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Screening of immuno-modulatory potential of different herbal plant extracts using striped catfish (*Pangasianodon hypophthalmus*) leukocyte-based *in vitro* tests

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ABSTRACT

Many medicinal plants have been shown to possess biological effects, including immuno-modulatory activities on human and other mammals. However, studies about the potential mechanisms of plant extracts on the humoral and tissular immunities in fish have received less attention. This study aimed to screen the immunestimulating properties of 20 ethanol plant extracts on striped catfish *Pangasianodon hypophthalmus* leukocytes. The peripheral blood mononuclear cells (PBMCs) and head kidney leukocytes (HKLs) of striped catfish (50 ± 5 g per fish) were stimulated at 10 and 100 µg of each plant extract per mL of cell culture medium. Several humoral immune parameters (lysozyme, complement and total immunoglobulin) were examined at 24-h post stimulation (hps). Furthermore, the responses of four cytokine genes, namely *il1β*, *ifr*γ 2a and b, and *mhc* class II were assessed by quantitative real-time PCR at 6, 12, 24, and 48 hps. The results showed that lysozyme, complement as well as total immunoglobulin levels in both PBMCs and HKLs were regulated by some of the plant extracts tested in a concentration-dependent manner; some plant extracts induced the highest immune responses at the low dose (10 µg mL^{-1}) while others were more efficient at high dose (100 µg mL^{-1}). Among the extracts, five extracts including garlic *Allium sativum* L. (As), neem *Azadirachta indica* A. Juss (Ai), asthma-plant *Euphorbia hirta* L. (Eh), bhumi amla *Phyllanthus amarus* Schum. et Thonn (Pa), and ginger *Zingiber officinale* Rosc (Zo) induced significant changes in the expression of pro-inflammatory cytokine (*il1β*), antiviral cytokines (*ifr*γ 2a and b) and adaptive immune cytokine (*mhc* class II) in striped catfish cells. Pa always modulated the strongest expression of the four cytokines in PBMCs and HKLs over the whole experimental period ($p < 0.05$), whereas Zo did not stimulate the *mhc* class II expression in striped catfish leukocytes throughout experimental periods. These *in vitro* results demonstrated that some plant extracts could differently modulate great potential immune response in fish, supporting their applications in further *in vivo* experiments.

1. Introduction

In fish, there is an interaction between innate and acquired immunity, although the adaptive immune response is more limited than that of higher vertebrates [1,2]. It is well known that components of the immune response include humoral, cellular receptor molecules and

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Table 1
List of crude ethanol extracts.

No	Family	Scientific name	Abbreviation	Part studied
1	*Asteraceae*	*Ageratum conyzoides* L.	Ac	Leaves, stem
2	*Meliaceae*	*Azadirachta indica* A. Juss	Ai	Leaves
3	*Annonaceae*	*Annona reticulate* L.	Ar	Leaves
4	*Alliaceae*	*Allium sativum* L.	As	Bulbs
5	*Amaranthaceae*	*Alternanthera sessilis* (L.) A. DC	Ase	Leaves, twigs
6	*Apiaceae*	*Centella asiatica* (L.) Urb	Ca	Whole plant
7	*Vitaceae*	*Cayratia trifolia* (L.) Domin	Ct	Leaves, stem
8	*Euphorbiaceae*	*Euphorbia hirta* L.	Eh	Leaves, twigs
9	*Asteraceae*	*Eclipta prostrata* (L.) L.	Ep	Leaves, twigs
10	*Saururaceae*	*Houttuynia cordata* Thunb.	Hc	Leaves, stem
11	*Cucurbitaceae*	*Momordica charantia* L.	Mc	Leaves
12	*Fabaceae*	*Mimosa pudica* L.	Mp	Leaves, twigs
13	*Lamiaceae*	*Ocimum basilicum* L.	Ob	Leaves, twigs
14	*Phyllanthaceae*	*Phyllanthus amarus* Schum. et Thonn.	Pa	Leaves, twigs
15	*Piperaceae*	*Piper betle* L.	Pb	Leaves
16	*Lamiaceae*	*Perilla frutescens* (L.) Britt	Pf	Leaves, twigs
17	*Myrtaceae*	*Psidium guajava* L.	Pg	Leaves
18	*Portulacaceae*	*Portulaca oleracea* L.	Po	Whole plant
19	*Compositae*	*Wedelia chinensis* (Osbeck) Merr	Wc	Whole plant
20	*Zingiberaceae*	*Zingiber officinale* Rosc.	Zo	Bulbs

central organs whose main function is involved in immune defense [3,4]. The humoral immunity consists of lysozyme, hemolysin, immunoglobulins and complement molecules, whereas cellular immunity includes phagocytic cells, neutrophils, natural killer cells and lymphocytes [5–10]. Moreover, cytokines – including tumor necrosis factor-TNF-α, interferons- IFNs, and pro-inflammatory interleukins (*i.e.* IL-1β and IL-8) - also play an important role in the immune system. They could bind to specific receptors present in the cell membrane and lead to induction, enhancement or inhibition of a number of regulatory genes in the nucleus [11–13].

Striped catfish is one of the most important commercial fish species cultured in Vietnam in general, and Mekong Delta in particular. Like other fish species, striped catfish also suffers from many kinds of diseases including bacteria, fungi and parasites under stress conditions (*e.g.* poor water quality and/or high density of fish) [14,15]. Such diseases have been susceptible to cause enormous economic losses and reduced profit margins of the aquaculture sector [16]. In recent years, to resolve the mortality problem, many attempts have been made to strengthen striped catfish immune system. Administration of live or heat killed *Aeromonas hydrophila* by intraperitoneal injection strongly stimulated phagocytosis, respiratory burst, complement, lysozyme, plasma peroxidase, total IgM, and pathogen-specific antibody IgM [17]. Moreover, dietary supplementation with immunostimulants (*e.g. Escherichia coli* lipopolysaccharide, levamisole, and β-glucan) or probiotics (*e.g. Bacillus amyloliquefaciens* 54A and *Bacillus pumilus* 47B) significantly enhanced respiratory burst activity, lysozyme activity, complement activity, plasma anti-proteases, natural antibody titer and total protein. Furthermore, these compounds were also potentially involved in fish defense mechanism against *Edwardsiella ictaluri* infection [18–22].

Nowadays, herbal immunostimulants have been used with success as a more environmentally friendly approach in disease management. Herbal extracts are sources of many active components among which alkaloids, steroids, phenolics, tannins, terpenoids, saponins, and flavonoids that possess various biological activities [23–30]. These herbs have been supplied in fish diets to enhance the innate immune system, as general preventive measures in aquaculture [25,31–38]. Dietary supplementation of Zo improved the non-specific immunity of Asian sea bass (*Lates calcarifer*) as showed by Talpur et al. [39]; whereas, Pratheepa and Sukumaran [40] found out an increase of phagocytic activity in common carp *Cyprinus carpio* proportionally to the dietary level of Eh. Gobi et al. [41] also proved that the ethanol leaf extract from Pg could play a major role in enhancing growth, antioxidant enzymes and immune variables in *Oreochromis mossambicus* compared with aqueous leaf extracts.

Up to now, most studies have investigated the effects of dietary herbal extracts on some indicators of the immune response in different fish species. However, there is limited information regarding the potential mechanisms of action of plant extracts on humoral and/or anti-inflammatory effects in fish immune cells. Sen et al. [42] reported that a flavonoid fraction of *P. guajava* leaf extract attenuated of LPS inducible *tnfα*, *il1β*, nitric oxide synthase-*inos* and cyclooxygenase-*cox*2 mRNA expression levels via the inhibition of nuclear factor kappa-light-chain-enhancer - NF-κB of activated B cells in roho labeo (*Labeo rohita*) macrophages. Na-Phatthalung et al. [43] observed the induction of some cytokine genes (*il1β*, *il8*, *il10, tnfα* and *tgfβ*) on rainbow trout *Oncorhynchus mykiss* head kidney macrophages by stimulating with rose myrtle *Rhodomyrtus tomentosa* leaf extract and its active compound, rhodomyrtone. In this respect, *in vitro* approaches may provide interesting hypotheses on the potential mechanisms of the direct action of plant extracts (or their active compounds) on fish immune cells before their validation through *in vivo* experiments. Moreover, these tests can substantially reduce the time and the number of experimental animals for *in vivo* study as well as reduce the economic costs and the ethical problems.

Based on bibliography review data and on a survey in fish farms of Mekong Delta, 20 plants possessing potential immunostimulatory activities were selected for *in vitro* testing. In the present study, the immunomodulatory effects of ethanol plant extracts on the lysozyme and complement activities as well as on the total immunoglobulin in the striped catfish peripheral blood mononuclear cells -PBMCs and head kidney leukocytes- HKLs were analyzed. After this first screening on 20 plant extracts, mRNA expression of cytokines (*il1β*, *ifnγ* 2a and 2b, *a2 mhc* class II integral membrane protein alpha chain 2- *mhc* class II) belonging to the innate and adaptive immunity was investigated at several time points after different stimulations with the five best extracts selected on the basis of the *in vitro* immune responses.

2. Material and methods

2.1. Extracts preparation

Twenty fresh plants were collected from Mekong Delta in Vietnam (Table 1). The plants were authenticated at the Department of Biology, College of Natural Science, Can Tho University. All collected parts of the plants were washed away from mud and dust; the rotten and

Table 2
Primers used for *P. hypophthalmus* immune gene expression [21,54].

No.	Gene name		Primer Sequence (5′-3′)	Calculated efficiencies (%)	Length (bp)	Accession number	Ref. Seq_Species
	Adaptive response						
1	a2 MHC class II integral membrane protein alpha chain 2		F: GAGCTCAACACTCAGCCAGT R: CACACCAGGAAGCTCCACAT	105	172	30783	*Danio rerio*
	Cytokine						
2	Interleukin-1β		F: CAGAGGCTGAAGCACACTCA R: CCTTGTCCTGCCTGCTGTAA	99	148	100304696	*Ictalurus punctatus*
3	Interferon γ	2a	F: TATGTCACTGAGCTGCTGGC R: TTAGCTTGACGTCGTCTCCG	96	143	N185453	*Pangasianodon hypophthalmus*
4		2b	F: TCCCAACCCTGCCAAATTGT R: GCCTCATTCTCCATCCAGGT	96	150	JN18545	
	Housekeeping Gene (Reference gene)						
5	16S rRNA		F: TATCTTCGGTTGGGGCG R: CCTGATCCAACATCGAGG	98	223	FJ432682	*Pangasianodon hypophthalmus*

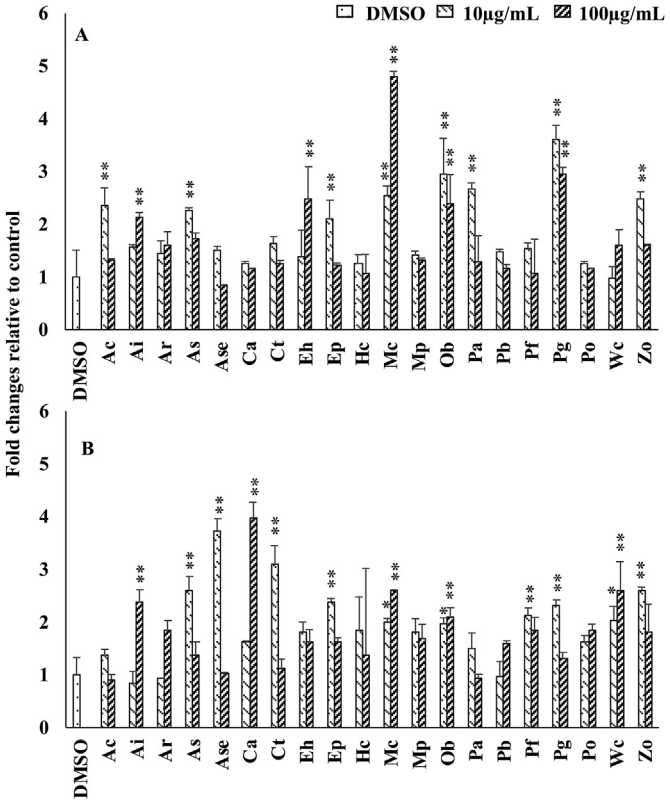

Fig. 1. Lysozyme activity of A) PBMCs and B) HKLs of the striped catfish in the plant extract treated groups and control group (DMSO). Asterisk indicates significant differences in lysozyme levels between stimulated and unstimulated cells at 24hps (*$p < 0.05$; **$p < 0.01$). Values are mean ± S.D. (n = 3).

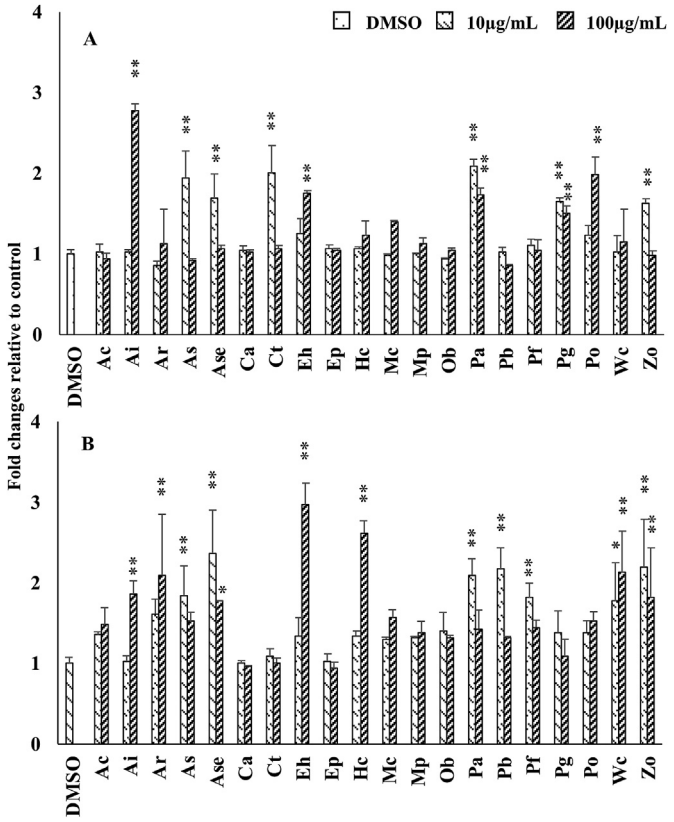

Fig. 2. ACH50 of A) PBMCs and B) HKLs of the striped catfish in the plant extract treated groups and control group (DMSO). Asterisk indicates significant differences in ACH50 levels between stimulated and unstimulated cells at 24hps (*$p < 0.05$; **$p < 0.01$). Values are mean ± S.D. (n = 3).

damaged parts were discarded. The plants were air dried in shade for several days and then in oven at about 60 °C until well-dried. After that, they were grounded into fine powder by blender and stored in sealed containers in a dry and cool place.

The dried powder was soaked in ethanol 96% (volume ratio 1: 20) for at least 24 h at room temperature with frequent agitation until the soluble matter had dissolved. The solvent-containing extracts were decanted and filtered. The ground samples were further extracted repeatedly four times with ethanol 96%. The filtrate from each extraction were combined and the 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 used. The extracts were re-dissolved in dimethyl sulfoxide (DMSO, Saint Louis, MO, US) in order to prepare stock solution at 20 and 2 mg mL^{-1} and use in the following assays.

2.2. Experimental fish

Total 150 striped catfish juveniles (body weight = 50 ± 5 g) were acclimated to laboratory conditions for 15 days at 28 ± 2 °C in composite tank (2000 L). Fish were fed twice (9 am and 3 pm) daily at a feeding rate of 1% of body weight with a commercial feed (30% crude proteins, 2.5 mm, Proconco) under a natural photoperiod prior to their use in the *in vitro* assay. The health status of experimental fish was checked following the method described by Biswas et al. [44] with slight modifications. Briefly, few randomly sampled animals were examined for the presence of any abnormal lesions or parasites on body

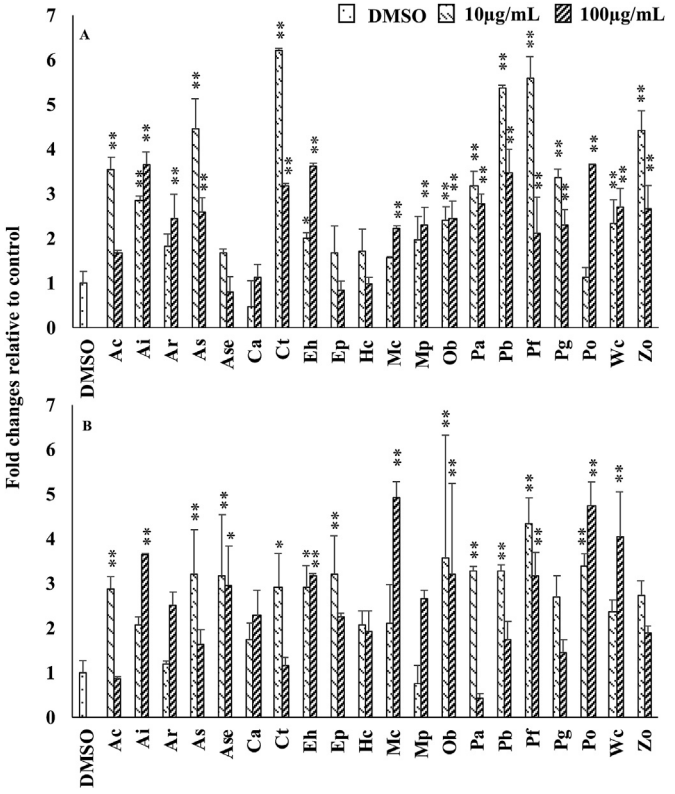

Fig. 3. Total Ig of A) PBMCs and B) HKLs of the striped catfish in the plant extract treated groups and control group (DMSO). Asterisk indicates significant differences in Ig levels between stimulated and unstimulated cells at 24hps (*$p < 0.05$; **$p < 0.01$). Values are mean ± S.D. (n = 3).

surfaces and internal organs [45]. Further, smears head kidney and blood from the same fish were cultured on tryptic soy agar plate (TSA, Merck) for 24–48 h at 28 °C for existence of any bacterial pathogens. Any colonies presented on TSA plate were used to perform PCR for detecting 16s RNA genes of commonly bacterial pathogens (*A. hydrophyla, E. ictaluri* and *Flavobacterium columnare*) in striped catfish [46]. Healthy fish which did not present any pathogenic bacteria, were used for experiment.

2.3. Isolation of PBMCs and HKLs

The isolation of PBMCs was performed according to the methods of Boyum [47] modified by Pierrard et al. [48]. Briefly, blood was aseptically collected from the caudal vein with a sterile heparinized syringe. 2.5 mL of heparinized blood were quickly diluted with 4 mL of phosphate-buffered saline (PBS 1X); the mixture was then poured over a layer of 6 mL Ficoll Paque Plus (1.077 g mL^{-1}, GE Healthcare, Uppsala, Sweden) and centrifuged (400 g, 20 min, 28 °C). The white cells at the interface were collected and slowly washed twice with 5 mL cold sterile PBS 1X at low speed centrifugation (1000 g, 7 min, 4 °C).

Head kidney tissue was aseptically excised from freshly euthanized striped catfish and gently pushed through a 40-μm nylon mesh (VWR International, LLC, Radnor, PA USA) with L-15 medium (pH 7.4, Sigma-Aldrich, St. Louis, MO, USA) supplemented with a 1% solution of 10,000 μg mL^{-1}streptomycin +10,000 U mL^{-1} penicillin (Invitrogen).

After washing, both PBMCs and HKLs were removed from residual erythrocytes by incubating them 5 min with an osmotic shock sterile red blood cell lysis buffer (pH 7.4). The suspension was neutralized by PBS 1X (v: v) and centrifuged as indicated previously, then the leukocytes were collected and suspended in L-15 medium supplemented with 5% fetal bovine serum (FBS; Invitrogen), 1% Hepes (20 mM, Sigma, USA) and 1% of a T-cell-specific mitogen agent, phytohemagglutinin A (PhA M form, Invitrogen).

Viable cells were adjusted to 5×10^6 cells mL^{-1} after enumeration using trypan blue stain (VWR, Leuven, Belgium) and seeded in wells of a 24 or 48-well plate (Greiner Bio-One, Vilvoorde, Belgium).

Table 3
Summary of the humoral immune responses in PBMCs and HKLs stimulated with the 20 selected plant extracts.

No.	Name	Dose (μg/ml)	Lysozyme		Complement		Total Ig	
			PBMCs	HKLs	PBMCs	HKLs	PBMCs	HKLs
1	Ac	10	**				**	**
		100						
2	Ai	10					**	
		100	**	**	**	**	**	**
3	Ar	10						
		100				**	**	
4	As	10	**	**	**	**	**	**
		100					**	
5	Ase	10		**	**	**		**
		100				*		*
6	Ca	10						
		100		**				
7	Ct	10		**	**		**	*
		100					**	
8	Eh	10					*	
		100	**		**	**	**	**
9	Ep	10	**	**				**
		100						
10	Hc	10						
		100				**		
11	Mc	10	**	*				
		100	**	**			**	**
12	Mp	10						
		100					**	
13	Ob	10	**	*			**	**
		100	**	**			**	**
14	Pa	10	**		**	**	**	**
		100			**		**	
15	Pb	10				**	**	**
		100					**	
16	Pf	10		**		**	**	**
		100					**	**
17	Pg	10	**	**	**		**	
		100	**		**		**	
18	Po	10						**
		100			**		**	**
19	Wc	10		*		*	**	
		100		**		**	**	**
20	Zo	10	**	**	**	**	**	
		100				**	**	

Data are presented as means ± SD. (*) Asterisks indicate significant increased between the experimental groups to control, when $p < 0.05$ (*), $p < 0.01$ (**). Yellow shading includes the extracts for which at least 5 immune responses were significantly increased compared to control at 24hps. PBMCs: peripheral blood mononuclear cells; HKLs: head kidney leukocytes.

2.4. In vitro stimulation of cells and detection of humoral immune parameters

After isolation of striped catfish HKLs and PBMCs, five hundred μL of cell suspension (5×10^6 cells mL^{-1}) in L-15 medium supplemented with 5% FBS, 1% Hepes and 1% of a T-cell-specific mitogen agent, phytohemagglutinin A were added to each well of 48-well plate (Greiner Bio-One, Vilvoorde, Belgium). Afterward, leukocytes stimulation was carried out with 20 ethanol plant extracts to reach final concentrations at 10 and 100 μg mL^{-1}. Cells cultivated in the same medium containing 0.5% DMSO served as control. Each experiment was realized in triplicates. The humoral immune response was assessed for 24 hps at 28 °C in a humidified atmosphere of 5% CO_2. Collected leukocyte membranes were disrupted by 50 μL lysis buffer (50 mM tris HCl, 150 mM NaCl, 0.1% Triton X 100, PMSF 0.1 μg mL^{-1}). Samples

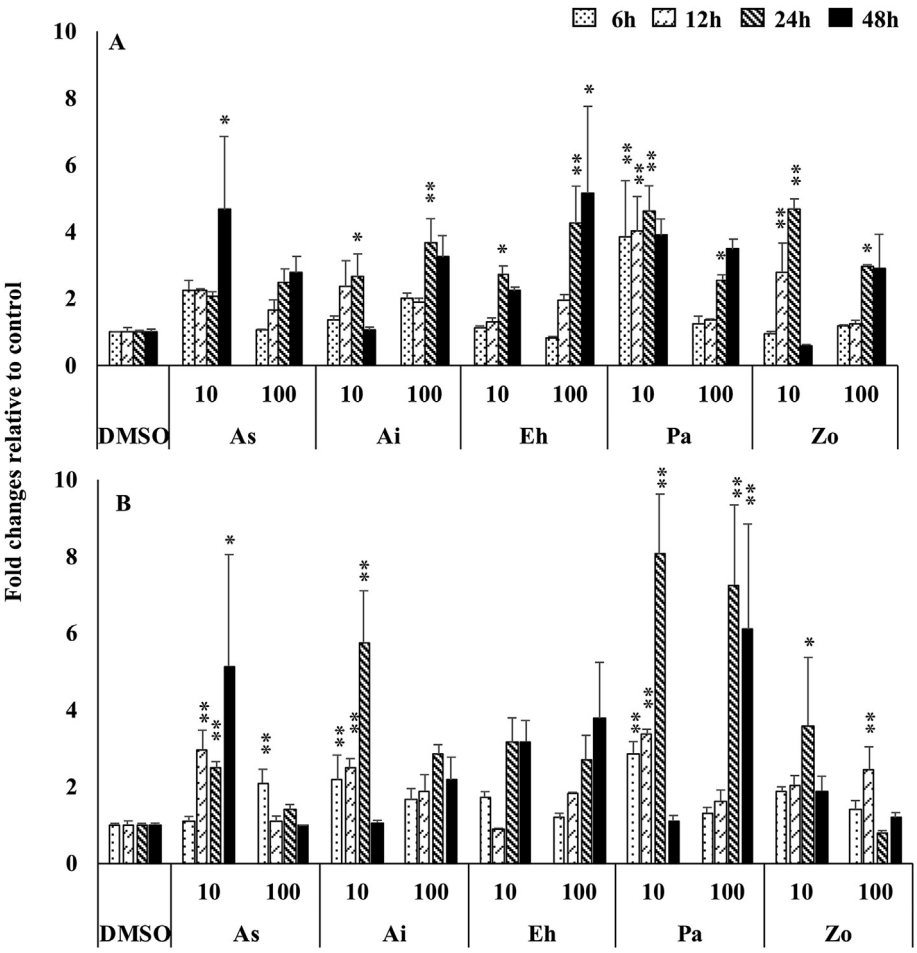

Fig. 4. Expression (mean ± SD; n = 3) of *il1β* gene at different time points in the striped catfish PBMCs (A) and HKLs (B) stimulated with five extracts (10 and 100 μg mL^{-1}). Bars with asterisks indicate significant differences in expression levels between stimulated cells and unstimulated control cells at a time point (*$p < 0.05$; **$p < 0.01$).

were centrifuged at 2000 g for 10 min to remove debris. Supernatants were collected for immune assays.

2.4.1. Lysozyme assay

The lysozyme assay protocols were adapted from Ellis [49] and Milla et al. [50]. Briefly, 0.3 μg mL^{-1} *Micrococcus lysodeikticus* (Sigma) was suspended in phosphate buffer, pH 6.2. In 96 wells microplates, the lysozyme activity was measured after mixing 30 μl of cell culture supernatant with 120 μL of *M. lysodeikticus* suspension. The difference in absorbance at 450 nm was monitored in every 30 s, between 0 and 30 min. The absorbance measurements were used to calculate lysozyme activity in units. One unit represents the amount of lysozyme that caused a 0.001 decrease in absorbance.

2.4.2. Complement assay

The alternative complement pathway (ACH50) was assayed using rabbit red blood cells (RRBC, Biomerieux, Craponne, France). The assay protocols were adapted from Sunyer and Tort [51] and Milla et al. [50]. Briefly, 3 μL of RRBC suspension (3%) diluted in veronal buffer (Biomerieux) were mixed with serial dilutions of cell culture supernatant (60 mL of total volume). After incubation for 120 min at 28 °C, the samples were centrifuged at 2000 g for 10 min at room temperature. The spontaneous hemolysis was obtained by adding 60 mL of veronal buffer to 10 mL of RRBC. The total lysis was obtained by adding 60 mL of distilled water to RRBC. The absorbance of these samples was then measured at 405 nm. Appropriate calculations served to estimate complement activity.

2.4.3. Total immunoglobulin assay

The total immunoglobulin (Ig) concentrations of samples were measured by the method of Siwicki and Anderson [52], modified by Milla et al. [50]. Briefly, immunoglobulins were precipitated with 10,000 kDa polyethylene glycol (PEG, Sigma). Serums were 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 before precipitation with PEG.

2.5. Detection of immunoregulatory genes production

Of the 20 herbal extracts tested, five strongly stimulated PBMCs and HKLs humoral immune responses were chosen for measuring immunoregulatory gene expressions. An *in vitro* study was performed to determine whether these five plant extracts can elicit different transcriptional profiles of the cytokines as well as antigen genes. Two mL of the PBMCs and HKLs suspension (5×10^6 cells mL^{-1}) in medium as mentioned above was added to each well of 12-well plate (Greiner Bio-One, Vilvoorde, Belgium), stimulated with five extracts as mentioned at 10 and 100 μg mL^{-1} and incubated for 6, 12, 24 and 48 h at 28 °C in a humidified atmosphere of 5% CO_2. Cells collected containing 0.5% DMSO served as a control. Each experiment was performed in triplicates. Sampling of incubated cells was conducted at the time points mentioned above and the cells were stored at −80 °C prior to RNA

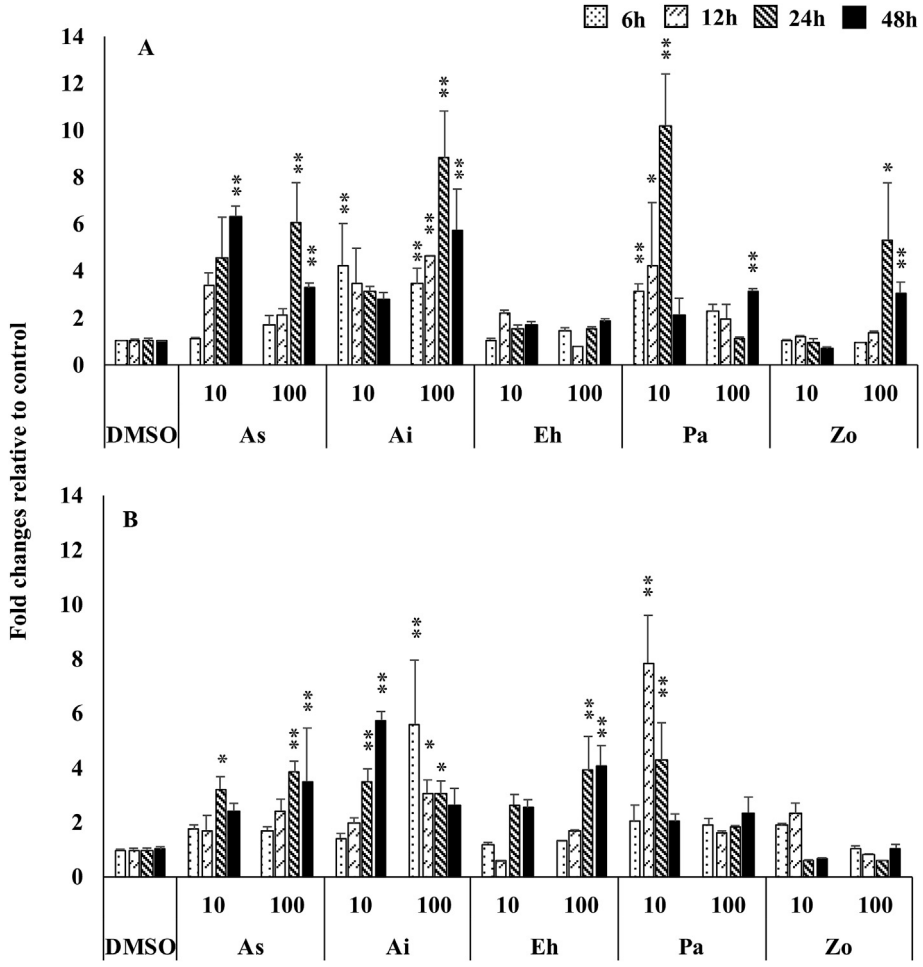

Fig. 5. Expression (mean ± SD; n = 3) of *ifn*γ 2a gene at different time points in the striped catfish PBMCs (A) and HKLs (B) stimulated with five extracts (10 and 100 μg mL^{-1}). Bars with asterisks indicate significant differences in expression levels between stimulated cells and unstimulated control cells at a time point (*$p < 0.05$; **$p < 0.01$).

extraction.

For expression analysis, total RNAs were extracted from cells using Extract-All (Eurobio, Courtaboeuf, France) according to the manufacturer's protocols. Samples were then DNAse treated (DNA-free kit, Ambion, Austin, USA). The extracted RNA was quantified by spectrophotometry using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). The RNA quality was assessed by the 260/280 and 260/230 ratios, while its integrity was evaluated by 1% agarose gel electrophoresis. Subsequently, total RNA was reverse transcribed using RevertAid HMinus First Strand cDNA synthesis Kit (Fermentas, Life Sciences, Germany). Briefly, 1 μg of total RNA and random hexamer primers were denatured for 5 min at 65 °C, then chilled on ice for 5 min. Reverse transcription was performed in 20 μL of a final volume containing 4 μL of PCR buffer 5 ×, 20 units of ribonuclease inhibitors (1 μL), 2 μL of 10 mM dNTP Mix and 200 units of M-MuLV reverse transcriptase (1 μL). The reaction was incubated for 60 min at 42 °C and stopped by heating at 70 °C for 5 min. The resulting cDNA was diluted 10x for initially testing the efficiency of primers combination or real-time quantitative PCR.

2.5.1. Gene expression analysis

Three μL of reverse transcription products (diluted 1/10) were used for each real-time PCR. Duplicates were run for each sample. Forward and reverse primers were used at a concentration of 600 nmol L^{-1} and added to SYBR Green PCR Master Mix (Applied Biosystem, Warrington, UK). The primers were designed for cytokine genes (*il1β*, *ifn*γ 2a and 2b) and adaptive immune cytokine (*mhc* class II) gene following previous paper of Sirimanapong et al. [21] and Sinha et al. [53] (Table 2). After a 2 min incubation step at 50 °C and a 10 min incubation step at 95 °C, 40 cycles of PCR were performed. The amplification parameters were as follows: 15 s of denaturing at 95 °C, 1 min annealing/extension at 60–64 °C depending on gene. The transcript abundance for each gene was calculated from the threshold cycle (CT) values using their respective standard curve followed by normalization with the geometric mean of ubiquitin and elongation factor. The expression was calculated according to the relative standard curve method of Plaffl [54], where $\Delta\Delta CT$ is $\Delta CT_{treatment} - \Delta CT_{control}$, ΔCT is $CT_{target\ gene} - C\ T_{16S,}$ and CT is the cycle at which the threshold is crossed. Data are presented as relative fold-change with 16S rRNA as internal control genes.

2.6. Statistical analysis

The statistical package for social science (SPSS) software (version 20.0, IBM Corp., Armonk, NY:IBM USA) was used to analyze differences between experimental and control groups. All data were expressed as mean ± standard deviation (S.D.). One-way analysis of variance (ANOVA) followed by Tukey's test was run to find out any difference in immune parameters.

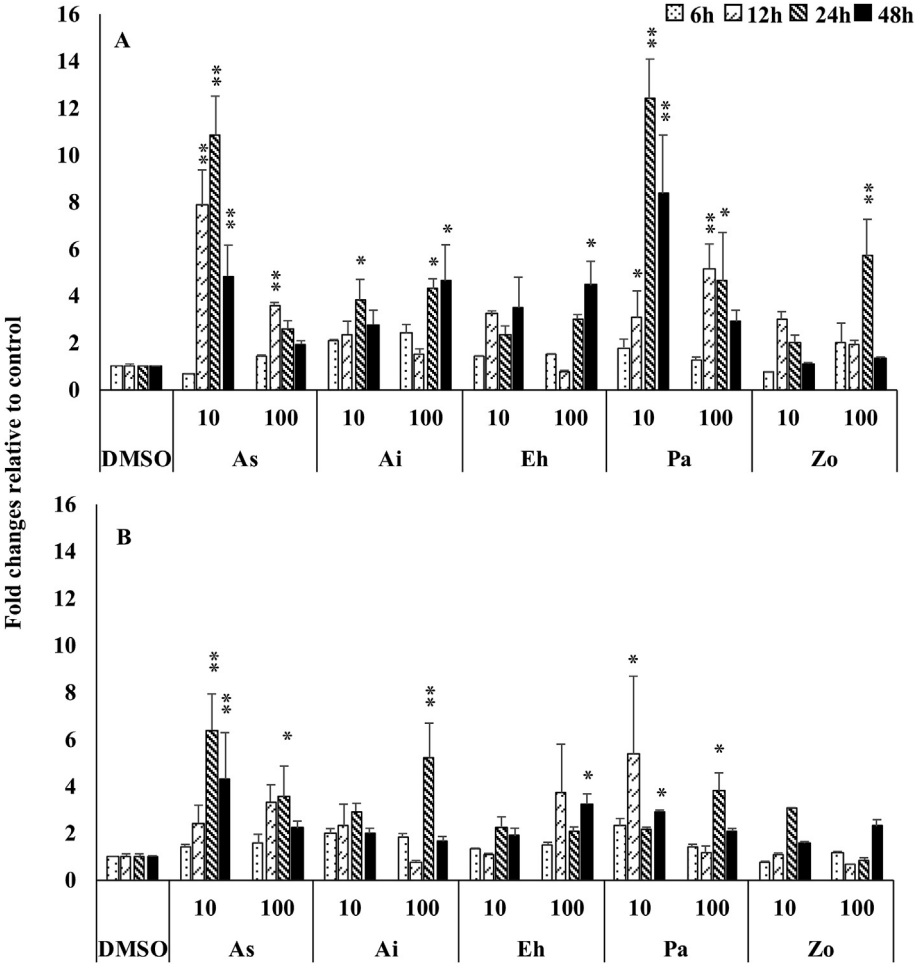

Fig. 6. Expression (mean ± SD; n = 3) of *ifn*γ 2b gene at different time points in the striped catfish PBMCs (A) and HKLs (B) stimulated with five extracts (10 and 100 μg mL^{-1}). Bars with asterisks indicate significant differences in expression levels between stimulated cells and unstimulated control cells at a time point (*$p < 0.05$; **$p < 0.01$).

3. Results

3.1. Humoral immune parameters in PBMCs and HKLs

3.1.1. Lysozyme activity

Most examined extracts, which were selected by their previously known for influence on the immune response, stimulated the release of lysozyme activity after 24 hps in PBMCs and/or HKLs (Fig. 1). The lysozyme levels in PBMCs ranged between 0.85 and 4.79 (at 100 μg mL^{-1} of Ase and Mc, respectively) times than those of the control. Both doses of 10 and 100 μg mL^{-1} of Mc, Ob and Pg extracts significantly enhanced the lysozyme levels compared with control ($p < 0.01$) in PBMCs, while only those of Mc, Ob and Wc were effective on HKLs. The strongest effect was observed in PBMC treated with 100 μg mL^{-1} of Mc and in HKLs treated with 100 μg mL^{-1} dose of Ca ($p < 0.01$). Five extracts including Ar, Hc, Mp, Pb and Po showed no statistical influence on lysozyme activity in both PBMCs and HKLs.

3.1.2. Alternative complement pathway (ACH50)

As shown in Fig. 2, the ACH50 levels increased in cells treated with several extracts compared with control treatment at 24 hps (Fig. 2). At 100 μg mL^{-1}, PBMCs stimulated with Ai displayed the highest ACH50 value ($p < 0.01$), whereas Eh induced the strongest ACH50 activity in kidney cells at the same concentration, compared with other treatments. No significant changes were observed in both PBMCs and HKLs stimulated with Ac, Ca, Ep, Mc, Mp and Op, while both doses 10 and 100 μg mL^{-1} of Pa and Pg showed significant effects on PBMCs and those of Ase, Wc and Zo were effective on HKLs.

3.1.3. Total immunoglobulin (Ig)

In both PBMCs and HKLs, total Ig activity was noticed to be statistically higher in plant extract treated groups at 24 hps compared with control group (Fig. 3) except Ca, Ep, Hc and Mc on PBMCs and Ar, Ca, Hc, Mp, Pg and Zo on HKLs. Most of the extracts could stimulate remarkably total Ig levels at single or both concentrations in PBMCs ($p < 0.01$). In case of HKLs, the level of total Ig was found to be the highest in Mc (at 100 μg mL^{-1}), Po (at 100 μg mL^{-1}), Pf (at 10 μg mL^{-1}), Ai (at 100 μg mL^{-1}) and Wc (at 100 μg mL^{-1}) ($p < 0.01$) treatments, while some seemed to decrease Ig activity (at one concentration Ca and Pa).

A compilation of the humoral immune results obtained in PBMCs and HKLs after stimulating with 20 plant extracts has been done (Table 3) in order to select the 5 best extracts, namely As, Ai, Eh, Pa and Zo and analyzed more in depth the gene expression of 4 cytokines (*il1β*, *ifr*γ 2a and 2b) and a2 *mhc* class II integral membrane protein alpha chain 2.

3.2. Gene expression

3.2.1. Expression of pro-inflammatory cytokine – il1β

The gene *il1β* was expressed in both cells types and its transcript levels were significantly enhanced by a stimulation with most plant

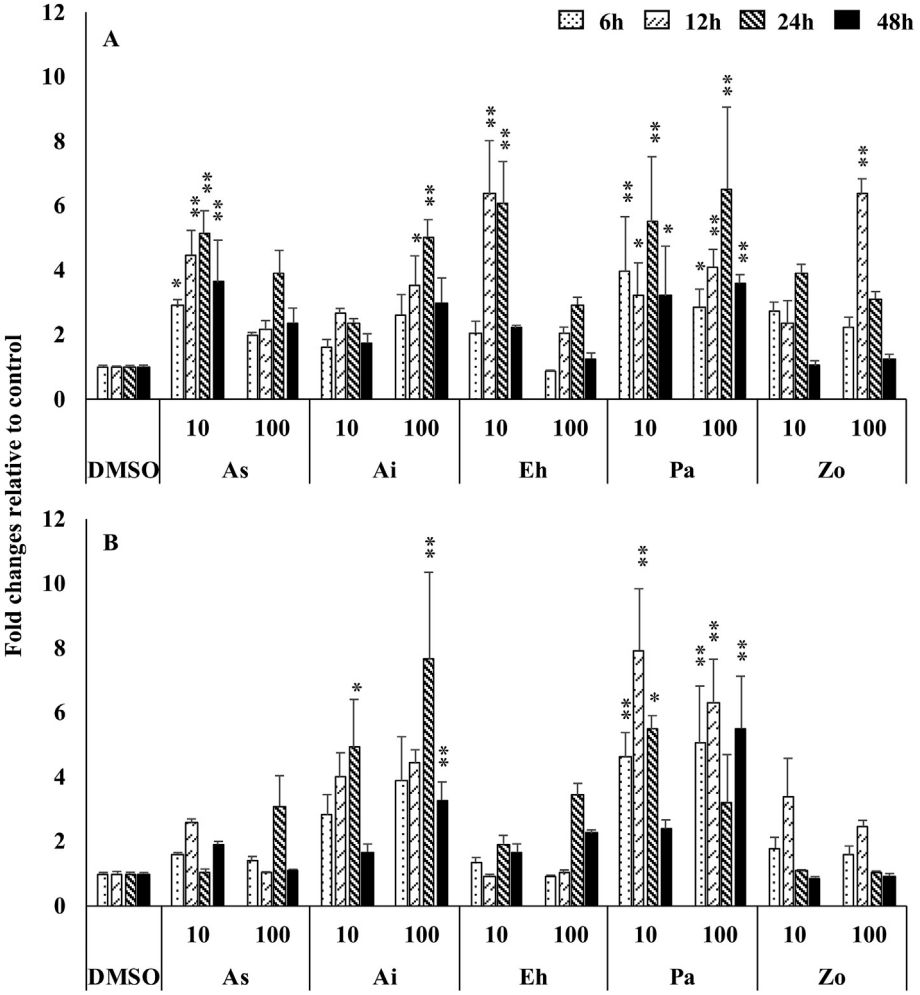

Fig. 7. Expression (mean ± SD; n = 3) of *mhc* class II gene at different time points in the striped catfish PBMCs (A) and HKLs (B) stimulated with five extracts (10 and 100 μg mL^{-1}). Bars with asterisks indicate significant differences in expression levels between stimulated cells and unstimulated control cells at a time point (*$p < 0.05$; **$p < 0.01$).

extracts at least at some time points (Fig. 4). The levels of *il1β* reached a peak after 24–48 hps mainly in PBMCs or HKLs. In PBMCs treatments, the expression of *il1β* was the highest in Eh (100 μg mL^{-1}) treated groups at 48 hps (Fig. 4A). When compared over time each individual treatment group, *il1β* levels displayed a wide variation in response according to the plant species used. An early upregulation (at 6 hps) of *il1β* expression was observed only in Pa (10 μg mL^{-1}) treated group, gradually increasing until 24 hps ($p < 0.01$) and the highest expression level was observed in HKLs after 24 h in both doses of Pa stimulated. Furthermore, the *il1β* levels increased in single or couple doses of several extracts at some time points in HKLs, only Eh appeared no statistical increase compared with control group ($p > 0.05$) (Fig. 4B).

3.2.2. Expression of antiviral cytokines

In the present study, an upregulation of *ifnγ* 2a and 2b expressions was observed in PBMCs and HKLs for most extracts at one or several concentration/time point except Eh on PBMCs and Zo on HKLs. The expression of *ifnγ* 2a ($p < 0.01$) in PBMCs stimulated with Ai (100 μg mL^{-1}) increased remarkably from initial period till the end of the study and then stabilized (or decreased) until 48 hps. In case of cells treated with Pa at 10 μg mL^{-1}, the highest peak of *ifnγ* 2a level was observed at 24 hps ($p < 0.01$) (Fig. 5A). Similarly, Pa (10 μg mL^{-1}) also stimulated a significant increase of *ifnγ* 2a gene expression in HKLs at 12 hps ($p < 0.01$) (Fig. 5B).

Significantly higher expression level ($p < 0.01$) of *ifnγ* 2b was detected in PBMCs and HKLs incubated with the low dose of As or Pa while the activity at 100 μg mL^{-1} was reduced (Fig. 6). High concentration of Ai also induced increased expression at some time points in both cell lines, while the effect of low dose was only significant after 24 hps on PBMCs at 10 μg mL^{-1}. Eh also showed some increase of *ifnγ* 2b gene at 100 μg mL^{-1} after 48 hps on both cell lines, and Zo at the highest concentration on PBMCs after 24 hps.

3.2.3. Expression of cytokines involved in adaptive immunity-mhc class II

Inductive expression of *mhc* class II was obtained in PBMCs and HKLs treated with several extracts, from 6 hps onwards and often gradually elevated till 24 hps as compared with non-stimulated groups. Specifically, the *mhc* class II levels were upregulated early in stimulated PBMCs with As (10 μg mL^{-1}) and with both doses of Pa, then a prolonged and stable increase in their expression was observed until 24 hps, although there was a very slight decrease after 48 hps ($p < 0.05$) (Fig. 7). *mhc* class II expression in HKLs was lower in comparison with the one of PBMCs, only treated cells with both doses of Pa showed an immediate rise in the expression level of *mhc* class II, and for Ai in some conditions. However, it was noticed that three extracts (As, Eh and Zo) failed to stimulate the expression of *mhc* class II in HKLs, regardless of the dose used.

4. Discussion

Plant products are a great source of bioactive molecules and some have been identified as potential therapeutic treatments by modulating immunity as well as by preventing or controlling fish diseases [26,29,55]. In the past few years, aquaculture of striped catfish has been suffering many troubles due to the disease threats, especially, infections, caused mostly by bacteria (*e.g. A. hydrophila* and *E. ictaluri*) [56–59]. However, research about the effects of plant extracts on immune molecules as well as on humoral endpoints in striped catfish is rather limited. The present study showed the results of screening the putative immune-stimulatory properties of 20 plant extracts on the PBMCs and the HKLs, which are well known for their involvement in innate and acquired immunities. Functional evaluation through *in vitro* studies were performed by measuring the lysozyme, complement activities and total Ig as well as the cytokine gene expression with colorimetric assays and molecular tools. This research further determined the mode of actions of some ethanol plant extracts in striped catfish leukocytes by expression of some cytokines at different time points.

Playing a role as indicators of inflammatory response, humoral immune parameters (lysozyme, complement and total Ig) were measured in two types of striped catfish leukocytes stimulated with plant extracts at 24 hps. Among these parameters, lysozyme is important in host mediating protection against microbial invasion, and is mainly expressed in neutrophils, monocytes and in small amount in macrophages [60]. In the present study, Ai, As, Eh, Mc, Ob, Pg and Zo stimulated to increase significantly lysozyme levels in both striped catfish leukocytes at single or couple concentrations at 24 h. A similar result was documented by Giri et al. [61] who reported that significant levels of lysozyme started to display in roho labeo head kidney macrophages at 24 h post stimulating with intracellular products of the probiotics *Bacillus subtilis* VSG1 and *Lactobacillus plantarum* VSG3. Lysozyme activities are immune effectors that could eliminate the widespread bacteria and viruses [62,63]. There are two major c-type and g-type lysozymes caused degree of lysozyme activity, which could be induced by LPS, bacterial, fungal and some viral infection [64,65]. In HKLs of Japanese pufferfish (*Takifugu rubripes*) stimulation by LPS (20 μg mL^{-1}) and Nigericin (30 μM), however, the lysozyme activity was observed to increase at 24 h, later than phagocytosis and superoxide anion production, then the levels continued to increase until 48 hps [66]. Similar to lysozyme parameter, our study also demonstrated that Ai, As, Ase, Eh, Pa and Zo extracts remarkably induce complement activity in both striped catfish PBMCs and HKLs. Aside with mediating humoral immunity, fish complement and immunoglobulin can have a synergistic effect on the microbial opsonization [67–69]. IgM is the common immunoglobulin in both serum and mucus which plays a key role in systemic immune responses [70]. Klesius [71] and Magnadóttir et al. [72] indicated that the IgM levels are variable among fish and with size/age. For this reason, total Ig is used as an immune parameter evaluated for medical immunostimulants. Previous studies suggested that total Ig levels were well regulated in fish by dietary extracts including Astragalus (*Astragalus membranaceus)* and wolfberry (*Lycium barbarum*) [73], rose myrtle [74–76]. Similar result was observed in which our study, several plant extracts could statistically regulate the total Ig level in both striped catfish cell types, mostly after 24 hps. These results indicated that the modulation of humoral immune responses (lysozyme and complement activities, total Ig) was activated in striped catfish by plant extracts but might differ between the kinds of plant extracts and leukocyte types (PBMCs and HKLs).

Nevertheless, the understanding of the molecular mechanisms behind the effects of plant extracts on fish immune response is undoubtedly helpful for proposing appropriate applications in fish farming. In this study, 5 plant products (As, Ai, Eh, Pa and Zo) were selected from the lysozyme, complement and total Ig results to determine the mRNA levels of immune-related genes in PBMCs and HKLs (Table 3). This study showed that most of the immune cytokine genes were upregulated in PBMCs and HKLs stimulated with these extracts, but this stimulation often depends on the concentration, with the highest concentration sometimes less effective than the lowest one. Effects were also sometimes lower after 48 hps. Like in mammals, teleost IL-1β has been found to regulate other immune relevant genes in lymphocyte activation, phagocytosis and bactericidal activities after stimulation with its recombinant protein [77–80]. Macrophages are the primary source of IL-1β although it is produced by a wide variety of other cell types as well [81]. The incubation of trout anterior kidney leukocytes with recombinant Il-1β induces phagocytosis and chemotaxis [79,82]. In the present study, *il1β* expression levels in striped catfish leukocytes varied over the experimental period according to the type of extract treatment. Although the *il1β* gene started to significantly increase at the initial hours after stimulation by some extracts when compared with control, the highest peaks of *il1β* expression were observed in most plant extract-treated cells after 24 or 48 hps. Similarly, upregulated expression of *il1β* was noticed at the very early stage in Japanese pufferfish HKLs after incubation with heat-killed probiotics [44]. A study by Bilen et al. [66] also found that the expression of proinflammatory cytokine genes in Japanese pufferfish HKLs increased after treated with nigericin, a combination of nigericin and LPS. However, these authors demonstrated that the *il1β* levels of Japanese pufferfish HKLs significantly increased very early at 1 hps, and the upregulation was sustained until 24 hps. In addition, rainbow trout head kidney macrophages treated with rose myrtle led to the upregulation of *il1β* after 4 hps only [43]. Moreover, Chi et al. [83] also confirmed an upregulation of *il1β* and *tnfα* mRNA levels in HK macrophages of grass carp (*Ctenopharyngodon idella*) after a stimulation with wood ferm *Dryopteris crassirhizoma* extract at 2 and 8 hps. This could be explained that the regulation of *il1β* level was varied in cells according to stimulants and kind of cell types.

As a crucial cytokine in immune mechanism, IFNs also provides mediating cellular defense against viral infections. Especially, the type II IFN (IFN-γ) plays a major role in both innate and adaptive immunity, including the ability to activate respiratory burst activity, nitric oxide production and phagocytosis of bacteria in macrophages [84–86]. Channel catfish (*Ictalurus punctatus) ifn* expression increased at 2 h after exposure to an inactivated double-stranded RNA retrovirus and polyinosinic: cytidilic acid (poly I:C) [87]. In correlation with the increased *il1β*, the current results found that *ifn*γ 2a and 2b expressions were upregulated by extract treatments mostly at 12 hps. Biswas et al. [44] found that type I- *ifn1* gene expression in Japanese pufferfish HKLs was induced by both heat-killed probiotics (*Lactobacillus paracasei* spp. *paracasei* - strain 06TCa22 and *L. plantarum* - strain 06CC2), whereas *ifn*γ gene expression was increased by *L. paracasei* spp. *paracasei* at different time points (4, 8, 12, 24 and 48 hps). Furthermore, upregulation of *ifn*γ2 gene was also induced by yeast extract administration to common carp, whereas induction of type I *ifn* (*ifn*α) expression was not detected [88]. The increased *ifn*γ levels observed in this study demonstrated that extract treatments could protect fish against viral infection and give striped catfish leukocytes the ability of killing intracellular pathogens by *ifn*γ mediation. On the other hand, expression of *mhc* class II gene was also obtained in PBMCs and HKLs treated with several extracts, although HKLs were less stimulated in comparison with PBMCs. Class II MHC plays a vital role by mediating antigen recognition in macrophages, B-cells and dendritic cells [89]. The *mhc* genes have been isolated and characterized in various fish species, including zebrafish (*Danio rerio*), rainbow trout, channel catfish, turbot *(Scophthalmus maximus)*, Nile tilapia (*Oreochromis niloticus*), sea bass (*Centropristis striata*) and half smooth tongue sole (*Cynoglossus semilaevis*) [90] and European eel (*Anguilla anguilla*) [91]. Jiang et al. [92] found that channel catfish head kidney monocytes/macrophages were stimulated by three flagellins (rFlaA, rFlaB and rFlaC). These flagellins could dramatically upregulate *mhc* class *II* level at 8 hps. The *mhc* class II genes were highly expressed compared with the other examined genes (namely toll-like receptor 5- *tlr5m, tlr5s, nfκB, il1β, tnfα, il8, inos1*).

Furthermore, class II *mhc* levels were strongly induced in LPS stimulated-macrophage of half smooth tongue sole at 12 hps [93].

Plant extracts possess many medicinal functions that could be due to alkaloids, steroids, phenolics, tannins, terpenoids, saponins, and flavonoids presented in the extracts [23–30]. A study by Catap et al., [94] indicated that the presence of tannins and alkaloids in the extract could produce the immunomodulatory effects in lymphocytes and macrophages. Likewise, alkaloids induced the proliferation of lymphocytes, but not at a significant level [94]. Furthermore, an *in vitro* study using Leishmania-infected RAW 264.7 cells suggested that tannins played as an indicator in activating macrophages, especially during infections [95]. Tannins have been reported to enhance innate immunity through proliferation of γδ T lymphocytes as a result of providing host protection against pathogens [96]. These could be explained why striped catfish leukocytes can improve the immune systems after being stimulated with plant extracts.

In conclusion, our results suggest a positive contribution of several herbal extracts to increase humoral immune responses in a dose dependent manner in striped catfish PBMCs and HKLs after 24 h. Nevertheless, the humoral immune responses cannot adequately explain the stimulatory effects of these extracts. Several extracts induced a strong upregulation of 4 cytokines (*il1β*, *ifnγ* 2a and 2b, and *a2 mhc* class II) according to the concentration, time points and kind of leukocytes. The specific enhancement of the gene expression by the plant extracts might promote the activity of fish macrophages leading to an early protective immunity during disease infection. Furthermore, this study demonstrates the ability of these plant extracts to enhance the activation of immune response and suggests a new mechanism explaining their important biological characteristics of plant extracts (*e.g.* antibacterial, antiviral, anti-inflammatory or immune-stimulating properties in fish).

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