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Effects of Bitter Melon Fruit Fraction with Fourier Transform Infra-Red (FTIR) Spectroscopy on Histopathological Changes of Koi Fish (*Cyprinus carpio*) Liver Infected with *Aeromonas salmonicida*

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ABSTRACT

Bitter melon is widely recognized for its medicinal properties, including its potential health benefits in various species. The present study investigated the impact of the bitter melon fraction on liver histological changes of koi fish infected with *Aeromonas salmonicida (A. salmonicida)*. Maceration of bitter melon fruit was carried out for 4 days, then filtered and evaporated to obtain a thick extract. Phytochemical testing included column chromatography for the separation of active compounds and UV-Vis spectrophotometry for the analysis of the absorbance spectra of the fractions. The *A. salmonicida* bacterial infection was performed five days prior to the fish being soaked in a bitter melon fraction, at a bacterial density of 10^7 cells/ml for 48 hours. A completely randomized design was employed, featuring five treatments, including no bitter melon fraction with no infection (K -), other groups infected with *A. salmonicida* and supplemented with 75 ppm of bitter melon (A), 95 ppm of bitter melon (B), 115 ppm of bitter melon (C), and 5 ppm chloramphenicol (antibiotic, K +). Each treatment group consisted of 15 fish (average length and weight of Koi fish were 8 cm and 12 grams) with three replications, which were immersed in their respective solutions for 48 hours. The current study adopted the maceration method with 98% ethanol for the extraction of bitter melon fruit. To determine liver histological damage in Koi fish, the authors employed the scoring method. The findings indicated slight liver damage in histology parameters with an average score of 1.2-1.4 for treatments A and B, respectively; however, treatment C and the positive control exhibited moderate damage with an average necrosis score of 2. According to the obtained data, the bitter melon fruit fraction at a concentration of 95 ppm had the highest preventive effects on the liver of the Koi fish infected with *A. salmonicida*.

Keywords: Bitter melon fruit, Fraction, Koi fish, Liver

INTRODUCTION

The intensive carp production system provides numerous business opportunities, such as seeding, nursery operations, and the expansion of fish farming. In East Java, the aquaculture production value peaked in 2018 (KKP, 2021). However, intensive carp farming increases the risk of disease outbreaks (Kusrini, 2015). One of the primary pathogens causing disease in carp is *Aeromonas salmonicida (A. salmonicida*). The ability of this bacterium to infect fish is closely linked to its capacity to generate toxins. Semwal et al. (2023) classified *A. salmonicida* as a highly virulent pathogenic bacterium. Buller (2004) indicated that the virulence of *A. salmonicida* is influenced by its capacity to generate particular enzymes and toxins that aid in the invasion and infection of fish.

In the management of diseases, especially bacterial infections, a range of antibiotics has been employed, such as chloramphenicol, oxytetracycline, and erythromycin. A significant number of these antibiotics contribute to the development of resistance against new strains in the treatment of the disease (Sumampouw, 2018). Addressing the disease with natural ingredients is essential, emphasizing eco-friendly alternatives such as bitter melon fruit, instead of depending exclusively on saponins and rotenones. Bitter melon is rich in several secondary metabolites that exhibit antimicrobial properties, including alkaloids, flavonoids, tannins, terpenoids, and saponins (Andayani et al., 2023). Alkaloids and flavonoids in bitter melons are known to damage microbial cell membranes, while tannins and saponins inhibit the growth of microorganisms by disrupting their metabolic processes and cell walls. In addition, the phenolic compounds in bitter melon act as antioxidant agents that protect the body from bacterial and fungal infections. The antimicrobial properties of bitter melon allow it to serve as a natural component and a substitute treatment for fish (Hasanah, 2018). According to Ghosh et al. (2018), some alkaloids can increase immunity and vitality in fish. A study by Andayani et al. (2023) demonstrated the capability of bitter melon leukocytes to improve differentials, positioning it as a potential immunostimulant that can enhance the immune response in fish. Additionally, bitter melon fruit is rich in

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polyphenols, which are recognized for their antioxidant and anti-inflammatory effects. These substances can mitigate oxidative stress and bolster immune function by stimulating leukocyte activity. An increase in leukocyte differentials, including lymphocytes and neutrophils, would enhance the capacity to combat infections and facilitate disease recovery in fish. Consequently, bitter melon emerges as a promising natural immunostimulant for the enhancement of fish health and resilience (Andayani et al., 2023).

While bitter melon is known for its antimicrobial and anti-inflammatory properties, there is a lack of data about its impact on infected fish with *A. salmonicida*, specifically at the histological level. The present study investigated and contributed new insights into the potential of bitter melon as a natural treatment for bacterial infections in aquaculture, particularly in promoting liver health and mitigating damage caused by bacterial infection. Consequently, valuable information was obtained regarding the use of medicinal plants as alternatives for treating bacterial infections in fish, with a focus on their effects on vital organs such as the liver.

MATERIALS AND METHODS

Ethical approval

All experimental procedures involving animals were conducted in accordance with the ethical standards of the University of Brawijaya, Malang, East Java, Indonesia, and followed national and international guidelines for the care and use of laboratory animals. Every effort was made to minimize the number of animals used and to reduce their suffering during the experimental procedures.

Animals

In the present study, Koi fish were used as a sample, a domesticated variety of the common carp (*Cyprinus carpio*) sourced from a single broodstock at the Blitar Cultivation Center in Blitar City, Indonesia. To maintain the well-being of the fish, it is essential to ensure appropriate water quality, prevent overcrowding, observe for indications of illness, and conduct routine physical assessments. These assessments should include behavioral observation as well as thorough inspections of the skin, fins, gills, and eyes. The housing density of 15 fish per 15 liters, with an average initial length of about 8 cm and an average initial weight of around 12 grams, effectively mitigated stress and inhibited the transmission of infections. Disease prevention strategies, including regular water changes, proper filtration, and maintaining optimal water conditions, such as temperature, pH, and oxygen levels, were regularly controlled. The fish were acclimated over a two-week period, during which 10-20% of the water was replaced weekly and were fed with commercial feed three times daily, offered *ad libitum*.

Bitter melon fruit fractionation

Bitter melon fruit was fractioned in the Brawijaya University laboratory, Indonesia, using the maceration method, where the peeled fruit was blended into a mash and mixed with methanol PA in a 1:1 ratio. This fractionation was repeated three times with 500 ml of fruit and 500 ml of methanol each time, resulting in different fraction weights for yield calculation (Valencia, 2012).

Application of bitter melon fraction

The bitter melon fraction was tested through immersion (Laili, 2016). The immersion setup consisted of three different treatment groups, including treatment A (75 ppm), treatment B (95 ppm), and treatment C (115 ppm). Each container held 8 liters of water. Subsequently, 15 fish were immersed in each treatment container for 48 hours. The *A. salmonicida* bacteria were obtained from the laboratory of FPIK Universitas Brawijaya, Malang, Indonesia. Infection with *A. salmonicida* derived from pure culture was performed 5 days after immersion in the bitter melon fraction. A bacterial density of 10^7 cells/ml was used, then a pure culture was introduced into an appropriate medium, and incubated at a temperature range of 30-37°C for a duration of 24-48 hours. The density was confirmed with plate count or spectrophotometry.

Experimental design

After pre-research, the Lethal Dose 50 (LD50) and Lethal Concentration 50 (LC50) results were obtained. The assessment of toxicity to ascertain the dose or concentration of a substance that results in mortality for 50% of the exposed population of organisms is referred to as LD50 and LC50 (Rand and Petrocelli, 1985). The LD50 measures the dose of a substance given, while the LC50 measures the concentration of a substance in the air that is deadly to 50% of organisms. In the present study, the results of LD50 and LC50 were used to design the randomized experiments, and 3 replications were taken to ensure the accuracy and reliability of the results and to test the toxic effects of substances on

fish under controlled conditions. Fish were exposed to various concentrations of the substance, and their responses were observed under controlled conditions. This approach facilitated understanding the potential risks associated with chemical exposures to aquatic life. The experiment was carried out under a completely randomized design with three replications per treatment. It means that all experimental units were randomly assigned to different treatments without any blocking or grouping. Each treatment was applied to three separate units (replications) to ensure the reliability of the obtained results and to reduce the effect of random variation. The treatment (A) with 75 ppm bitter melon fraction with *A. salmonicida* infection, treatment (B) with 95 ppm bitter melon fraction with *A. salmonicida* infection, treatment (C) with 115 ppm bitter melon fraction with *A. salmonicida* infection, K(-); negative control, neither infected with *A. salmonicida* nor administered bitter melon fraction, serving as the baseline control group, and K(+); positive control group, infected with *A. salmonicida* and treated with 5 ppm chloramphenicol, served as a reference for antibiotic treatment efficacy (Laili, 2016).

Sampling and histopathological examination

The process of removing fish liver started with ethically euthanizing the fish. This was done by using a scalpel or another sharp instrument to quickly and effectively insert it into the brain area, just behind the eyes, ensuring instant death (Sneddon et al., 2014). After the fish was euthanized, it was placed on its back, and an incision was made in the abdominal cavity, extending from below the gills to the stomach using either a knife or scissors. The internal organs, including the liver, were then carefully separated using tweezers or small scissors. Three fish from each treatment A, B, C, K (+), and K (-) were sampled for liver tissue. Then, the samples were put into a microtube containing Davidson preservative solution. The histopathological preparations were made through fixation, dehydration, clearing, impregnation, embedding, staining, and mounting. Histological examination of the Koi fish liver was carried out after 5 days of administration of bitter melon extract. Histological observations were conducted to examine the liver tissue structure of Tilapia subjected to different stocking densities, followed by scoring the extent of tissue damage. According to Izzah (2019), each parameter was evaluated to determine the level of assessment of the network by measuring the extent of damage and calculating the percentage. The reading scoring histology of liver Koi fish starts from the left edge (according to the position of the tail of the preparation) towards the head, then goes down and shifts towards the tail again (zig-zag motion). Each field of view was observed for the level of tissue damage with the criteria of congestion and necrosis, then the percentage of scoring liver fish was assigned a score from 1 to 4 for microscopical evaluation. According to Maftuch et al. (2015), the scoring value for the percentage of damage histology of the liver of the Koi fish is presented in Table 1.

Table 1. Scoring value according to damage percentage in the liver of Koi fish

Scoring value	Damage percentage	Information
0	0	Not broken
1	1-25	Slight
2	26-50	Moderate
3	51-75	Lots
4	76-100	Huge

Remarks: "Huge" can be interpreted as more severe than "lots", because "huge" emphasizes the magnitude of the impact or scale, not just the amount.

Electron microscope examination method

Scanning electron microscopy (SEM) is a high-resolution imaging technique that utilizes a focused beam of high-energy electrons to scan the surface of a specimen, producing detailed topographic and compositional images. In this study, SEM was used to observe the liver tissue of Koi fish. The process began with sample collection and preparation, in which fish livers were aseptically isolated and fixed in a 2.5% glutaraldehyde solution in phosphate-buffered saline (PBS). After fixation, the samples were washed with PBS and subjected to a graded ethanol dehydration series ranging from 30% to 100%. The samples underwent critical point drying to prevent structural deformation. To enhance conductivity, samples were then coated with a thin layer of gold or platinum using a sputter coater. Imaging and analyzing were conducted under high vacuum conditions using the SEM. The monochromatic images obtained, as a result of the electron beams' non-visible wavelength, were examined to evaluate hepatocyte morphology, the integrity of the cell membrane, and indications of tissue stress or damage. Two primary types of images were produced by SEM, including surface topography and compositional maps. Both types were derived from electron-sample interactions that generate signals carrying information about surface structure and material properties. These signals were then detected and processed to form high-detail images for further examination (Masta, 2020).

Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) spectrophotometry is a spectroscopic technique that utilizes infrared light and incorporates a Fourier transform for spectrum analysis. The FTIR analysis in this case determined the functional groups that are characteristic of a compound and are found in a fraction (Smith, 2011). Testing of Koi fish liver samples using the FTIR method began with taking fish livers, which were then frozen and dried using a freeze-dryer to remove air content. The dried sample was crushed into a fine powder, directly analyzed, or mixed with Potassium Bromide and pressed into a transparent tablet. The sample was then placed on an FTIR spectrometer and scanned at a distance of 4000-400 cm^{-1} to detect functional groups based on the infrared spectrum produced. The spectrum peaks were described with standard references to identify the biochemical composition in Koi fish liver, such as proteins, lipids, and carbohydrates.

RESULTS AND DISCUSSION

Fourier transform infrared spectroscopy of bitter melon fruit

Figure 1 illustrates the wavelength absorption results obtained from the FTIR spectrophotometric analysis of the bitter melon fruit fraction (*Momordica charantia*), along with the absorption band data derived from the same analysis. As depicted in Figure 1, the FTIR spectrum displays several absorption peaks that signify the presence of functional groups within the sample. A prominent peak near 3000 cm^{-1} suggests the existence of -OH or -CH groups, whereas the absorption observed around 1700 cm^{-1} points to the presence of carbonyl (C=O) groups. The range of 1500-500 cm^{-1} serves as a distinctive molecular fingerprint region for each compound. The identification of functional groups within the bitter melon fruit fraction was conducted using FTIR. The spectral data acquired displayed a range of absorption bands that are associated with various chemical bonds and functional groups. A summary of this data is presented in Table 2. The results from the infrared spectrometry identification suggest that the compound under analysis possesses multiple functional groups, each of which contributes to an absorption band located in a specific region. The detection of a C-O band implies the potential presence of functional groups such as alcohol, ether, or carboxylic acid. Additionally, a peak absorption band is noted at a wavelength of 1180-1360 cm^{-1}, indicating the presence of a C-N functional group, specifically amine/amide. Furthermore, the presence of the C-H stretching vibration, which is characteristic of alkanes, is confirmed by the absorption peak observed in the range of 2850-2970 cm^{-1}. The wavelength of 1300-1370 cm^{-1} was the absorption of the NO_2 in the fungus group. The C=O group, which was an aldehyde/ketone / carboxylic acid/ester group, is located at the peak absorption wavelength of 1690-1760 cm^{-1} (Hercos et al., 2021). The C=O group at a wavelength of 1637 cm^{-1} indicated a phenol compound in the sample. The presence of hydroxyl groups (OH) and aromatic hydrocarbon groups in the sample indicated the presence of phenyl propanoic compounds (Sariningsih et al., 2015). A previous study by Coates (2000) announced that the presence of OH (hydroxyl), C=O, aromatic C=C, and aliphatic CH functional groups indicates the presence of flavonoid compounds. The detection of hydroxyl (OH) and aromatic hydrocarbon groups in the sample suggests the presence of phenylpropanoid compounds (Khowas, 2021). Accordingly, it can be found that the dominant content in the third fraction of bitter melon is phenol and its derivatives, namely phenylpropanoids.

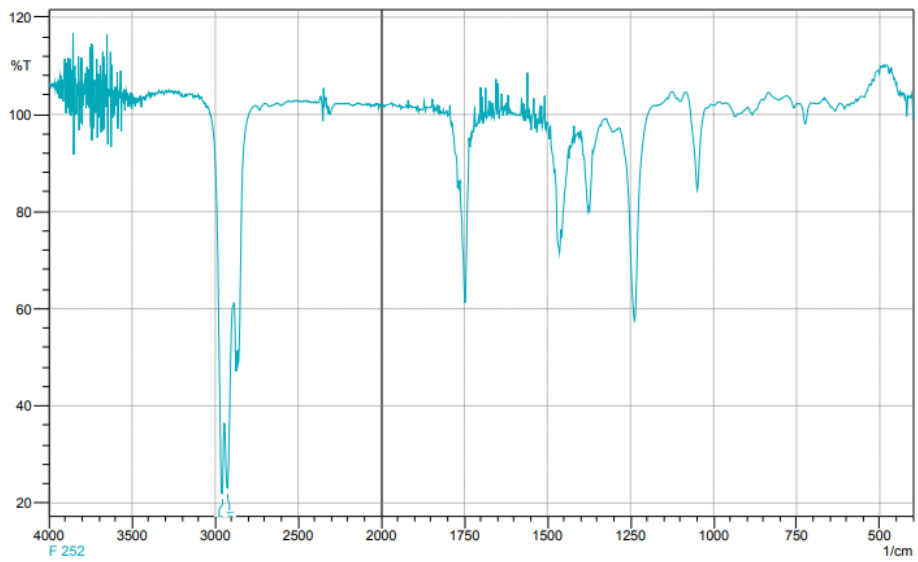

Figure 1. Fourier transform infrared spectroscopy spectral analysis results of bitter melon fruit

Table 2. The Fourier transform infrared spectroscopy spectral data for bitter melon fruit

No	Frequency of absorption band (cm^{-1})	Functional groups (bonds)	Group	Literature
1	1050 - 1300	C-O	Alcohols and Aliphatic Ethers	(Prata and da Costa, 2024)
2	1180 - 1360	C-N	Alkane	(Kristiningrum, 2016)
3	2850 - 2970	C-H	Alkane	(Kristiningrum, 2016)
4	1300 - 1370	NO_2	Aromatic Hydrocarbon	Saputra et al. (2016)
5	1690 - 1760	C=O	Carbonyl	Saputra et al. (2016)

Electron microscopy analysis of the liver

The SEM test provides detailed structural insights into the liver tissue of Koi fish. By analyzing the surface morphology at a microscopic level, SEM assists in identifying cellular integrity, tissue organization, and potential abnormalities (Goldstein et al., 2017). The SEM examination results are presented in Figure 2, highlighting key structural features of the Koi fish liver. The results of the SEM examination of the liver of the control group indicate a visible and complete part composition. These findings included the nucleus, sinusoids, and hepatocytes. The liver damage caused by the bacterial infection treatment showed organ necrosis and discoloration of the tissue, indicating congestion and bleeding.

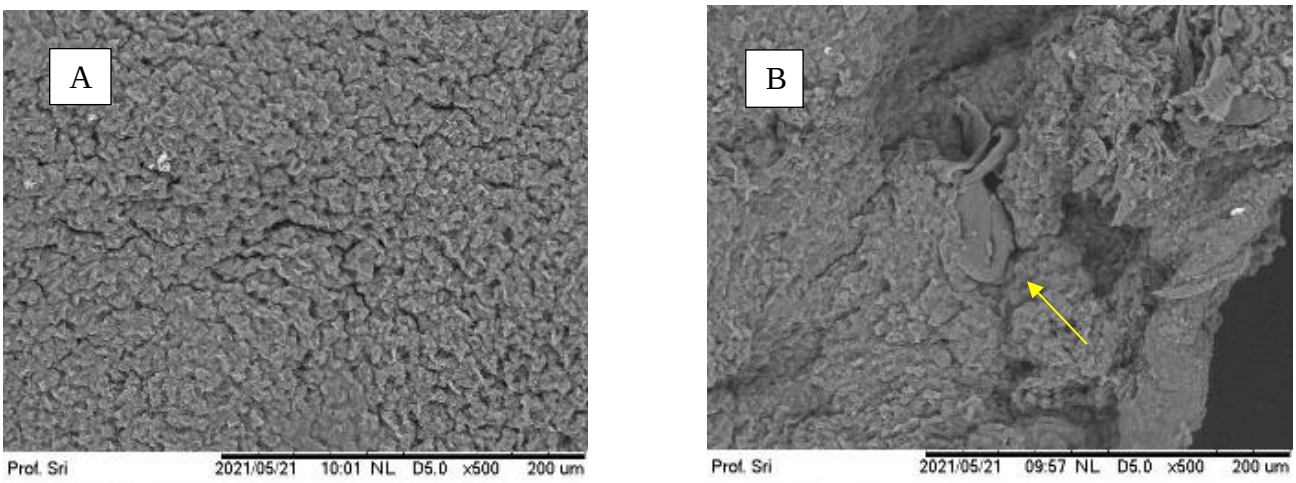

Figure 2. Scanning electron microscopy test of the Koi fish liver. Normal liver (**A**) and damaged liver (**B**, yellow arrow), Koi fish infected with *A. salmonicida* (**B**); 500x.

The results of the histopathological examination of the liver

The present study investigates the histopathological changes in the liver of Koi fish in response to experimental conditions. The results of the histological examination are demonstrated in Figure 3. The study results indicated that treating Koi fish with bitter melon fruit fraction significantly improves the negative impact on the liver of fish infected with *A. salmonicidae* bacteria (Figure 3). The fish appeared more active after being given the bitter melon fruit fraction. The Koi fish treated with bitter melon fruit exhibited minimal histological damage compared to those treated with antibiotics (without the bitter melon fruit fraction). The immune-stimulating effect of bitter melon fraction is linked to its natural components, such as polyphenols, alkaloids, and saponins, which enhance the body's resistance.

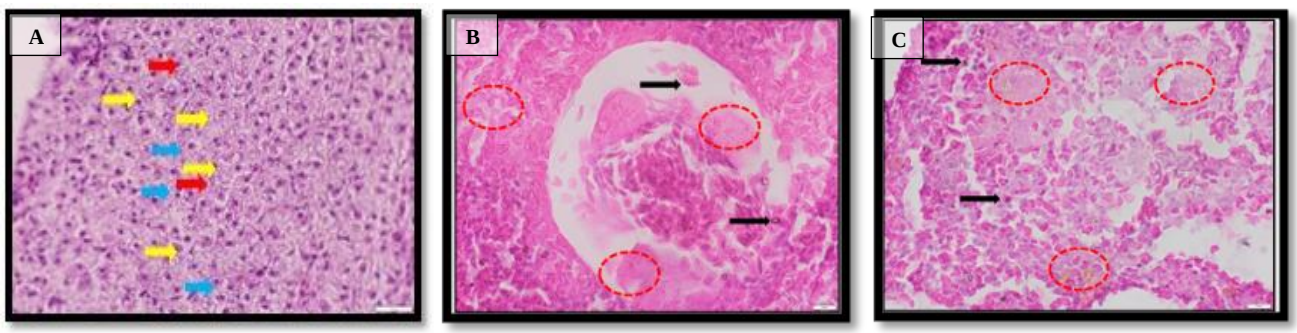

Figure 3. The histopathological examination of the Liver of Koi fish (400x). Normal liver (**A**), Livers with antibiotic treatment (**B**, without bitter melon fruit fraction treatment), Livers with bitter melon fruit fraction treatment (**C**). Yellow arrows: Core cells. Red arrows: Hepatocytes. Blue arrows: Sinusoids. Black arrows: Congestion. Dotted circles: Necrosis.

The immune-boosting effects of bitter melon fraction were considered by Nurandi et al. (2024), who indicated that Alkaloids contribute to detoxification and reduce toxicity in fish infected by bacteria. Saponins act as antibacterial and antiviral agents, contributing to improved immunity and overall health (Syihan, 2024). Moreover, Boshtam et al. (2016) and Maryani et al. (2018) highlighted that saponins boost the immune system by forming hydrogen bonds with cell membranes. This interaction leads to the production of complex compounds that compromise the integrity of bacterial cell membranes, resulting in bacterial cell death and enhanced immune function. Saponins enhance immune function by stimulating immune cell activity, increasing cytokine production, and acting as adjuvants in the immune response. In addition, saponins disrupt the integrity of bacterial cell membranes by forming hydrogen bonds, causing increased permeability and eventual lysis of bacterial cells, thus helping to effectively fight infection. Additionally, the polyphenols and phenolic derivatives found in the bitter melon fraction possess antioxidant properties. These polyphenols function as scavengers of free radicals and antioxidants, and they can also enhance the body's immune response by stimulating cytokine production, activating immune cells, and protecting cells from oxidative stress (Brandão et al., 2013; Sadowska et al., 2020).

In the control group, the liver lobule structure looks normal, characterized by the presence of clear cell nuclei, neatly arranged hepatocyte cells, and sinusoids that appear without any damage or pathological changes. The liver organ observed in fish treated with *A. salmonicidae* bacteria and bitter melon was found to have tissue damage in the form of congestion and necrosis. Congestion refers to the accumulation of excess blood within the small blood vessels or sinusoids of the liver, leading to impaired circulation and potential tissue damage. This condition occurs when blood flow is obstructed or slowed, causing dilation of the sinusoids and an increase in pressure within the hepatic vasculature. As a result, red blood cells may accumulate, leading to the formation of blood clots and reduced oxygen supply to liver tissues. Prolonged congestion can contribute to cellular stress, inflammation, and in severe cases, liver dysfunction (Sartijo et al., 2014). Additionally, necrosis is a condition characterized by the death of cells or tissues, which occurs after the loss of their normal function. It typically results from severe damage, often due to bacterial invasion, which irreparably harms the structure and function of the affected cells (Nurandi et al., 2024).

In the current study, necrosis was caused by infection with the bacteria *A. salmonicida*, which attacked the liver of Koi fish. Necrosis in liver cells is caused by the cytolysis or phagocytosis activity of lymphocytes or histocytes, which causes shrinkage/reduction in the size of the nucleus as a whole (sporadic, Bernet et al., 1999). Necrosis is often seen in cases of severe infections, where the liver tissue changes and looks damaged. This condition causes a decline in overall physiological function and death if it is not treated immediately. The results of scoring histological observations of the liver after treatment with bitter melon fruit fraction are presented in Tables 3 and 4.

Table 3. The score of histological observations of the liver after treatment with bitter melon fruit fraction in Koi fish for 48 hours (congestion)

Treatment	Congestion	Description
A	2	Moderate
B	1.6	Slightly damaged
C	1.8	Slightly damaged
K (+)	2	Moderate
K (-)	0.6	Not broken

Table 4. The score of histological observations of the liver after treatment with bitter melon fruit fraction in Koi fish for 48 hours (necrosis)

Treatment	Congestion	Description
A	1.4	Slightly damaged
B	1.2	Slightly damaged
C	1.8	Slightly damaged
K (+)	2	Moderate
K (-)	0.2	Not broken

The results of the histological scoring test of the liver of the Koi fish showed minor damage in group B. It is indicated that the organ remained largely intact with minimal structural alterations. The limited damage is attributed to the presence of polyphenols in bitter melon, which exhibit strong antibacterial and antioxidant properties. Polyphenols reduced oxidative stress by neutralizing free radicals, thereby preventing lipid peroxidation and cellular damage in liver tissues. Furthermore, these bioactive compounds played a crucial role in modulating the immune response, enhancing the fish's ability to combat bacterial infections, and promoting overall liver health (Andayani et al., 2023). By improving cellular defense mechanisms, polyphenols contribute to maintaining liver integrity and function, ensuring better resilience against environmental stressors and potential pathogens. Additionally, polyphenols helped modulate the immune response, enhancing the fish's ability to combat bacterial infections and maintain overall liver health.

Furthermore, these bioactive compounds played a crucial role in modulating the immune response, enhancing the fish's ability to combat bacterial infections and maintain overall liver health. By improving cellular defense mechanisms, polyphenols contributed to preserving liver integrity and function, promoting resilience against environmental stressors and potential pathogens (Andayani et al., 2023).

CONCLUSION

The bitter melon fruit fraction effectively reduced pathogenic bacterial infestation and improved liver tissue integrity in Koi fish. The optimal result was achieved at a dose of 95 ppm (Treatment B), which indicates minimal liver congestion, scoring 1.6. The present findings suggest the potential of bitter melon fruit as a natural antibacterial and hepatoprotective agent for sustainable aquaculture. Further studies in other fish breeds are needed to indicate the impact of bitter melon fruit fraction on other internal organs.

DECLARATIONS

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Availability of data and materials

All data produced in this study are relevant and have been incorporated into the published article. Additional details or inquiries will be available through reasonable requests from the corresponding author.

Ethical considerations

All authors have thoroughly examined the manuscript for any ethical concerns, including plagiarism, research misconduct, data fabrication or falsification, and redundant publication.

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Authors' contributions

Sri Andayani was responsible for data collection and analysis, as well as writing the original manuscript. Mohamad Fadjar, Yenni Risjani, Muhamad Sulaiman Dadiono, Vina Nur Nadiro, and Nadiah Nurandi contributed to data analysis, manuscript preparation, and revisions. All authors reviewed and approved the final version of the manuscript.

Competing interests

The authors did not declare any conflict of interest.

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