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Psidium guajava L.- dichloromethane and ethyl acetate fractions ameliorate striped catfish (*Pangasianodon hypophthalmus*) status via immune response, inflammatory, and apoptosis pathways

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ABSTRACT

Psidium guajava L. is known to possess immune-modulatory properties in humans and other mammals. Although the positive effects of *P. guajava*-based diets on the immunological status have been shown for some fish species, the underlying molecular mechanisms of its protective effects remain to be investigated. The aims of this study were to evaluate the immune-modulatory effects of two guava fractions from dichloromethane (CC) and ethyl acetate (EA) on striped catfish with *in vitro* and *in vivo* experiments. Striped catfish head kidney leukocytes were stimulated with 40, 20, 10 and 0 μg/ml of each extract fraction, and the immune parameters (ROS, NOS, and lysozyme) were examined at 6 and 24 h post stimulation. A final concentration of each fraction at 40, 10 and 0 μg/fish was then intraperitoneally injected into the fish. After 6, 24, and 72 h of administration, immune parameters as well as the expression of some cytokines related to innate and adaptive immune responses, inflammation, and apoptosis were measured in the head kidney. Results indicated that the humoral (lysozyme) and cellular (ROS and NOS) immune endpoints were regulated differently by CC and EA fractions depending on dose and time in both, *in vitro* and *in vivo* experiments. With regards to the *in vivo* experiment, the CC fraction of the guava extract could significantly enhance the TLRs-MyD88-NF-κB signaling pathway by upregulating its cytokine genes (*tlr1*, *tlr4*, *myd88*, and *traf6*), following the upregulation of inflammatory (*nfκb, tnf*, *il1β,* and *il6*) and apoptosis (*tp53* and *casp8*) genes 6 h after injection. Moreover, fish treated with both CC and EA fractions significantly enhanced cytokine gene expression including *lys* and *inos* at the later time points - 24 h or 72 h. Our observations suggest that *P. guajava* fractions modulate the immune, inflammatory, and apoptotic pathways.

1. Introduction

Guava (*Psidium guajava* L.), a traditional herbal medicine, used as food but also as folk medicine [1]. Leaves, shoots, bark, flower buds, and roots of guava are widely applied to treat several diseases including diabetes, hypertension, caries, wounds, pain relief, and fever [2]. Guava contains various antioxidants and phytochemicals, including essential oils, polysaccharides, minerals, vitamins, enzymes, as well as triterpenoid acid alkaloids, steroids, glycosides, tannins, flavonoids and saponins [3]. These compounds are potentially responsible for bioactivities, including immune stimulation, anti-microbial, anti-inflammatory, anti-cancer along with anti-oxidant activities [4]. In addition, guava is a rich source of pectin and an important dietary fiber [5]. In pharmaceutical science, the guava leaves are considered the most promising

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part of the guava tree to explore its medicinal value [6,7]. Recently, guava was developed as food additive reagents in fish industry, aiming to improve growth performances, immune responses, antioxidant activities as well as resistance to infectious pathogens [8,9].

One of the potential mechanisms of guava intakes may involve the attenuation of inflammation in animal species [1]. After stimulating an inflammation with lipopolysaccharides (LPS), an ethanol extract from fermented guava leaves (125 μg/mL) significantly inhibited the expression level of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 protein levels by down-regulating the transcriptional activity of the nuclear factor-κB (NF-κB) in mouse macrophage cells [10]. A flavonoid fraction (200 μg/mL) of guava leaf extracts reduced the activation of NF-κB in head-kidney macrophages of rohu (*Labeo rohita*) after LPS stimulation [11]. Moreover, guava stimulated the immune system of the cultivated animal. *P. guajava* bark extracts induced apoptosis in leukemia CCRF-CEM cells via the activation of caspases 3/7, 8 and 9. The production of reactive oxygen species (ROS) was also significantly increased in those cells 24 h after stimulation with 2 × the half-maximal inhibitory concentration (IC50 = 6.35 ± 1.74 μg/mL) of a guava bark extract [12]. Diets based on guava leaf powder improved the non-specific immune response in skin mucus and serum and further significantly upregulated the expression level of interleukin 8 (*il8*) and interleukin 1 beta (*il1β*) in common carp (*Cyprinus carpio*) fingerlings [13]. A previous study of Giri et al. (2015) demonstrated that diets based on 0.5% guava leave extracts could upregulate the levels of *il1β* and the tumor necrosis factor alpha (*tnfα*), and downregulate *inos*, *nfkb*, cyclooxygenase-2 (*cox2*), as well as transform expression levels of the growth factor beta (*tgfβ*) in the head kidney, intestine and hepatopancreas of rohu [8]. These studies partially revealed a molecular basis for the promising immuno-properties of guava extracts.

We previously found that *P. guajava* extract-based diets were not only potentially interesting for modulating immune responses but also for improving resistance to the pathogenic bacterium *Edwardsiella ictaluri* in striped catfish (*Pangasianodon hypophthalmus*) [14]. To the best of our knowledge, the mechanism of guava leaf extracts acting in the fish immune response has not yet been reported. Moreover, its biological activities during inflammation processes in fish are largely unknown. The present study examines the early functional contributions of different fractions including dichloromethane (CC) and ethyl acetate (EA) from *P. guajava* on the immune response in striped catfish using *in vitro* and *in vivo* dynamic models. We observed these effects at different time points via *in vitro* and *in vivo* experiments. Inducible enzymes were selected for the *in vivo* experiment, namely respiratory burst activity (RBA), nitric oxide synthase (NOS), lysozyme, as well as cytokine genes related to immune response, inflammatory and apoptosis pathways.

2. Material and methods

2.1. Extract preparation

The collection CC and EA fractions from *P. guajava* ethanol extract has already been described in a previous study [15]. Briefly, the dry powder of guava leaves (1 kg) was macerated with ethanol (3 × 5 L) at room temperature for 24 h, then filtered with paper filter (Whatman™ 1001-400 Grade 1 Qualitative Filter Paper, Diameter: 40 cm, Pore Size: 11 μm). The solutions were concentrated under reduced pressure with a rotatory evaporator at 45 °C until a dark syrup was obtained which was then lyophilized into guava crude extracts. To obtain their fractions, the extracts were continuously separated three times using liquid-liquid partitioning with dichloromethane and ethyl acetate. The products were then re-dissolved in dimethyl sulfoxide (DMSO, Saint Louis, MO, US) to prepare stock solutions at 8, 4, and 2 mg/mL for the *in vitro* study, and at 400 and 100 μg/mL for the *in vivo* study. The stock solutions were stored at – 20 °C until use.

2.2. Experimental fish

Striped catfish juveniles of 50 ± 5 g (*in vitro*) and 15 ± 5 g (*in vivo*) were acclimated to laboratory conditions at 28 ± 2 °C in a composite tank (2000 L) for 15 days. Fish were fed twice (9 a.m. and 3 p.m.) daily at a feeding rate of 1% body weight with a commercial feed (30% crude proteins, 2.5 mm, Proconco, Vietnam) under natural photoperiod prior to their use in the *in vitro* assay. The health status of experimental fish was checked following the method described in a previous study [16]. Fish were randomly examined for the presence of abnormal lesions or parasites on their body surface and organs. Further, smears from head kidneys and blood from the same fish were cultured on tryptic soy agar plates (TSA, Merck) for 24–48 h at 28 °C for the occurrence of any bacterial pathogens. Healthy fish without abnormal clinical or pathogenic bacteria were used for the experiment.

2.3. In vitro experiment

HKLs were collected following the method of Boyum [17], modified by Pierrard et al. [18]. After isolation of striped catfish HKLs, 2 mL of cell suspension (5×10^6 cells/mL) in L-15 medium supplemented with 5% FBS, 1% Hepes and 1% of a T-cell-specific mitogen agent, phytohemagglutinin A, was added to each well of a 24-well plate (Greiner Bio-One, Vilvoorde, Belgium). Afterward, leukocyte stimulation was carried out with three doses (10, 20, and 40 μg/mL) of each extract fraction. Cells cultivated in the same medium containing 0.5% DMSO served as control. Each experiment was realized in triplicate. The HKLs were incubated at 28 °C in a humidified atmosphere of 5% CO_2. The humoral immune response as well as cytokine expression were assessed at 6 and 24 h post-stimulation (hps). At each time point, a volume of 200 μL of cells was taken for nitric oxide species (NOS) and respiratory burst assays (RBA). The residual cell suspension was collected by centrifugation at 10,000×*g* at 4 °C for 5 min for gene expression analysis, while the supernatant was used for lysozyme activity testing.

2.4. In vivo treatment

After two weeks of acclimation, a total of 150 striped catfish were randomly divided into 4 treated groups and one control group. Fish were intraperitoneally injected with 100 μL of each fraction, resulting in final concentrations of 40 and 10 μg/fish, respectively. The control treatment was injected with 1X PBS; each treatment was given in triplicate. Samples were collected at 6, 24, and 72 h for analysis. Briefly, 3 fish per tank (9 fish per treatment) were randomly collected and anaesthetized using 0.1 ppm M222 (Sigma–Aldrich, MO, USA). Blood plasma was sampled individually for lysozyme. The fresh spleen was mashed in L-15 medium through a 100 μM nylon mesh and then used for NOS and RBA analysis. The head kidney was collected for gene expression analyses.

2.5. Immune variables

2.5.1. Lysozyme activity assay

The protocol of the lysozyme activity assay was adapted from Ellis [19] and Milla et al. [20], which was then adapted for HKLs and serum of striped catfish. In 96-well microplates, the lysozyme activity assay was initiated by mixing 10 μL of plasma or 30 μL of cell suspension 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 cells). The difference in absorbance at 450 nm was monitored between 0 and 30 min for plasma (0 and 15 min for HKLs) and used to calculate units of lysozyme activity. One unit is the amount of lysozyme required to generate a 0.001 decrease in absorbance.

2.5.2. Respiratory burst activity assay

The respiratory burst activity assay was adapted from Rook et al. [21]. Spleen leukocytes as well as HKLs were washed twice in L-15

medium (1000 g, 5 min, 28 °C). The culture media were then replaced by a corresponding fresh medium containing 2 mg/mL NBT. Cells were incubated for 1 h at 28 °C in a light-protected environment. After 1 h, the cells were washed twice in PBS and the reaction was stopped by adding 200 μL of methanol. The cells were rinsed by centrifugation (1000 g, 10 min, 4 °C) and finally air dried for 10 min. The resulting formazan was dissolved in 240 μL of KOH 2 M and 280 μL of N-dimethylformamide. The absorbance of the final supernatant was measured at 550 nm. A standard curve was created by serially diluting NBT directly in KOH 2 M and N-dimethylformamide. The samples and the cell-free negative control were run twice. Activity was measured via protein concentrations in cell suspensions using the Bradford assay.

2.5.3. Nitric oxide species assay

The Griess reaction was used to measure NOS production. First, 100 μL cell suspension was incubated for 1 h at 28 °C with 5 μL E. *ictaluri* suspension (OD 2) resuspended in equivalent culture media. Following the addition of 100 μL Griess reactant, the solutions were incubated for another 15 min. Absorbance was measured at 540 nm. A standard curve was generated with serial dilutions $NaNO_3$. The culture media (without cells) incubated with *E. ictaluri* suspension and Griess reactant served as negative control. Activity was recorded via protein concentrations in cell suspensions measured with the Bradford assay.

2.5.4. Immunoregulatory gene expression

For expression analysis, total RNA was extracted from fresh head kidneys 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). RNA quality was assessed using the 260/280 and 260/230 ratios, while its integrity was evaluated through 1% agarose gel electrophoresis. Subsequently, total RNA was reverse transcribed using RevertAid HMinus First Strand cDNA synthesis Kit (Fermentas, Life Sciences, Germany). The resulting cDNA was diluted 5x to initially test the efficiency of primer combinations and 25x for real-time quantitative PCR, respectively.

A total 2.5 μL of reverse transcription products (diluted 1/25) were used for each real-time PCR. Each sample was run twice. Forward and reverse primers were used at a concentration of 500 nmol/L and added to Sso Advanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). The relative expression of several immune-related genes was investigated by RT-qPCR, including genes involved in the immune response (toll-like receptor - *tlr1*, *tlr2,* and *tlr4*, myeloid differentiation primary response 88 - *myd88*, tumor necrosis factor receptor-associated factor 6 - *traf6*, c type lysozyme - *lys*, *inos*, class II major histocompatibility complex - *mhc class II*); in the inflammatory response (*nfκb1* and *nfκb2*, tumor necrosis factor - *tnf*, *il1β* and interleukin 6 - *il6*); and in the apoptosis process (*casp3*, *casp8* and tumor protein 53 - *tp53*). The 16 S ribosomal RNA (*16srna*) and beta actin served as internal control genes. These primers were designed using Primer3 software, and their quality was validated against sequences of common carp published on Genbank using Amplifix software. Primer sequences and gene functions are presented in Table 1. Before analysis, the efficiency of each gene was confirmed. Thermal cycles and fluorescence detection were performed using a StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) under the following conditions: 10 min of initial denaturation at 95 °C, followed by 40 cycles at 95 °C for 30 s and 60 °C for 30 s. The transcript abundance for each gene was calculated by subtracting the threshold cycle values from their respective standard curves followed by normalization with the geometric mean of ubiquitin and the elongation factor. The expression was

Table 1
The primers used for realtime PCR analysis.

Name	Sequence	Product size	GeneBank Number
Inducible nitric oxide synthase - *inos*	GGTCTTGAACCAGAGGTCC ACCCAGATGGCTAACCAGGA	103	XM_026935143.1
C type lysozyme - *lys*	ACGCTATGAACGGTGTGAGC CCGGTGTTGTAGTCAGACTCG	120	KU601195
Myeloid differentiation primary response 88 – *myd88*	AGGATCGAGGCATCACCGTA GGTTGTAGTCGGTCTGCTCC	129	XM_026938292.1
Toll like receptor 1 - *tlr1*	CTGGACCATTGCAGTCCCAT CCGTGTCATCTGGCAAGTCT	161	XM_026922777.1
Toll like receptor 2 - *tlr2*	CCGGAGTTAGAAAGCGCTGA CGAAGTTCTCCGACAGGACG	142	XM_026929176.1
Toll like receptor 4 - *tlr4*	GCAGGTCCTGGATCTCACAAG CAGGCCAATGTCCACGAGAA	175	XM_026932800.1
Tumor necrosis factor receptor associated factor 6 - *traf6*	GGGAGTCGTACCTAAGCCCT TCAGGCAGATGGGACACTCA	135	XM_026937676.1
Class II of major histocompatibility complex - *mhc class II*	CGCATGCTCAGACTCGGATAA TGAGTCTTGGCGGTCTCGTA	153	XM_026945809.1
Nuclear factor kappa light chain enhancer of activated B cells- 1 - *nfkb1*	ACGTAGAGGTTCAGGAGCGA CTGCTGCGATGTGAAGAGGT	156	XM_026947061.1
Nuclear factor kappa light chain enhancer of activated B cells- 2 - *nfkb2*	TTCCGCAACCCTATGACCAC AGAAGTTCGGCCCATCCAAG	159	XM_026939857.1
Interleukin 1 beta - *il1β*	TTGGCCATGAGTGGCAGATG TCCTGGTCAGTGAACTCCGT	155	XM_026943671.1
Tumor necrosis factor - *tnf*	AGACCAGTCTTTCGCTTCGG CCCTCGGACTCATTATCGGC	129	XM_026942329.1
Interleukin 6 - *il6*	TGATAAGGTTCACCCAACCTCCT TCATGAAGTCTGCGAAGTTGTGC	104	XM_026930094.1
Caspase 8 - casp8	GGTACCGTGCTAGGGACTGA TCCATCGTGCCTCAACACAC	156	XM_026933210.1
Caspase 3 - casp3	CTGGCATCGAGGTTGACAGT ACAGGGACTGCATGAACCAC	141	XM_026947691.1
Tumor protein 53 - *tp53*	TCCAGGTGCGTGGGAAAGAG GCGATACTTCTCCTGGTCAGC	101	XM_026911853.1
16sRNA	TTACAACTGCCGACCAACGG CCTTAATAGCGGCTGCACCA	134	MF346571.1
Beta-actin	GAAATTGCCGCACTGGTTGTT TGTCTTGGGCGACCCACAAT	110	XM_026929614.1

calculated according to the relative standard curve method of Pfaffl [22], where $\Delta\Delta CT$ is $\Delta CT_{treatment} - \Delta CT_{control}$, ΔCT is $CT_{target\ gene} - CT_{housekeeping\ gene}$, and CT is the cycle at which the threshold is crossed. Data are presented as fold-change relative to internal control genes.

2.6. Statistical analysis

All statistical analyses were performed using SPSS version 20 (IBM Corp., Armonk, NY:IBM USA). Shapiro-Wilk's and Levene's tests served to check for normal distribution of the data and homogeneity of variances between groups. Results are presented as mean ± standard deviation (S.D.) for *in vitro* tests or as mean ± SEM (standard error of the means) for the *in vivo* experiment. Differences between immunological variables in fish from the different plant extract treatments and the control were determined with a one-way ANOVA (analysis of variance) followed by a post hoc Duncan's multiple range test at a confidence level of 95% ($p < 0.05$).

3. Results

3.1. CC and EA fractions differentially modulate the RBA, NOS and lysozyme activities in HKLs

In HKLs, both CC and EA fractions could differentially enhance the level of RBA, NOS, as well as lysozyme activities depending on dose and time (Fig. 1). All concentrations of the EA extract significantly increased the RBA activity compared to the control after a short time (6 h) of stimulation ($p < 0.05$), while the CC extract did not affect the RBA activity at this time point ($p > 0.05$) (Fig. 1A). Nevertheless, after 24 h, samples treated with 20 μg/mL of the CC extract showed the highest levels of RBA activity. Compared to the control, treatment with fractions significantly induced RBA activity at 24 h ($p < 0.05$) in most of the HKLs, except when treated with 40 μg/mL of the EA.

Also, NOS activity was considerably increased in the majority of the extract treatments at both time points ($p < 0.05$). Only the treatment with 10 μg/mL of CC statistically lowered NOS activity at 6 h ($p < 0.05$) (Fig. 1B).

The lysozyme activity was raised early (at 6 h) in HKLs treated with 40 μg/mL of CC and 10 μg/mL of EA, then increased continuously in most extract treatments compared to the control until 24 h ($p < 0.05$) (Fig. 1C).

3.2. Time and dose-dependent modulation of RBA, NOS and lysozyme activities of striped catfish stimulated by CC and EA fractions

The results indicate that spleen RBA, spleen NOS, and serum lysozyme activity at 6 h were not significantly increased by CC and EA fractions (Fig. 2). After 24 h of injection, the CC fraction at 40 μg/fish significantly inhibited the activity of RBA and NOS in the spleen, whereas the EA fraction (10 μg/fish) could significantly raised the levels of RBA, NOS and lysozyme activities when compared to the control ($p < 0.05$). Fish treated with high doses of CC statistically enhanced RBA and NOS activities in the spleen at 72 h ($p < 0.05$). Similarly, a significant increase of the NOS level was observed in fish treated with high doses (40 μg/fish) of the EA fraction ($p < 0.05$). Serum lysozyme activity only significantly increased at 72 h when treated with the EA fraction (10 μg/fish).

3.3. Time and dose-dependent modulation of cytokine expression in head kidney of striped catfish treated with CC and EA fractions

3.3.1. Expression of cytokines involved in innate and adaptive immune responses

After stimulation with fractions, mRNA levels of *lys*, *inos*, *tlr1*, *tlr2*, *tlr4*, *myd88*, *traf6* and *mhc II* in the head kidney were measured at different time points to assess the innate immune status of striped catfish (Fig. 3). *Lys* expression showed significant variations depending on dose and sampling time. Both, CC and EA fractions did not affect the mRNA level of *lys* after 6 h of injection. However, the *lys* expression was significantly upregulated at 24 h in fish treated with 10 μg/fish of EA fraction ($p < 0.05$). Similar augmentations were observed after 72 h when treated with a high dose of the CC fraction.

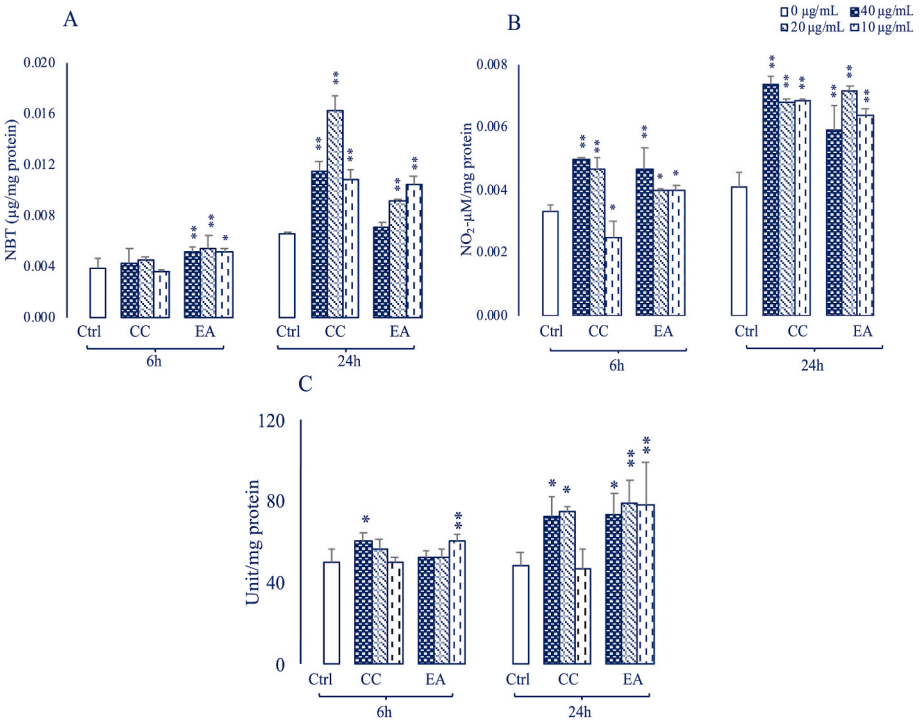

Fig. 1. Immune parameters A) Respiratory burst activity- RBA, B) Nitric oxide synthase- NOS, and C) lysozyme of striped catfish head kidney leukocytes stimulated with different concentrations of each dichloromethane (CC) or ethyl acetae (EA) extract fraction. Values are presented as means ± SD.

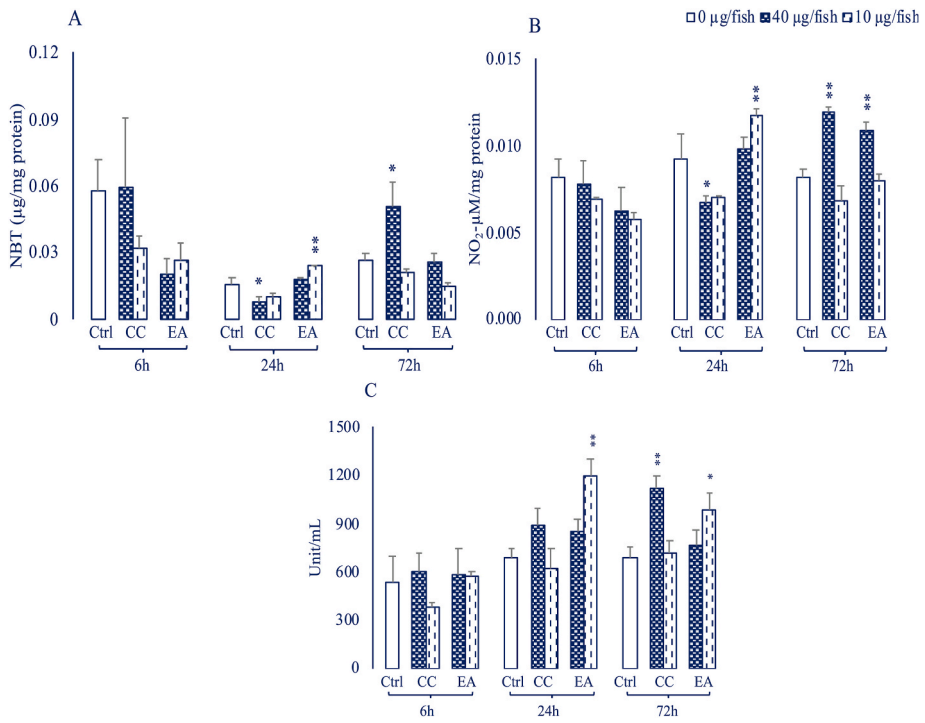

Fig. 2. Immune parameters A) Respiratory burst activity- RBA, B) Nitric oxide synthase- NOS, and C) lysozyme of striped catfish were intraperitoneally injected with different concentrations of each dichloromethane (CC) or ethyl acetae (EA) extract fraction. Values are presented as means ± SEM.

Treatment with both doses of the CC fraction, resulted in a significant increase in the mRNA level of *myd88* compared to the control after a short time - 6 h ($p < 0.05$), whereas *myd88* levels did not change following treatment with EA fractions. In most treatments, *myd88* expression reverted to its original levels after 24 and 72 h.

The mRNA expression of *tlrs* and *tlr1* reached maximum levels after 6 h in fish treated with the high dose of the CC fraction; levels then returned to baseline at 24 and 72 h post stimulation. None of the fractions significantly affected the *tlr2* expression throughout the sampling time. Additionally, at the early time points, the mRNA level of *tlr4* in the head kidney was significantly enhanced in fish treated with the high dose of the CC fraction. Moreover, the *tlr4* mRNA level was significantly upregulated in fish treated with both doses of the EA fraction at 72 h compared to the control ($p < 0.05$).

Compared to the control ($p < 0.05$), the maximum level of *traf6* expression was recorded in striped catfish treated with the low dose of the CC fraction after 6 h. Conversely, the *traf6* expression was significantly downregulated in fish treated with the high dose of CC after 24 h ($p < 0.01$). In all other experimental treatments, the *traf6* expression was similar to the control after 72 h ($p > 0.05$).

At the early time (6 h) of stimulation, the fractions had no effect on the mRNA level of *inos* ($p > 0.05$). However, after 24 h, significantly higher expression levels ($p < 0.05$) of *inos* were detected in fish treated with low doses of EA fractions. Further, at 72 h, high doses of the CC fraction significantly increased the *inos* level compared to the control ($p > 0.05$).

None of the doses of CC and EA fractions significantly enhanced the mRNA expression of *mhc class II* throughout the sampling period.

3.3.2. Expression of cytokines related to inflammatory responses

The levels of inflammatory cytokines varied depending on the fraction used (Fig. 4). Fish treatment with CC and EA fractions did not affect the mRNA expression of *nfκb1* at any time point. However, shortly after stimulation (6 h), the expression of *nfκb2* was upregulated in fish treated with 10 µg/fish of EA compared to the control ($p < 0.01$), the level returning to the baseline at the later time points. Contrarily, the level of *nfκb2* showed no effect at 6 h following stimulation with the EA fraction and then increased significantly after 24 h compared to the control to finally revert to initial levels at 72 h post stimulation.

mRNA levels of both, *il1β* and *il6,* were statistically higher at 6 h post treatment with CC fractions compared to the control ($p < 0.01$), and then returned to normal immune homeostasis after 24 h. Similarly, fish treatment with a high dose of the CC fraction significantly enhanced the *tnf* in the head kidney ($p < 0.05$). However, no differences were detected in the expression of *il1β, il6* and *tnf* in fish treated with EA fractions throughout the sampling period ($p > 0.05$).

3.3.3. Expression of cytokines related to apoptosis process

In the head kidney of striped catfish, CC and EA treatments did not influence the relative expression of *casp3* throughout the sampling time ($p > 0.05$) (Fig. 5). Whereas the level of *casp8* was significantly upregulated 6 h after stimulation with both doses of the CC fraction ($p < 0.05$). Similar observations were made after 24 h at the low dose of CC and at both doses of EA fractions ($p < 0.05$). Furthermore, treating fish with both doses of the CC fraction could significantly decrease the expression of *casp8* at 72 h compared to the control ($p < 0.05$). Additionally, only fish treated with the low dose of the CC fraction significantly upregulated *tp53* expression at 6 h ($p < 0.05$).

4. Discussion

Plant products have been identified as potential therapeutic treatments by modulating immunity and preventing or controlling fish diseases [23]. The levels of bioactive compounds in the extract may vary greatly due to the polarity of the extraction solvents, resulting in a large range of extraction yields [24]. To the best of our knowledge, the phytochemical constituents of the CC fraction of guava ethanol extracts have not yet been published. The CC fraction of guava methanol extract contained a moderate amount of flavonoids and low levels of phenolics and tannins. Specifically, the CC fraction mainly consisted of 2^1, 6^1-dihydroxy-4^1-methoxychalcone, caryophyllene, 1-methylindene, pseudocumene, and α-copaene [25]. Whereas the components of the

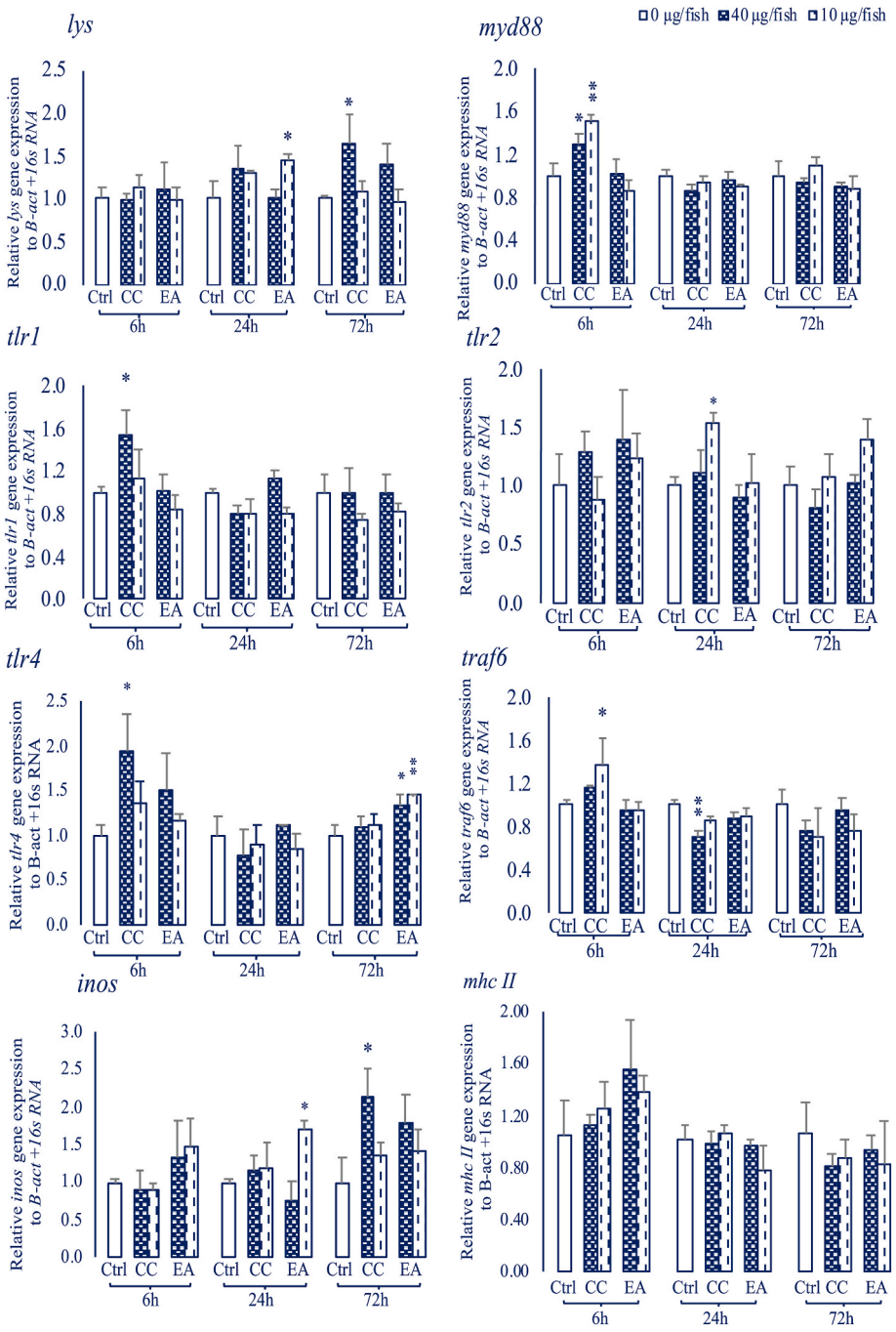

Fig. 3. Expression of cytokine genes related to immune response in head kidney of striped catfish at 6, 24 and 72 h after intraperitaneous injection of P. guajava extract fractions (40 and 10 µg per fish). Bars represent the mean ± SEM (n = 3) of relative mRNA expression as a fold change relative to beta actin and 16s RNA. Significant differences compare to control treatment: *p < 0.05, **p < 0.01. CC: Dichloromethane, EA: ethyl acetate.

EA fraction of guava ethanol extract included quercetin, avicularin, ellagic acid, gossypetin, 5,6,2′-trimethoxyflavone, epicatechin, catechina, procyanidin B2, myricetin-3-O-pentoside, chebuloside II, (10E, 15E)-9,12,13-trihydroxyoctadeca-10,15-dienoic acid, 9,12,13-trihydroxyoctadec-10-enoic acid, kaempferol and epicatechin gallate [26]. In the present study, both *in vitro* and *in vivo* tests indicated that the CC and EA fractions acted as immunostimulators via their capacity to enhance RBA, NOS and lysozyme activities. ROS primarily appears during phagocytosis, and is mainly released on the endosomal membranes of the phagocytosing cells (*i.e.* neutrophils and macrophages) [27]. Nitric oxide is an important mediator of biological and immune functions, including inflammation and cytotoxicity against invading organisms. Moreover, the nitric oxide response is linked to the respiratory burst via a tryptophan degradation response [28]. Here, we observed that the RBA activity in HKLs was significantly increased at an early time point (6 h) after stimulation with the EA fraction, whereas the RBA level in CC treatments significantly increased at the later time point, 24 h post stimulation. Conversely, HKLs stimulated with most fractions significantly enhanced the NOS activity after 6 and 24 h. However, intraperitoneal administration of both fractions to fish did not affect the RBA level after 6 h. The up- or downregulation of RBA and NOS activities depended on time and dose. In line with our results, Mbaveng et al. (2018) indicated that a guava bark extract significantly increased the production of ROS in leukemia CCRF-CEM cells after 24 h

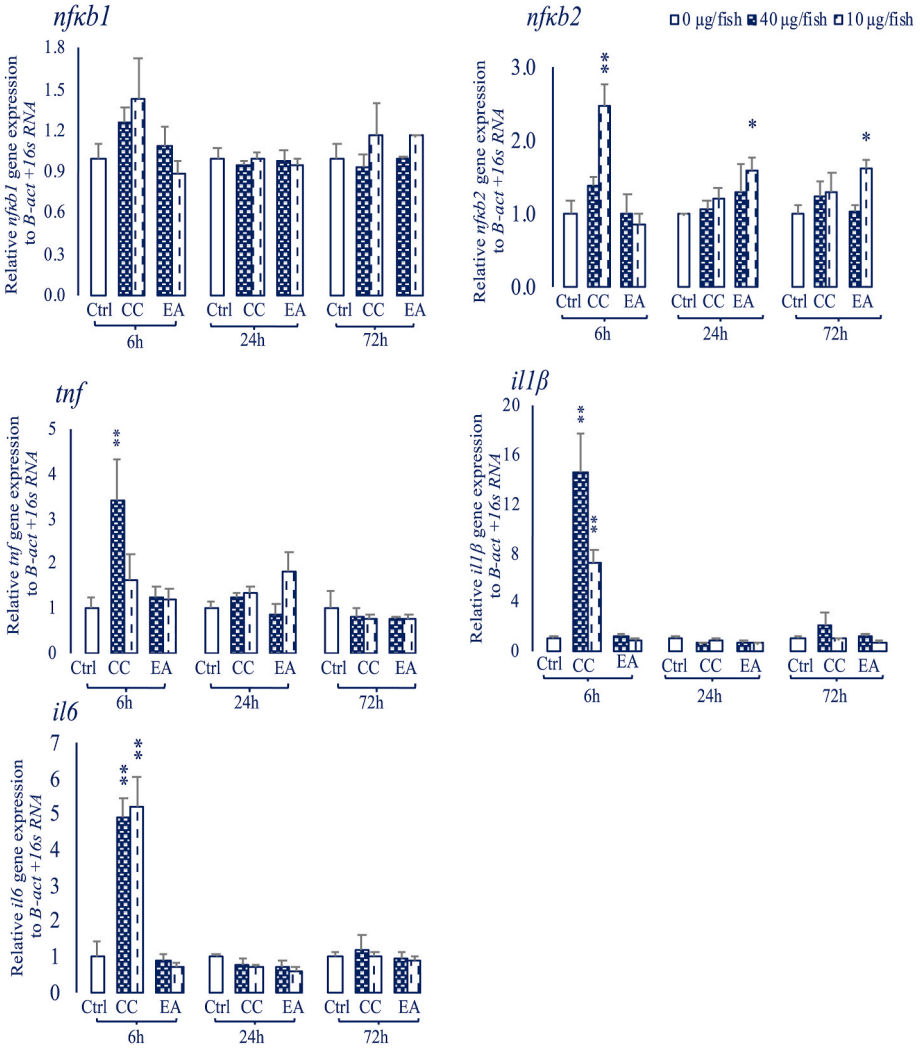

Fig. 4. Expression of cytokine genes related to inflammatory in head kidney of striped catfish at 6, 24 and 72 h after intraperitaneous injection of P. guajava extract fractions (40 and 10 μg per fish). Bars represent the mean ± SEM (n = 3) of relative mRNA expression as a fold change relative to beta actin and 16s RNA. Significant differences compare to control treatment: *p < 0.05, **p < 0.01. CC: Dichloromethane, EA: ethyl acetate.

[12]. Nguyen et al. (2019) demonstrated that guava leave extracts also significantly enhanced the ROS activity in the human hepatocellular carcinoma cell line HepG2 at 48 h [29]. Moreover, ethyl acetate extracts from murtilla (*Ugni molinae* Turcz) increased the availability of nitric oxide in bovine aortic endothelial cells via the activation of the protein kinase B (AKT) and eNOS phosphorylation [30]. Additionally, we also found that the CC fraction significantly inhibited the release of ROS and NOS activities, whereas the EA fraction significantly enhanced these activities after 24 h. In conclusion, the biological activities related to immune responses may be strongly affected by the kind of extract solvent. Moreover, the amount of tannin in the extract may influence the expression of immune markers. Plant-derived tannins directly enhanced innate immunity via proliferation of γδ T lymphocytes [31] and activated macrophages in RAW 264.7 cells infected with *Leishmania* [32].

Lysozyme, which belongs to the humoral immune response and is mainly produced in the neutrophil [33], plays a more important role in the host by mediating protection against microbial invasion. In the present experiment, both, CC and EA fractions significantly increased the lysozyme activity in *in vitro* testing, whereas only the low dose of the EA fraction could significantly stimulate the lysozyme level in the *in vivo* experiment. As mentioned above, the activation of macrophages not only triggered RBA and NOS activities but also increased lysozyme activity [34]. However, the lysozyme results in the present study did not always correspond with the RBA or NOS activity discussed earlier. This may be due to the fact that the *P. guajava* extract contains some phenolic compounds (*i.e.* tannins, terpenoids and alkaloids) [35], which are capable to bind and inhibit proteins such as enzymes [36].

Aside from the humoral and cellular immune responses, our study also provided evidence that both guava fractions could positively regulate the cytokine-related gene expressions in striped catfish shortly after stimulation. Indeed, relative *inos* and *lys* expressions were differentially enhanced depending on dose and time. Consequently, the level of *inos* and *lys* increased accompanied by NOS and lysozyme activities in the spleen and serum of striped catfish. Similar to mammals, the immune defense of fish recognizes pathogens via various types of cell surface pattern recognition receptors, such as toll like receptors (TLRs) [37]. Our study highlighted that mRNA levels of *tlr1* and *tlr4* were significantly increased at high doses of CC after 6 h. Similarly, the *tlr2* expression was statistically increased in fish treated with low doses of CC fractions after 24 h. In agreement with our results, Chen et al. (2017) reported that polysaccharides in a water extract of *Salvia miltiorrhiza* Bunge significantly enhanced transcript levels of *tl1*, *tlr2* and *tlr4* in peripheral blood T lymphocytes of cancer patients [38]. In the present study, the *myd88* expression was found to be significantly upregulated in fish treated with both doses of the CC fraction after 6 h. Later, the *myd88* transcript was again reduced to the basal immune homeostasis. Su et al.

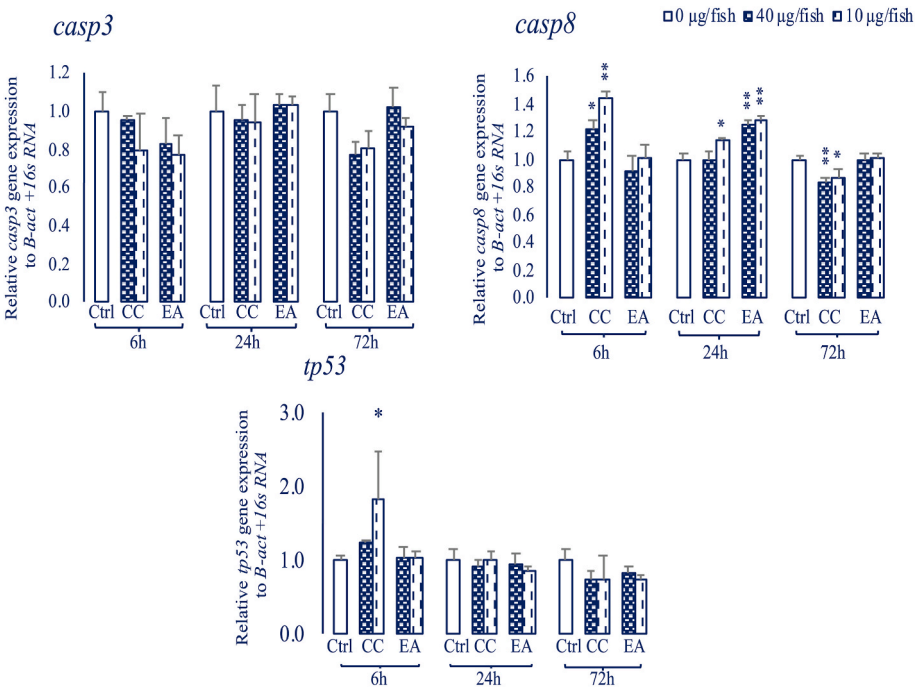

Fig. 5. Expression of cytokine genes related to immune response in head kidney of striped catfish at 6, 24 and 72 h after intraperitaneous injection of P. guajava extract fractions (40 and 10 µg per fish). Bars represent the mean ± SEM (n = 3) of relative mRNA expression as a fold change relative to beta actin and 16s RNA. Significant differences compare to control treatment: *p < 0.05, **p < 0.01. CC: Dichloromethane, EA: ethyl acetate.

(2011) also demonstrated that mRNA levels of *myd88* in head kidney cells of grass carp (*Ctenopharyngodon idella*) treated with polycytidylic acid considerably increased after short time (2 and 8 h) and then returned to control levels after 24 h [39]. A similar increase of *myd88* expression was observed in grass carp head kidney macrophages stimulated with kaempferol 3-O-L-(4-O-acetyl) rhamnopyranoside-7-a-L-rhamnopyranoside after 8 h [40]. We also found that CC and EA fractions could regulate the expression of *traf6* depending on time and dose. Arain et al. (2019) reported that the mRNA level of *traf6* was significantly upregulated in peripheral blood leukocytes of goats stimulated with *Debaryomyces hansenii* β-glucan [6]. However, our results showed no effect on the mRNA expression level of *mhc class II* in fish treated with CC and EA fractions, suggesting that *P. guajava* fractions may not stimulate the adaptive immune response following the first hours after treatment.

Activating TLRs signaling pathways following recruitment of *myd88* and *traf6* adaptor proteins results in the production of proinflammatory cytokines and adaptive immunity [39]. Among the inflammatory cytokines, the nuclear transcription factor *nfκb* has been considered important in regulating the expression of various genes that are vital in apoptosis and immunomodulation [41]. In the current study, the *nfκb2* transcript level was significantly upregulated early (6 h) in fish treated with the CC fraction, whereas the low dose of the EA treatment could significantly stimulate the *nfκb2* expression at the later time point (24 h). The level continued to increase gradually until 72 h. RualaCap, a bioactive fraction isolated from *Russula alatoreticula,* potentially enhanced the transcript level of *nfκb* in RAW 264.7 murine macrophages [42]. Also studying RAW 264.7 macrophages, Shen et al. (2017) suggested that polysaccharides isolated from wheat bran rapidly upregulated mRNA levels of *nfκb* via *tlr4* activation [43]. Additionally, simultaneous increase of *il1β* and *tnfα* could enhance the production of iNOS [44] and notably regulate the expression of IL-6 [45]. Interestingly, our results show that the mRNA levels of *tnf*, *il1β* and *il6* were considerably upregulated after 6 h in fish treated with the CC fraction. A similar upregulation of *il1β* transcript was observed in common carp (*Cyprinus carpio*) fingerlings fed with diets enriched with guava leaf powder [13]. In addition, a significant upregulation of levels of *il-1β* and the tumor necrosis factor alpha (*tnfα*) and downregulation of expression genes of *inos*, *nfkb*, cyclooxygenase-2 (*cox2*), and the transforming growth factor beta (*tgfβ*) was observed in different tissues of rohu after feeding a diet supplemented with 0.5% guava leaf extract [8]. However, high concentrations of guava leaves could significantly inhibit the expression level of iNOS and cyclooxygenase-2 protein levels by down-regulating NF-κB in mouse macrophage cells following an inflammation stimulated by LPS [10]. Flavonoid fractions of guava leaf extracts could reduce the NF-κB activation in head kidney macrophages of rohu *Labeo rohita* following inflammation stimulated by LPS [11]. These studies combined with our results confirm that guava extracts not only activate proinflammatory cytokines during the onset of inflammations, but also enhance/reinforce anti-inflammatory cytokines during the resolution of an inflammation. Further studies should optimize the employed doses to achieve the best immunotherapeutic applications of guava extracts.

Apoptosis plays an important role in limiting the spread of pathogens and helps to protect the integrity of surrounding tissues by removing infected cells [46]. Our results showed that *casp3* transcript levels were not significantly affected by the two fractions. However, CC and EA fractions could differentially regulate the expression of *casp8* in the head kidney of striped catfish. Mbaveng et al. (2018) also reported that CCRF-CEM cells stimulated with a *P. guajava* bark extract could activate the CASP3/7, 8, and 9 activities, and then induce apoptosis [12]. Similarly, a significant increase of CASP8 and CASP9 activities was observed in SW480 cells stimulated with *Teucrum chamaedrys* L. extract [47]. CASP8 activation happened via both the death receptor pathway and the mitochondrial pathway. Moreover, the P53 family, including the tp53 transcript, is important in upregulating caspase activation [48]. In the present study, only a low dose of the CC fraction significantly enhanced the mRNA level of *tp53* in the head kidney of striped catfish after 6 h, returning to a basal level after 24 h.

5. Conclusions

Our study highlighted via *in vivo* and *in vitro* experiments that CC and EA fractions positively enhanced humoral (lysozyme) and cellular (NOS and ROS) immune responses in striped catfish. Moreover, these fractions potentially upregulated the expression of cytokine genes involved in immune, inflammatory, and apoptotic responses at an early time point. The CC fraction could upregulate the cytokine genes related to immune responses (*myd88*, *tlr1*, *tlr4*, and *traf6*), inflammation (*nfκb2, tnf, il1β,* and *il6*) *and* apoptosis (*tp53* and *casp8*) 6 h after injection into fish, depending on concentration. Besides, two genes (*nfκb2* and *casp8*) were upregulated in fish treated with the AE fraction. The results support the possibility of using *P. guajava* as a natural pharmaceutical in fish. However, additional studies are still needed to investigate mechanisms by which the active compounds present in this plant could induce an immunomodulatory response.

CRediT authorship contribution statement

Truong Quynh Nhu: Investigation, Formal analysis, Validation, Writing – original draft. **Hang Bui Thi Bich:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Huong Do Thi Thanh:** Conceptualization, Methodology. **Marie-Louise Scippo:** Conceptualization, Methodology. **Phuong Nguyen Thanh:** Conceptualization, Methodology. **Joëlle Quetin-Leclercq:** Conceptualization, Methodology, Writing – review & editing. **Patrick Kestemont:** Conceptualization, Methodology, Writing – review & editing, Supervision.

Data availability

Data will be made available on request.

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