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Plant extract-based diets differently modulate immune responses and resistance to bacterial infection in striped catfish (*Pangasianodon hypophthalmus*)

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ABSTRACT

A feeding trial was performed to compare the effects of five ethanol herbal extracts (bhumi amla, *Phyllanthus amarus* Schum and Thonn [Pa]; guava, *Psidium guajava* L. [Pg]; sensitive plant, *Mimosa pudica* L. [Mp]; neem, *Azadirachta indica* A. Juss [Ai] and asthma plant, *Euphorbia hirta* L. [Eh]) on the immune response and disease resistance against *Edwardsiella ictaluri* infection of striped catfish (*Pangasianodon hypophthalmus*). Fish were fed diets supplemented with two doses of each plant extract (0% [basal diet], 0.4% Eh [Eh0.4], 2.0% Eh [Eh2.0], 0.2% Pa [Pa0.2], 1.0% Pa [Pa1.0], 0.2% Pg [Pg0.2], 1.0% Pg [Pg1.0], 0.4% Mp [Mp0.4], 2.0% Mp [Mp2.0], 0.4% Ai [Ai0.4], 2.0% Ai [Ai2.0]) for 8 weeks. Results showed that hematological parameters (total red blood cells, white blood cells, lymphocytes, monocytes, and neutrophils) of fish fed extract-based diets were significantly higher than in those fed the control diet ($p < 0.05$) after 4 and 8 weeks. Plasma lysozyme activity increased in fish whose diets contained both doses of Eh ($p < 0.05$) in week 4 (W4), whereas lysozyme activity increased in fish fed 0.2% Pa and Pg, and 2.0% Ai and Eh ($p < 0.05$) in week 8 (W8). The lysozyme levels in skin mucus did not significantly differ between treatments ($p > 0.05$) in W4 and after the bacterial challenge test. At the end of the feeding trial, levels of ACH50 significantly increased in most of extract groups compared to the control group ($p < 0.05$). Total immunoglobulin increased considerably in both the plasma and skin mucus of fish fed extract-supplemented diets after 8 weeks. In addition, dietary supplementation with Pg, Mp, Pa0.2, Eh2.0, and Ai0.4 for 8 weeks considerably reduced the cumulative mortality against *E. ictaluri* infection in striped catfish. The results suggest that plant extracts possibly modulate the striped catfish immune response in a time and dose dependent manner. Specifically, diets enriched with extracts of *P. guajava* at 0.2 and 1.0%, or *M. pudica* at 2.0% for 8 weeks, have great potential for improving striped catfish health by enhancing the immune system and reducing mortality against bacterial challenges.

1. Introduction

The striped catfish (*P. hypophthalmus*) is a migratory riverine species that has become an economically important fish and is widely cultured in several Asian countries. Striped catfish aquaculture has been developing rapidly in Vietnam, with an annual production that exceeded 300 thousand tons in 2004 to more than 1.2 million tons in 2018 [1,2]. However, intensive striped catfish production induces environmental stressors due to high stocking densities, and consequently increases mortality rates due to bacterial infections. Recent studies have reported that *E. ictaluri* is one of the most common bacterial pathogens of striped catfish and is responsible for huge economic losses at fingerling stages

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Table 1
Composition of experimental diets.

Ingredients (100 g of feed)	Control diet	Experimental diets									
		Pa0.2	Pa1.0	Pg0.2	Pg1.0	Ai0.4	Ai2.0	Mp0.4	Mp2.0	Eh0.4	Eh2.0
[a]Soybean meal (g)	32.62	32.62	32.62	32.62	32.62	32.62	32.62	32.62	32.62	32.62	32.62
[b]Rice bran (g)	29.5	29.5	29.5	29.5	29.5	29.5	29.5	29.5	29.5	29.5	29.5
[c]Casava (g)	18.36	18.16	17.36	18.16	17.36	17.96	16.36	17.96	16.36	17.96	16.36
[d]Fishmeal (g)	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0
[e]Fish oil (g)	1	1	1	1	1	1	1	1	1	1	1
[f]Premix* (g)	3	3	3	3	3	3	3	3	3	3	3
[g]Gelatin (g)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
[h]Butylated hydroxytoluene (BHT)	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Plant extracts (g)											
Pa	–	0.2	1.0	–	–	–	–	–	–	–	–
Pg	–	–	–	0.2	1.0	–	–	–	–	–	–
Ai	–	–	–	–	–	0.4	2.0	–	–	–	–
Mp	–	–	–	–	–	–	–	0.4	2.0	–	–
Eh	–	–	–	–	–	–	–	–	–	0.4	2.0

Pa: *Phyllanthus amarus*, Pg: *Psidium guajava*, Mp: *Mimosa pudica*, Ai: *Azadirachta indica*, Eh: *Euphorbia hirta*.

[a] Wilpromil R Soy Protein Concentrate, Yihai (Fangchenggang) Soybeans Industries, (Wilmar Group), Fangchenggang, China.

[b] Cai Lan Oils & Fats Industries Company, Can Tho Branch, Can Tho City, Vietnam.

[c] Hong Ha Company, Can Tho City, Vietnam.

[d] Minh Tam, Can Tho, Vietnam.

[e] Vegetable oil (Simply, Vietnam) and squid oil (Vemedim, Vietnam) at a ratio of 1:1.

[f] The vitamin/mineral premix (Unit/kg) from Vemedim, Can Tho, VietNam: vitamin A, 6000 IU; vitamin D3, 5600 IU; vitamin E, 160 IU; vitamin B1, 10 mg; vitamin B6, 20 mg; vitamin B12, 0.03 mg; vitamin K, 0.3 mg; riboflavin, 60 mg; vitamin C, 300 mg; pantothenic acid, 60 mg; folic acid, 8 mg; nicotinic acid, 184 mg; biotin, 0.3 mg; iron, 50 mg; copper, 10 mg; iodine, 9 mg; zinc, 34 mg; selenium, 0.4 mg; manganese, 30 mg.

[g] Xilong Chemical Industry Incorporated (China).

[h] Honshu Chemical Industry Company, Japan.

[3,4]. Understanding how the specific and non-specific immune responses modulate fish health is a key to improve productivity and reduce losses in the intensive aquaculture sector. Several immunostimulants used as diet additives could enhance striped catfish defense mechanisms and thus prevent losses from diseases [5–8]. Moreover, boosting the immune response with ecologically friendly compounds is an effective strategy to promote sustainable aquaculture.

From this perspective, bio-products resulting from natural plants have attracted considerable attention as a source of eco-friendly prophylactic compounds for using in the aquaculture industry. To date, many medicinal plant products have been demonstrated to possess a wide range of active components such as alkaloids, steroids, phenols, tannins, terpenoids, saponins, glycosides, and flavonoids [9,10], although their ingredients have not yet been completely described [11]. These compounds are responsible for potential bio-activities in fish [12], including growth promotion, appetite stimulation, immune stimulation, antimicrobial, and anti-stress effects [13–15]. Previous research reports that plant extracts have been successfully applied to improve aquatic organisms' immunity and disease resistance [16–19]. In fish, three different routes of administration have been tested, including injection, immersion, and oral, and the latter tends to be more practical than the others [20–22].

Innate and adaptive immune responses are considered to be the essential functions of fish defense mechanisms. The epithelial/mucosal barrier, and humoral and cellular immunity are the three main components of fish immunity [23]. The innate humoral parameters are represented by lysozyme, hemolysin, and complement molecules, etc., which are commonly high in fish plasma [24–26]; the key humoral parameter of the adaptive system is the immunoglobulins, expressed mainly as B-lymphocyte receptors or secreted in plasma [23]. Additionally, the mucosal layer of the fish skin also functions as the primary barrier between a fish and its external environment, and thus plays an important role in fish health protection. Physical properties of the skin mucus (including lysozyme, immunoglobulins, complement, agglutinins, and lysins) act as biologically active molecules in fish [27,28]. Earlier studies have reported that dietary supplemented with ginger (*Zingiber officinale* Roscoe) [29], garlic (*Allium sativum)* [30] and extracts of date palm fruit (*Phoenix dactylifera* L.) [31] promoted skin mucosal immune responses in rohu (*Labeo rohita*), Caspian roach (*Rutilus rutilus*), and common carp (*Cyprinus carpio*), respectively. 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 [32]. Vietnam has plenty of wild plant resources distributed in the different eco-regions [33]. However, the use of natural products in aquaculture is not yet popular in the country. Farmers lack knowledge regarding the existence of such bio-active products or their efficacy in fish. This study was conducted to investigate whether five ethanolic herbal extracts (*E. hirta, P. amarus*, *P. guajava*, *M. pudica* and *A. indica*) affect the modulation of innate and adaptive immune responses and disease resistance against *E. ictaluri* infection in striped catfish.

2. Material and methods

2.1. Extract preparation

Fresh parts of *E. hirta* [Eh] (leaves, twigs), *P. amarus* [Pa] (leaves, twigs), *M. pudica* [Mp] (leaves, twigs), *P. guajava* [Pg] (leaves), and *A. indica* [Ai] (leaves) were collected from the Mekong Delta in Vietnam. The plants were authenticated at the Department of Biology, College of Natural Science, Can Tho University. 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 °C until well-dried. Plants were then ground to fine powders in a blender and stored in sealed containers in a cool, dry place.

The dried powder (100 g) was soaked in ethanol 96% (800 mL) for at least 24 h at room temperature with frequent agitation. The solvent-containing extracts were then decanted and filtered. The ground samples were further extracted 4 times with ethanol 96%. 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 °C

until use.

2.2. Diet preparation

The basal diet was prepared according to Table 1. The diet contained 30% crude protein, 6.66% crude lipid, 10.58% ash, 3.21% fibre, and 4.41 kcaL/g energy. Fishmeal, soybean meal, cassava, and rice bran were mixed and sterilized at 110 °C for 10 min (Mixture A). Butylated hydroxytoluene (BHT), vitamins and minerals were well mixed with each of the plant extract concentrations (Mixture B). Then mixture A was mixed with mixture B and fish oil. The final mixture was extruded through a mini-extrusion machine (Can Tho University, Vietnam) at 70 °C without steaming. Basal diets were supplemented with the different plant extracts at two concentrations for each plant extract as described above. The experimental feeds were then dried at 60 °C for 24 h to achieve 11–12% moisture. Pellets of 2 mm were stored at −20 °C until use.

2.3. Bacteria preparation

E. ictaluri strain- Ed1 isolated from striped catfish in Mekong Delta of Vietnam were cultured on tryptic soy agar plates (Merck, MA, USA) for 48 h at 28 °C following the method of Hang et al. [5]. Then, a single colony was collected and harvested into tryptic soy broth (Merck, MA, USA). This suspension was shaken overnight, 180 rpm at 28 °C. Then, bacteria were centrifuged at 5000 rpm at 4 °C for 5 min and washed 3 times with 0.85% NaCl solution. The mean colony count was found using the optical density method [34] by spectrophotometer (Thermo spectronic, USA) at 590 nm, and OD value was adjusted to 0.1. This suspension was diluted 1000 times with NaCl solution and injected to the fish.

2.4. Fish acclimatization and feeding trial

Farm-raised striped catfish juveniles (15–20 g) were obtained from a local fish farm in Vinh Long province, Vietnam, and transported to the laboratory in plastic bags filled with oxygenated water. The fish were acclimatized to laboratory conditions for 15 days then maintained into composite tanks (250 L) under a flow-through freshwater supply system, and fed twice a day with the formulated diets at a rate of 2% of their body weight/day.

For the feeding trial of plant extract-based diets, fish were randomly divided into 11 treatments, with each treatment given in triplicate. Fish were fed the experimental diets for 8 weeks, at 2% of body weight and three times (8 am, 12 am, and 5 pm) daily. Tank capacity was 250 L, and each tank contained 50 fish. The photoperiod was of 12 h light: 12 h dark. Water temperature, dissolved oxygen, and pH were monitored daily and maintained throughout the experimental period at 30 ± 2°C, 5.7 ± 0.01 mg/L, and 7.5 ± 0.02 respectively.

After 8 weeks of feeding, all groups (45 fish per group) fed plant extract-based diets were injected intraperitoneally (i.p.) with 0.1 mL LD50 of *E. ictaluri* suspension. At the same time, the control groups were divided into two small groups, the first group was the control injected with 0.1 mL of 0.85% NaCl solution and the second group was challenged with 0.1 mL LD50 of *E. ictaluri*. All groups were maintained in triplicate, 15 fish per tank. Cumulative mortality was recorded daily for 14 days after the challenge test. To ensure that mortalities were due to bacterial infection, *E. ictaluri* was re-isolated and identified by PCR confirmation.

2.5. Sample collection

The skin mucus samples were collected at W4 and W8 of the feeding trial, and 3 days post injection (dpi) according to the method by Ross et al. [35] with slight modification. Briefly, 3 fish per tank were randomly collected and anaesthetised using 0.1 ppm M222 (Sigma–Aldrich, MO, USA). Fish surfaces were individually washed with distilled water and then transferred into polyethylene bags containing 1 mL of PBS 1X. After 2 min of gentle shaking, mucus was collected, transferred to 2.0 mL sterile eppendorf tubes and centrifuged (1500 × *g* for 10 min at 4 °C). The supernatant was stored at −80 °C for further analysis. Similarly, blood samples were obtained from the caudal vein of individual fish (9 fish per treatment, 3 fish per tank) and centrifuged at 4000 rpm for 10 min. The plasma supernatant was collected into new eppendorf tubes and kept at −80 °C until analysis.

2.6. Hematology

Total red blood cell (RBC) counts were made with a Neubauer hemocytometer using Natt-Herrick solution as a diluent stain [36]. First, 10 μL of each blood sample was diluted in 1990 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. White blood cell (WBC) types were identified by smearing a small drop of whole blood on a microscope smearing slide (cover glasses 22 × 22 mm, Germany). The slide smear was quickly dried, fixed in methanol (95%, Merck, MA, USA) for 1–2 min and stained with Wright's or Giemsa (Merck, MA, USA) [37]. Classification of blood cell types was determined following Supranee et al. [38]. Results for each blood cell were calculated according to Hrubec et al. [39].

2.7. Lysozyme assay

The lysozyme assay protocol was adapted from Ellis [40] and Milla et al. [41]. In 96-well microplates, the lysozyme activity assay was initiated by mixing 10 μL of plasma or 20 μL of skin mucus with 130 μL of lyophilized *Micrococcus lysodeikticus* (Sigma–Aldrich, MO, USA) suspension in phosphate buffer, pH 6.2 (0.6 mg/mL for plasma and 0.3 mg/mL for skin mucus). The difference in absorbance at 450 nm was monitored between 0 and 30 min for plasma (0 and 15 min for the skin mucus) and used to calculate units of lysozyme activity. One unit represents the amount of lysozyme that caused a 0.001 decrease in absorbance.

2.8. Complement assay

The plasma alternative complement pathway was assayed using rabbit red blood cells (RRBC, BioMérieux, France) as targets following Sunyer and Tort [42] and adapted by Milla et al. [41]. Briefly, 10 μL of RRBC suspension (3%) diluted in veronal buffer (BioMérieux, Marcy l'Étoile, France) were mixed with serial dilutions of plasma (60 μL total volume). After incubation for 100 min at 28 °C, the samples were centrifuged at 2000 × *g* for 10 min at room temperature. Spontaneous hemolysis was obtained by adding 60 μL of veronal buffer to 10 μL of RRBC. Total lysis was obtained by adding 60 μL of distilled water to RRBC. The absorbance was measured at 405 nm. Appropriate calculations served to estimate complement activity.

2.9. Total Ig assay

The total immunoglobulin concentration of each sample was measured using the method of Siwicki and Anderson [43], modified by Milla et al. [41]. Briefly, immunoglobulins were precipitated with 10,000 kDa polyethylene glycol (PEG, Sigma–Aldrich, MO, USA). Plasma or skin mucus was mixed with 12% PEG solution (v:v) for 2 h at room temperature under constant shaking. After centrifugation at 1000 × *g* for 10 min, the supernatant was collected and assayed for its protein concentration. The total immunoglobulin concentration was calculated by subtracting this value from the total protein concentration in the plasma or mucus before precipitation with PEG.

2.10. Bacteria detection

2.10.1. DNA extraction

Head kidneys of fish were mixed and ground with 600 μL lysis buffer (0.5 M NaCl, 0.1 M Tris–HCl at pH 8.0, 1% sodium dodecyl sulfate, and 0.1 mM EDTA) and 2.5 μL of proteinase-K solution (40 mg/mL). All mixtures were mixed well and incubated for 15 min at 37 °C and then 2.5 μL of RNase (2 mg/mL) was added, mixed and incubated for 30 min at 37 °C. Upon addition of 600 μL chloroform: isoamylalcohol (24:1, v:v), the mixtures were centrifuged at 13,000 rpm for 15 min at 4 °C. The supernatant was collected in a new tube. Following addition of 600 μL phenol: chloroform: isoamylalcohol (25:24:1), the mixtures were mixed well and centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatant was collected and quickly mixed with 500 μL cold isopropanol, and centrifuged at 13,000 rpm for 10 min. The DNA pellets were washed once with 70% ethanol and dried. Before PCR, DNA was dissolved in TE buffer (10 mM Tris–HCl and 0.1 mM EDTA at pH 8.0) and stored at −20 °C.

2.10.2. PCR amplification

A PCR reaction was performed to amplify a 407 bp specific DNA fragment of *E. ictaluri* with forward primer 5′-GTA GCA GGG AGA AAG CTT GC-3′ and reverse primer 5′-GAA CGC TAT TAA CGC TCA CAC C-3′ [44]. Each 25 μL reaction contained 1.5 mM $MgCl_2$, 0.2 nM dNTPs, 0.4 μM of each primer, 2.5 U of Taq polymerase (Promega, Madison, USA), and 100 ng of DNA extracted from fish head kidneys. PCR amplification was performed using a thermocycler (Applied Biosystems). The cycling parameters consisted of an initial denaturation at 95 °C for 4 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 45 s and extension at 72 °C for 30 s, and a final extension at 72 °C for 10 min. PCR amplicons were resolved by agarose gel electrophoresis in 1% agarose in 40 mM Tris–acetate, 1 mM EDTA, and stained with 1 mg/mL ethidium bromide.

2.10.3. Statistical analyses

All statistical analyses were performed using SPSS version 20 (IBM Corp., Armonk, NY:IBM USA). The normality of the data and the homogeneity of variance between groups were tested using Shapiro-Wilks and Levene tests. Results are presented as means ± SEM (standard error of the means). One-way ANOVA analysis of variance Duncan's multiple range test at a confidence level of 95% ($p < 0.05$) was used to determine significant differences between immunological variables in fish from the different plant extract treatments and control treatment.

3. Results

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

The hematological parameters significantly increased ($p < 0.05$) in a dose-dependent manner in all extract treatments compared to those of the control (Fig. 1). The number of RBCs did not show any significant differences between groups at W4, but the value was statistically higher than control group in the Pg1.0 group in W8. The RBC abundance tended to decrease in all experimental groups after *E. ictaluri* infection. Pa0.2 group significantly reduced the number of RBCs, whereas the RBC counts was statistically higher in the Mp2.0 group compared to control treatment ($p < 0.05$) (Fig. 1A).

For all sampling times, the WBC numbers statistically increased in Pg groups and in the Mp2.0 group compared to control. Extract-based diets with Pa and Ai did not enhance the number of WBCs in W4 and W8. WBC counts increased considerably in Eh0.4 group in W4 and both doses of Eh (Eh0.4 and Eh2.0) groups in W8, while no significant differences were observed between Eh versus control group after injection with bacteria (Fig. 1B).

Statistical analysis showed that the number of lymphocytes in Pg, and Mp2.0 groups were more abundant than the control treatment in W4 ($p < 0.05$). In contrast, no significant differences between treated and control groups were observed in the number of lymphocytes in W8. After challenge with *E. ictaluri*, the quantity of lymphocytes decreased in all treatments, but this value still remained significantly higher in Pg, Mp, Ai0.4, and Eh2.0 groups than in the control treatment (Fig. 2A).

The highest abundance of neutrophils was observed in fish fed Pg0.2 in W4 and in the Mp2.0 group in W8 (Fig. 2B). Although decreasing after the bacterial challenge test, the number of neutrophils was still significantly higher in most extract-supplemented groups compared to control ($p < 0.05$), except for Pa0.2.

Compared to the control, the number of monocytes considerably increased in Pg0.2 and Eh0.4 groups in W4. Similarly, monocytes also increased in fish fed Pa0.2, Mp0.4, and both doses of Ai compared to control in W8 ($p < 0.05$). After challenge test, the number of monocytes statistically increased in both doses of Pg, Mp and Eh0.4 compared to control group (Fig. 2C).

3.2. Effect of extract supplemented diets on the humoral and mucosal immune response of striped catfish

3.2.1. Lysozyme activity

Plasma lysozyme activity in fish fed Eh (Eh0.4 and Eh2.0) and Ai2.0 was higher than in other groups and significantly differed compared to control in W4 ($p < 0.05$). At the end of the experiment, a significant increase in the plasma lysozyme activity was observed in Pa0.2, Pg0.2, Mp2.0, and Eh2.0 groups. The plasma lysozyme level in most treatments tended to increase after the challenge test. In particular, lysozyme activity in fish fed Pg1.0, Ai (Ai0.4 and Ai2.0), and Eh0.4 diets increased in comparison to the levels in control fish ($p < 0.05$) (Fig. 3A).

The extract supplemented diets did not influence the lysozyme activity in skin mucus after 4 weeks of feeding (Fig. 3B). However, the lysozyme activity in fish skin mucus increased significantly in Pa0.2, Pg0.2 and Eh2.0 groups in W8. In contrast, the lysozyme activity in skin mucus decreased and did not show any significant difference between extract-enriched diets and the control group after the bacterial challenge test.

3.3. Plasma natural hemolytic complement activity

The plasma natural complement activity of striped catfish fed with different experimental diets is shown in Fig. 4. Among the 5 extracts, fish fed with Pa0.2, Pg0.2, and Mp2.0 had a substantially increased plasma natural complement level in W4. At the end of the feeding trial, the ACH50 level was comparatively increased in most of extract groups, which increased relative to the feeding period. Moreover, the ACH50 activity was still higher in Pg and Mp groups compared to other extract groups and statistically different to the control at 3 dpi ($p < 0.05$).

3.4. Total immunoglobulin

The plasma total Ig started to significantly increase in fish fed diets containing Pa, Mp, Eh, Pg1.0, and Ai2.0 (Fig. 5A) in W4. After 8 weeks of feeding, plasma total Ig was significantly higher in Pa1.0, Mp0.4, and Pg groups than in other groups, while no significant differences in total Ig were observed between treatments after injection with *E. ictaluri*.

The maximum total Ig was recorded in skin mucus of fish fed Pa1.0 compared to other groups in W4. The total Ig significantly increased in Pa, Pg, Mp2.0, and Eh2.0 groups compared to control in W8. Striped catfish fed Pg, Mp2.0, and Eh0.4 supplemented diets showed higher total Ig compared to other diets after the bacterial challenge test (Fig. 5B).

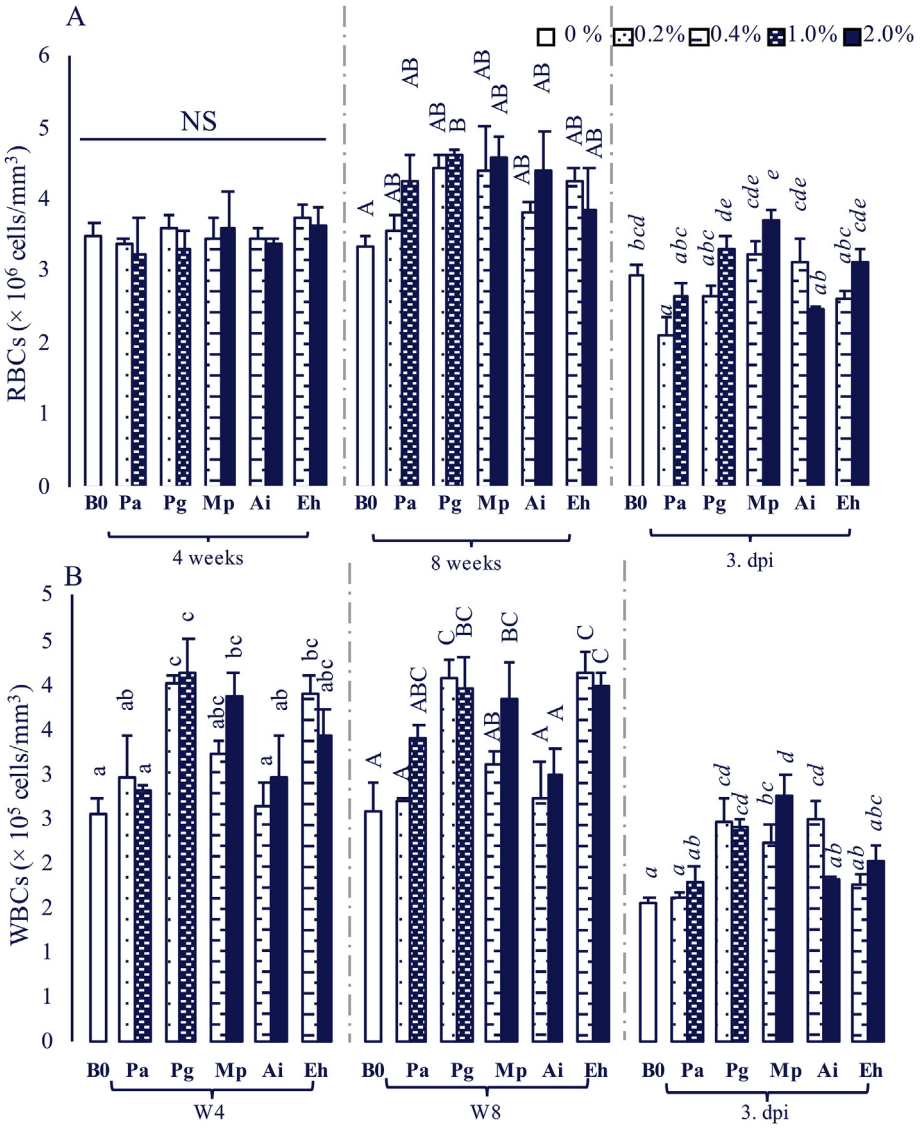

Fig. 1. Effects of dietary plant extract administrations on A) total RBCs and B) total WBCs of striped catfish at different sampling times (W4, W8 and 3 dpi). Values are mean ± SEM, different letters indicate significant differences between treatments ($p < 0.05$), NS: non-significant.

3.5. Disease resistance of striped catfish against E. ictaluri

Mortality of striped catfish occurred from day 4 to day 8 after infection with *E. ictaluri* (Fig. 6). A maximum of 92.6% mortality rate was recorded in the control group. Mortality was significantly reduced in fish fed plant extract-based diets, especially in Pg, Mp, Pa0.2, Eh2.0, and Ai0.4 groups. Of the different plant extract diets, Mp0.4 and Mp2.0 displayed the highest survival rate post-challenge (58.3% and 55.6%, respectively). No mortalities were recorded in the negative control group. Moreover, bacterial identification found that *E. ictaluri* was detected in all bacterial infection samples (Fig. 7).

The differential effects of striped catfish extract-based diets on immune parameters and resistance to *E. ictaluri* were summarized in Table 2.

4. Discussion

In the present study, the immunomodulatory effects of different plant extracts were assessed by investigating the changes in 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 vitro* screening study. In the previous study, we found that *E. hirta, P. amarus*, *P. guajava* and *A. indica* extracts could strongly stimulate some immune markers, while *M. pudica* did not affect the immune responses in head kidney leukocytes of striped catfish after 24 h of *in vitro* culture. 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 [9,10]. Moreover, Pa extracts contain three important bioactive constituents (phyllanthin, hypophyllanthin, and corilagin) [45], while guajaverin, quercetin, avicularin, and guavinoside have been found in Pg extracts [46]. Due to the presence of the various compounds and secondary metabolites, the plant species were reported to display immunomodulatory activities [5–8,47,48]. Extraction efficiency is partly affected by the type of solvents with varying polarity in the same conditions of pH, temperature as well as extraction time [49,50]. Total phenol and flavonoid contents of rice paddy-*Limnophila aromatica* (Lamk.) Merr in pure ethanol extract were higher than that in pure acetone, pure methanol and water extracts, although the extraction yield was highest in methanol and decreased following by water, ethanol and acetone [50]. The biological compounds in the extracts including phenols, gallic acid, myricetin were also variable after extraction in different solvents [51]. Moreover, methanol and ethanol were the best solvents for extraction of biological

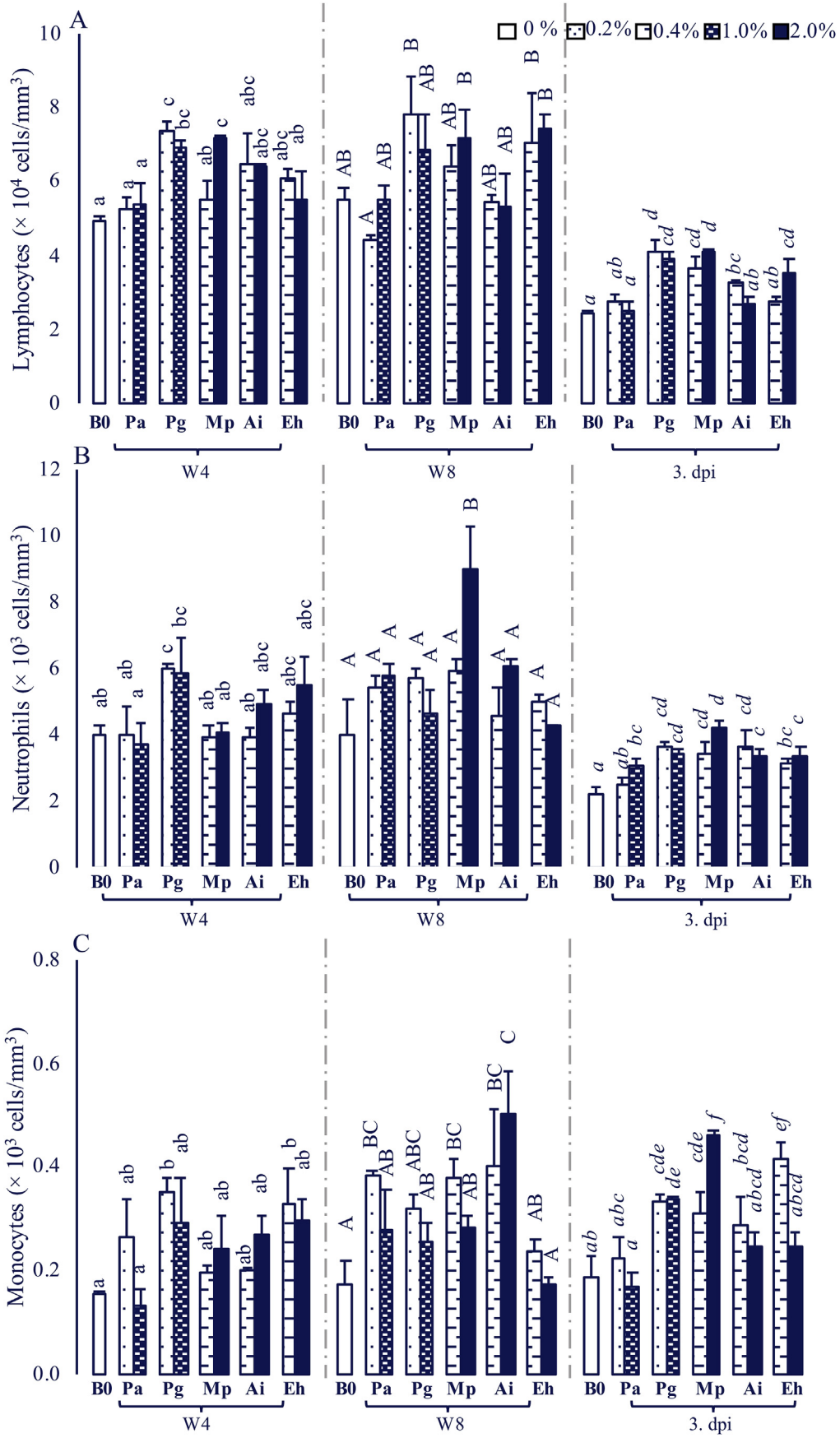

Fig. 2. Effects of dietary plant extract administrations on A) lymphocyte, B) neutrophil and C) monocyte numbers of striped catfish at different sampling times (W4, W8 and 3 dpi). Values are mean ± SEM, different letters indicate significant differences between treatments ($p < 0.05$).

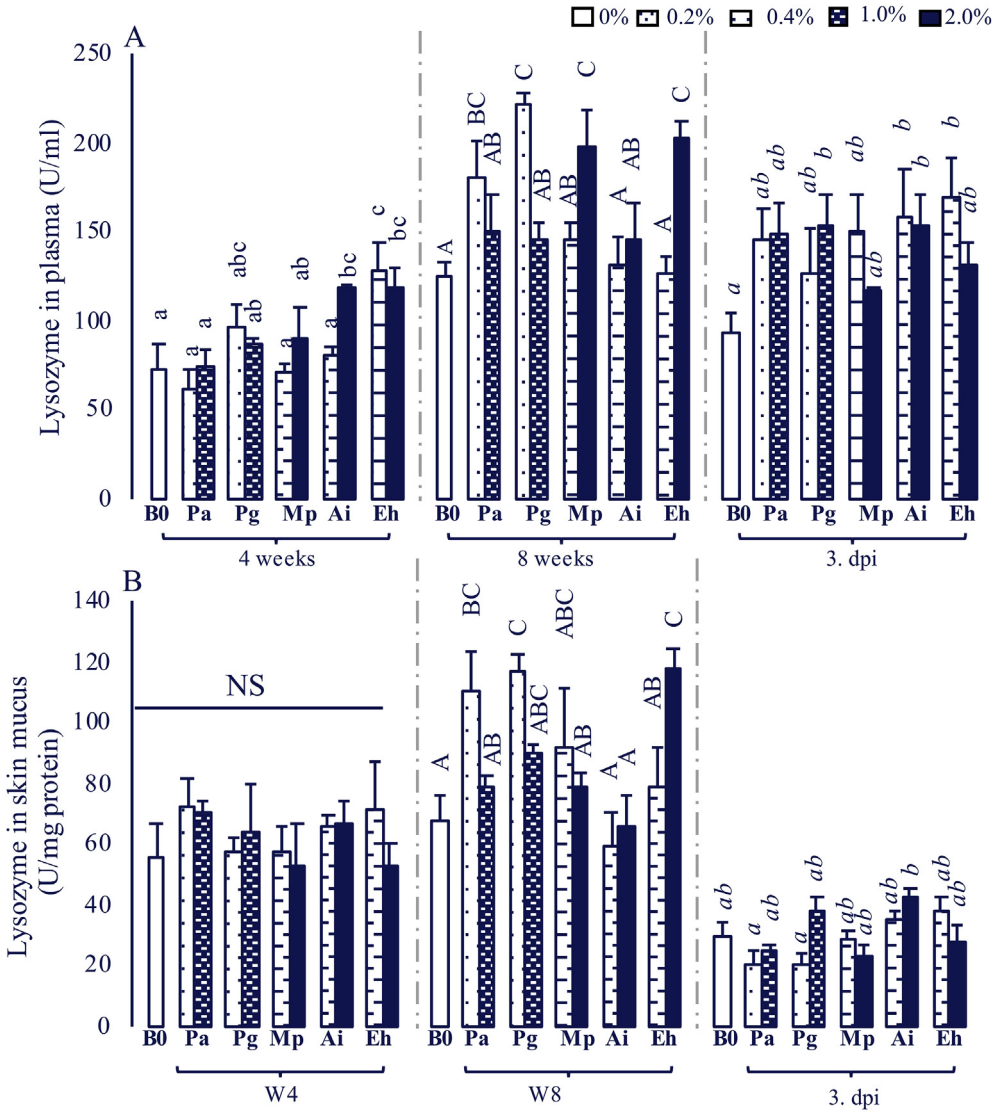

Fig. 3. Effects of dietary plant extract administrations on A) plasma and B) skin mucus lysozyme activities of striped catfish at different sampling times (W4, W8 and 3 dpi). Values are mean ± SEM, different letters indicate significant differences between treatments ($p < 0.05$). NS: non-significant.

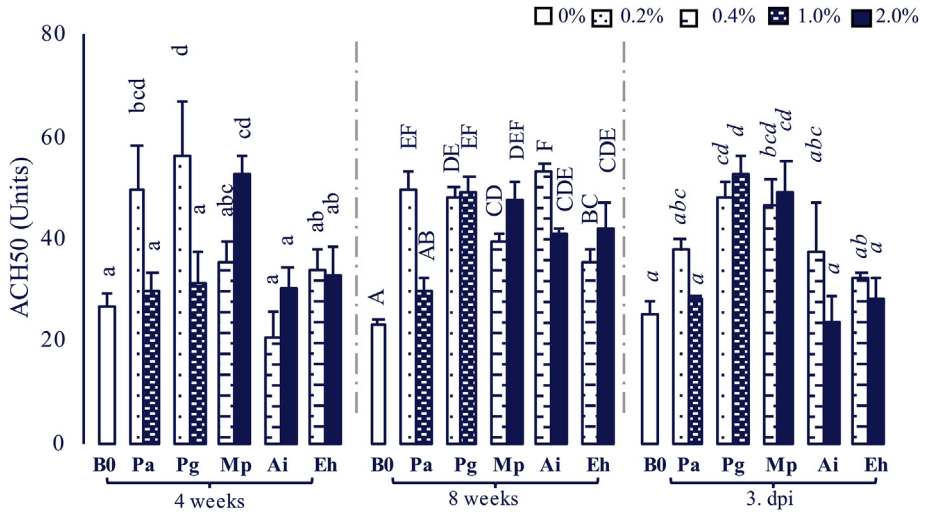

Fig. 4. Effects of dietary plant extract administrations on ACH50 activity of striped catfish at different sampling times (W4, W8 and 3. dpi). Values are mean ± SEM, different letters indicate significant differences between treatments ($p < 0.05$).

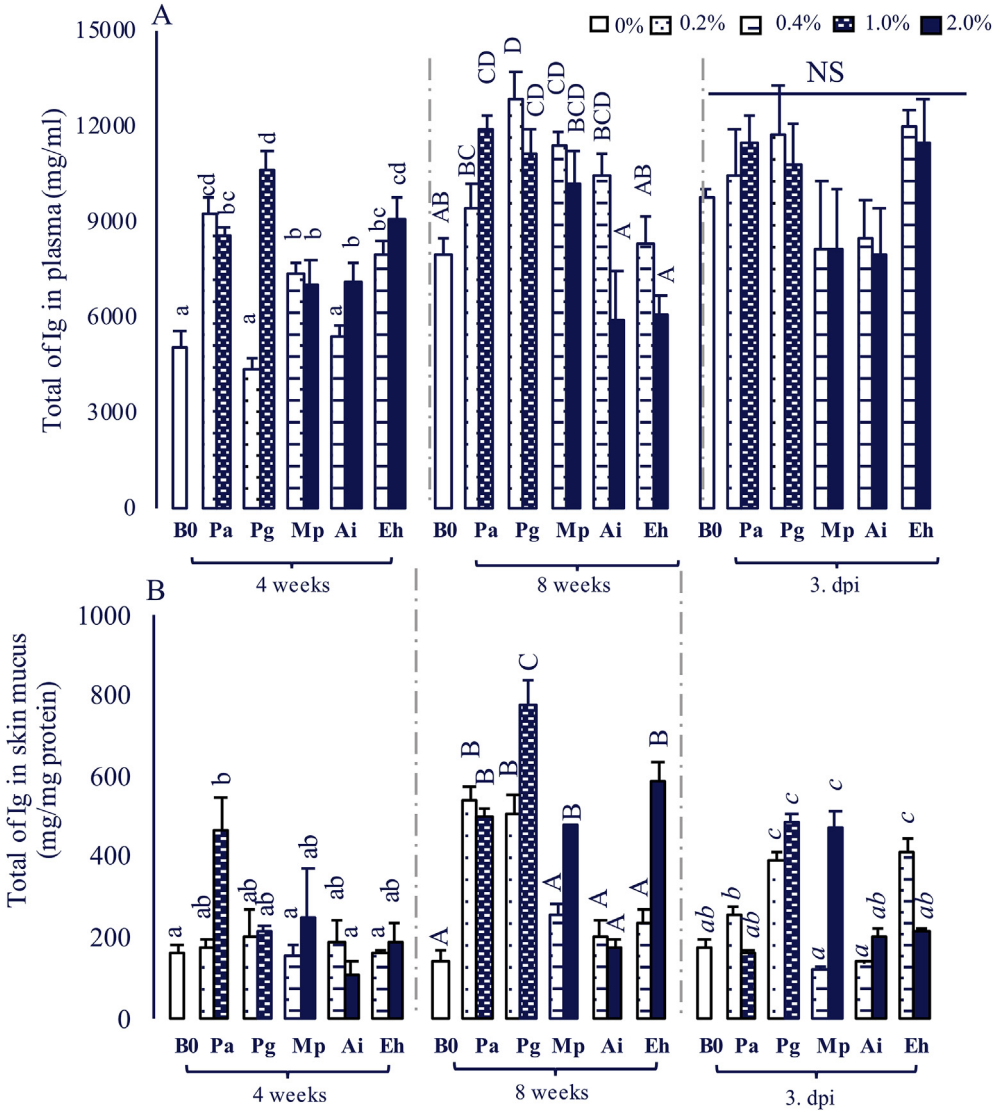

Fig. 5. Effects of dietary plant extract administrations on A) plasma and B) skin mucus total immunoglobulin of striped catfish at different sampling times (W4, W8 and 3 dpi). Values are mean ± SEM, different letters indicate significant differences between treatments ($p < 0.05$). NS: non-significant.

components, which mainly functions in immunostimulatory properties and antibacterial activity [52,53].

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 [54]. In this study, plant extract-enriched diets did not affect the total RBCs in W4, whereas WBCs, lymphocytes, neutrophils, and monocytes started to increase in a dose dependent manner in fish fed enriched diets from W4. The RBC counts were significantly reduced in Pa groups at 3 dpi compared to controls. This may be due to the presence of higher quantities of tannins and saponins in the ethanolic Pa extract than in other extracts. These compounds have previously been associated with a significant reduction in the number of RBCs [55]. In addition, plants containing polysaccharides usually induce a proliferation of lymphocytes [56–58], 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 Pg, Mp, and Eh stimulated a significant increase in blood indices in W4, W8, and 3 dpi. A study by Panase et al. [59] demonstrated that WBC numbers were significantly different in hybrid catfish (*Clarias macrocephalus* × *C. gariepinus*) fed Eh leaf extract, although RBCs did not significantly increase on days 30 and 90 of the experiment. Yeganeh et al. [60] also found that RBCs, WBCs, and haemoglobin significantly increased in rainbow trout (*Oncorhynchus mykiss*) after oral diets containing *Spirulina platensis* over 10 weeks. A similar increase in WBCs was reported in common carp when this species was daily dip treated for 10 min over 30 days with an aqueous Ai leaf extract at 1 g/L [61]. 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 triggering of striped catfish immunity.

Lysozyme is an important indication of the non-specific humoral immune response. It is primarily released by monocytes and macrophages, and this is higher in macrophages [62]. The present results showed that fish in the groups fed extracts had dose-dependent enhanced plasma lysozyme levels compared to the control throughout the 8-week feeding experiment. Measurement of lysozyme activity is a way to determine whether non-specific immune responses are boosted by immunostimulants. As mentioned earlier, plasma lysozyme level was shown to increase in striped catfish after injection of LPS and levamisole [5,6]. Similarly, Giri et al. [63] found that the level of plasma lysozyme gene expression significantly increased in juvenile rohu after they were fed Pg supplemented diets for 60 days. The same results were

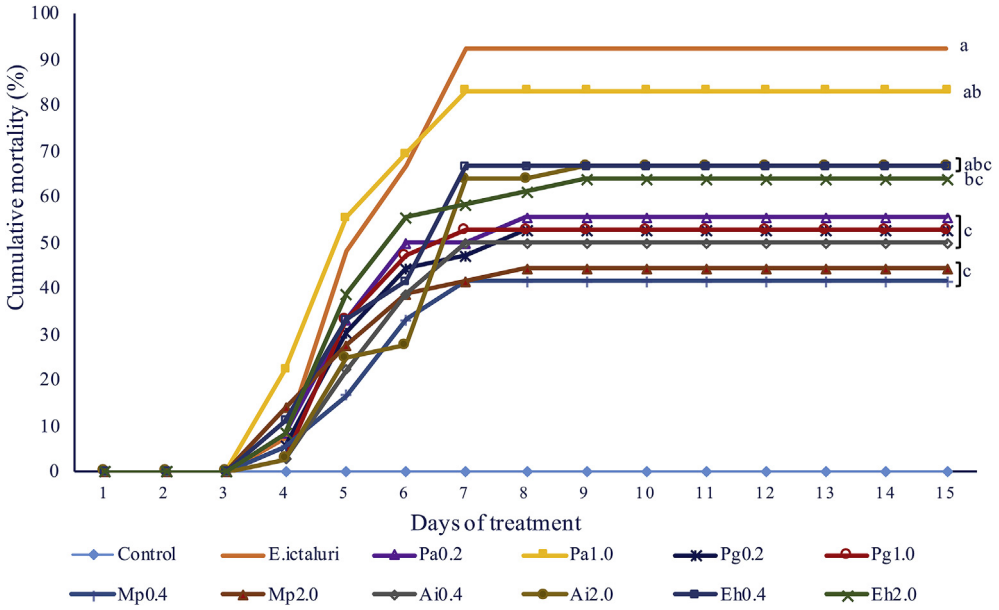

Fig. 6. Fish cumulative mortality of challenge test with *E. ictaluri* at W8. Values are mean ± SEM, different letters indicate significant differences between treatments ($p < 0.05$).

presented by Gobi et al. [16] when tilapias (*Oreochromis mossambicus*) were fed diets containing guava leaf extract for 30 days. Hoseinifar et al. [64] also reported that the enrichment of diets with 0.25% Pg increased the plasma lysozyme activity in common carp fingerlings in an 8-week feeding trial. However, the lysozyme activity does not always increase in fish after being stimulated. Some plant extracts may act as an immunosuppressor of the innate immune system [65,66]. This may be due to the presence of some phenolic compounds in the plant extracts which could inhibit the enzyme activity. Compounds including flavonoids, for example, strongly bind to lysozyme due to the number and position of hydrogen and type and position of glycosides [67].

Besides the non-specific humoral immune system, lysozyme possesses both bactericidal and opsonin effects that activate the complement system and phagocytes which prevent infectious diseases [62]. In the present study, the plasma complement (ACH50) levels were not significantly enhanced before W4 in most of groups fed with extract-enriched diets, except in Ai2.0 and Eh (Eh0.4 and Eh2.0) groups. In contrast, a statistical increase of ACH50 activity was noted when fish were fed with both doses of Pg, Mp, Ai, Eh, and 0.2% of Pa in W8. This outcome could suggest that the increment of plasma ACH50 activity was time dependent and affected by the length of the extract feeding period. These results are consistent with reports of tilapia fed guava leaf extract-enriched diets, which could significantly enhance ACH50 levels after 60 days of feeding [63]. However, Gobi et al. [16] found that guava leaf extract (0.5 and 1.0%) could considerably raise the ACH50 activity in tilapia in a shorter 30-day feeding trial. A similar result was also reported by Bahi et al. [68], these authors indicate that dietary administration of fenugreek (*Trigonella foenum graecum*) seeds, alone or combined with *Bacillus licheniformis* (TSB27), *Lactobacillus plantarum*, or *Bacillus subtilis* (B46) strongly enhanced ACH50 activity after 2 and 3 weeks of a feeding experiment. Concerning the fish fed diets supplemented with Pa extracts, the humoral innate immune responses (lysozyme and complement activities) were only found to considerably increase at the lowest level of inclusion. There was lack of increment in lysozyme and complement levels in the groups fed with Pa at higher concentrations throughout the experimental period. Therefore, boosted immune responses were also affected by the dose of all the extract-supplemented diets.

Aside from the humoral innate immune responses, total immunoglobulin plays an essential role in host defense mechanisms and acts as biomarker for the fish adaptive immune response [69]. In the present study, total Ig significantly increased in extract-enriched diets in both W4 and W8, whereas the total Ig level did not significantly differ between the treatments after injection with *E. ictaluri*. The total Ig activity was also found to be significantly enhanced in koi carp (*Cyprinus carpio koi*) fingerlings after feeding with edible eryngii mushroom powder (*Pleurotus eryngii)* for 63 days [70]. Similar increments of total Ig activity were observed in common carp fed jujube (*Ziziphus jujube*) fruit extract for 8 weeks [34]. Moreover, Laltlanmawia et al. [71] showed that the total Ig content greatly increased for a shorter duration of experiment, 15 and 30 days, when rohu were fed diets supplemented with a mixture of *Withania somnifera* (Ashwagandha) root extract and vitamin C. With regard to Eh extracts, the high and low dose Eh groups had a significantly enhanced total Ig level in W4 compared to control, while Eh did not affect the total Ig after 8 weeks of feeding. Thus, the duration of feeding should also be considered for each plant extract.

Skin is the first physical barrier against the invasion of environmental pathogens in fish [72]. Moreover, skin mucus possesses various

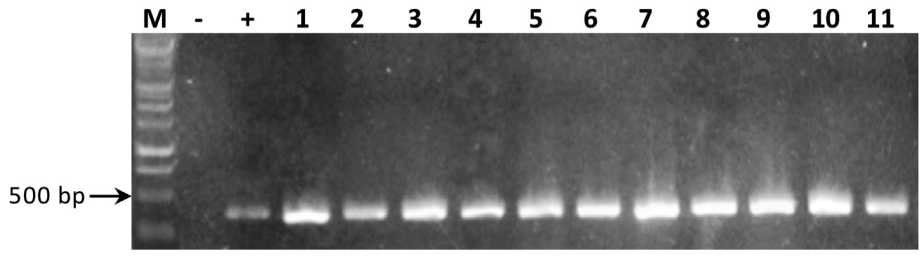

Fig. 7. Bacteria confirmation by PCR (M: marker; (−): negative control, (+): positive control, 1–11: fish in each diet were injected with bacteria).

Table 2
Summary results of the analysis of striped catfish immune parameters in short- and long-duration of striped catfish extract-based diets at different doses.

Plant extract	Conc. (%)	Time	Blood parameters					Lysozyme		ACH50	Total Ig		Mortality
			RBCs	WBCs	Lymphocytes	Monocytes	Neutrophils	Skin	Serum	Serum	Skin	Serum	
Pa		W4	NS	NS	NS	NS	NS	NS	NS	+	NS	+	
	0.2	W8	NS	NS	NS	+	NS	+	+	+	+	NS	
		3.dpi	NS	NS	NS	NS	NS	NS	NS	NS	+	NS	+
		W4	NS	NS	NS	NS	NS	NS	NS	NS	+	+	
	1.0	W8	NS	NS	NS	NS	NS	NS	NS	NS	+	+	
		3.dpi	NS	NS	NS	NS	+	NS	NS	NS	NS	NS	NS
Pg		W4	NS	+	+	+	+	NS	NS	+	NS	NS	
	0.2	W8	NS	+	NS	NS	NS	+	+	+	+	+	
		3.dpi	NS	+	+	+	+	NS	NS	+	+	NS	+
		W4	NS	+	+	NS	NS	NS	NS	NS	NS	+	
	1.0	W8	+	+	NS	NS	NS	NS	NS	+	+	+	
		3.dpi	NS	+	+	+	+	NS	+	+	+	NS	+
Mp		W4	NS	NS	NS	NS	NS	NS	NS	NS	NS	+	
	0.4	W8	NS	NS	NS	+	NS	NS	NS	+	NS	+	
		3.dpi	NS	+	+	+	+	NS	NS	+	NS	NS	+
		W4	NS	+	+	NS	NS	NS	NS	+	NS	+	
	2.0	W8	NS	+	NS	NS	+	NS	+	+	+	NS	
		3.dpi	+	+	+	+	+	NS	NS	+	+	NS	+
Ai		W4	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	
	0.4	W8	NS	NS	NS	+	NS	NS	NS	+	NS	NS	
		3.dpi	NS	+	+	NS	+	NS	+	NS	NS	NS	+
		W4	NS	NS	NS	NS	NS	NS	+	NS	NS	+	
	2.0	W8	NS	NS	NS	+	NS	NS	NS	+	NS	NS	
		3.dpi	NS	NS	NS	NS	+	NS	+	NS	NS	NS	NS
Eh		W4	NS	+	NS	+	NS	NS	+	NS	NS	+	
	0.4	W8	NS	+	NS	NS	NS	NS	NS	+	NS	NS	
		3.dpi	NS	NS	NS	+	+	NS	+	NS	+	NS	NS
		W4	NS	NS	NS	NS	NS	NS	+	NS	NS	+	
	2.0	W8	NS	+	NS	NS	NS	+	+	+	+	NS	
		3.dpi	NS	NS	+	NS	+	NS	NS	NS	NS	NS	+

+: increase significant level at $p < 0.05$, NS: non-significant. W4: week 4th, W8: week 8th and 3. dpi: three-day post infection; Pa: *Phyllanthus amarus*, Pg: *Psidium guajava*, Mp: *Mimosa pudica*, Ai: *Azadirachta indica*, Eh: *Euphorbia hirta*; RBCs: Red blood cells, WBCs: White blood cells, ACH50: Alternative complement pathway hemolytic activity; Total Ig: Total immunoglobulin.

cellular (i.e., B, T, and mast cells, macrophages, and granulocytes) and humoral (i.e., complement proteins, immunoglobulins, lectins, lysozyme, proteases, and antimicrobial peptides) immune parameters [73]. In this study, the changes in lysozyme and total Ig were evaluated in the skin mucus of striped catfish. The results showed that dietary administration of plant extracts did not enhance lysozyme activity in skin mucus after 4 weeks, whereas the levels of lysozyme increased significantly in Pa0.2, Pg0.2, and Eh2.0 groups after 8 weeks. In addition, the extract supplemented diets induced stronger effects on total Ig activity of skin mucus in W4, W8, and 3 dpi. In line with our results, Hoseinifar et al. [74] demonstrated that medlar (*Mespilus germanica*) leaf extract stimulated the increase of lysozyme and total Ig activities in skin mucus of common carp after 49 days of a feeding trial. Oral administration of myrtle (*Myrtus communis*) significantly improved mucosal immune responses (the activity of lysozyme, total Ig, and protease) in zebrafish (*Danio rerio*) for 60 days [75]. In contrast, Taee et al. [76] showed no significant differences in skin mucus lysozyme activity between myrtle extract treatments and control groups in rainbow trout during a 60 day feeding period. It can be concluded that treatment with the same immunostimulants may have varying impacts on immune parameters in different animal species.

The elevation of blood parameters in conjunction with the enhanced immune response (humoral and mucosal) possibly 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* in a dose dependent manner. 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 Mp groups at 41.7% and 44.4% respectively. To the best of our knowledge, no publications so far have reported the effects of Mp extract-enriched diets on disease resistance in aquatic animals. In parallel, Pg extract diets showed significantly improved survival after 8 weeks of feeding. These results are not only comparable but they also confirm earlier studies that reported a significantly reduced mortality rate after *Aeromonas hydrophila* infection in rohu [63,77] and tilapia [16] fed guava leaf extract-based diets. Moreover, PCR confirmation detected the 16s RNA gene of *E. ictaluri* in most treatments (except the negative control group), suggesting that *E. ictaluri* mainly caused the mortality in striped catfish after the challenge test.

In conclusion, this study confirmed the positive effects of dietary supplementation with plant extracts by improving the immune response of striped catfish. The results demonstrated that both hematological parameters and humoral immune responses of striped catfish were differently enhanced according to the dose and type of extract, as well as to the duration of feeding. Extract dietary administration of *P. guajava* at 0.2 and 1.0%, and *M. pudica* at 2.0% for 8 weeks have the optimal potential for modulating blood parameters and immune responses (humoral and mucosal) in striped catfish, and provided better protection against *E. ictaluri* infection. Further studies are needed to determine the most suitable dose and duration of plant extract treatment in boosting striped catfish immune responses. Moreover, the appetence of the different plant extract-based diets should be investigated, as this can influence feed intake and thus the resulting growth and protection efficiency against pathogens.

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