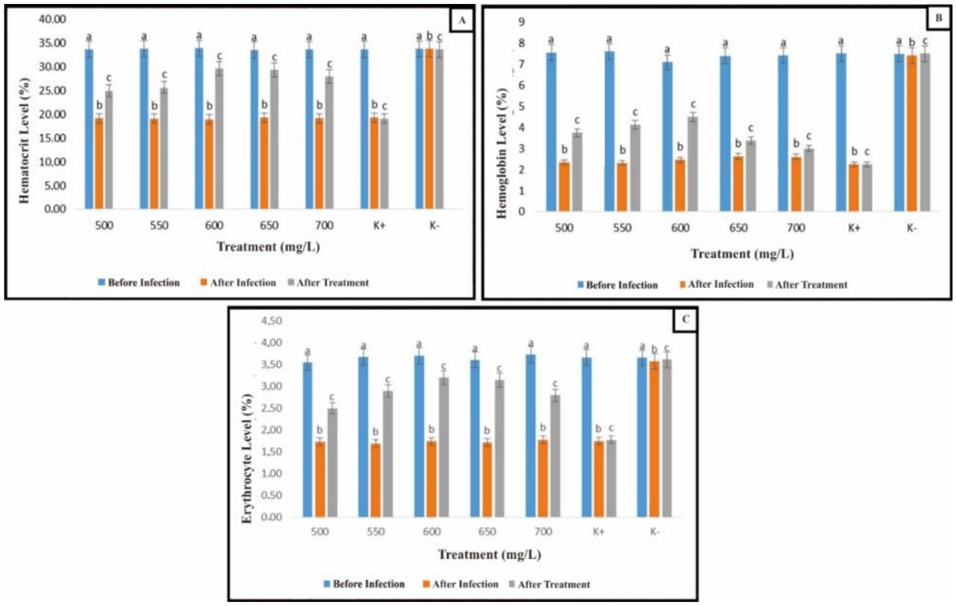

Fig. 3. (A) The value of hematocrit, **(B)** hemoglobin, and **(C)** erythrocytes from gourami fish in this study. K+: Normal (non-infected), K-: Infected fish (not-treated)

Treatment with different doses of *C. variegatum* also induced varied effects on leukocyte, lymphocyte, monocyte, and neutrophil values in the fish samples (Fig. 4). Following the administration of *C. variegatum*, there was a noticeable decrease in leukocyte values. The most effective dose observed during the study was 600mg/ L, which reduced the leukocyte count from 15.7 x 10^4 to 9.7 x 10^4 cells/mm^3. Regression analysis indicated that the 600mg/ L dose of *C. variegatum* had a 90% positive impact on enhancing leukocyte values.

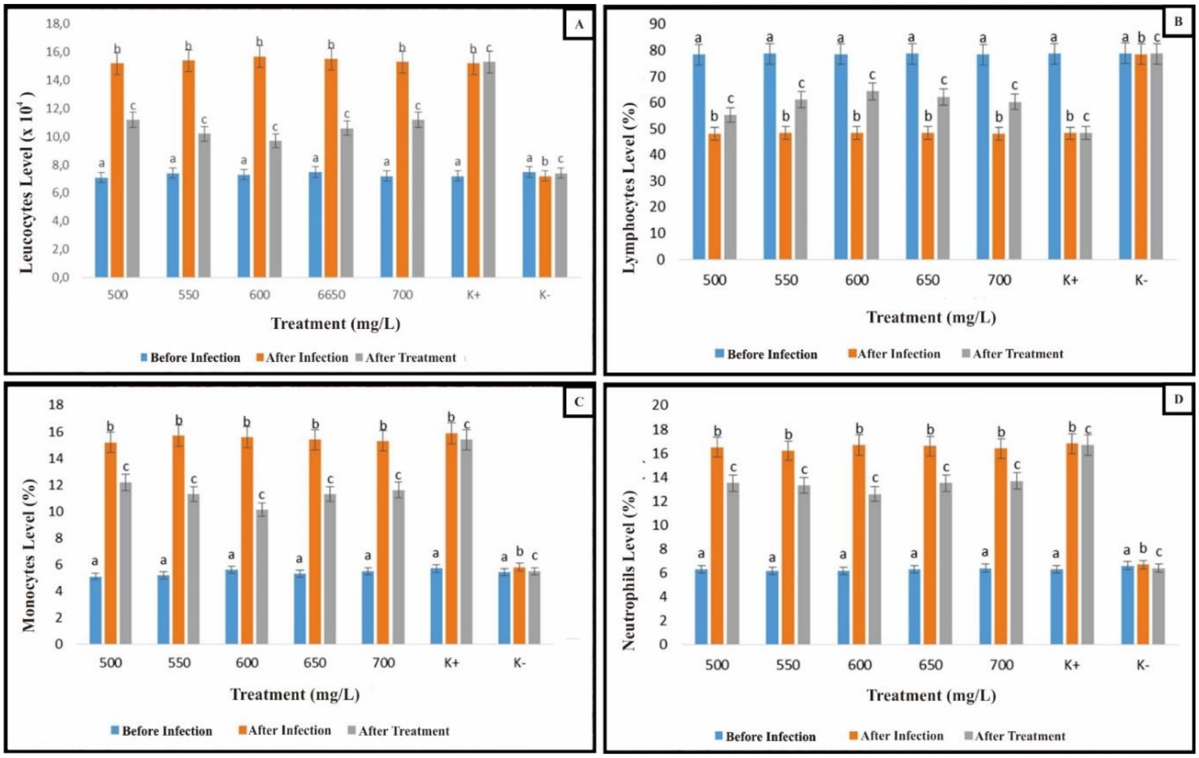

Fig. 4. (A) The value of leukocytes, **(B)** lymphocytes, **(C)** monocytes, and **(D)** neutrophils from gourami fish in this study. K+: Normal (non-infected), K-: Infected fish

After fish were infected with bacteria, lymphocyte values notably decreased by 48.5%. However, following treatment, lymphocyte values started to rise, with the most significant increase observed at an extract concentration of 600mg/ L, reaching a 64.4% increase. After treatment with *C. variegatum* extract, both monocyte and neutrophil values decreased. The most significant reduction in monocyte values was observed at a concentration of 600mg/ L, declining from 15.2 to 10.1%. Similarly, the highest reduction in neutrophil activity occurred at a treatment concentration of 600mg/ L, decreasing from 16.8 to 12.6%. Leukocyte, lymphocyte, monocyte, and neutrophil values did not show optimal improvement at doses of 650 and 700mg/ L.

4. Survival rate test

Following the application of *C. variegatum* treatments, the experimental groups' survival rate values were recorded (Fig. 5). The treatment of 600mg/ L showed the best result (83%). This value was close to the negative control (95%). However, a value of 22% from positive control treatment was the lowest number of survival rate in this study.

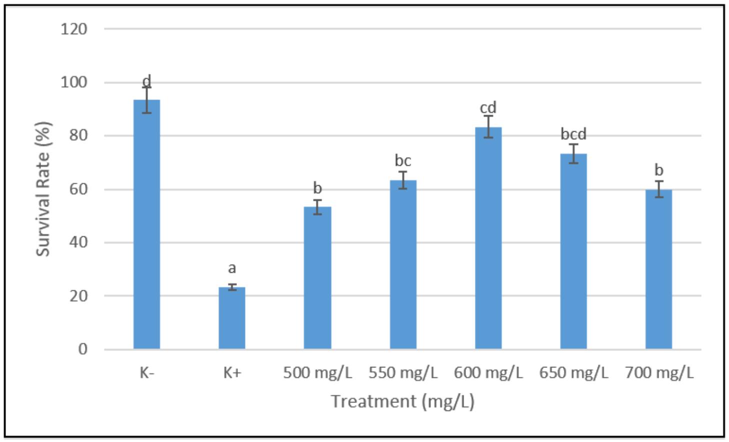

Fig. 5. Survival rate of gourami fish after treatment. K+: Normal (non-infected), K-: Infected fish (not-treated)

DISCUSSION

The findings in phytochemical test results of *C. variegatum* leaves align with the results reported by **Bijekar and Gayatri (2014)**, which indicated that puring leaves were abundant in flavonoids, phenols, and terpenoids, three compounds that play a role in overcoming bacterial infections. This has been confirmed through FTIR in this study that the contents present in *C. variegatum* were flavonoids, alkaloids, terpenoids, and tannins. **Rusmawanto *et al.* (2019)** added that flavonoids are secondary compounds from plants or hydroxylated phenolic substances that are synthesized in response to microbial infections. Another compound, alkaloids, can damage cell walls of bacteria while tannins have a mechanism of action by damaging bacterial cell membranes **(Nimah *et al.*, 2012; Widowati *et al.*, 2014)**. Apart from that, tannin works by causing a decrease in the adhesion of bacteria and inhibiting protein synthesis for cell wall formation. While, terpenoids exhibit antibacterial properties that can help inhibit the replication of bacteria **(Hidayati *et al*., 2023)**.

Bacterial infections cause injury to the liver, including congestion, hemorrhage and necrosis. The reddish color observed in liver cells of infected fish is indicative of congestion resulting from an increased presence of blood clots within the blood vessels, as explained by **Triadayani *et al.* (2010)** that congestion in the liver starts from the central vein which then extends to the irregularly arranged sinusoids and inside which there are erythrocytes due to blockage in the hepatic vein. If this blockage lasts long enough, liver cells appear to be lost due to pressure and disruption of the delivery of nutrients. This is because the blood flowing from the periphery of the liver lobules to the center (central vein) has mostly lost nutrients when it arrives in the middle of the lobules, so that in the middle of the lobules it becomes deficient in nutrients. **Mutiara *et al.* (2013)** added that congestion can develop into necrosis, describing a situation where there is a decrease in network activity which is characterized by the loss of several parts of cells from one tissue, which results in death within a short time.

In terms of histopathology, the utilization of *C. variegatum* had been shown to inhibit *E. tarda* infection in gourami fish. Previous studies have investigated the use of *C. variegatum* in treating pathogenic bacteria such as *Staphylococcus aureus*, *Enterococcus faecalis, Enterobacter aerogenes,* and *Escherichia coli* in fish *in vitro* **(Vennila & Udayakumar, 2015)**. However, this study was the first to demonstrate the efficacy of *C. variegatum* in treating *E. tarda* infection in gourami fish. Significantly, liver necrosis was found to increase in average scores at the 650 and 700mg/ L treatments, suggesting that the extract reached its maximum effectiveness for treatment following *E. tarda* infection. According to **Sujono *et al.* (2012)**, liver cell damage is caused by toxic metabolites from *C. variegatum* extract which are formed in excessive amounts or doses. As a result, the amount of bioactive compounds from duan puring extract available is not sufficient to neutralize these toxic compounds, so they will be associated with liver cell macromolecules, resulting in liver cell necrosis.

The administration of *C. variegatum* extract seems to have a beneficial effect on reducing the degree of liver necrosis as compared to the positive control group. The bioactive substances found in the crude extract of *C. variegatum*, such as flavonoids, which have the strongest antibacterial qualities, are responsible for this beneficial impact. According to **Maharani and Sutrisno (2016)**, one of the compounds that contain antibacterial properties is flavonoids. There are several flavonoid compounds that contain antibacterial properties, including 7,8-dihydrosiflavon, daticetin, robinetin, myricetin, and epigallocatechin.

At higher doses, the extract of *C. variegatum* leaves may become toxic to gourami fish. The increase in tissue damage at doses of 650 and 700mg/ L suggests that the effectiveness had peaked at the optimal dosage. According to **Ridwan *et al.* (2020)**, toxic substances can cause damage to liver cells. Essentially, every chemical compound can be toxic if administered in excessive doses. This toxic effect arises from biochemical interactions between the toxic substances in the extract and the cells in the body's organs. This study also demonstrated that no test animals succumbed during the treatment process, as the density of bacteria and the concentration of the extract dose used had undergone acute LD_{50} and LC_{50} toxicity tests to assess the acute safety of the drug or substance to be used.

The rise in hematocrit, hemoglobin, and erythrocyte levels in the blood of gourami fish can be attributed to the bioactive properties of the extract from *C. varieganum*, such as terpenoids. **Minaka *et al.* (2012)** explained that the process of rupture of red blood cells is caused by bacteria producing toxins, one of the functions of which is to produce the hemolysin enzyme which is responsible for lysing red blood cells. **Alamanda *et al.* (2007)** added that anemia has an impact on inhibiting fish growth, because the low number of erythrocytes results in a reduced food supply to cells, tissues and organs, so that the fish's metabolic process will be hampered. **Hardi *et al.* (2011)** elaborated that a rise in total erythrocytes in fish blood signifies a homeostatic reaction within the fish's body. This reaction is aimed at generating more blood cells to replenish erythrocytes that might be lysed due to infection. **Sundaryono (2011)** stated that terpenoids have the capacity to stimulate erythropoiesis, which is the process of erythrocyte formation in the bone marrow.

The decrease in hematology (erythrocyte, hemoglobin, and hematocrit) levels at 650 and 700mg/ L treatment was ascribed to the fish being stressed by the high dosages. Every chemical compound is basically toxic if given in excessive doses. This is in accordance with **Castilhos *et al.* (2017)**, which state that several secondary metabolite compounds are toxic because they are part of the self-defense mechanism. The higher the dose of extract given indicates the greater the damage due to the toxicity.

This decline in leukocyte count suggested that the *C. variegatum* extract contributed to the treatment process of gourami fish following their infection with *E. tarda* bacteria. Because the infection stops, the presence of leukocytes as phagocytes is no longer needed, so the number of leukocytes returns close to normal. According to **Sumadewi and Puspaningrum (2018)**, the bacteriostatic properties of the flavonoid and triterpenoid compounds contained in the crude extract of croton leaves will inhibit bacterial growth so that leukocyte cells are no longer produced in large numbers.

This elevation in lymphocyte count post-treatment suggested that the compounds found in the crude extract of *C. variegatum* possessed the capability to bolster the immune system of gourami fish in combating *E. tarda* bacterial infection. **Ismail (2019)** stated that lymphocytes function as producers of antibodies to deal with disease disorders. Lymphocytes play a major role in the formation of humoral and cellular immunity to attack and destroy disease agents. An increase in the number of lymphocytes in the blood circulation will be balanced by a low number of neutrophils, and vice versa. According to previous study, natural substances can boost immunity and can improve defenses against infections when taken in the proper quantity **(Kim & Yang, 2018)**.

The crude extract of *C. variegatum* leaves demonstrated the ability to inhibit the growth of *E. tarda* bacteria, resulting in a decrease in the number of monocytes. Monocytes are stronger phagocytic compared to neutrophils and can phagocytize larger particles, mature monocytes are called macrophages **(Afifah *et al*., 2014)**. Leukocyte, lymphocyte, monocyte, and neutrophil values did not show optimal improvement at doses of 650 and 700mg/ L. This is consistent with earlier research findings, such as those by **Rand *et al.* (2015)**, who elucidated that with escalating doses of extract administration, the extent of tissue cell damage also escalates.

The extract of *C. variegatum* with a dose of 600mg/ L successfully increased the survival rate of gourami fish by up to 83%. Since the test fish already have immunological memory, giving them a booster, such as a herbal extract, can increase the generation of antibodies and cause a stronger immune response **(Kenconojati *et al*.,**

2023). The content of antibacterial activity such as tannins, terpenoids, alkaloids, and flavonoids will reduce the impact of bacteria and reduce mortality in gourami fish **(Nimah *et al.*, 2012; Bijekar & Gayatri, 2014; Widowati *et al.*, 2014)**. The provided dose had peaked, as seen by the decline in gourami fish survival rates in the 650 and 700mg/ L treatment groups. According to **Setyani *et al*. (2018)**, high mortality happens when bacterial infections cause substantial organ damage and when the extract dose is sufficient to have a serious effect on the organism. According to **Awad and Awaad (2017)**, using many extracts does not always improve the effectiveness of therapy for bacterial infections. If the concentration of an extract's active ingredients rises over a particular point, the extract may become poisonous to test animals.

CONCLUSION

In summary, the findings of this study demonstrate that *C. variegatum* leaves contain abundant flavonoids, alkaloids, terpenoids, and tannins, suggesting their potential use as a natural remedy for bacterial infections in fish. It was discovered that 600mg/ L was the most efficient dosage for preventing *E. tarda* infection in gourami fish. Numerous parameters, such as hematological, histopathological, and survival rate showed better results using this dosage. This would provide deeper insights into its potential benefits and practical feasibility in real-world aquaculture settings.

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