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ADMINISTRATION OF *Curcuma* spp. EXTRACT TO CONTROL *Aeromonas hydrophila* INFECTION IN STRIPED CATFISH (*Pangasianodon hypophthalmus*)

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ABSTRACT

Curcuma spp. is a phytobiotic with potential application in fish farming. This study aimed to evaluate the potential of Curcuma zedoaria, Curcuma aeruginosa and Curcuma mangga extracts in striped catfish infected with Aeromonas hydrophila. The study used a complete randomized design (CRD) consisting of six treatments: KN (negative control), KP (positive control, fish infected without treatment), PE (C. zedoaria extract 6.25 g kg^{-1}), HE (C. aeruginosa extract 6.25 g kg^{-1}), ME (C. mangga extract 6.25 g kg^{-1}) and KE (combination of C. zedoaria extract 2.1 g kg^{-1} + C. aeruginosa extract 2.1 g kg^{-1} + C. mangga extract 2.1 g kg^{-1}). A total of 360 striped catfish, 10 ± 0.5 cm were kept in 18 aquariums measuring 50 × 40 × 35 cm^3 and fed the treatment for 30 days. The challenge test was conducted on day 31 by injecting A. hydrophila suspension (10^6 colony-forming units (CFU) mL^{-1}) intramuscularly into fish. The results showed that the treatment administered were able to stimulate the expression of interleukin-1β, interferon-γ 2a, 2b genes, increase the number of red and white blood cells, hematocrit, hemoglobin, phagocytosis activity, respiratory burst, reduce the population of A. hydrophila in the intestine, and reduce tissue damage in striped catfish. Striped catfish treated with the extracts showed significantly higher survival rates ($p<0.05$) compared to the positive control group. The survival rates were: KN (100.00 %), KP (53.33%), PE (93.33 %), HE (91.67 %), ME (93.33 %), and KE (88.33 %). In conclusion, the treatment administered were able to enhance the immune response and resistance of striped catfish infected with A. hydrophila.

KEYWORDS: *Aeromonas hydrophila*; *Curcuma aeruginosa*; *Curcuma mangga*; *Curcuma zedoaria*, phytobiotic; striped catfish

INTRODUCTION

Striped catfish (*Pangasius hypophthalmus*) farming is an important sector that contribute significantly to Indonesia's national economy (KKP, 2024), but it often faces challenges in the form of Motile *Aeromonas* Septicemia (MAS) disease, which is caused by infection with *Aeromonas hydrophila* (Le *et al.,* 2018; Radkhah *et al.,* 2021; Radkhah & Eagderi, 2022; Erickson, 2023). MAS disease has high mortality rates, reaching up to 80%. Currently, MAS disease control generally uses antibiotics and synthetic chemicals, but the use of antibiotics in aquaculture is increasingly restricted owing to bacterial resistance and food safety concerns (Zdanowicz *et al.*, 2020). Therefore, safer and more effective alternatives are needed to replace antibiotics for the prevention of infectious diseases in catfish farming.

Bioactive compounds from plants are potential alternatives to antibiotics and other synthetic compounds for fish disease control (Van Hai, 2015; Awad & Awaad, 2017; Elumalai *et al*., 2020; Radkhah *et al*., 2021). Citarasu (2010) stated that bioactive compounds found in plants can function as antimicrobial, immunostimulatory, antistress, and anti-inflammatory agents. In addition, Harikrishnan *et al.* (2011) reported that bioactive compounds in plants, such as alkaloids, flavonoids, pigments, phenols, terpenoids, steroids, and essential oils can support overall fish health by increasing the immune response against pathogens. One plant with a variety of bioactive compounds is *Curcuma* sp.

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Curcuma sp. is a genus of herbal plants rich in bioactive compounds, such as curcuminoids, volatile oils, flavonoids, and alkaloids, which have been shown to have various pharmacological benefits, including antimicrobial, antioxidant, and immunostimulatory activities (Rajkumari & Sanatombi, 2018; Yuandani *et al*., 2021). Thus, *Curcuma* sp. has potential as phytobiotic. Phytobiotics are bioactive compounds derived from plants that can provide health and growth benefits to animals (Kikusato, 2021; Mary & Gudipati, 2023). As a phytobiotic, *Curcuma* sp. extract plays a role in stimulates the immune system of fish by increasing phagocytic activity, lysozyme production, and respiratory burst response (Citarasu, 2010; Harikrishnan *et al*., 2011; Khieokhajonkhet *et al.,* 2023). In addition, bioactive compounds in *Curcuma* sp. can inhibit the growth of pathogenic bacteria, thereby reducing the risk of infection (Baldissera *et al*., 2018; Riauwaty *et al*., 2021). The use of *Curcuma* sp. extract as a supplement in fish feed is a safer and environmentally friendly alternative to synthetic antibiotics (Mishra *et al*., 2018; Purbomartono *et al*., 2021).

Previous studies have shown that feeding 2.5% *C. longa* supplemented diets can improve the growth and immune response against *A. hydrophila* bacterial infection (Adeshina *et al*., 2017). Mahmoud *et al.* (2017) also reported that curcumin supplementation from *C. longa* in tilapia (*Oreochromis niloticus*) increased antioxidant capacity by increasing glutathione (GSH) and catalase (CAT) activity. Curcumin-supplemented tilapia had a lower population of *Aeromonas* spp. in the gastrointestinal tract and a relative survival rate of 100%. Riauwaty *et al.* (2021) stated that Catfish (*Clarias batrachus*) supplemented with turmeric showed fewer abnormalities during infection with *A. hydrophila*. In addition, Saefudin *et al.* (2022) demonstrated that *C. xanthorriza* can protect gold fish (*Cyprinus carpio*) larvae when challenged with *A. hydrophila*. Curcumin in *Curcuma* sp. can increase membrane permeability and cause intracellular leakage, although its role is not as effective as enrofloxacin (Zhang *et al*., 2022). Curcumin can also reduce the expression of serine protease and metalloprotease genes in *A. hydrophila* (SobhZahedi *et al*., 2023).

The genus *Curcuma* sp. has a variety of species spread across various regions of Indonesia, most of which are utilized as raw materials for herbal medicine for humans (Setiadi *et al*., 2017; Syafi'i *et al.,* 2018). However, the potential of *Curcuma* sp. to be utilized in fish farming, especially as a phytobiotic agent, has still not been widely explored. Research on the use of bioactive compounds in *C. zedoaria*, *C. aeruginosa*, and *C. mangga* to treat fish diseases is limited. Samad *et al.* (2022) reported that *C. zedoaria* extract at a dose of 0.5 g kg^{-1} had the ability to enhance immune response and had a positive effect on nonspecific immunity in tiger grouper (*Epinephelus fuscoguttatus*) against *Vibrio alginolyticus* and *V. parahaemolyticus* infections. Zhang *et al.* (2020) used curdione from *C. zedoaria* to control *Gyrodactilus kobayashii* infection in gold fish (*Carassius auratus*). To date, research on the potential of *C. zedoaria*, *C. aeruginosa*, and *C. mangga* extracts to overcome *A. hydrophila* infection in striped catfish has never been conducted. Therefore, this study aimed to evaluate the potential of *C. zedoaria*, *C. aeruginosa*, and *C. mangga* extracts in increasing the immune response and resistance of catfish to *A. hydrophila* infection.

MATERIALS AND METHODS

Preparation of *Curcuma* extract

Thie research was conducted from December 2022 until Mei 2023 at the Laboratory Aquatic Organism Health, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, IPB University. The rhizomes of *C. zedoaria*, *C. aeruginosa*, and *C. mangga* were obtained from the Research Center for Medicinal Plants and Aromatics (Balitro) Bogor, West Java. Rhizomes were washed, thinly sliced, and oven dried at 60 °C for 48 h. After drying, the sample was crushed until smooth and sieved. The resulting flour was extracted by the maceration method for 72 h using 70% ethanol solvent at a ratio of 1:5 (b/v). The maceration products were then filtered using Whatman qualitative filter paper, Grade 1 and concentrated using a rotary evaporator (Tanvir *et al*., 2017).

Preparation of *A. hydrophila* bacteria

The *A. hydrophila* isolate was resistant to 50 µg mL^{-1} rifampicin antibiotic as a marker (0.25 g rifampicin, 9.5 mL ethanol absolute, 0.5 mL distilled water) on triptic soy agar (TSA) media. *A. hydrophila* stock was cultured on TSA media that had been given rifampicin for 24 hours at 28 °C. Furthermore, the growing isolate was taken as much as 1 ose transferred to 25 mL of Triptic soy broth (TSB) media and incubated on a waterbath shaker for 24 hours. A total of 1 mL of the suspension was transferred to a 1.5 mL Eppendorf tube and centrifuged (5000 rpm, 5 min, 4° C). The supernatant was discarded and the bacterial precipitate formed was resuspended in phosphate buffered saline solution (PBS, 8 g NaCl, 1.5 g Na_2HPO_4, 0.2 g KCl, 0.2 g KH_2PO_4, 100 mL distilled water) at a density of 10^8 cfu mL^{-1} (Munaeni *et al*., 2020). Further dilution was performed to obtain a density of 10^6 cfu mL^{-1} (the density found in the LD_{50} test) for the chal-

lenge test.

Maintenance of striped catfish

A total of 360 striped catfish, 10 ± 0.5 cm in length and 14 ± 0.75 g in weight were reared in 18 aquariums with dimension of 50 × 40 × 35 cm^3 and a water volume of 44 L. Each aquarium was filled with 20 fish. Fish were reared for 38 days. The treatment feed was prepared using the repelleting method. Commercial feed (32% protein, 5% fat, 6% crude fiber, 12% ash, 12% moisture content) was crushed into flour and then mixed with *C. zedoaria*, *C. aeruginosa* and *C. mangga* extracts according to the treatment and cellulose filler. It was then molded and oven dried at 60 °C. The treatment feed was provided for 30 days. On day 31, the fish were fed commercial feed without the addition of extracts. The fish were fed morning and evening at satiation. On day 31, a challenge test was conducted using *A. hydrophila* Rf^R (10^6 cfu mL^{-1}). A suspension of *A. hydrophila* Rf^R was injected intramuscularly 0.1 mL. Water quality during rearing were temperature 27.3 – 29.7 °C measured with thermometer, dissolved oxygen 4.8 – 6.1 mg L^{-1} measured with a DO meter, pH 6.4 – 7.0 measured with pH meter, and total ammonia nitrogen (TAN) 0.15 – 0.25 mg L^{-1} was read using a spectrophotometer (Thermo Scientific Genesys 10S) at a wavelength of 630 nm.

Research design and sampling schedule

The study used a completely randomized design (CRD) with six treatments and each treatment was repeated three times. The concentration used is based on previous research on the ability of *C. zedoaria*, *C. aeruginosa*, and *C. mangga* extracts to inhibit the population of *A. hydrophila* in vitro (Sasanti *et al.*, 2025). The treatments included the addition of extracts with treatment codes, as shown in Table 1.

Samples for gene expression observation were taken on day 32 of rearing with the kidney as the target organ. Blood samples and bacterial population observations were obtained on days 32, 34, 36 and 38 of rearing. Bacterial population samples were obtained from the intestine, kidneys, and liver. Samples for histopathological observation were taken on days 32 and 38 of rearing, with the kidney and liver as target organs. The survival value of the striped catfish was calculated on day 38 of rearing.

Non-specific immune response

Non-specific immune response parameters observed included total red blood cells, total white blood cells, hematocrit, hemoglobin, phagocytic activity and respiratory burst. Blood samples were taken from the vein near the base of the tail using a 1 mL syringe that had been rinsed with 10% ethylenediaminetetraacetic acid (EDTA) anticoagulant. Red and white blood cells were counted using a Neubaur' hemocytometer. The blood sample was aspirated with a pipette to a scale of 0.5, hayem solution was added to a scale of 101 for red blood cell counts and Turk's solution was added to a scale of 11 for white blood cell counts. The pipette was shaken for 5 min. The first drop in the pipette was discarded and the next drop was dropped using a hemocytometer (Blaxhall & Daisley, 1973). Total red and white blood cells were observed and counted under a microscope at 400 × magnification.

Hematocrit (%) was measured by placing the blood samples into microhematocrit tubes up to ¼ volume and then centrifuging at 8000 rpm for 5 min. Hematocrit values were determined by comparing the volume of solids and the blood volume using a microhematocrit reader (Anderson & Siwicki, 1995). Hemoglobin measurement refers to Sahli's method with a haemometer and is expressed in % on a yellow scale (Wedemeyer & Yasutake, 1977). Briefly, 0.1 N HCl solution was put into the haemometer tube until scale 10 (red scale) and blood was put up to scale 5

Table 1. Treatment of the addition of *Curcuma* extract to striped catfish feed

Code	Description
KN	No extract and no pathogen injection
KP	No extract and pathogen injection
PE	*C. zedoaria* extract 6.25 g kg^{-1} feed and pathogen injected
HE	*C. aeruginosa* extract 6.25 g kg^{-1} feed and pathogen injected
ME	*C. mangga* extract 6.25 g kg^{-1} feed and pathogen injected
KE	*C. zedoaria* extract 2.1 g kg^{-1} + *C. aeruginosa* extract 2.1 g kg^{-1} + *C. mangga* extract 2.1 g kg^{-1} feed and pathogen injected

and allowed to stand for 3 min. Distilled water was added until the color of the solution in the tube was the same as that on both sides of the tube. The yellow scale was used to determine the hemoglobin levels.

Phagocytosis activity was measured based on the percentage of phagocytic cells that showed the phagocytosis process (Hampton *et al.,* 2020). Blood samples as much as 50 µL were dripped into 96 microplate wells and then 50 µL of *Staphylococcus aureus* suspension (10^7 CFU mL^{-1}) was added. The suspension was incubated for 1 h at 30 °C. A total of 5 µL of the suspension was used for review preparation. The preparations were dried, fixed in 100% methanol for 5 min, and aerated. The preparations were then immersed in the Giemsa dye for 20 min, rinsed with distilled water, and aerated. Observations were made using a microscope at 400 × magnification.

Respiratory burst activity was determined based on nitroblue tetrazolium (NBT) reduction to determine O_2^- production (Wu *et al.*, 2016). The blood samples were taken as much as 50 µL and put into a microplate and incubated for 1 hour at room temperature. The blood was then removed, and the microplate containing the blood sample was rinsed with 100 µL PBS and repeated 3 times. A total of 50 µL of nitro-blue tetrazolium (NBT solution 0.2%) was added to the microplate and incubated for 1 h at room temperature. The NBT was removed and the microplate was fixed with 50 µL of 100% methanol for 3 min. After fixation, the microplate was rinsed with 30% methanol and aerated then 60 µL KOH and 70 µL dimethyl sulfoxide (DMSO) were added. Respiratory burst value was measured using a microplate reader at a wavelength of 630 nm.

Gene expression

The immune-related gene expression observed consisted of interleukin-1β (IL-1β), interferon γ-2a (IFγ-2a) and 2b (IFγ-2b). Quantitative real-time reverse transcription (RT)-polymerase chain reaction (qRT-PCR) was used. Total RNA from kidney samples was extracted and purified using GENEzol™ reagent (Geneaid, Taiwan) according to the manufacturer's instructions. The purity of the total RNA was analyzed spectrophotometrically (λ = 260 nm and 280 nm). First-strand complementary DNA (cDNA) was synthesized from total RNA using the ReverTraAce® q PCR RT Master Mix with a gDNA remover Kit (Toyobo Co., Ltd., Japan) according to the manufacturer's instructions. The specific primer pairs used based on Nhu *et al.* (2019) (Table 2). qPCR analysis was performed using a Rotorgene 6000 machine (Corbett, USA) with the KAPA SYBR® FAST Qpcr (KAPA, USA).

Amplification was performed in a reaction volume of 20 µL (10 µL KAPA SYBR® FAST qPCR Kit (KAPA, USA) Green Master Mix; 1.6 µL specific forward and reverse primers (0.8 µL each); 4 µL template cDNA; and 4.4 µL nuclease free water (NFW). NFW was used as the negative control. The qPCR program consisted of pre-denaturation at 95 °C for 2 min, followed by 40 cycles at 95 °C for 15 s (denaturation), 60 °C for 15 s (annealing) and acquiring to cycling, 72 °C for 20 s (elongation) and a melting curve in the temperature range of 72 - 95 °C for 15 s. Gene expression was quantified using the relative quantification method with a reference gene. Gene expression was quantified using the relative quantitation method with a reference gene (Livak & Schmittgen, 2001).

Table 2. Primers used for *P. hypophthalmus* immune gene expression

No	Gene name	Primer Sequence (5 '-3')	Length (bp)	Accession number	Ref. Seq_Species
1	Interlekuin-1β	F: CAGAGGCTGAAGCACACTCA R: CCTTGTCCTGCCTGCTGTAA	148	100304696	*Ictalurus punctatus*
2	Interferon γ 2a	F: TATGTCACTGAGCTGCTGGC R: TTAGCTTGACGTCGTCTCCG	143	N185453	*Pangasianodon hypophthalmus*
3	Interferon γ 2b	F: TCCCAACCCTGCCAAATTGT R: GCCTCATTCTCCATCCAGGT	150	JN18545	*Pangasianodon hypophthalmus*
4	Reference gene 16S rRNA	F: TATCTTCGGTTGGGGCG R: CCTGATCCAACATCGAGG	223	FJ432682	*Pangasianodon hypophthalmus*

Calculation of bacterial population

The calculated bacterial population included total bacteria, *Aeromonas* sp., and total *A. hydrophila* Rf^R. The spread plate method was used in this study. The method used is adapted from (Munaeni *et al*., 2020). The target organ for the calculation of total bacteria and *Aeromonas* sp., was the intestine. The target organ for the calculation of total *A. hydrophila* Rf^R consist of the intestine, kidney and liver. Target organs (0.1 g) were collected aseptically and suspended in 0.9 mL of sterile PBS solution for serial dilution. A 50 µL suspension was inoculated on TSA medium for total bacteria count, Rhimler Shorts medium (R-S medium) agar for total *Aeromonas* sp., and R-S medium agar with 50 µg mL^{-1} rifampicin added to *A. hydrophila* Rf^R. The cells were incubated for 24 h at 30 °C.

Histopathology

Kidney and liver samples were collected from each treatment and then fixed with 10% Neutral-Buffered-Formalin (NBF) solution for 24 h. Subsequently, rough trimming of ± 2 mm thickness was performed on the organs and followed by dehydration using 70%, 80%, 90% and 95% absolute alcohol for 2 h. After dehydration, the clearing process was carried out using xylol for 2 h. The next step involves embedding and sectioning. The sectioning process uses a 5 µm thick microtome. After cutting, the preparation was attached to a glass object and the mounting process was continued. The preparations were stained with hematoxylin-eosin (Bancroft & Layton, 2019). The finished preparations were observed under a microscope at 400 × magnification (Munaeni *et al*., 2020).

Survival rate

Survival rate was calculated based on the formula used by (Zhang *et al*., 2020). The survival rate was calculated as follows: survival rate (%) = (number of fish survived / initial number of fish) × 100.

Data analysis

Data analysis utilized Microsoft Excel 2019 software to tabulate the data. The data obtained were tested for homogeneity and normality using the Kolomogorov-Smirnov and Shapiro-Wilk tests. Data were analyzed by analysis of variance (ANOVA) using SPSS version 25. If the results were found to be significantly different, Duncan's test was performed at 95% confidence interval. The histopathological parameters were analyzed descriptively.

RESULTS AND DISCUSSIONS

Gene expression

IFγ-2a gene expression at the 24th hour after the challenge test showed significantly different values in all extract treatments when compared to the positive control ($P<0.05$). IFγ-2a gene expression levels in the PE, HE, and ME treatments showed values that were not significantly different ($P>0.05$), with observed expression levels of 0.241 ± 0.03 fold, 0.295 ± 0.02 fold, and 0.274 ± 0.03 fold, respectively. The KE treatment had the lowest expression level with an observed value of 0.109 ± 0.01 fold (Figure 1).

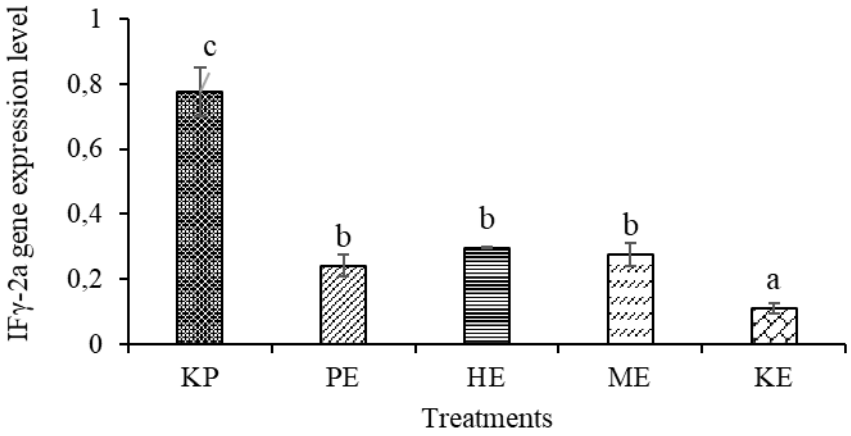

Figure 1. The IFγ-2a gene expression levels in striped catfish kidney organs at 24 h after challenge with *A. hydrophila*. Different superscript letters in each bar (mean ± standard deviation) indicate statistically significant differences ($P < 0.05$). KP (Positive control), PE (*C. zedoaria* extract), HE (*C. aeruginosa* extract), ME (*C. mangga* extract), KE (Combination of the three extracts).

IFγ-2b gene expression at 24 h post-challenge test showed significantly different values in all extract treatments when compared with the positive control ($P < 0.05$). PE and KE treatments showed values that were not significantly different ($P > 0.05$) from the observed gene expression of 0.188 ± 0.04 fold and 0.160 ± 0.033 fold, respectively. The level of IFγ-2b gene expression observed in the HE treatment was 0.204 ± 0.06 fold and IFγ-2b gene expression observed in ME treatment was 0.524 ± 0.043 fold (Figure 2).

IL-1β gene expression 24 h after the challenge test showed that the level of IL-1β gene expression in the PE treatment was not significantly different from that in the ME treatment with values of 0.435 ± 0.10 fold and, 0.616 ± 0.08 fold, respectively. The HE and KE treatments had IL-1β gene expression levels that were not significantly different from the observed values of 0.204 ± 0.06 fold and 0.160 ± 0.11 fold, respectively. All extract treatments showed significant differences from the positive control (0.819 ± 0.18 fold) (Figure 3).

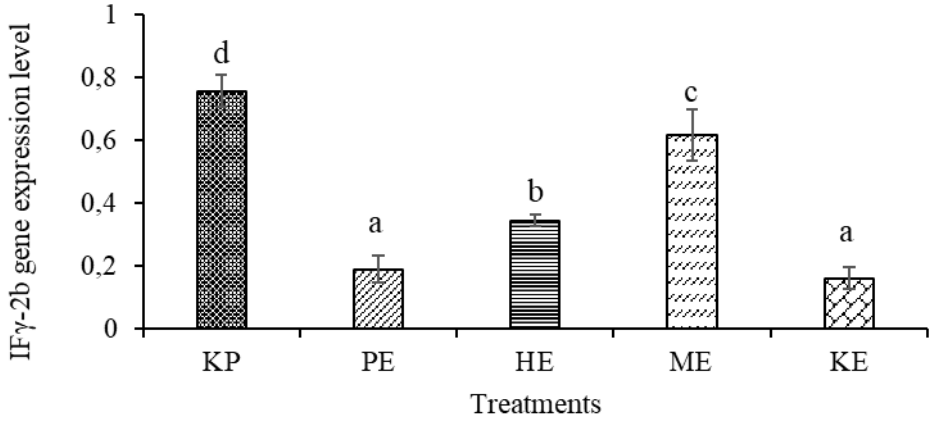

Figure 2. IFγ-2b gene expression levels in striped catfish kidney organs at 24 h after challenge with *A. hydrophila*. Different superscript letters in each bar (mean ± standard deviation) indicate statistically significant differences ($P < 0.05$). KP (Positive control), PE (*C. zedoaria* extract), HE (*C. aeruginosa* extract), ME (*C. mangga* extract), KE (Combination of the three extracts).

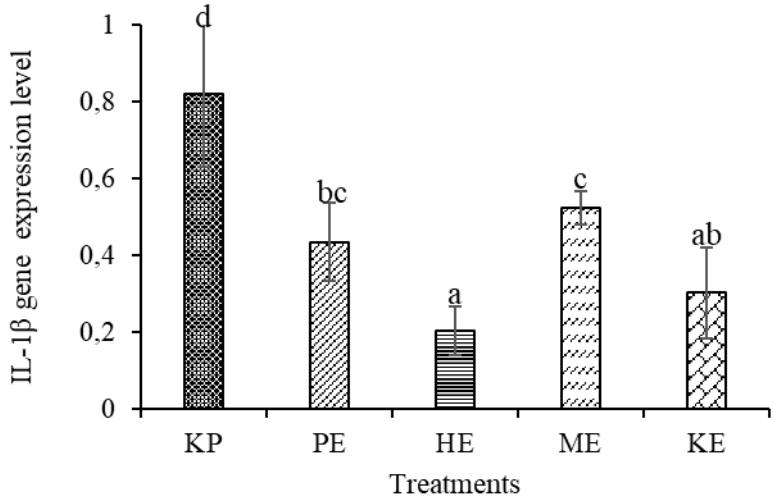

Figure 3. IL-1β gene expression levels in striped catfish kidney organs at 24 h after challenge with *A. hydrophila*. Different superscript letters in each bar (mean ± standard deviation) indicate statistically significant differences ($P < 0.05$). KP (Positive control), PE (*C. zedoaria* extract), HE (*C. aeruginosa* extract), ME (*C. mangga* extract), KE (Combination of the three extracts).

Medicinal plants can stimulate cellular and humoral immune responses in fish (Elumalai *et al.*, 2020), as well as the expression of immunity-related genes (Nhu *et al.*, 2019) and increase the resistance of fish to pathogens (Mahmoud *et al.,* 2017). According to An *et al.* (2019), digestive enzyme commonly found in the digestive tract of the host are generally unable to directly break down polysaccharides and other components found in herbal plants. Polysaccharides in plants are fermented by bacteria in the host's digestive tract. The result of this fermentation is short-chain fatty acids (SCFAs). Based on Hasan *et al.* (2019), The SCFAs formed also bind to specific reeptors, namely G-proteins (GPR 41/43), which regulate the host's immune response. SCFAs can diffuse through intestinal epithelial cells and interact with dendritic cells, stimulating T cells to activate anti-inflammatory cytokines. This study demonstrated that administration of *C. zedoaria*, *C. aeruginosa*, and *C. mangga* extracts can stimulate the expression of IL-1β genes, which are associated with inflammatory responses. The IL-1β, IFγ-2a, and IFγ-2b genes observed in this study play important roles in the body's immune defense mechanism. The IL-1β is a major proinflammatory cytokine that mediates the innate immune response by stimulating inflammation and activating immune cells, such as neutrophils and macrophages, and plays a role in the body's protective mechanisms. On the other hand, IFγ-2a, and IFγ-2b are antiviral cytokines that increase macrophage activity, activate T and NK (natural killer) cells, and strengthen recognition of infected cells through increased expression of MHC class I and II. Both types of interferons play a crucial role in adaptive immune responses, especially in eliminating virus-infected cells (Critchlow *et al.*, 2024). *C. zedoaria*, *C. aeruginosa*, and *C. mangga* extracts has been shown to stimulate the gene expression of IL-1β, IFγ-2a, and IFγ-2b. This finding is in line with the results of research by Nhu *et al.* (2019), who stated that bioactive compounds from plants can increase the gene expression of proinflammatory cytokines, such as interleukin-1ß, as well as antiviral cytokines, including interferon-ã 2a and 2b. Although in this study the expression of immune genes decreased 24 h post-infection (Figures 1, 2 and 3), this could be explained by the initial immune response to the pathogen that peaked before this period and then decreased. This finding is consistent with those of previous studies, which showed that the expression of immune system-related genes peaked in the early phase of infection and, then decreased significantly after 24 h (Zhou *et al.*, 2024).

Immune response

Before the challenge test, striped catfish that received feed with *C. zedoaria*, *C. aeruginosa* and *C. mangga* extracts, had higher values of total erythrocytes, hemoglobin, hematocrit, total leukocytes, respiratory burst and phagocytosis activity and were significantly different from the control group ($P < 0.05$). After the challenge test, on the 32nd day there were decreases in total erythrocytes, hemoglobin, and hematocrit. Treatment with the extract resulted in higher values and was significantly different from the positive control. After post-challenge test, day 34, 36 and 38 showed an increase in total erythrocytes, hemoglobin and hematocrit from day 32 post-challenge test. The parameters of total leukocytes, respiratory burst and phagocytic activity of striped catfish increased after the challenge test on day 32. All treatments included the addition of *C. zedoaria*, *C. aeruginosa* and *C. mangga* yielded better results and was significantly different from that of the positive control ($P < 0.05$). A decrease in total leukocytes, respiratory burst and phagocytosis activity occurred after the challenge test on days 34, 36 and 38. All treatments included the addition of *C. zedoaria*, *C. aeruginosa* and *C. mangga* extract yielded better results than did the positive control ($P < 0.05$) (Figure 4).

In the host's digestive tract, bioactive compounds derived from herbal plants interact with the microbiota present in the host's digestive tract to produce SCFAs (An *et al.*, 2019). SCFAs are capable of activating T cells. Activated T cells can increase the host's immune tolerance. This immunomodulatory effect supports the balance between the innate and adaptive immune systems, thereby increasing the fish's resistance to pathogens (Hasan *et al.*, 2019). In this study, *C. zedoaria*, *C. aeruginosa*, and *C. mangga* extracts were found to enhance innate immune response by increasing phagocytic activity, respiratory burst, total white blood cell and total red blood cell.

In addition, the immune response measured through the parameters of total erythrocytes, hemoglobin, hematocrit, total leukocytes, respiratory burst, and phagocytosis activity showed better results in the treatment with *Curcuma* spp. extract than in the control. This finding is in line with the research of Kondera *et al.* (2021) who stated that bioactive compounds and biological properties of phytobiotics can enhance immune responses through certain mechanisms, which ultimately strengthen the immune response of fish. After *A. hydrophila* RF^{R} infection, there was a significant reduction in total erythrocytes, hemoglobin, and hematocrit on day 32. However, these parameters increased again by day

36 following the challenge test. This reduction is likely due to the hemolytic properties of *A. hydrophila* RF^R, which can damage erythrocytes and result in significant alterations to the hematological profile of fish (Citterio & Biavasco, 2015; Sani *et al.,* 2023). Despite the reduction in total erythrocytes, hemoglobin, and hematocrit, striped catfish treated with extracts of *C. zedoaria*, *C. aeruginosa*, and *C. mangga* exhibited higher values than those in the positive control group (Figure 4). These results indicate that the extracts of the three types of *Curcuma* spp. provide protection to striped catfish during infection by *A. hydrophila* RF^R. This protective effect is associated with the ability of bioactive compounds in the extracts to reduce erythrocyte damage caused by the hemolytic properties of the pathogen.

Total leukocyte count, phagocytic activity and respiratory burst increased on day 32 post-challenge, indicating an acute immune response to infection by *A. hydrophila* RF^R. Leukocytes, the primary immune cells, increased to combat the bacterial infection through phagocytosis, a process in which these cells engulf and destroy pathogens. At the same time, the increase in the respiratory burst, a process in which phagocytic cells produce reactive oxygen species to kill microorganisms, is more active, indicating that the innate immune response is active at this stage (Ellis, 2001). The reduction that occurred on days 36 and 38 post-challenge indicated that the recovery phase after the infection began to be controlled. During this phase, leukocyte counts and phagocytic activity begin to decline as the number of pathogens in the fish decreases (Korni *et al*., 2017). This process indicates that the host immune system is responding effectively to the infection, and that the acute inflammatory phase is transitioning to the resolution phase (Abdelhamid *et al*., 2021; Syawal *et al*., 2021). The observed decrease may also be attributed to the effectiveness of the *Curcuma* spp. extract in modulating the immune response, preventing its progression to chronic inflammation, which can be detrimental.

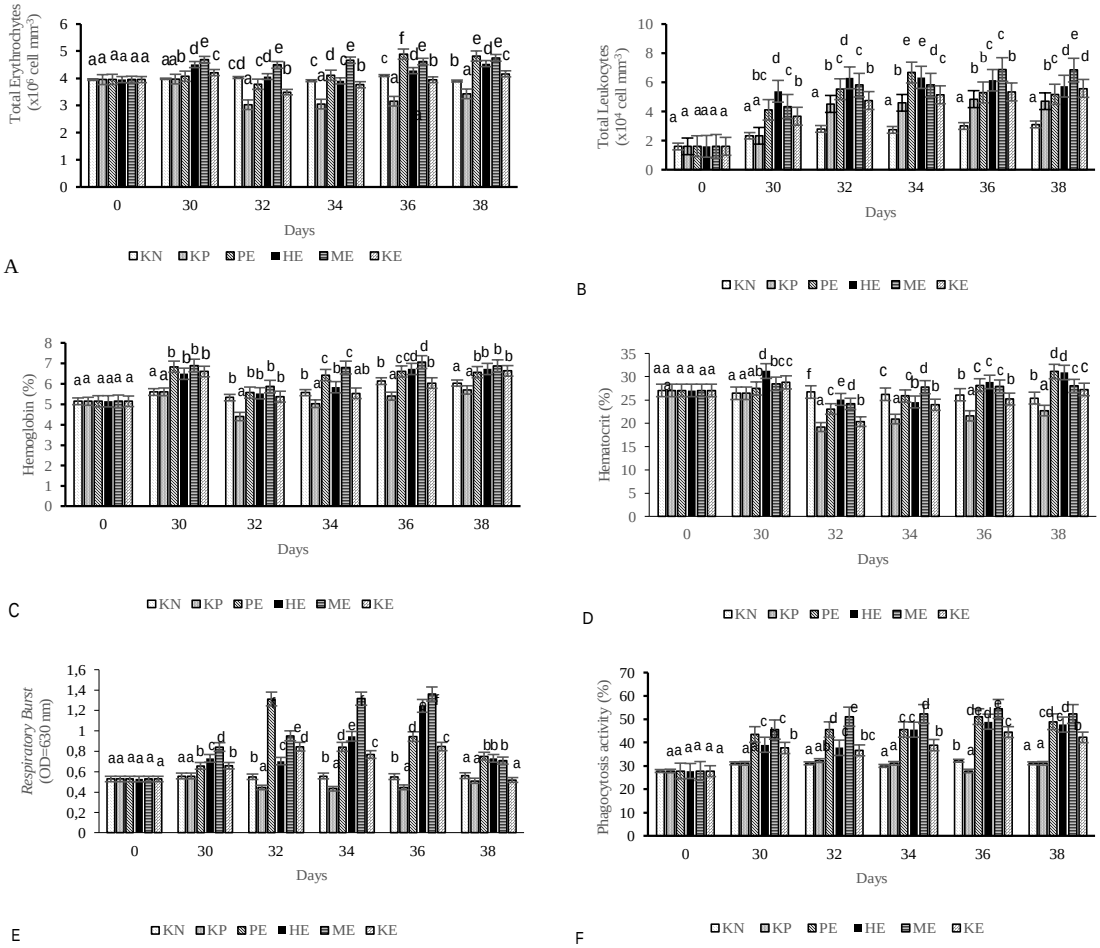

Figure 4. Total erythrocytes (A), total leukocytes (B), hemoglobin (C), hematocrit (D), respiratory burst (E), phagocytic activity (F) of striped catfish before and after challenge with *A. hydrophila* Rf^R. Different superscript letters in each bar (mean value ± standard deviation) indicate statistically significant differences ($P < 0.05$). KP (Positive control), PE (*C. zedoaria* extract), HE (*C. aeruginosa* extract), ME (*C. mangga* extract), KE (Combination of the three extracts).

Bacterial population

Total bacteria in the gut of catfish treated with extracts of *C. zedoaria*, *C. aeruginosa*, and *C. mangga* were higher than those in the positive control ($P < 0.05$). The total bacterial count of *A. hydrophila* Rf^R after the challenge test on days 32, 34, 36 and 38 showed that all extract treatments had lower values than the positive control treatment ($P < 0.05$). Further observations were made by calculating the population of *A. hydrophila* Rf^R in the target organs (the liver and kidney) after the challenge test. The results showed that in each target organ, the population of *A. hydrophila* Rf^R in all extract treatments was lower than that in the positive control (Figure 5).

The administration of *C. zedoaria*, *C. aeruginosa* and *C. mangga* extracts in this study, suppressed the population of *Aeromonas* sp. in the intestinal organs, and *A. hydrophila* Rf^R in the kidney and liver of striped catfish after the challenge test (Figure 5). Another study also showed that the use of herbal ingredients can reduce the population of *A. hydrophila* in the intestines of fish after a challenge test (Semwal *et al.*, 2023).

The reduction in the population of *Aeromonas* sp. in the intestine and *A. hydrophila* RF^R in the kidney and liver of striped catfish following administration of *C. zedoaria*, *C. aeruginosa*, and *C. mangga* extracts is attributed to the bioactive compounds present in these plant extracts, such as curcuminoids, flavonoids, and essential oils, which possess antibacterial properties. These compounds inhibit bacterial growth through mechanisms such as damaging the bacterial cell wall, disrupting membrane function, and inhibiting the synthesis of proteins or enzymes essential for bacterial survival (Semwal *et al.,* 2023). In addition, the immunostimulatory properties of *Curcuma* spp. extract can also enhance the immune response of fish, helping to suppress the population of pathogenic bacteria such as *A. hydrophila*. The increased activity of immune cells, such as macrophages and neutrophils, improves pathogen elimination, thereby reducing infection rates in important organs such as the kidneys and liver (Zhou *et al*., 2024).

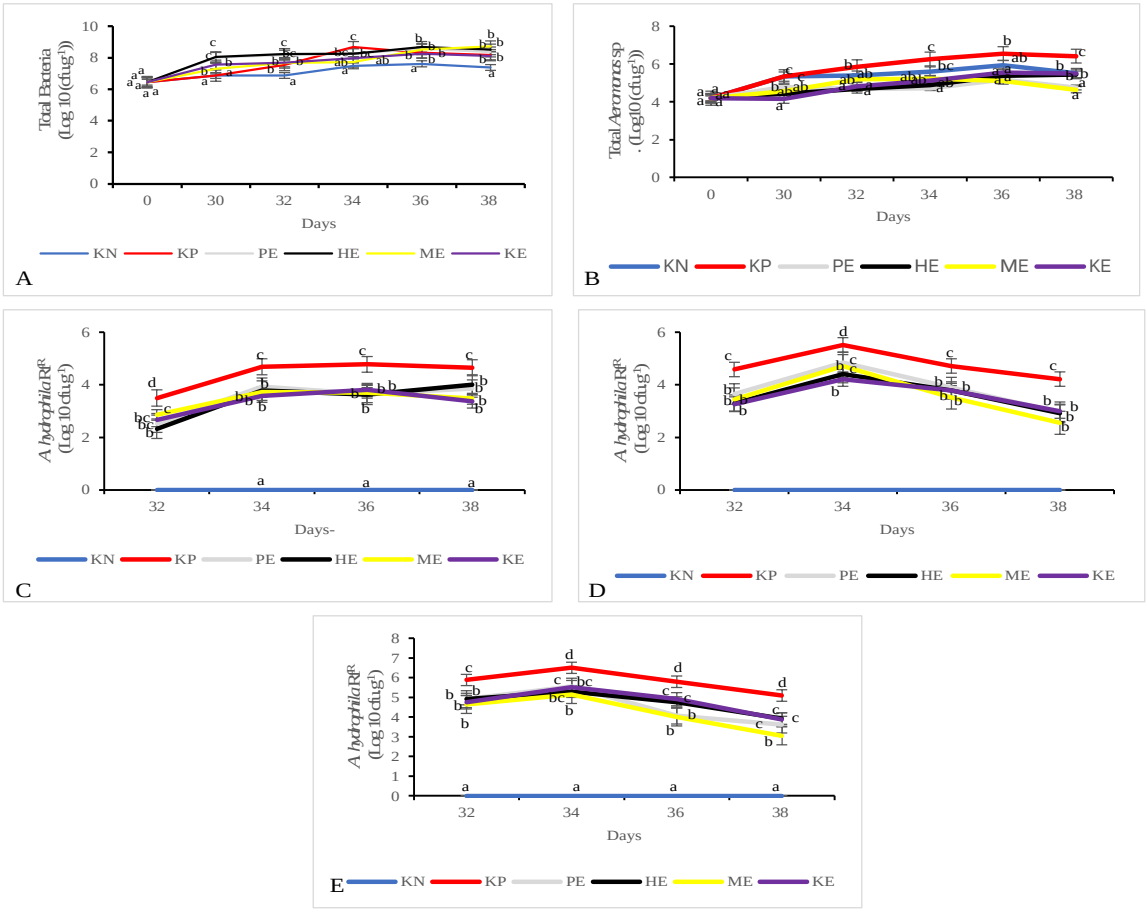

Figure 5. Total bacteria (A), total *Aeromonas* sp. (B), total *A. hydrophila* Rf^R in intestine (C), total *A. hydrophila* Rf^R in liver (D), and total *A. hydrophila* Rf^R in kidney (E) of striped catfish after challenge with *A. hydrophila*. Different superscript letters in each bar (mean ± standard deviation) indicate statistically significant differences ($P < 0.05$). KP (Positive control), PE (*C. zedoaria* extract), HE (*C. aeruginosa* extract), ME (*C. mangga* extract), KE (Combination of the three extracts).

Histopathology

Histopathological analysis of the liver and kidney organs (Figure 6) showed abnormalities in the form of vacuolization, necrosis, inflammatory cell infiltration, and hydropic degeneration. The most severe damage was observed on day 32 after the challenge test. However, the condition of the organs began to improve by day 38 after the challenge, as indicated by the restoration of organ structure towards normal conditions. This suggests that the fish had entered the recovery phase.

The population of *A. hydrophila* is thought to have a significant effect on the health of the liver and kidney of striped catfish. Histologically, results on day 32 after the challenge showed cellular abnormalities, with the most severe damage observed in the positive control treatment. These abnormalities included necrosis, vacuolization, hydropic degeneration, and inflammatory cell infiltration, which were identified in all treatments (Figure 6). This finding is in line with the research of Abd El-Salam *et al.* (2018), who stated that *A. hydrophila* infection can cause damage to organs such as the kidneys, liver, and spleen. In general, the reduced population of *A. hydrophila* RF^R in the liver and kidney of catfish treated with *Curcuma* sp. extract indicated the potential for protection against cell damage in both organs. Syawal *et al.* (2021) also found similar results, where the administration of herbal ingredients to catfish was able to reduce the negative effects of infection. Rajkumari & Sanatombi (2018) suggested that chemical compounds in medicinal plants can accelerate the process of cell regeneration, which contributes to organ recovery. Therefore, the administration of *Curcuma* spp. extract in this study not only reduced the population of *A. hydrophila*, but also contributed to the repair of liver and kidney cell damage, while maintaining the hematological condition of catfish during infection.

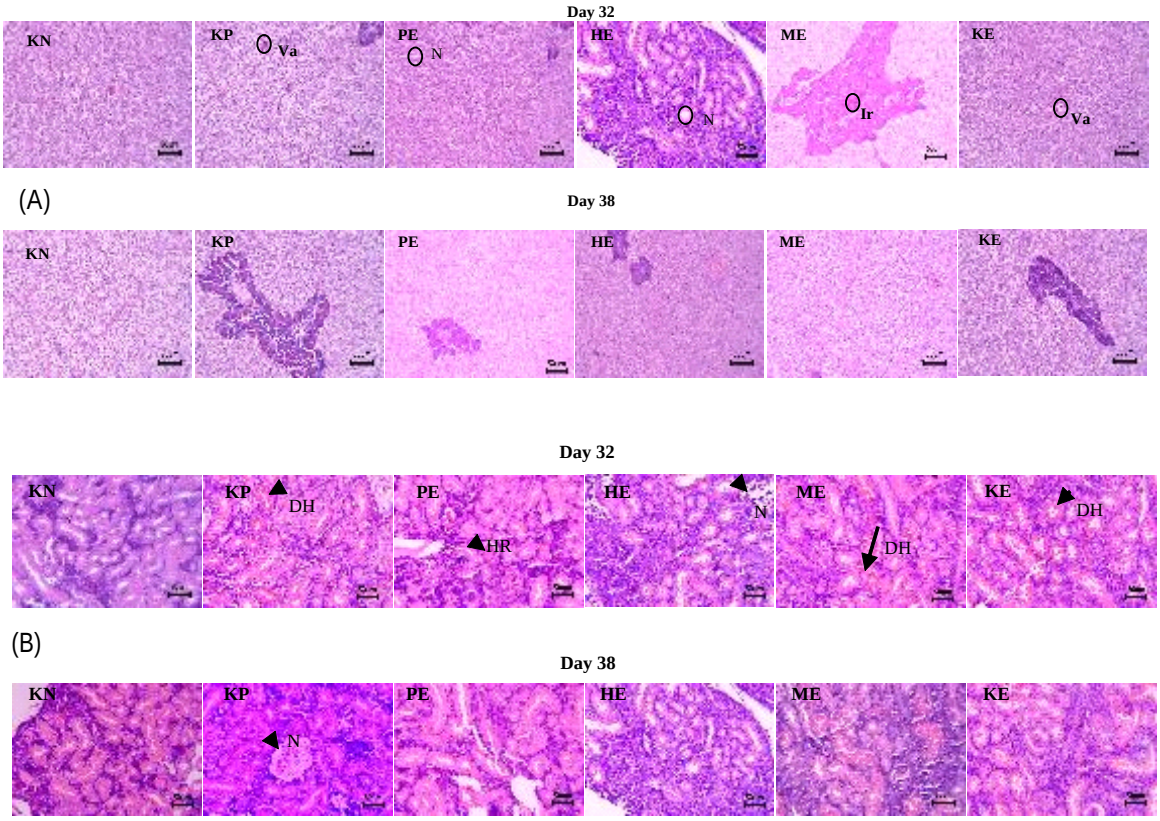

Figure 6. Histology of (A) liver and (B) kidney of striped catfish after challenge with *A. hydrophila* RF^R (day 32 and day 8). Staining using hematoxcylin and eosin (HE), magnification 400x. N (Necrosis), Va (Vacuolization), DH (Hydropic degeneration), Ir (Inflammatory cell infiltration). KP (Positive control), PE (*C. zedoaria* extract), HE (*C. aeruginosa* extract), ME (*C. mangga* extract), KE (Combination of the three extracts).

Post-challenge survival rate

The survival rate of striped catfish after the challenge test with *A. hydrophila* RF^R showed that all extract treatments significantly increased the survival rate compared to that of the positive control ($P < 0.05$). The survival rates of striped catfish after challenge tests were: KN (100%), KP (53.33%), PE (93.33%), HE (91.67%), ME (93.33%), and KE (88.33%) (Figure 7).

The high survival rate of striped catfish treated with *C. zedoaria*, *C. aeruginosa*, and *C. mangga* extracts after the challenge test (Figure 7) indicates the significant role of the extracts in enhancing the immune response in fish. The administration of *C. mangga* and *C. zedoaria* extracts resulted in a survival rate of 93.33%, which was not significantly different from that of the group that received the *C. aeruginosa* extract or the combination of extracts. This finding is in line with the research of Abdelhamid *et al.* (2021) and Khieokhajonkhet *et al.* (2023), which states that the use of herbal ingredients can increase immune response and survival in farmed fish. This increase is due to the content of bioactive compounds in the *Curcuma* spp. extract, which has the potential to modulate the immune system of fish, thereby increasing the ability of fish to deal with stressors and pathogens (Syawal *et al.*, 2021; Purbomartono *et al.*, 2021).

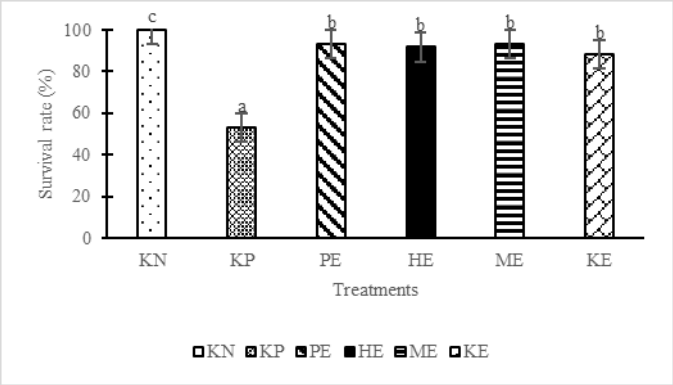

Figure 7 Survival of catfish after challenge with *A. hydrophila* RF^R. Different superscript letters in each bar (mean ± standard deviation) indicate statistically significant differences ($P < 0.05$). KP (Positive control), PE (*C. zedoaria* extract), HE (*C. aeruginosa* extract), ME (*C. mangga* extract), KE (Combination of the three extracts).

CONCLUSIONS

Extracts of *C. zedoaria*, *C. aeruginosa* and *C. mangga* at a concentration of 6.25 g kg^{-1} feed were shown to enhance the immune response and resistance of striped catfish infected with *A. hydrophila* by stimulating the expression of interleukin-1β, interferon-γ 2a and 2b genes. This led to increased red blood cell and white blood cell count, hematocrit, hemoglobin, phagocytic activity, and respiratory burst, while also reducing the population of *A. hydrophila* in the intestine, minimizing tissue damage in infected catfish, and improving the survival rate of the fish.

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