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USE OF DIETARY GARLIC (*Allium sativum* L.) AND VIETNAMESE BALM (*Elsholtzia ciliata*) EXTRACT FOR PREVENTION OF BACILLARY NECROSIS IN PANGASIUS (BNP) IN STRIPED CATFISH (*Pangasianodon hypophthalmus*)

Luu Tang Phuc Khang[1], Truong Vinh[2], Luong Thi Le Tho[1], Nguyen Xuan Tong[3,*], Tran Thi Phuong Dung[1]

[1]Ho Chi Minh City University of Education, Vietnam
[2]International School Ho Chi Minh City, Vietnam
[3]Institute of Environmental Science, Engineering and Management - Industrial University of Ho Chi Minh City, Vietnam

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ABSTRACT

Garlic (*Allium sativum*) and Vietnamese balm (*Elsholtzia ciliata*) are well-known herbs that have been widely used in Vietnamese traditional medicines. However, studies on the effects of these plants in improving the immune system of fish have received less attention. This study aimed to investigate the effect of ethanolic leave extracts from garlic and Vietnamese balm leaves on the growth and immune response of striped catfish (*Pangasianodon hypophthalmus*) for the prevention of Bacillary Necrosis of Pangasius. Bacillary Necrosis of Pangasius (BNP) caused by *Edwardsiella ictaluri* is the most serious disease occurring in farmed striped catfish in Vietnam. The experiment was in a completely randomized design with five triplicated treatments including 0% (control); 2% and 4% of garlic and 2% and 4% of Vietnamese balm leaves extract. Fish were soaked with *E. ictaluri*. Samples were collected on the days 7th and 14th of the growth experiment and days 3rd, 5th and 7th of the challenge test. Fish mortalities and immune response among challenge tests were also recorded. The results suggest that plant extracts possibly modulate the striped catfish's immune response in a time and dose dependent manner. Specifically, diets enriched with extracts of *A. sativum* at 4% have great potential than Vietnamese balm extraction for improving striped catfish health by enhancing the immune system. After challenging with *E. ictaluri*, the accumulative mortality of fish in garlic and Vietnamese balm leaves treatments was lower than that of the control. The mortality of fish fed with 4% garlic extract was the lowest. These results indicated that supplementation of 4% garlic extract significantly improved the resistance to the BNP infection in striped catfish and has the ability to add fish feed in reality.

Keywords: Bacillary Necrosis in Pangasius, immune response, *Allium sativum*, *Elsholtzia ciliata*, striped catfish.

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[*]*Corresponding author email*: nguyenxuantonghui@gmail.com

INTRODUCTION

The striped catfish (*Pangasianodon hypophthalmus*) is a migratory fish that is one of the species with high economic value that is widely cultured in the Mekong Delta (Phan et al., 2009). The popularity of striped catfish is due to its relatively fast growth rate, high flesh quality, tolerance to low oxygen and crowding, as well as its ability to adapt well to various culture systems. It also presents an efficient feed conversion rate and accepts manufactured feed (De Silva & Nguyen, 2011). In 2021, the production of striped catfish in Vietnam reached 1.56 million metric tons and a turnover of approximately 1.61 billion USD (VASSEP, 2022). There has been a phenomenal shift from an extensive to an intensive culture of carps and catfishes in the last three decades. In addition to the rapid increase in farming areas, the increasing density of cultured fish is also one of the causes of water pollution and disease outbreaks in farmed fish. Diseases that are common and have a great influence on intensive pangasius farming in Vietnam (Le & Cheong, 2010) are especially Bacillary Necrosis in Pangasius (BNP) caused by *Edwardsiella ictaluri* (Crumlish et al., 2002). Striped catfish farming households in the Mekong Delta tend to use antibiotics or special chemicals to limit diseases caused by bacteria. Research by Luu et al. (2021) showed that, on fish farms in Vietnam, 23 different antibiotics and chemicals (belonging to 11 groups) were used, each pangasius farming household used at least 17 drugs and chemicals. The excessive use of chemicals and antibiotics is recommended to be limited due to a number of reasons, such as the possibility of multi-drug resistance of bacterial species (Tu, 2010), impaired environmental degradation, antibiotic residues in aquatic products adversely affect human health (Sarter et al., 2007).

Vietnam has plenty of wild plant resources distributed in the different eco-regions (Punitha et al., 2008). The mode of action of these herbs is usually the enhancement of the immune response through the elevation of immune parameters, and control of infectious diseases by mitigation of many side effects involving the synthesis of antimicrobials (Gull et al., 2012). However, the use of natural products in aquaculture is not yet popular in the country. Farmers lack knowledge regarding the existence of such bioactive products or their efficacy in fish. Garlic (*Allium sativum*) is a herb containing many compounds with strong biological effects such as allicin or sulfur compounds and polyphenols (Gull et al., 2012; Rahman et al., 2012) and should be widely used in veterinary medicine (Bui & Nguyen, 2009). In addition, Vietnamese balm (*Elsholtzia ciliata*) is also a herb with an antibacterial role due to the presence of phenolic compounds, such as flavonoids (Dormans & Deans, 2000) and caryophyllene, 8,13-epoxy-androst-14-en -3-one, p-menth-1-en-4-ol (Dongsa et al., 1992). Based on the reference information, this study was carried out to evaluate the effect of adding garlic and Vietnamese balm extracts to the feed on the antibacterial ability of *E. ictaluri* to prevent BNP in striped catfish. This study was conducted to investigate whether two ethanoic herbal extracts (*A. sativum*, *E. ciliata*) affect growth indices, adaptive immune responses and disease resistance against *E. ictaluri* infection in striped catfish.

MATERIALS AND METHODS

Material

Plant materials

Fresh parts of *A. sativum* [As] and *E. ciliata* [Ec] (leaves) were collected from the Can Gio district, Ho Chi Minh City, Vietnam. The plants were authenticated at the Department of Biology, Ho Chi Minh City University of Education, Vietnam.

Fish

A total of 270 healthy striped catfish juveniles (15–20 g) were procured from National Feeding Center for Southern Freshwater Aquaculture located in Tien Giang province.

Bacteria

The bacterial strain *Edwardsiella ictaluri* Gly09M isolated from BNP catfish in An Giang province in 2009 and preserved in the bacterial collection of the Research Institute for Aquaculture No.2 (RIA2) was used in this research. The bacteria were restored and re-isolated from healthy striped catfish three times prior challenge.

Methods

Plant extraction

All collected plant parts were washed to remove mud and dust, and rotten and damaged parts were discarded. Plants were air dried in shade for several days and then in an oven at about 60 ^{o}C until well-dried (humidity < 10%). Plants were then ground to fine powders in a blender and stored in sealed containers in a cool, dry place.

The dried powder (4 g) was soaked in ethanol 70% (250 mL) for 72 hours at room temperature with frequent agitation. The solvent containing extracts were then decanted and filtered. The filtrate from each extraction was combined and the excess solvent was evaporated under reduced pressure using a rotary evaporator to give crude ethanol extracts. All the well-dried crude ethanol extracts were stored at -20 ^{o}C (Vongsak et al., 2013).

Diet preparation

The feed used in the experiment was industrial feed with 32% protein, 2 mm/pellets (Grobest, Vietnam). Proportional extracts of each treatment were added to the feed by diluting with 10 mL of DMSO and continuing with 10 mL of water, spraying and mixing until the extract was absorbed into the feed, and allowed to dry naturally for 4 hours. Then coat the feed pellets with squid oil and continue to dry naturally for 8 hours at room temperature. Pellets were stored at 4 ^{o}C during the experiment.

Growth experiment

The fish were acclimatized to laboratory conditions for fifteen days then maintained in composite tanks (90 L) and fed twice a day with the formulated diets at a rate of 3–4% of their body weight/day.

For the feeding trial of plant extract-based diets, fish were randomly divided into five treatments (control, As 2%, As 4%, Ec 2%, Ec 4%), with each treatment given in triplicate. Fish were fed the experimental diets for two weeks, at 3–4% of body weight and three times (7 am and 4 pm) daily. The tank capacity was 90 L, and each tank contained 15 fishes. The photoperiod was 12 hours light: 12 hours dark. Water parameters (pH, total ammonium concentration and temperature) were frequently monitored, and the temperature was maintained at 26 ^{o}C.

Challenge test

E. ictaluri Gly09M were cultured on tryptic sheep blood agar plates for 48 hours at 28 ^{o}C following the method of Le et al. (2013). Then, a single colony was collected and harvested into Brain Heart Infusion (BHI) broth (Merck, Germany). This suspension was shaken overnight, at 85 rpm at 28 ^{o}C. The mean colony count was found using the optical density method (Hoseinifar et al., 2018) by spectrophotometer (Thermo spectronic, USA) at 590 nm, and the OD value was adjusted to 0.1 (~10^{9} CFU/mL). This suspension was diluted 1000 times with NaCl solution and soaked fish for 3 hours (Dang et al., 2014). There are six experiments: soaked with *E. ictaluri* (As 2%, As 4%; Ec 2%; Ec 4%); control (soaked with bacteria); negative control (0.85% NaCl) (Bui et al., 2021). Each treatment consisted of 45 animals, repeated two times. After soaking, fish in each experiment get the switch to a 90 L tank for two weeks of monitoring. To ensure that mortalities were due to bacterial infection, *E. ictaluri* was re-isolated and identified by PCR confirmation.

Sample collection and analysis parameters

At the end of the experiment, which lasted 14 days, fish in each aquarium were weighted and counted for analyse of growth indices.

Blood samples were collected from the fish caudal vein by a sterile syringe containing

EDTA as an anticoagulant nine fishes per treatment, three fishes per tank) at day 7 (D7), day 14 (D14), three day-post infection (dpi), 5 dpi, 7 dpi. Blood was used for erythrocyte count, total and differential leukocyte count and hematocrit value.

Growth indices

Growth parameters such as final length and weight, net gain in length and weight, percentage final weight, average daily weight and length gain (ADG) and specific growth rate (SGR) were measured using the following formulas (Tok et al., 2016).

1. Weight gain (WG) = (Final weight – initial weight).

2. Percentage specific growth rate (%SGR) = (Log_e final body weight – log_e initial body weight)/Culture days × 100

3. Average daily weight gain (ADG) = (Final weight – initial weight)/Days of culture

4. Percentage length gain (%WG) = (Final length – initial length)/(Initial length) × 100.

5. Percentage specific growth rate ($\%SGR_L$) = (Log_e final body length–log_e initial body length)/Culture days × 100

6. Average daily length gain (ADG_L) = (Final length – initial length)/Days of culture

Total red blood cells count

Total red blood cell (RBC) counts were made with a Neubauer hemocytometer using Natt-Herrick solution as a diluent stain (Natt & Herrick, 1952). First, 10 µL of each blood sample was diluted in 1,990 µL of Natt and Herrick's solution and mixed gently for at least 3 min. The cell suspension was put into the chamber and allowed to settle for 2–3 min before initiating a count under the microscope light. The RBCs were counted in 5 out of the 25 small areas. The total RBC was calculated as RBC = A × 10,000 (A: the number of RBC counted) (Budiari et al., 2021).

Total and differential leukocyte count

White blood cells (WBCs) were calculated using a hemocytometer. Total leukocyte was determined manually with the improved Neubauer counting chamber. In addition, the differential count of WBC including neutrophils (NEU), lymphocytes (LYM), monocytes (MONO) was performed by preparing and staining the blood smear according to the method recommended by Borges et al. (2004) under an optical microscope (Alphaphot-2 YS2, Nikon, Japan). The total WBC was calculated as B × 50 (B: the number of WBC counted) (Budiari et al., 2021).

Quantification of NEU, MONO, LYM was performed according to Hrubec et al. (2000) with modifications. First, we counted the total number of each white blood cell (NEU, MONO, LYM) per 100 cells. The density of each type of these white blood cells (cell/mL) was calculated as the number of each type × density of WBC count in percentage (Hrubec et al., 2000).

Hematocrit

The measurement of hematocrit using a hematocrit capillary pipette was collected by filling 4/5 parts of the hematocrit capillary with blood. Then, both sides of the hole were closed using a Critoseal (wax cover). Next, the centrifuge was done at the speed of 12,000 rpm for 4 minutes. After 4 minutes, the centrifuge cover was removed to observe the result by reading the hematocrit table scale expressed in percentage (Zuhrawati et al., 2014).

Statistical analysis

The data of growth parameter, immune response of striped catfish at each time point were tested. The one-way ANOVA with a significant level of 5% ($P < 0.05$) was used to compare the mean values at each time point. These analyses were performed on the SPSS statistical software to test the significant difference at probability $P < 0.05$. All data were presented as mean ± standard errors (SE).

RESULTS AND DISCUSSION

Effect of extract-supplemented diets on growth indices of striped catfish

Results of the effect of extract-supplemented diets on growth indices of striped catfish at different sampling times (D7, D14) are given in Figure 1.

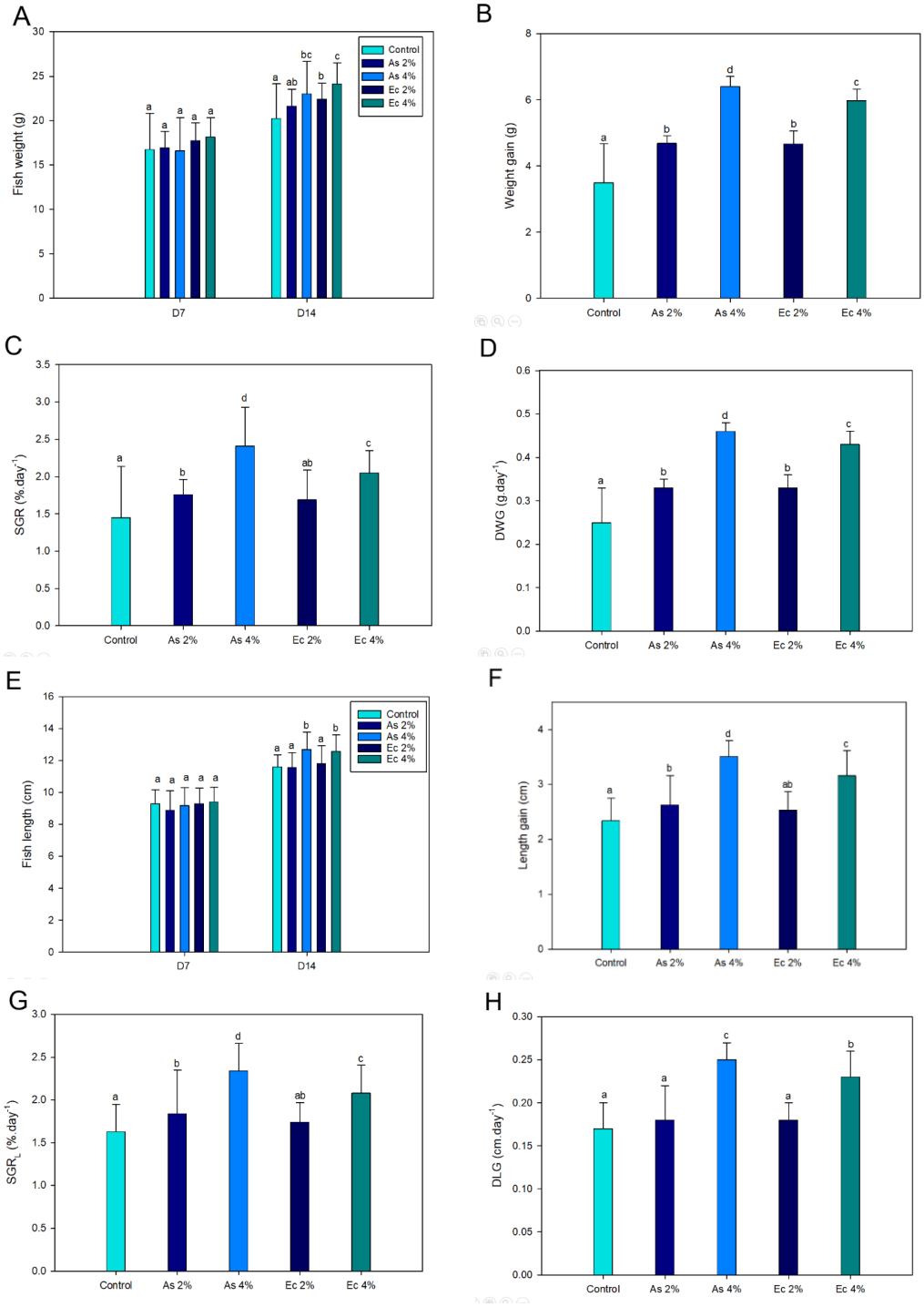

Figure 1. Effects of dietary plant extract administrations on (A) fish weight; (B) weight gain (WG); (C) percentage specific growth rate (%SGR); (D) daily weight gain (DLG); (E) fish length; (F) length gain (LG); (G) percentage specific growth rate (%SGR_L); (H) daily length gain (DLG) at different sampling times (D7, D14). Values are mean ± SE, different letters indicate significant differences between treatments ($P < 0.05$), NS: non-significant

After 14 days of the experiment, striped catfish in As 2%, As 4%; Ec 2%; Ec 4% had total weight gain (WG) ranged from 4.67 ± 0.39 to 6.40 ± 0.31 g; percentage specific growth rate (SRG) and daily weight gain (DWG) of fish in the treatments supplemented with *A. sativum* and *E. ciliata* extracts ranged from 1.69 ± 0.40 to 2.41 ± 0.52 %, 0.33 ± 0.02 to 0.46 ± 0.02 g. Total length gain (LG) of striped catfish in As 2%, As 4%; Ec 2%; Ec 4% ranged from 2,53 ± 0,34 to 3,51 ± 0,29%; percentage specific growth rate (SRG_L) and daily length gain (DLG) from 1,74 ± 0,23 to 2,34 ± 0,32 % and 0,18 ± 0,02 to 0,25 ± 0,02 cm, significantly different from fish in the control treatment. Growth parameters of striped catfish fed diets supplemented with garlic extract were higher than those of Vietnamese balm extract. Which, fish supplemented with 4% garlic extract had the highest growth and absolute growth rate (Fig. 1).

Effect of extract-supplemented diets on blood parameters of striped catfish

Results of total red blood cell count (RBCs), total white blood cell count (WBCs); monocytes (MONO); neutrophils (NEU); lymphocytes (LYM); hematocrit value (HCT) at different sampling times (D7, D14) are given in Figure 2.

The hematological parameters significantly increased ($P < 0.05$) in a dose-dependent manner in all extract treatments compared to those of the control (Fig. 2). The number of RBC, WBC, MONO, NEU, LYM, HCT was statistically higher than the control group in D14. For all sampling times, the WBC numbers statistically increased in fish fed on diets containing garlic and Vietnamese balm. Fish supplemented with 4% garlic extract had the highest hematological parameters (RBC: 17.50 ± 0.71×10^5 cell/mL; WBC: 20.07 ± 0.43×10^4 cell/mL; MONO: 1.96 ± 0.08×10^3 cell/mL; NEU: 1.83 ± 0.03×10^3 cell/mL; LYM: 15.41 ± 0.39 10^3 cell/mL; HCT: 39.60 ± 2.37 %).

The hematological parameters significantly changed ($P < 0.05$) in a dose-dependent manner in all extract treatments compared to those of the control (Fig. 2). The RBC abundance tended to decrease in all experimental groups after *E. ictaluri* infection. The Control group significantly reduced the number of RBCs, whereas the RBC counts were statistically higher in the As 4% group compared to the control and Ec treatment ($P < 0.05$) (Fig. 2A). For all sampling times, the WBC numbers statistically increased in As groups and in the Ec 4% group compared to the control. WBC counts increased considerably in As 4% group in 3 dpi and both doses of Ec (Ec 2% and Ec 4%) and As 2% groups in 3 dpi, while no significant differences were observed between Ec 2% versus the control group after injection with bacteria (Fig. 2B). Compared to the control, the number of monocytes considerably increased in As 4% and Ec 4% groups in 5 dpi. Similarly, monocytes also increased in fish fed As 2% compared to control in 3 dpi ($P < 0.05$). After the challenge test, the number of monocytes statistically increased in both doses of As and Ec compared to the control group (Fig. 2C). The highest abundance of neutrophils was observed in fish fed As 4% in 3 dpi (Fig. 2D). The number of neutrophils was still significantly higher in most extract-supplemented groups compared to the control ($P < 0.05$). Statistical analysis showed that the number of lymphocytes in As, and Ec groups were more abundant than the control treatment in 3 DPI ($P < 0.05$). After the challenge with *E. ictaluri*, the quantity of lymphocytes increased in all treatments, but this value remained significantly higher in As, Ec groups than in the control treatment (Fig. 2E). The hematocrit value in As and Ec groups changed, the addition of garlic and Vietnamese bahm extracts in the diet, especially garlic at a concentration of 4 % was found to limit the decrease in hematocrit if compared to the control (Fig. 2).

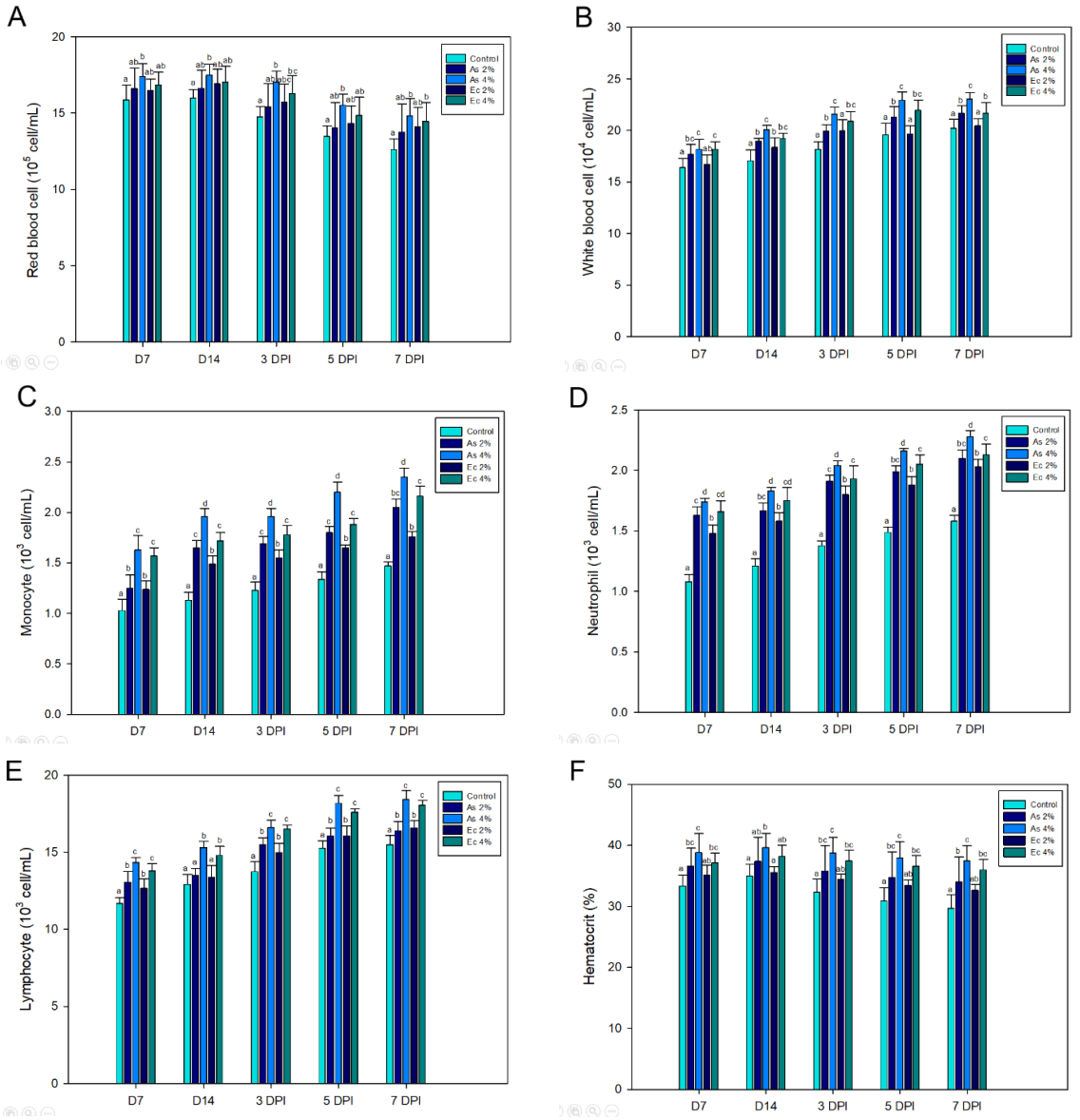

Figure 2. Effects of dietary plant extract administrations on (A) red blood cells (RBCs); (B) white blood cells (WBCs); (C) monocytes (MONO); (D) neutrophils (NEU); (E) lymphocytes (LYM); (F) hematocrit value (HCT) at different sampling times (D7, D14). Values are mean ± SE, different letters indicate significant differences between treatments ($P < 0.05$)

Disease resistance of striped catfish against *Edwardsiella* ictaluri

Affected fish in experiments showed behavioural changes including erratic swimming in a spiral motion and stopped feeding prior to mortality. Internally, the affected fish presented grossly with white lesions (1–2 mm diameter) observed distributed throughout the spleen and the kidney (Fig. 3). Later, white lesions also occurred on the liver of infected fish. The body cavity was swollen and ascites were present. The spleen and kidney were enlarged.

Pure cultures of bacteria identified as *E. ictaluri* were only recovered from moribund and freshly dead fish.

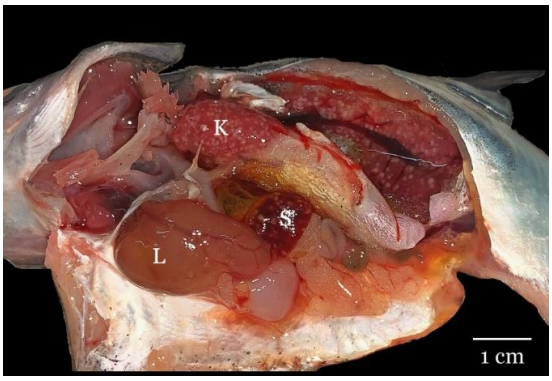

Figure 3. White lesions were presented in the kidney (K) and spleen (S), liver (L) was enlarged

Mortality of striped catfish occurred from day 4 to day 14 after infection with *E. ictaluri* (Fig. 4). A maximum of 100% mortality rate was recorded in the control group. Mortality was significantly reduced in fish fed plant extract-based diets, especially in As (2%, 4%) and Ec (2%, 4%) groups. Of the different plant extract diets, As 4% and Ec 4% displayed the highest survival rate post-challenge (56.67% and 50.0%, respectively). No mortalities were recorded in the negative control group. Moreover, bacterial identification found that *E. ictaluri* was detected in all bacterial infection samples. The cumulative percentage of daily mortalities in the refined immersion challenge study is presented in Figure 4. There were no mortalities in the control group throughout the study period (Fig. 4).

The first mortality occurred on day 4 (Fig. 4) and the second mortality was observed in the treatment group on day 5 post bacterial challenge. The duration of this immersion challenge had an effect on time to death between treatments from day 3 to day 4 post challenges (Fig. 4). However, from day 5 post-bacterial exposure the mortalities occurred in all treatment groups except the control (Fig. 4). The highest percentage of cumulative mortality was found in the treatment group (Fig. 4). The mortality curves for each of the treatment groups were similar (Fig. 4).

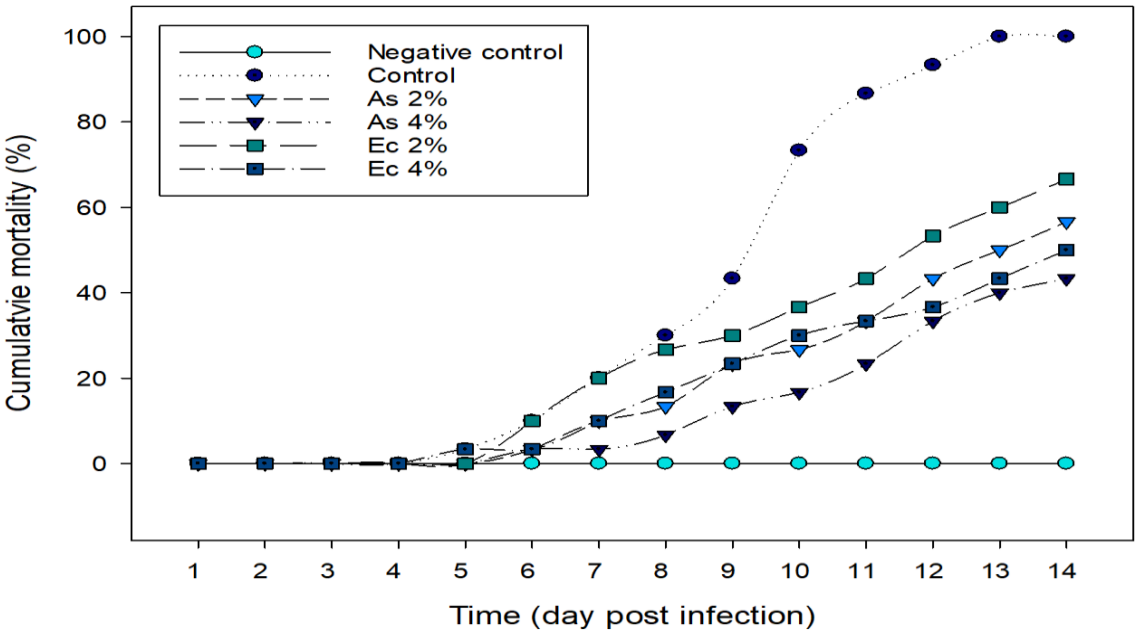

Figure 4. Cumulative mortality percentage of challenge experiment (CM)

Discussion

In the present study, the immunomodulatory effects of different plant extracts were assessed by investigating the changes in growth parameters, immune parameters and disease resistance against *E. ictaluri* in striped catfish. The plant species and extract concentrations used in the study were selected based on a preliminary in the previous *in vitro* study (Tran et al., 2022). Many plants have been reported to possess a wide range of active components such as alkaloids, steroids, phenols, tannins, terpenoids, saponins, glycosides, flavonoids, and many other compounds such as polysaccharides (Sivaram et al., 2004; Harikrishanan et al., 2011). Garlic (*Allium sativum*) is famous for centuries may be due to its dietary important for human health and medicinal properties. It contains antimicrobial properties (Harriss et al., 2001). Allicin is one of the active components of garlic performs antibacterial activity against disease causing pathogens *Aeromonas* sp., *Pseudomonas* sp. and *Edwardsiella tarda* (Lee & Gao, 2012). *E. ciliata* is known to have various chemical components, such as essential oil, elsholtzia ketone, flavonoids, steroids, and triterpenes (Dembitskii et al., 1993). Due to the presence of various compounds and secondary metabolites, the plant species were reported to display immunomodulatory activities (Kassuya et al., 2005; Jantan et al., 2014). The biological compounds in the extracts including phenols, gallic acid, myricetin were also variable after extraction in different solvents (Kumar et al., 2013). Moreover, methanol and ethanol were the best solvents for the extraction of biological components, which mainly functions in immune stimulatory properties and antibacterial activity (Guo et al., 2001; Ashraf et al., 2016).

The use of plant extracts in aquaculture to promote growth and immunity has been gaining momentum (Rahman et al., 2012). Abdel-Hakim et al. (2010) added garlic to tilapia feed for 22 weeks increased survival, growth promotion and feed conversion. Similarly, the addition of herbs such as *Sesbania grandiflora*, *Moringa oleifera*, *Plectranthus amboinicus*, *Ocimum basilicum* to the feed also promoted growth in *Oreochromis mossambicus* (Lee & Yao, 2012). Through the obtained results, garlic and Vietnamese balm extractions affected the weight and length of fish in the fingerling stage. During this stage, the growth in weight increases rapidly, the growth in length also increases accordingly.

The findings of this study clearly show that oral administration of ethanol extracts could improve fish health status, which was indicated by a higher immune response than in fish fed diets without plant extracts. Hematological parameters are clinical indicators of health and disease conditions (Kelada et al., 2012). In this study, plant extract-enriched diets affected the total RBC, WBC, LYM, NEU, MONO and hematocrit values started to increase in a dose dependent manner in fish fed enriched diets from D7. The RBC counts were significantly reduced in As groups at 3 dpi compared to controls. In addition, plants containing polysaccharides usually induce a proliferation of lymphocytes (Shen et al., 2013; Li et al., 2014), possibly explaining the significantly increased number of lymphocytes in striped catfish fed extract-based diets for all sampling times. Among the extracts, diets supplemented with As and Ec stimulated a significant increase in blood indices in D7, D14, and 3–7 dpi. The significant increase in the total WBCs after extract stimulation, including various leukocytes such as lymphocytes, monocytes, and neutrophils, could be a good indicator of the triggering of striped catfish immunity. This may be due to the presence of higher quantities of allicin in the methanolic As extract than in other extracts.

The elevation of blood parameters in conjunction with the enhanced immune response contributes to enhancing the defense mechanism against bacterial infection in striped catfish after a plant extract feeding period. The current results revealed that plant extract-based diets significantly increased the survival rates of striped catfish injected with

E. ictaluri. Mortality was mostly recorded from day 4 to day 8 after the challenge for most treatments. The minimum cumulative mortality in striped catfish was observed in As 4% and Ec 4% groups at 43.33% and 50.0% respectively. In this study, the mortality rate of fish after infection with *E. ictaluri* was lowest in the treatment with garlic supplemented with 4%. This is completely consistent with the test results of the immune parameters of the striped catfish. Fish supplemented with 4% garlic had the best immune response shown by stimulating the production of RBC, WBC, MONO, NEU, LYM and hematocrit values.

CONCLUSION

This study demonstrated that there were significant differences in the growth parameter (weight gain, daily weight gain, specific growth rate, length gain, daily length gain) and immune response (specifically red blood cells, white blood cell, neutrophils, monocytes, hematocrit values), between As (2%, 4%) and Ec (2%, 4%) groups to bacteria *E. ictaluri* causes BNPs in striped catfish. In addition, the cumulative mortality of fish was collected, fish supplemented with 4% garlic had the best growth parameters and immune response.

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