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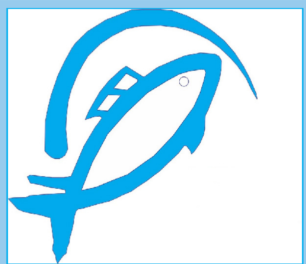

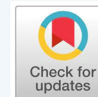

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Short Communication

Effect of *Andrographis paniculata* Leaf Crude Extract Against *Edwardsiella tarda* and Histopathological Profile of the Liver of *Osphronemus gouramy* Juvenil

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Abstract

Edwardsiellosis caused by bacterium *E. tarda* is a serious challenge faced by Gourami farmers. This study aimed to determine the antibacterial activity of *A. paniculata* leaf crude extract on the growth of *E. tarda* bacteria and its effect on the histopathology of gourami liver which was carried out in two stages. In the first stage, the active compound was identified in the leaves of *A. paniculata* followed by the Minimum Inhibitory Concentration (MIC) test at five different doses and two controls. In-silico test was conducted to determine the antibacterial activity of crude extract of *A. paniculata* leaf. In the second stage, the Lethal Dosage (LD50) and Toxicity (LC50) tests were carried out. Histopathological test of gouramy liver was carried out by taking the liver of fish that had been exposed to *E. tarda* bacteria. The active compounds contained in the crude extract of *A. paniculata* leaf are flavonoids, alkaloids, tannins, triterpenoids, and saponins. The Lethal Dosage (LD50) was 10^6 CFU/ml, and a toxicity test showed a dose of 250 mg/L caused the most death compared to other treatments. Histopathological test of gouramy liver showed that crude extract of *A. paniculata* leaf could improve liver function optimally at a dose of 300 (mg/L). The results of this study indicate that crude extract of *A. paniculata* leaf can affect the histology profile of gouramy liver and can cause toxicity if used inappropriately.

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1. Introduction

The production of gourami cultivation continues to increase by 121,544 tons in 2015 to 187,950 tons in 2019 (KKP, 2019). From a business perspective, gourami cultivation is a more profitable fishery business compared to other types of freshwater fish. This is indicated by the selling price of gourami which is more expensive and more stable in the market (Pratama *et al*., 2018). Along with the development of technology, the method generally used to increase production is an intensive cultivation system. The intensive cultivation system employs high stocking density and high protein feed (Azhari and Tomasoa, 2018). The application of this technology certainly does not escape problems, where there is a decrease in water quality which can lead to the emergence of pathogens in fish. One of the obstacles faced in the cultivation of gourami (*Osphronemus gouramy*) is Edwardsiellosis disease caused by the bacterium *E. tarda* (Prastiti *et al*., 2015). This is generally influenced by inadequate fish farming environmental conditions with high temperatures and high concentrations of organic matter (Davies *et al*., 2018).

One of the organs that can be used as an observation indicator when a bacterial infection occurs is the liver due to it being crucial for the metabolism process, as a means of secretion in the detoxification process and functions to phagocytize foreign objects that enter the liver. The liver is an organ that often suffers damage or histological structural abnormalities, the largest digestive gland and is composed of parenchymal cells (hepatocytes) and interwoven fibers (Faccioli *et al*., 2014; Wolf and Wheeler, 2018). Liver arteries and veins empty wastes into the liver then the bile ducts brings it from the liver to the intestines (Safratilofa, 2017; Araújo *et al*., 2019). To overcome bacterial infections in cultured *O. gouramy*, prevention, treatment, and control efforts are needed. Efforts to control *E. tarda* infection generally use synthetic antibiotics and chemicals. However, the use of synthetic antibiotics in the long term will have a negative impact on both cultivated biota and the surrounding environment (Chanda *et al*., 2011). The use of antibiotics can cause bacteria to become resistant to generic drugs which in turn will increase mortality (Aly and Albutti, 2014; Pepi and Focardi, 2021). Currently, the use of antibiotics and chemicals has been limited by the government and it is recommended to reduce their use (Polianciuc *et al*., 2020). Based on these problems, the use of natural ingredients can be used as an appropriate alternative medicine to overcome existing problems. *A. paniculata* leaf crude extract has antibacterial properties because it contains alkaloids, flavonoids, saponins and tannins. In several studies, *A. paniculata* leaf crude extract has been proven as an antibacterial that can inhibit the growth of *Staphylococcus aureus*, *E. coli* (Retnowati *et al*., 2011; Sawitti *et al*., 2013). This study aims to determine the antibacterial activity of *A. paniculata* leaf crude extract on the growth of *E. tarda* bacteria and its effect on the histopathology of gourami liver.

2. Materials and Method

2.1 Time and Place

This research was conducted in February - April 2022 at the Fish Health Laboratory, Faculty of Fisheries and Marine Sciences, Universitas Brawijaya Malang.

2.2 Tools and Materials

The tools used in this study were analytical scale, autoclave, beaker glass, bunsen burner, digital scale, Erlenmeyer flask, film bottle, funnel, hot plate, incubator, laminar air flow (LAF), measuring cup, ose needle, oven, refrigerator, petri dish, pipette, rotary vacuum evaporator, section set, spatula, spectrophotometer, spray bottle, suction ball, syringe, test-tube, test-tube rack, tray, and vortex mixer.

The material used is *A. paniculata* leaf obtained by Materia Medika, Batu City, East Java. The solvent for maceration is ethanol with pro-analytical quality (PA), Whatman No. 42 trademark filter paper, aluminum foil and plastic wrap, DMSO and hydrobath. The bacteria used for this research activity were obtained from the Faculty of Medicine, Universitas Brawijaya. Culture and bacterial rejuvenation media are in the form of Tryptic Soy Agar (TSA), Tryptone Soya Broth (TSB), distilled water, and Alcohol. The test animals used in this study were *O. gouramy* seeds with a length of 7-10 cm and bacteria obtained from the Faculty of Medicine, Universitas Brawijaya, Malang. For culture media and bacterial rejuvenation using Tryptone Soya Broth (TSB) media.

2.3 Research Design

This research was carried out in two stages, namely the in vitro bacterial activity test and continued with the in vivo test. The method used in this study refers to the study of Budianto *et al*. (2015), with little modification and using a Completely Randomized Design with three replications, positive, and negative controls.

2.4 Working Procedure

2.4.1 Extract production

A. paniculata leaves were air dried in a room then ground and sieved to obtain a fine powder. A total of 100 grams of *A. paniculata* leaves powder was macerated by means of simplicia in 1000 ml of ethanol solvent (1:10 ratio) for 2x24 hours at room temperature. The extract was filtered using filter paper and then evaporated with a rotary vacuum evaporator at a temperature of 35-40^{0}C and the yield was calculated.

2.4.2 Identification of active compound extract

The compound identification test was carried out to determine the active compounds in the *A. paniculata* leaf crude extract and to determine the dominant compound in the *A. paniculata* leaf crude extract. The methods used in this study include the phytochemical test where a color change of the test solution signifies a specific compound, and the Fourier transform-infrared (FTIR) spectrophotometer test. This type of analysis can be used to characterize samples in the form of liquids, solutions, pastes, powders, fibers, and gases (Nandiyanto *et al*., 2019).

2.4.3 Bacterial culture and rejuvenation of E. tarda

Pure cultures of *E. tarda* were obtained from the Faculty of Medicine (FK) Universitas Brawijaya, Malang. The medium used for the culture and rejuvenation of *E. tarda* bacteria was TSB. 9 ml of agar medium was put into a test tube, then sterilized with an autoclave for 15 minutes at a temperature of 121^{0}C and a pressure of 1 atm. The pure culture of *E. tarda* bacteria was planted with aseptic ose on TSB media. After planting, the media was then incubated at 31^{0}C for 24 hours (Riyadi *et al.,* 2021).

2.4.4 MIC test

The MIC test was carried out by preparing TSB media and cultured bacteria in test tubes then adding 0.5 ml of *A. paniculata* leaf extract to each test tube. Test tubes 1 to 5 were filled with *A. paniculata* leaf extract with different concentrations of 25, 50, 100, 200, and 400 ppm. The 6th and 7th tubes were filled with two controls, namely positive and negative controls. Positive control was 5 ppm of synthetic antibacterial (Chloramphenicol) with a volume of 0.5 ml while for negative control the cultured bacteria were left as is in the media (Fauziyyah *et al.,* 2021).

2.4.5 In-silico test

The method used in the in-silico test utilizes computational chemistry applications that can display the active compound in three dimensions (3D) and perform comparisons with other compounds that are known to have high activity. Based on a 3D comparison equipped with similarity and energy calculations, it provides an overview of the potential parts and groups that can be developed from the compounds used. Then various derivative compounds and analogues are described according to the requirements of the computer application used.

2.4.6 LD_{50} (Lethal dosage 50) test

The Lethal Dosage 50 (LD_{50}) test was used to determine the density and duration of *E. tarda* bacteria required to kill 50% of the fish tested. Bacteria were cultured on TSB media as much as 10^9 CFU/ml. Then, the dilution was carried out in stages to a density of 10^8, 10^7, 10^6, 10^5, and 10^4 CFU/ml. The calculation of the bacterial suspension was carried out using the Thompson-Weil method referring to Janik-Spiechowicz and Wyszyńska (1999), with the following formula:

$$V_1 \times N_1 = V_2 \times N_2$$

N1: Density of bacterial population in the media (cells/ml)
N2: Desired bacterial population density (cells/ml)
V1: Volume of bacterial suspension in required medium
V2: Volume of water medium in fish rearing container

2.4.7 Extract toxicity test

Toxicity test was carried out by soaking the leaf of *A. paniculata* in the test fish for 96 hours which aims to determine the concentration of extract that is toxic to fish. In this study, the determination of the toxicity test dose used the development of the extract dose according to the results of the disc test, namely 50 ppm, 100 ppm, 150 ppm, 200 ppm, and 250 ppm (Rusmawanto *et al.,* 2021).

2.4.8 Liver histopathological observations

According to Zulfadhli *et al*. (2016), histopathological observations were carried out by preparing samples in the form of fish that were dissected and the tissues of the organs were taken. The tissue taken was then cleaned using distilled water and put into a film bottle containing 10% formalin. Then histopathological preparations were made by fixation of the liver to be observed followed by the stages of dehydration, clearing, impregnation, embedding, tissue staining, mounting, and scoring.

3. Result and Discussion

3.1 Extract Production

The extraction process in this study used 70% ethanol as a solvent. The use of 70% ethanol was chosen because of the nature of ethanol which can attract

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polar compounds, which are the intended compounds contained in *A. paniculata* leaf. According to Riwanti *et al*. (2020), 70% ethanol is a solvent that is more polar than 96% ethanol and more non-polar than 50% ethanol, and so, polar compounds will dissolve more in 70% ethanol. Yield results obtained using a ratio of 1:10 using 100 g of *A. paniculata* leaf powder and 1 L of ethanol followed by evaporation process to produce 11.15 g of extract.

3.2 Phytochemical Screening

The results of phytochemical tests on *A. paniculata* leaf crude extract showed that there were flavonoid compounds, alkaloids, tannins, triterpenoids, and saponins (Table 1).

Table 1. Phytochemical test results on *A. paniculata* leaf crude extract.

Compound Identification	Characteristics	Result
Flavonoid	Orange, Brick Red, Pink, Dark Red	(+) Positive
Alkaloid		
Mayer	White precipitate	(+) Positive
Dragendrof	Orange precipitate	(+) Positive
Tanin	Dark Chocolate, Dark Blue	(+) Positive
Terpenoid		
Steroid	Blue Green	(-) Negative
Triterpenoid	Orange, Orange Brown	(+) Positive
Saponin	Permanent Foam	(+) Positive

Note: (+) there is a chemical content, (-) there is no chemical content.

3.3 FTIR Test

From the results of phytochemicals and FTIR, it could be seen that there were similarities in the results of the analysis on the content of active compounds contained in *A. paniculata* leaf.

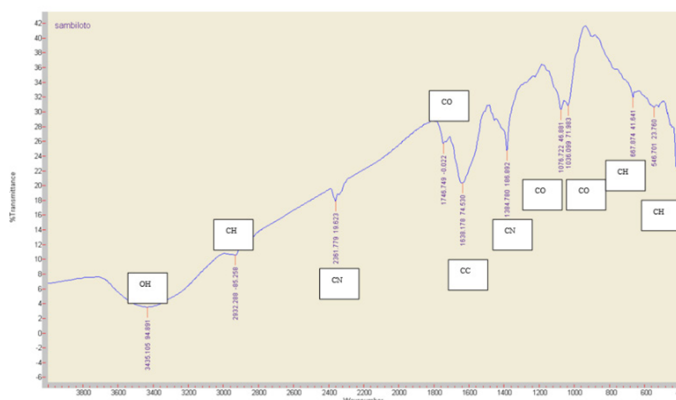

Figure 1. Results of FTIR test analysis of *A. paniculata* leaf

The results obtained from the FTIR test showed that there were 9 absorption band frequency regions with different functional groups indicating the presence of flavonoids, alkaloids, and tannins (Figure 1).

3.4 MIC Test

The MIC test was seen from the absorbance value of the bacteria that had been given the extract. The absorbance value indicated the ability of *A. paniculata* leaf crude extract to inhibit the growth of *E. tarda* bacteria.

A. paniculata leaf crude extract with ethanol solvent at a concentration of 100 mg/L had the lowest absorbance value with a value of 0.455 which was closest to the Negative Control (Table 2). A dose of 100 mg/L of *A. paniculata* leaf extract can be used as a standard minimum dose for the disc test due to an increase in the absorbance value again at doses of 200 and 400 mg/L.

Table 2. MIC test results

Concentration (mg/L)	Absorbance
Control (+)	0.893
25	0.512
50	0.493
100	0.455
200	0.461
400	0.475
Control (-)	0.021

Description : Positive control (+) bacteria only, Negative control (-) using chloramphenicol 5 mg/L.

According to Riyono (2006), the measurement of absorbance values using the spectrometer method has weaknesses where the sensitivity of the instrument used is low and the spectrometer also cannot distinguish the level of pigment turbidity from the extract and turbidity from bacterial cells so that the Optical Density (OD) value obtained is a combination of both. To acquire more accurate results, further tests were carried out in the form of disc tests based on data from the MIC test.

3.5 In–Silico Test

The in-silico test is a method that can be used to predict preclinical toxicological end points, clinical side effects, and computerized drug substance metabolism (Valerio Jr., 2009). In the in-silico test Extracellular Target Protein (ECP) in *E. tarda* bacteria is DegP (serine protease) and EvpC protein (virulence protein). The protein sequences of *E. tarda* DegP (serine protease) and EvpC protein. BothFASTA protein sequences were modeled in swissmodel to obtain the 3D structure of the protein. According to Fardiyah *et al*. (2020), the compounds present in the leaf extract of *A. paniculata* are quercetin type flavonoids, carpaine alkaloids and tannins. The results obtained showed that all compounds could inhibit DegP and EvpC proteins in the same area in chloramphenicol, except for tannin compounds which could not inhibit the growth of DegP protein but showed the greatest inhibition on Evpc protein.

3.6 LD_{50} (Lethal Dosage 50) Test

Based on the results of the LD50 test using the bacterium *E. tarda* which was used in the challenge test process on *O. gouramy* for 96 hours, 50% death occurred at a bacterial concentration of 10^6 (Table 3).

Table 3. *E. tarda* pathogenicity test (LD_{50})

Bacterial Density	Total Test Fish	Total Dead Fish
10^4	10	1
10^5	10	3
10^6	10	4
10^7	10	5
10^8	10	7

In this study, the main factors that affect the number of fish deaths are the pathogenicity of bacteria that produce toxins and the speed at which bacteria multiply. According to Olga (2012), the higher the dose of the bacterial suspension that is infected, the more severe the symptoms and the higher mortality in fish.

3.7 Toxicity Test

This toxicity test aims to determine the level of toxicity of the extract used on the test fish (Table 4).

Table 4. Extract toxicity test results

Extract Concentration (mg/L)	Total Test Fish	Total Dead Fish
50	12	0
100	12	0
150	12	1
200	12	2
250	12	3

Toxicity test was carried out by observing the effect of the extract used on the test fish in the form of mortality or immobilization, results of which could be further processed in lethality and immobilization test. The results of the toxicity test show that a dose of 250 mg/L caused the most mortality (Table 5).

Table 5. Extract toxicity values

Relative Toxicity	Dose (mg/L)
Too toxic	0.01-0.1
Very toxic	0.1-1
Quite toxic	1-10
Slightly toxic	10-100
Practically non-toxic	100-1000
Relatively harmless	>1000

Source : (El-Harbawi, 2014)

It can be said that the extracts used are still in the practically harmless category for use. The higher the dose used, the more toxic it will be to fish and can cause death (Table 5).

3.8 Liver Histopathological Observations

To determine liver damage in *O. gouramy* which has been exposed to *E. tarda* bacteria for 7 days, histopathological tests were carried out (Figure 2).

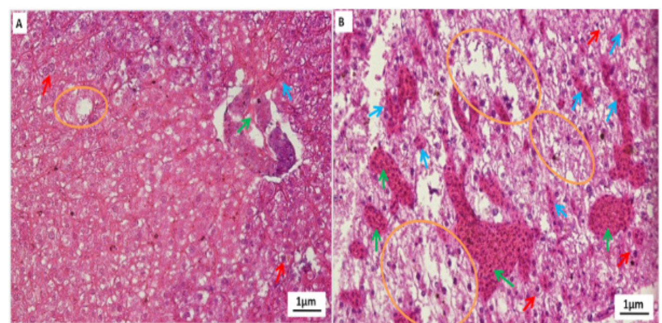

Figure 2. Histopathology of healthy and sick gourami liver

With a magnification of 400 times, the condition of healthy gourami liver (A) shows that the tissue is still intact and is still in the good category (Figure 2). Meanwhile, liver infected with *E. tarda* bacteria (B) showed a lot of damage, namely congestion (green arrow), hemorrhage (blue arrow), degeneration (red arrow), and necrosis (orange circle).

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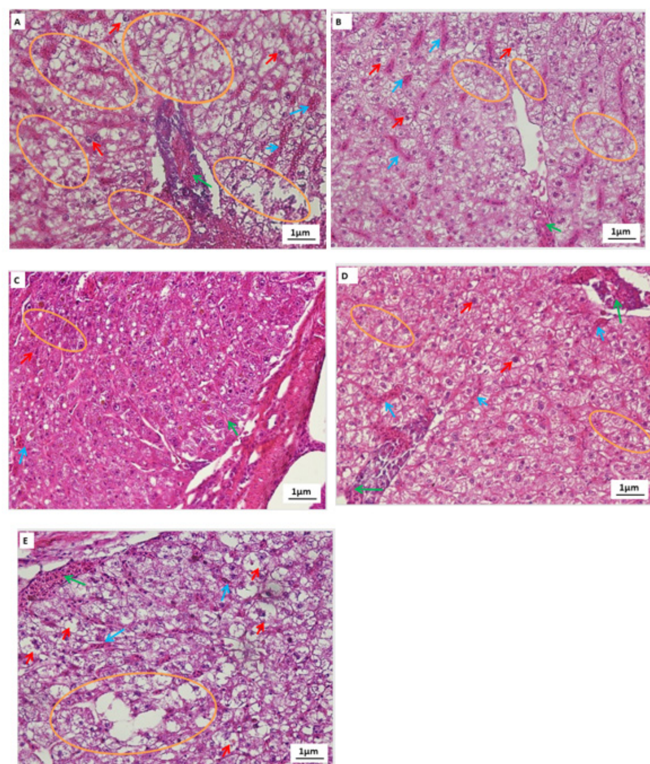

Figure 3. Histopathology of *O. gouramy* liver after treatment with 400x magnification microscope for 7 days. (A) 250 mg/L, (B) 275 mg/L, (C) 300 mg/L, (D) 325 mg/L, and 350 mg/L.

Analysis of liver damage infected with *E. tarda* bacteria after treatment using *A. paniculata* leaf crude extract showed damages (Figure 3). The damages are described below:

3.8.1 Degeneration

Treatment of *O. gouramy* using *A. paniculata* leaf crude extract (*A. paniculata*) after exposure to the bacterium *E. tarda* showed liver tissue damage in the form of degeneration (Table 6).

Table 6. Average degeneration score

Treatment (mg/L)	Degeneration
0 (K+)	2.66 ± 0.11^{d}
250 (A)	2.20 ± 0.20^{c}
275 (B)	1.93 ± 0.11^{c}
300 (C)	1.20 ± 0.20^{b}
325 (D)	1.46 ± 0.30^{b}
350 (E)	1.93 ± 0.11^{c}
0 (K-)	0.86 ± 0.11^{a}

Description: Values with the same Superscript in the row indicate no significant difference (P>0.05).

One-way ANOVA test with a 95% confidence level showed the results of damage to the liver in the form of significant degeneration (P<0.05) (Table 4). The administration of *A. paniculata* leaf crude extract can reduce the value of degeneration damage in the liver of *O. gouramy* infected with *E. tarda* bacteria with the lowest damage value in treatment C using a dose of 300 mg/L, which is 1.20.

This shows that treatment C is the best treatment in this study. There was an increase in the value of damage to liver degeneration again in treatments D and E where the doses used were 325 and 350 mg/L. This indicates that the given extract has reached its optimum point in the treatment of *O. gouramy* after being infected with *E. tarda* bacteria. The use of inappropriate doses can be a contributing factor to the occurrence of tissue damage in fish to be treated (Mauro *et al*., 2021).

3.8.2 Congestion

Treatment of *O. gouramy* using *A. paniculata* leaf crude extract after exposure to the bacterium *E. tarda* showed liver tissue damage in the form of congestion (Table 7).

Table 7. Average congestion score

Treatment (mg/L)	Congestion
0 (K+)	2.53 ± 0.11^{d}
250 (A)	1.60 ± 0.20^{c}
275 (B)	1.46 ± 0.11^{c}
300 (C)	1.00 ± 0.20^{ab}
325 (D)	1.33 ± 0.11^{bc}
350 (E)	1.60 ± 0.40^{c}
0 (K-)	0.66 ± 0.11^{a}

Description: Values with the same Superscript in the row indicate no significant difference (P>0.05).

Based on the results of the one-way ANOVA test with a 95% confidence level, there was a damage to the liver in the form of significant congestion (P<0.05) (Table 7). This indicates that the administration of *A. paniculata* leaf crude extract can reduce the value of congestion damage in the liver of gourami infected with *E. tarda* bacteria with the lowest damage value in treatment C using a dose of 300 mg/L, which is 1.00.

This shows that treatment C is the best treatment in this study. There was an increase in the value of liver congestion damage again in treatments D and E where the doses used were 325 and 350 mg/L. This indicates that the given extract has reached its optimum point in the treatment of *O. gouramy* after being infected with *E. tarda* bacteria.

3.8.3 Necrosis

Treatment of *O. gouramy* using *A. paniculata* leaf crude extract after exposure to the bacterium

E. tarda showed liver tissue damage in the form of necrosis (Table 8).

Table 8. Average necrosis score

Treatment (mg/L)	Necrosis
0 (K+)	2.46 ± 0.11^{e}
250 (A)	1.73 ± 0.30^{d}
275 (B)	1.53 ± 0.46^{bc}
300 (C)	1.33 ± 0.11^{b}
325 (D)	1.40 ± 0.20^{bc}
350 (E)	1.46 ± 0.30^{cd}
0 (K-)	1.00 ± 0.00^{a}

Description: Values with the same superscript in the row indicated no significant difference ($P>0.05$).

Based on the results of the one-way ANOVA test with a 95% confidence level, there was a damage to the liver in the form of significant necrosis ($P<0.05$) (Table 8). This indicated that the administration of *A. paniculata* leaf crude extract could reduce the value of necrosis damage to the liver of gourami infected with *E. tarda* bacteria with the lowest damage value in treatment C using a dose of 300 mg/L is 1.33.

This showed that treatment C was the best treatment in this study. There was an increase in the value of liver congestion damage again in treatments D and E where the doses used were 325 and 350 mg/L. This indicated that the given extract had reached its optimum point in the treatment of *O. gouramy* after being infected with *E. tarda* bacteria.

4. Conclusion

The active compounds contained in *A. paniculata* leaf crude extract are flavonoids, alkaloids, tannins, triterpenoids, and saponins. The MIC and in-silico test results showed that *A. paniculata* leaf crude extract could inhibit the spread and metabolism of *E. tarda* bacteria. The LD_{50} test on fish with a bacterial density of 10^7 CFU/ml can kill as much as 50% of fish. Toxicity test showed that the *A. paniculata* leaf crude extract was not toxic. The results of the histopathological test of gourami liver showed that *A. paniculata* leaf crude extract could optimally improve the liver at a dose of 300 (mg/L).

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Authors' Contributions

All authors have contributed to the final manuscript. The contribution of each author as follow, GP; collected the data, drafted the manuscript and designed the figures. AP and TDS; devised the main conceptual ideas and critical revision of the article. All authors discussed the results and contributed to the final manuscript.

Conflict of Interest

The authors declare that they have no competing interests.

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