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Fraction and Antibacterial Activity of Bitter Melons (*Momordica charantia)* in Koi Fish (*Cypinus carpio*) Infected with *Aeromonas salmonicida* Bacteria and its Effect on Gills Histopathology

Nadiah Nurandi[1*], Sri Andayani[2], Yuni Kilawati[2]

[1] Master's Degree Program in Faculty of Fisheries and Marine Science, University of Brawijaya, Malang, Indonesia.
[2] Department of Aquaculture, Faculty of Fisheries and Marine Science, University of Brawijaya, Malang, Indonesia

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Corresponding Author:
Nadiah Nurandi
yanik@ub.ac.id

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Abstract: Koi fish (*Cyprinus carpio*) is a type of freshwater ornamental fish with significant economic value, attracting widespread public interest due to its vibrant colors and agile movements. Therefore, this study introduces a novel approach by evaluating the antibacterial activity of bitter melon extract fraction (*Momordica charantia*) against *A. salmonicida* infection in koi fish and its impact on gill histopathology. The novelty of this study lies in the exploration of bitter melon extract fraction as a natural, eco-friendly antibacterial alternative to synthetic antibiotics in aquaculture. This research is crucial as it addresses the growing global concern over antibiotic resistance and the urgent need for sustainable, natural solutions in fish health management. Antibacterial tests were conducted to assess the ability of the bitter melon extract to inhibit bacterial growth, while histopathological analysis aimed to observe the specific changes in gill tissues of infected koi fish. The results revealed that the bitter melon extract fraction significantly reduced bacterial count and improved gill tissue integrity. Histopathological improvements were observed, including reduced hyperplasia, lamellar fusion, edema, and necrosis, indicating tissue recovery. This finding highlights the potential of bitter melon extract fraction as a natural antibacterial agent for controlling bacterial infections in fish, offering an alternative to chemical antibiotics. Further research is needed to determine the optimal dosage and to explore its antibacterial mechanism, ensuring its safe and effective application in aquaculture.

Keywords: Antibacterial; Fractionation; Histopathology; Momordica charantia

Introduction

Bitter melon (*M. charantia*) has been known as one of the medicinal plants that has various pharmacological properties. This plant is widely found in Asia and Africa and has long been used in traditional medicine to treat various diseases, including bacterial infections. Bitter melon contains active compounds such as saponins, alkaloids, flavonoids, and tannins, which have the potential to be antibacterial agents. Previous research has shown that bitter melon extract is able to inhibit the growth of various types of pathogenic bacteria. This is the basis for further exploring the potential of bitter melon in the treatment of fish diseases, especially those caused by bacterial infections (Muribeca et al., 2022).

In the context of fish farming, diseases due to bacterial infections are one of the main problems faced by fish farmers. One pathogenic bacteria that often attacks fish is *A. salmonicida*, which can cause infections in freshwater fish such as koi (*C. carpio*). This infection

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often causes disturbances in the gills, which has an impact on the respiratory system of the fish. This condition requires immediate medical action so that the fish can survive and minimize the economic losses experienced by the farmer (Apriliyanti et al., 2013).

One of the main advantages of using natural ingredients is their ability to reduce the risk of antimicrobial resistance, a growing problem due to the overuse of synthetic antibiotics. In aquaculture, the use of antibiotics is often necessary to overcome bacterial infections, but this can trigger bacterial resistance and negatively impact aquatic ecosystems. As an alternative solution, natural ingredients such as bitter melon (*M. charantia*) offer the potential for safer and more environmentally friendly treatments. The active compounds contained in bitter melon are known to have strong antibacterial properties, so they can help control bacterial infections without causing adverse side effects such as resistance. This study became important to evaluate the effectiveness of bitter melon fractions in the treatment of bacterial infections in koi fish (*C. carpio*), especially those caused by *A. salmonicida* (Laoli et al., 2024).

Bitter melon (*M. charantia*) contains various compounds that function as antibacterial and antioxidants, such as flavonoids, lectins, saponins, polyphenols, vitamin C, cucurbitacin glycosides, momordicin, as well as charantin, which are useful for fighting free radicals. Some studies show that bitter melon extract has strong antibacterial abilities, but its effectiveness against *A. salmonicida* is still not widely studied. In this study, fractions from bitter melon extract will be tested to evaluate its effectiveness in treating infections caused by *A. salmonicida*. The research method involves the isolation of the active fraction of bitter melon extract, as well as testing for antibacterial activity against bacteria taken from infected koi fish. The purpose of this test was to see how much potential bitter melon has in treating bacterial infections in koi fish, specifically infections that can affect the health of the gills and the overall condition of the fish (Rini et al., 2021).

In addition to testing the antibacterial ability, this study also evaluated the effect of treatment using bitter melon fraction on the histopathological condition of koi fish gills. Gill is a vital organ that functions in gas exchange and osmoregulation, so damage to the gills can be fatal for fish. Histopathological analysis provides an overview of the structural changes in the gills due to bacterial infection and the improvements that occur after treatment (Irshath et al., 2023).

Aside from the antibacterial effectiveness, it is also important to understand how these treatments affect the overall well-being of the fish. This study will observe the physical condition of koi fish after being treated using bitter melon fractions, including changes in weight, mortality rate, and feeding activity. This aims to ensure that the treatment given is not only effective in treating the infection, but also safe for the fish (Bortolotti et al., 2019).

With the increasing awareness of the importance of environmentally friendly approaches in the treatment of fish diseases, this research is expected to be a reference in the development of sustainable natural treatment methods. This research not only focuses on the effectiveness of antibacterial, but also the ecological and economic impacts of the use of bitter melon in aquaculture. The results of this research are expected to provide alternative solutions that can be implemented by koi fish farmers around the world (Lê et al., 2024).

Method

Location and Time

This research was carried out in the period of February 2024 to June 2024 at the Fish Farming Laboratory, Division of Fish Disease and Health, Universitas Brawijaya. The selection of this location is based on complete laboratory facilities as well as access to the koi fish (*C. carpio*) needed for testing. In this laboratory, various scientific procedures such as sampling, antibacterial tests, and histopathological observations can be carried out with high research standards. Koi fish infected with Aeromonas salmonicida bacteria will be observed to determine the effectiveness of bitter melon fraction (*M. charantia*) as an antibacterial agent. The entire series of research is carried out with strict control of environmental conditions, such as temperature and water quality, to ensure the accuracy of the results.

Sample Collection and Preparation

This research material involves the observation of histopathological observations of koi fish gills that have been treated with bitter melon fruit extract (*M. charantia*) to treat *A. salmonicida* bacterial infection. Koi fish samples were randomly selected and infected with the bacteria before being given bitter melon extract as a treatment. This extract is obtained from the processing of bitter melon and tested at various doses to determine the most effective dose. The main parameters observed included fish survival, water quality during the study, as well as histopathological observations of fish gills. This technique aims to evaluate whether bitter melon extract is able to increase the survival rate of koi fish infected with bacteria.

Extraction and Identification of Active Compounds

The extraction method used in this study is the maceration method. 7 kg of bitter melon is ground and processed until it becomes a powder. Soaking (maceration) was carried out in a ratio of 1:3 where 500 grams of bitter melon powder was macerated using 98% Analyst Pro ethanol, after 4 days the maserat was filtered from the pulp with a Buchner funnel that had been coated with filter paper. The maserat formed is evaporated with a rotary evaporator at a temperature of 60°C until a thick extract (Maftuch et al., 2018).

This phytochemical test usually uses a color reagent that will produce a color reaction in plants that have certain chemical contents. Phytochemical tests must pay attention to the extraction method and also the solvent to be used. Phytochemical tests are carried out to identify the presence of several compounds such as phenols, saponins, triterpenes, flavonoids, alkaloids and steroids (Emilia et al., 2023).

The phytochemical testing methods used are column chromatography and UV-Vis spectrophotometer. Column chromatography aims to separate the active compounds contained in bitter melon extract (*M. charantia*) into fractions. For UV-Vis, it can be done by measuring the spectrum of absorbance wavelength of ultra violet radiation fraction 2 with a resolution spectrophotometer of 1 nm (from 200-800 nm) on a normal quartz bowl with a trace length. Where fraction 2 of the column chromatography results are taken as much as 3 ml and put into the cuvette, then the spectrophotometer is scanned with a wavelength starting from 200 nm (Akhter et al., 2012).

Anti-Bacterial Activity Test in Vitro

The antibacterial activity test was carried out in vitro with two methods, namely the Minimum Inhibition Concentration (MIC) and the disc test. The MIC test was carried out by mixing bitter melon extract in a test tube containing sterile TSB with varying doses. Positive controls use synthetic antibacterials, such as Chloramphenicol, while negative controls are not given extracts (Nurjannah et al., 2013). Aeromonas salmonicida bacterial isolate was then added and the tubes were incubated for 24 hours at 32°C. Furthermore, the level of turbidity of the media was measured using a spectrophotometer at a wavelength of 600 nm. In addition, the disc test was carried out using a sterile disc that had been soaked in a bitter melon extract fraction and placed on a agar medium that had been inoculated with bacteria, referring to the method (El-Sayed et al., 2020; Juwitaningsih et al., 2021).

In this in vitro test, it was carried out using a sterile TSB inserted in a 5 ml reproduction tube. Then bitter melon extract (*M. charantia*) was added to the test tube with a different dose in each tube. In this test, 2 control treatments were also given, namely positive control treatment with the administration of synthetic antibacterial (Chloramphenicol) as much as 5 ppm, while for negative control no extracts were given. Then, each tube was given 0.1 ml of bacterial isolate (107 CFU/ml) and then incubated at 32°C for 24 hours. Then, the test medium was checked for turbidity and its absorption was measured with a spectrophotometer (wavelength of 600 nm).

The disc test can be done by preparing a sterile blank disc that has been immersed in bitter bitter fraction according to the dosage, which is then inserted into TSA media. After that, the 107 density bacteria were put into the medium and incubated for 24 hours then observed and measurements were made for the presence of the inhibitory zone (Maleta et al., 2018).

Anti-Bacterial Activity Test in Vivo

The in vivo test began with the adaptation process of koi fish measuring 5-7 cm for seven days. Each jar was filled with 10 liters of water and five koi fish for testing. After adaptation, the fish were treated with bitter melon extract according to the dosage obtained from the MIC test. An acute toxicity test (LD50) was conducted to determine the symptoms of poisoning, the cause of death, and the dose of the extract that killed 50% of fish in a short time. The data obtained were then used to determine the LC50 value, which is the dose of the extract that causes the death of 50% of fish within 96 hours. Observations were made on fish behavior, time of death, and number of dead fish (Murwantoko et al., 2013).

The acute toxicity test (Lethal Dose 50% or LD50) is intended to obtain information about the symptoms of poisoning, the cause of death, the sequence of the death process and the range of doses that kill the test animal in a short time. The purpose of the acute toxicity test is to detect the presence of toxicity of a substance, determine the target organ and its sensitivity, obtain hazard data after acute administration of a compound and to obtain preliminary information that can be used to determine the required dose level (Tr et al., 2016).

The LC50 value is determined based on the dosage of the MIC test results. Koi fish (*C. carpio*) are prepared as many as 20 and divided into 5 containers with a capacity of 10 L of water each. The fish are acclimatized for 7 days and fed with pellets. After the acclimatization process, the extract was inserted into each container and observed for 96 hours. The results of dead fish, the time of death and behavior are recorded as data from the LC50 test.

After conducting LC50 and LD50 toxicity tests, observation of the survival rate of koi fish was carried

out, the number of dead and live koi fish was calculated from each treatment. The number of dead and live fish will be calculated using the SR (Survival Rate) formula (Aniputri & Hutabarat, 2014) and the last is the observation of the histopathology of the dose of gills according to the results of the LC50 test.

Data Analysis

The data obtained from this study was analyzed using the ANOVA statistical test to determine the difference between treatments. Before the analysis, the data is tested for normality first. The F test (ANOVA) is used to determine whether there is a significant difference between different treatments, both in vitro and in vivo trials. The analysis was carried out using SPSS software to ensure that the results were valid and could be interpreted appropriately. This method allows researchers to evaluate the effectiveness of bitter melon extract in overcoming bacterial infections in koi fish and its impact on fish survival rates.

Result and Discussion

Identification of Active Compounds Using Column Chromatography and UV-Vis Spketrophotometer

Phytochemical screening showed that bitter melon fruit extract (*M. charantia*) contained important compounds such as flavonoids, alkaloids, tannins, terpenoids, and saponins. These compounds have the potential to inhibit the growth of microorganisms. Study by Hardani et al. (2023) reinforced these findings, stating that bitter melon extract may inhibit the growth of *Staphylococcus aureus* and *Escherichia coli bacteria*, suggesting that the active compounds in bitter melon are effective against various bacterial pathogens (Zaini & Shufiyani, 2017).

Figure 1. Results of Fractionation of Bitter Melons Extract (*M. charantia*) with Column Chromatography

In the fractionation of bitter melon extract by column chromatography, eluene n-hexane and ethyl acetate (17:3) were used as the phase of motion. This process is a continuation of Thin-Layer Chromatography (KLT) to separate the best fractions from the extracts. The fractionation results will be used in antibacterial tests. At this stage, fractional separation aims to obtain an optimal concentration of active compounds for further testing, ensuring effectiveness against *A. salmonicida*.

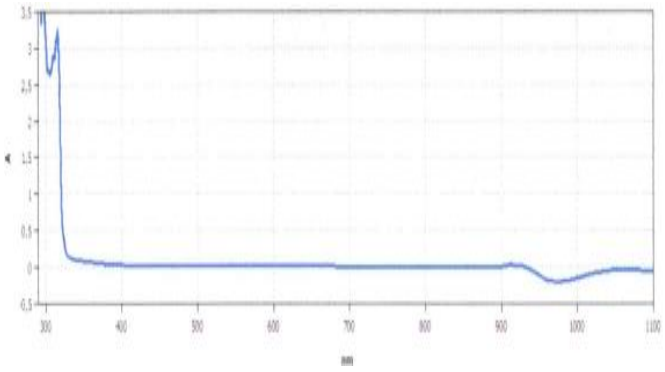

Figure 2. Results of UV-Vis Analysis Fraction 3

Based on the results of UV-Vis analysis fraction 2 of bitter melon fruit extract (*M. charantia*) at wavelengths of 297 nm and 320 nm.

Anti-Bacterial Activity Test in Vitro

In vitro antibacterial activity tests against Aeromonas salmonicida were carried out using MIC test and disc test. In the MIC test, the absorbance value was measured at a wavelength of 600 nm to determine the ability of the extract to inhibit bacteria. The results showed that a concentration of 31.25 ppm gave an absorbance value of 1.203, close to a positive control, indicating that at this concentration, the extract was able to inhibit bacterial growth with a slightly cloudy visual result.

Table 1. Minimum Inhibition Cocentration Result

Concentration (ppm)	Absorbance Value	Media Color
1000	1.12	Turbid
500	1.17	Turbid
250	1.04	Turbid
125	1.27	Slightly Cloudy
62.5	0.98	Slightly Cloudy
31.25	1.20	Slightly Cloudy
K (+)	1.22	Slightly Clear
K (-)	1.37	Slightly Clear

Based on Table 1, it can be seen that bitter melon extract (*M. charantia*) with a concentration of 31.25 ppm has an absorbance value of 1.203 which can inhibit the growth of *A. salmonicida* bacteria because at a concentration of 32.5 ppm the absorbance value is closest

to a positive control value with a slightly cloudy visual result.

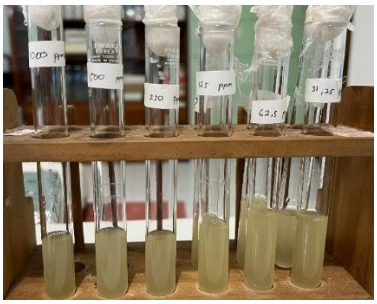

Figure 3. *MIC Test Results*

Next, a disc test or inhibition test is carried out. The bacterial inhibition test can be done by the diffusion method in the disc test. In this study, a disc test was used to obtain the best fraction of bitter melon extract (*M. charantia*) to inhibit *A. salmonicida bacteria*.

It can be seen from the results of this study that the higher the concentration value used, the larger the diameter of the inhibition zone formed. Some of the factors that affect the size of the inhibitory zone formed are growth sensitivity, environmental pH, media components, incubation temperature, reactions between active ingredients, size of the inoculum, and metabolic activity of microorganisms (Asfi et al., 2023).

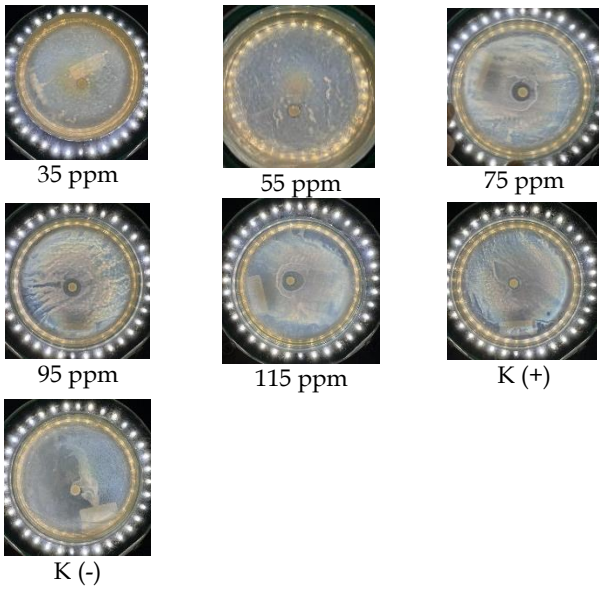

Figure 4. Disc Test Results

Table 2. Inhibitory Power Test Results

Concentration (ppm)	Deuteronomy 1	Deuteronomy 2	Total	Average±SD	Classification of Inhibition Zone Response
K (-)	0	0	0	0±0A	Weak
35 ppm	3.7	4.0	7.7	3.85±0.21b	Weak
55 ppm	4.3	4.8	9.1	4.55±0.35C	Weak
75 ppm	11.4	11.7	23.1	11.55±0.21d	Strong
95 ppm	11.9	12.5	24.4	12.2±0.42e	Strong
115 ppm	13.2	15.3	28.5	14.25±0.48f	Strong
K (+)	5.0	5.1	10.1	5.05±0.07g	Keep

Anti-Bacterial Activity Test in Vivo

The in vivo test began with *the Lethal Concentration 50* (LC50) test using bitter melon extract (*M. charantia*) concentrations of 35 ppm, 55 ppm, 75 ppm, 95 ppm, and 115 ppm respectively. The results were obtained that the LC50 toxicity test test at doses of 35 ppm and 55 ppm has not shown high toxicity because at that dose only killed 1-2 koi fish. At doses of 75 ppm, 95 ppm and 115 ppm fish mortality began to increase. The dose of 75 ppm killed 3 fish at the 18th hour, while the doses of 95 ppm and 115 ppm showed the same result, namely 4 dead fish at the 20th and 22nd hours. Based on these results, for the next stage, a dose of 55 ppm was used, namely 75 ppm, 95 ppm, and 115 pm which are suspected to be still safe in administering doses to test animals because fish mortality is still below 50. The use of extracts or compounds in a test animal must be ensured so that the extract does not kill the test animal itself because the level of toxicity of the compounds in the extract is too high (Gruber et al., 1997; Tr et al., 2016).

The results of the Lethal Dossage 50 (LD50) test of koi fish (*C. carpio*) against *A. salmonicida* with a density of 107 CFU/ml obtained data that the higher the density of the infecting bacteria, the higher the mortality value of the test animal. Doses of 108–1010 CFU/ml make fish die above 50% before 24 hours, with a total of 10 initial fish to 3 fish. Generally, several clinical symptoms were found in fish that were given a bacterial density above 107 CFU/ml, namely decreased appetite, passive movements tended to be more at the base of the maintenance medium, the release of some fish scales, then some fish experienced damage to the fins, and bleeding on the body. For the 107 CFU/ml test, the fish still looked normal in the first 24 hours, but after 24 hours began to show abnormal signs such as being more

passive, appetite began to decrease. Half of the 6 test fish died during the 96-hour test.

Observation of clinical symptoms was carried out after *A. salmonicida infection* and after soaking bitter melon extract (*M. charantia*). These observations include fish movement, fish response to food and physical damage arising from *A. salmonicida infection*. Some changes in fish behavior can be seen by the movement of fish that begin to passive, appetite decreases, and tends to gather closer to aeration. Meanwhile, physical or morphological damage that can be seen is that the gills of the fish appear pale, there are wounds on the fins and scales, and the fish produces more mucus. This is in accordance with (Singh et al., 2017) which states that damage to fish scales or skin can make it easier for pathogens to infect the gills. The clinical symptoms are in accordance with the research of (Kumar et al., 2017) which states that fish infected with *A. salmonicida* bacteria will experience decreased appetite, swim passively, exfoliate scales and gill organs will look pale.

Histopathological Analysis of Koi Fish Gills (C. carpio)

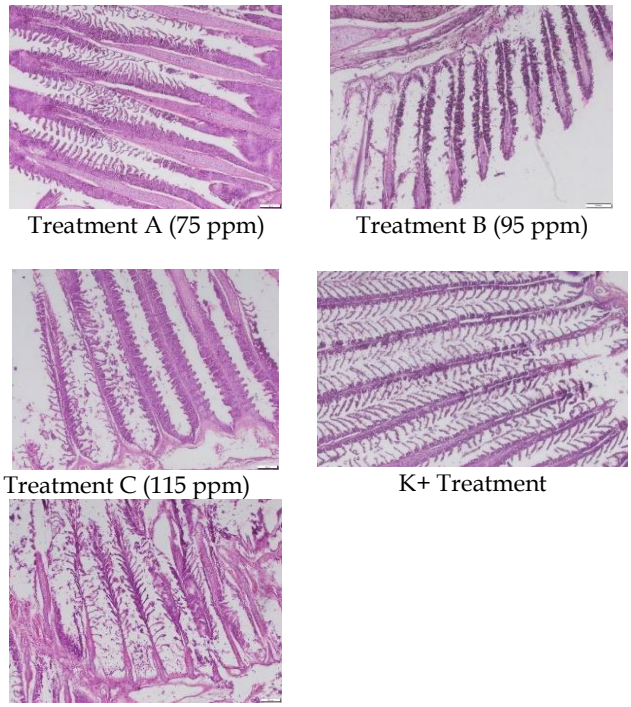

Figure 5. Histology Results of Koi Fish Gill (C. carpio)

Histopathological examination aims to provide information on changes due to infection of test animals by pathogens by detecting abnormal changes at the tissue level (Wardhani & Kusdarwati, 2017). One of the factors that causes histological damage is bacteria. Bacteria that cause histological damage generally attack fish in waters are from the Aeromonas group (Chung et al., 2006). Histopathological changes provide an overview of damage to the target organ, namely the gills. The difference in gill description between healthy fish and sick fish gills in koi fish (*C. carpio*) is presented in Figure 5.

Damage to hyperplasia, lamella fusion, edema and necrosis

The damage that occurs to the gills of koi fish (*C. carpio*) after infection with the bacterium *A. salmonicida* involves several significant histopathological changes, one of which is hyperplasia. Hyperplasia is a condition in which there is an increase in the number of cells, especially in the gill tissue. This increase in the number of cells leads to thickening of the gill tissue, which results in disruption of the gas exchange process. In infected fish, this is particularly detrimental because the ability of the gills to absorb oxygen and expel carbon dioxide decreases. This thick gill tissue will inhibit oxygen diffusion, causing the fish to have difficulty breathing. This condition can be observed from the behavior of fish that tend to be near water aeration sources more often.

In addition to hyperplasia, lamella fusion is also an important damage observed in the gills of infected fish. Lamella fusion occurs when a secondary lamella, a small structure in the gills that functions in gas exchange, undergoes union or fusion. This union reduces the surface area of the gills available for respiration, so the fish's ability to take oxygen from the water becomes very limited. This lamella fusion also causes fish to become more passive and less responsive to stimuli, as an inadequate oxygen supply affects the fish's metabolism and physiological activity.

Edema also often appears in the gills of fish infected with *A. salmonicida*. Edema refers to the excessive accumulation of fluid in the tissues of the body, in this case in the gill tissues. This condition causes swelling in the gills of fish and interferes with blood flow that should be running smoothly in the gill capillaries. As a result of this swelling, the ability of the gills to absorb oxygen from the water is reduced, which ultimately has an impact on the decline in the metabolic performance of the fish. This edema is often seen in conjunction with other clinical symptoms such as increased mucus production and decreased appetite, which indicates a disturbance in the fish's physiological system.

Necrosis is one of the most serious forms of damage to fish gills due to bacterial infections. Necrosis is the death of a cell or tissue that occurs due to severe damage, usually caused by an invasion of bacteria that irreversibly damages the structure and function of the cell. In fish gills, necrosis causes certain parts of the gills to undergo complete degeneration, so that they can no longer function in the respiratory process. Necrosis is often seen in cases of severe infections, where the gill

tissue changes color to blackish and looks damaged. This condition causes fish to experience a decline in overall physiological function and leads to death if not treated immediately (Kamar et al., 2021).

Histopathological damages such as hyperplasia, lamella fusion, edema, and necrosis are all indications of serious impairment of the fish's respiratory function due to *A. salmonicida* infection. The impact of this damage is significant, considering that the gills are vital organs for fish to breathe and maintain internal balance. The inability of the gills to function properly due to this damage causes the fish to become weak, susceptible to other diseases, and ultimately leads to death. Therefore, early treatment of these bacterial infections is essential to prevent more severe damage to gill tissue and improve fish survival rates.

Conclusion

The results revealed that the bitter melon extract fraction significantly reduced bacterial count and improved gill tissue integrity. Histopathological improvements were observed, including reduced hyperplasia, lamellar fusion, edema, and necrosis, indicating tissue recovery. This finding highlights the potential of bitter melon extract fraction as a natural antibacterial agent for controlling bacterial infections in fish, offering an alternative to chemical antibiotics. Further research is needed to determine the optimal dosage and to explore its antibacterial mechanism, ensuring its safe and effective application in aquaculture.

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Author Contributions
Conceptualization, Nurandi, N and Andayani, S.; methodology, Nurandi, N, Andayani, S and Kilawati.; software, Nurandi, N.; validation, Andayani, S and Kilawati.; formal analysis, Andayani, S.; investigation, Nurandi, N., Andayani, S and Kilawati; resources, Andayani, S and Kilawati.; data curation, Nurandi, N and Andayani, S.; writing—original draft preparation, Nurandi, N., Andayani, S and Kilawati; writing—review and editing, Nurandi, N., Andayani, S and Kilawati; visualization, Nurandi, N.; supervision, Andayani, S. and Kilawati; project administration, Andayani, S.; funding acquisition, Andayani, S.

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Conflicts of Interest
The author stated that there was no conflict of interest. The funder has no role in the design of the study, the collection, analysis, or interpretation of the data, the writing of the manuscript, nor the decision to publish the results of this research.

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