Fish and Shellfish Immunology 89 (2019) 603–613

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Effects of *Moringa oleifera* leaf extract on growth performance, physiological and immune response, and related immune gene expression of *Macrobrachium rosenbergii* with *Vibrio anguillarum* and ammonia stress

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ARTICLE INFO

Keywords:
M. rosenbergii
M. oleifera
Ammonia stress
Immunity
Growth

ABSTRACT

In order to study the effects of *Moringa oleifera* leaf extract on *Macrobrachium rosenbergii* under high ammonia exposure, freshwater prawns were randomly divided into five groups: a control group was fed with basal diet, and four treatment groups fed with basal diet supplemented with 0.25%, 0.5% and 1.0% *M. oleifera* leaf extract and 0.025% Enrofloxacin for 60 days, respectively. Then, freshwater prawns were exposed to high ammonia stress for 72 h and *Vibro anguillarum* infection. The growth, antioxidant capabilities, related immune genes as well as resistance to infection by *V. anguillarum* were determined. The results showed that compared with the control group, the weight gain, specific growth rate and protein efficiency rate, haemolymph catalase (CAT), superoxide dismutase (SOD) and inducible nitric oxide synthase (iNOS) increased while feed conversion ratio, haemolymph aspartate aminotransferase, alanine aminotransferase, nitrogen oxide (NO), hepatopancreas heat shock proteins (HSP70), immune deficiency (IMD) expression levels decreased in the group of 0.5% *M. oleifera* leaf extract before the stress. After ammonia stress, the group of 0.5% *M. oleifera* leaf extract also could improve the haemolymph SOD, glutathione peroxidase, NO, iNOS, hepatopancreas HSP70 expression levels and reduce haemolymph CAT, hepatopancreas peroxiredoxin 5 and NF kappa B inhibitor alpha expression level compared with the control group. The rate of mortality of the prawns challenged with *V. anguillarum* was lower in the supplemented groups in comparison with the control group with the lowest being in the group of 0.5% *M. oleifera* leaf extract. Antioxidant activities as well as biochemical parameters in the enrofloxacin group (0.025% E) were not significantly enhanced both pre and post challenge in comparison with the *M. oleifera* leaf extract groups, showing the superiority of the natural herb over the synthetic antibiotic. In summary, this study suggested that at an inclusion rate of 0.5%, *M. oleifera* leaf extract could increase the growth performance, even has positive effects on physiological and immune function and prevents high ammonia stress in the Freshwater prawn, *M.rosenbergii*.

1. Introduction

Aquaculture over the years despite its many benefits in producing an affordable source of protein to feed the world population has not been without its own share of problems. Some of these problems have increased in intensity and occurrence due to the intensification drive of increasing aquaculture production. One of the consequences in aquaculture is the prevalence of diseases. This led to huge economic losses as a result of disease outbreaks [1,2]. In order to curb and treat diseases, antibiotics have widely been employed [3]. The frequent and often unregulated use of antibiotics has led to its abuse among the farmers all over the world. Some of the consequences of this behavior have been

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https://doi.org/10.1016/j.fsi.2019.03.039
Received 22 January 2019; Received in revised form 12 March 2019; Accepted 14 March 2019
Available online 19 March 2019

the development of anti-resistant pathogens [4] especially close to aquaculture sites. The uses of antibiotics are not without their own consequences [5,6]. The European Union (EU), recognizing the devastating effects of antibiotics, has since the year 2006 placed a ban on the use of antibiotics as feed additives in the production of food animals [7]. One of the most common antibiotics used in shrimp aquaculture to deal with bacterial diseases is enrofloxacin [8]. It belongs to the Quinolone family of antibiotics and have been used both prophylactically and chemotherapeutically in dealing with infections caused by bacterial agents [9–11]. The misuse of this and other synthetic antibiotics in aquaculture leads to resistance to antibiotics.

As an alternative to antibiotics, natural, environmentally safe as well as cost effective solutions have been sought after and encouraged in recent years. This has shone the light on the application of herbal medicines as very viable alternatives with superior properties. In China, there is a long history of the application of traditional herbs in the treatment of various human ailments [12,13]. An important herbal plant that has been recognized in aquaculture over the years has been *Moringa oleifera*. Originally from the Himalayas and Northern India, *Moringa oleifera* Lam is a softwood tree which has been widely investigated and found to have very useful applications in humans [14]. It belongs to the family Moringaceae [15] and in Africa the highest cultivation rate comes from countries such as Senegal, Ghana and Malawi [16]. It is believed in traditional Ayurvedic medicine that in excess of 300 diseases can be prevented by *M. oleifera* [17]. The leaves, immature pods, fruits, and other parts of this tree have been used for their nutritional benefits in many countries since they are edible [18]. The leaves are rich in carotenoids, vitamin A and C, potassium and protein [19]. Having a great number of bioactive compounds including polyphenols (phenolic acids and flavonoids), carotenoids, the leaves of *M. oleifera* tend to be the most used part of the tree [14,20].

In mammals, studies have been conducted to evaluate the effects of *M. oleifera* on the immune responses [21], as well as anti-inflammation [22]. In aquaculture, *M. oleifera* leaf meal can be used to partially replace protein in the diet for Nile tilapia [23], improve the immunity of *Oreochromis niloticus* [24] and enhance antibacterial properties for *Macrobrachium amazonicum* [25]. However, application of high quantity of *M. oleifera* seed powder in aquaculture leads to mortality of fish due to the presence of toxic substances or anti-nutritional factors [26,27]. Up to now, whether *M.oleifera* leaf extract could improve the growth performance and innate immunity of *Macrobrachium rosenbergii* is still unknown.

In shrimp culture and aquaculture in general, one of the environmental pollutants that poses a major concern with regards to the survival of shrimps is ammonia [28]. Ammonia has been linked to the facilitation of increases in reactive oxygen species leading to oxidative stress in organisms [29]. It is produced as a main nitrogenous product formed as result of the breaking down of protein in aquatic organism and also through the decay of uneaten food as well as faeces in the water environment [30]. Reduction in growth, tissue erosion and degeneration, immune suppression and high mortality of the fish could arise as result of the accumulation of high amounts of ammonia in the body fluids of aquatic organisms [31–33]. It is therefore imperative that more natural, sustainable and environmentally safe alternatives are identified to help to reduce the ammonia stress and disease.

M.rosenbergii also referred to as the giant freshwater prawn is of importance as a crustacean culture species the world over. Its place of origin is Northern Ocean, Western pacific Islands, South Pacific Countries, South East Asia [34,35]. It is one of the most cultured freshwater prawns in the world. Globally, the total production of *M.rosenbergii* was 198 000 tons in 2012 and this rose to 234 000 tons in 2016 [36,37]. Of the total production of *M.rosenbergii,* China accounted for 132 678 tons, representing nearly 60% of the total production for that year. Its significance as a culture species stems from the fact that it is highly tolerable to different environments, has high growth performance [38,39]. However, similar to other aquatic organisms, their growth and well-being is influenced by various physical, chemical and biological factors [40], which have caused significant economic losses. To date, the effect of *M.oleifera* leaf extract on *M.rosenbergii* physiological responses under ammonia stress has hardly been found in research reports. In view if this, this work seeks to determine the effects *M.oleifera* leaf extract will have on the growth, physiological and immune response together with their related genes expressions in *M.rosenbergii* under ammonia, hopefully serving as a better alternative to synthetic chemicals and antibiotics.

2. Materials and methods

2.1. Animals and experimental system

The freshwater prawns were supplied by Zhejiang South Taihu Lake Freshwater Fish Breeding co. Ltd., Huzhou, Prawns of similar sizes (0.22 ± 0.001g) were selected to be assigned into 15 concrete tanks (2.0 m × 1.50 m × 0.7 m) randomly and acclimatized for 7 days. Thereafter, freshwater prawns were randomly divided into five groups: one control and four treatment groups. Triplicate groups of *M. rosenbergii* (3 tanks, 50 individuals per tank) were fed with the basal diet (see Table 1) and the basal diet supplied with 0.25% *M. oleifera* leaf extract (0.25M), 0.5% *M. oleifera* leaf extract (0.5M), 1.0% *M. oleifera* leaf extract (1.0M) and 0.025% Enrofloxacin (0.025E), respectively. There was a random arrangement of three tanks and these were assigned to each of the test diets.

2.2. Preparation of diets

The diets were formulated to contain the same nutrients with the different levels of *M. oleifera* (0, 0.25%, 0.5% and 1.0%) and 0.025%

Table 1
Ingredients and proximate chemical composition of the experimental diets.

Ingredients	Diet 1 0%	Diet 2 0.25% *M. oleifera* leaf extract	Diet3 0.5% *M. oleifera* leaf extract	Diet4 1.0% *M. oleifera* leaf extract	Diet5 0.025% Enrofloxacin
Fish meal	35	35	35	35	35
Soybean meal	20	20	20	20	20
Rapeseed meal	10	10	10	10	10
Shrimp powder	8	8	8	8	8
α-starch	14	14	14	14	14
Soybean oil	3	3	3	3	3
Squid paste	3	3	3	3	3
Lecithin Powder	1	1	1	1	1
Cholesterol	0.3	0.3	0.3	0.3	0.3
Ecdysone	0.2	0.2	0.2	0.2	0.2
Choline chloride (50%)	1	1	1	1	1
Vitamin additive	1	1	1	1	1
Mineral additives	1	1	1	0.5	1
Bentonite	0.5	0.25	0	0	0.48
Calcium dihydrogen phosphate	2	2	2	2	2
Moringa leaves extract	0	0.25	0.5	1	
Enrofloxacin					0.025
Total	100	100	100	100	100
Proximate Composition					
Dry matter	90.15	90.15	90.15	90.15	90.15
Crude protein (%)	40.54	40.54	40.54	40.54	40.54
Ether extract (%)	8.11	8.11	8.11	8.11	8.11
Nitrogen Free extract	26.43	26.43	26.43	26.43	26.43

Note: Vitamin and mineral additives were provided by Wuxi Hanove Animal Health Products Co.,Ltd.

Enrofloxacin. Fish meal, shrimp powder, soybean meal, rapeseed meal were the main protein sources for the diets. All the ingredients were ground into powder and thoroughly mixed with the oil and water and then pelleted (1.5 mm and 2.0 mm in diameter) using Lab pelletizer (Science and Technology Industrial Factory of South China University of Technology, Guangzhou, China). Pellets were air-dried to approximately 10% moisture, sealed in plastic bags and stored at −15 °C until used.

M. oleifera leaf extract was commercial product supplied by Xi'an Realin Biotechnology Co., Ltd., Xi'an, China.

According to the reference of Brilhante et al. (2015) [25], the extraction process of Moringa oleifera leaf extract made the appropriate modifications below. First, the leaves of *M. oleifera* were washed and dried using dryer. Then *M. oleifera* leaves was crushed into powder. The coarse powder of the dried *M. oleifera* leaves was soaked using 75% ethanol a Soxhlet extractor for 2 h, then *M. oleifera* leaves was extracted by 75% ethanol with heat reflux extraction twice, 3h per reflux extraction. After this, liquid mixture was separated and concentrated at 65–75 °C. The extracts were concentrated and sterilized, and then was stored in airtight container in cool place and used throughout the study. Enrofloxacin with a purity of above 99% as supplied by Wuxi zhongshui fishery medicine co. Ltd., Wuxi, China.

2.3. Husbandry practices

The freshwater prawns of an initial weight of 0.22 ± 0.001g were selected after acclimatization and restocked at a density of 50 prawns per tank. After, three tanks were randomly selected and assigned to each of the test diets. The prawns were fed with the experimental diets at a feeding rate of about 6.0%–8.0% body weight to near satiation. Feeding was done three times daily (7:00–7:30, 12:00–12:30 and 17:00–17:30) for a period of 60 days. Water from an underground source was used. Daily, silt was siphoned before feeding with an exchange of one-third of water in each tank once every two weeks. The system was provided with oxygen throughout the entire period by the use of an aerator. Water temperature was measured daily and recorded in a data logger throughout the period. Water quality parameters recorded during the period were as follows: temperature range 25–32 °C, pH 7.8–8.5, NH_3 < 0.05 mg/L, DO > 5 mg L^1. The other parameters apart from temperature were measured weekly. Based on body weight measurements, the feeding amount was adjusted.

2.4. Challenge test

2.4.1. Vibrio anguillarum challenge

After feeding experiment, 10 prawns in each tank were stocked in plastic tanks containing 10 L of water with a total of 30 prawns per group (3 tanks per group). The LC_{50} was determined by intraperitoneal injection of three groups of shrimps with different concentrations of *V. anguillarum*, and the result showed that the LC_{50} was 5×10^7 CFU/mL. In order to reach the final concentration of 5×10^7 CFU/mL, *V. anguillarum* was activated twice and diluted by bacterial normal saline. This was carried out according to the method described by Su et al. (2008) [41]. The prawns were then injected with 0.05 mL of the bacterial solution in the abdominal cavity. The total number of dead prawns during the period was recorded. The mortality of the prawns was monitored throughout an entire period of 96 h.

2.4.2. Ammonia challenge

The prawns of uniform size were selected for the experiment of ammonia stress after rearing period. According to the reference of Ding et al. (2017) [33], a total of 24 prawns in the each group with triplication (8 prawns in each tank) were immersed in plastic tanks containing 10 L of water with the concentration of 16.7 mg/L total ammonia-nitrogen, with sufficient oxygen (DO > 5 mg/L), in a quite environment with no human interference. A stock solution of high purity NH_4Cl (1 g/L) was used as a source of the total ammonia-nitrogen, which was subsequently diluted to the desired concentrations of total ammonia-nitrogen. The concentrations of ammonia were determined every 12 h using the spectrophotometric method described by Solórzano (1969) [42]. Over a period of 72 h, mortality of shrimps in the various treatment groups was monitored. Samples were taken after the ammonia challenge for further analysis after the experiment.

2.5. Sample collection

At the conclusion of the culturing experiment, the prawns were starved for 24 h to get rid of the contents in the alimentary tract. The length and weight of each prawn was measured. The sampled prawns were then dissected gently and samples of hepatopancreas were collected and weighed for further analysis. 9 haemolymph samples (3 prawns per tank, 3 tanks per group) before or after ammonia challenge were taken from each group. Using a 1 mL syringe kept at 4 °C, the samples of haemolymph were taken from the abdominal cavity or the area around the heart ventricle. Alsever's solution was used as the anticoagulant, and the proportion of haemolymph to the anticoagulant was 1:1 [43]. The haemolymph samples were kept undisturbed in refrigerator for 1–2 h at 4 °C, and then centrifuged for 10 min (4 °C, 800 × *g*). The supernatant of haemolymph and hepatopancreas were stored at a temperature of −80 °C for further analysis.

2.6. Growth performance measurements

Progress in terms of the freshwater prawn growth was measured using indices such as specific growth rate (SGR), weight gain (WG). Parameters such as feed conversion ratio (FCR) and protein efficiency ratio (PER) were used to determine feed utilization. The formulae were as followed

Specific growth rate (SGR; %/day) = [(ln final body weight–ln initial body weight)/feeding days] × 100

Feed conversion ratio (FCR) = [feed intake (g) / weight gain (g)]

Weight gain rate (WG, %) = [(final body weight - initial body weight)/ initial body weight] × 100

PER = WG (g)/protein intake (g)

2.7. Haemolymph biochemical measurements

With the aid of the Mindray Auto Bio-chemical Analyzer (BS-400, Mindray, China) biochemical parameters such as haemolymph total protein content (TP), Albumin (ALB) content, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities of 9 haemolymph samples in each group were determined using the colorimetric method using test kits from Mindray Bio Medical Co., Ltd., Shenzhen, China.

2.8. Antioxidant activities and immune parameters determination

Using the methods enumerated by Rice-Evans and Miller (1994) [44], Wang et al.(1999) [45] and Xiang and Wang (1990) [46], the catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) of 9 haemolymph samples in each group were measured by spectrophotometric means using devices from Bio-tek Instruments, Inc., USA. Commercial tests kits were acquired from Nanjing Jiancheng Bioengineering Institute China. According to the method by Zhu et al. (2006) [47], the nitrogen oxide (NO) concentration and inducible nitric oxide synthase (iNOS) of 9 haemolymph samples in each group were determined by nitrate reductase method and colormetric method. The test kit for the tests was acquired from Nanjing Jiancheng Biological Engineering Research Institute.

Table 2
Real time PCR sequence.

Gene	Primer sequences (5′-3′)	Length (bp)	Amplification length (bp)	Sequence source
Relish	GATGAGCCTTCAGTGCCAGA	20	238	KR827675.1
	CCAGGTGACGCCATGTATCA	20		
NFκBI-α	AATCATACCGGAAGGACGGCGTTA	24	133	HQ668091.1
	TCACGGGTCTGGTTAATTGGGTCA	24		
IMD[1]	CGACCACATTCTCCTCCTCCC	21	184	Shi, 2016
	TTCAGTGCATCCACGTCCCTC	21		
Peroxiredoxin-5[2]	ACTGTGTCACCTTGCCATCTT	21	94	Transcriptome data
	AAATCCCTTGGGCTGGAACAA	21		
HSP70	TGACAAGGGTCGCCTCAGTA	20	158	EU884290.2
	CATTATCTTGTTGCGATCCTC	21		
β-actin	TCCGTAAGGACCTGTATGCC	20	96	AY651918.2
	TCGGGAGGTGCGATGATTTT	20		

Note: 1.The primer sequence of IMD was obtained from the reference of Shi, 2016 [48]. 2. The sequence of Peroxiredoxin-5 was obtained from the the transcriptome data of *Macrobrachium rosenbergii* in our group.

2.9. Real time PCR measurement of NF-κB pathway

In accordance with the partial cDNA sequence of the target gene using *M. rosenbergii* transcriptome analysis acquired from the Department of Fish diseases and Nutrition, Freshwater Fisheries Research Center, China, specific primers of NF kappa B inhibitor alpha (NFκBI-α, gene bank No. HQ668091.1), the NF kappa B factor Relish (Relish, gene bank No.KR827675.1), heat shock proteins (HSP70, gene bank No.EU884290.2), peroxiredoxin 5, immune deficiency (IMD) as well as β-actin (gene bank No.AY651918.2) the target genes were designed (Table 2). The cDNA sequence of peroxiredoxin 5 was obtained from the transcriptome data of *Macrobrachium rosenbergii* in our group.The cDNA sequence of IMD was obtained from the reference of Shi, 2016 [48].

Synthesization of all the primers was done by Shanghai Generay Biotech Co. Ltd., (Shanghai, China). RNAiso Plus kit (Takara, Dalian, China) was used in extracting total RNA from 9 hepatopancreas of *M. rosenbergii* of each group. With the use of agarose gel electrophoresis at 1% and spectrophotometry (A260: 280 nm ratio), assessment of the quality and quantity was carried out. Following that, in accordance with the manufacturer's instructions, a PrimerScript™ RT Reagent kit (Takara, Dalian, China) was used to synthesize cDNA. Briefly, in the presence of Prime Script TM RT enzyme mix I, 5 × Primer Script™ buffer, RNase free distilled water at 37 °C for 15 min, oligo dT primer (50 μM) were used to reverse transcribe the respective RNA. This was followed by inactivation at 84 °C for a period of 4 s.

The transcript expression levels were determined by Real-time fluorescent quantitation PCR (RT-PCR). In accordance with standard protocols using with SYBR® Premix ExmTaq. II (TliRNase Plus) Kit using ABI 7500 Real-time PCR System, the amplification was done. In short, 10 (μL) SYBR® Premix ExmTaq. ™ (2x), 0.4 μL ROX Reference Dye, 0.8 μL PCR Reverse Primer (10 μM) and 6 μL RNase -dH2O was reacted with 0.2 μL of cDNA with the final reaction volume of solution being 20 μL. The condition under which the reaction was carried out are as follows; 95 °C for 10 s, followed by 45 cycles consisting of 95 °C for 5 s, 62 °C for 15 s, 72 °C for 10s, after which the plate was read, and then at 72 °C for 3 min, the final step was done. The housekeeping genes β-actin of *M. rosenbergii* were used to normalize the expression levels of the target genes after the completion of the program. After making sure that the primers were amplified with an acceptable efficiency [49], the $2^{-\Delta\Delta CT}$method was employed in calculating the expression levels. The amplification frequency was between 95 and 105%.

2.10. Statistical analysis

Analysis of data was done by the use of the Statistical package for Social Scientists (SPSS) (version 20, Chicago, IL, USA) for windows. All the results were subjected to one-way analysis of variance (ANOVA) to determine the effect the different levels of *M.oleifera* leaf extract on the various treatments. Two-way analyses were also carried out to determine the effect of the diets and ammonia as the two factors in terms of significant interactions on the enzyme concentrations. Duncan's multiple range test was used to determine significant differences ($P < 0.05$) among the various treatments, shown by different small letters. The data was expressed as means plus or minus the standard error (X ± SE).

3. Results

3.1. Effects of M. oleifera leaf extract on growth performance and feed utilization

The growth as well as feed utilization indices are shown in Table 3. There were significant differences between the control group and the 0.25% *M.oleifera* extract, 0.5% *M.oleifera* extract, 0.025% Enrofloxacin groups with respects to final weight, WG, SGR, PER, and FCR ($P < 0.05$). The lower FCR was recorded by the shrimps fed with 0.25–0.5% *M. oleifera* extract and the higher FCR was recorded by the

Table 3
Growth performance and feed utilization of *M. rosenbergii* fed different levels of *M. oleifera* leaf extract for 60 days.

Parameters	Control	0.25% *M. oleifera* leaf extract	0.5% *M. oleifera* leaf extract	1.0% *M. oleifera* leaf extract	0.025% Enrofloxacin
Initial Weight(g)	0.21 ± 0.001	0.21 ± 0.002	0.22 ± 0.001	0.22 ± 0.001	0.22 ± 0.001
Final Weight (g)	12.41 ± 0.36^{a}	13.65 ± 0.38^{b}	13.77 ± 0.08^{b}	12.93 ± 0.31^{ab}	13.65 ± 0.21^{b}
WGR	5748.13 ± 203.93^{a}	6258.97 ± 192.44^{b}	6256.18 ± 36.69^{b}	5848.92 ± 126.77^{ab}	6238.77 ± 79.07^{b}
SGR	6.78 ± 0.06^{a}	6.92 ± 0.05^{b}	6.92 ± 0.01^{b}	6.81 ± 0.04^{ab}	6.92 ± 0.02^{b}
FCR	1.68 ± 0.05^{b}	1.52 ± 0.04^{a}	1.51 ± 0.01^{a}	1.61 ± 0.04^{ab}	1.52 ± 0.02^{a}
PER	164.10 ± 4.81^{a}	180.67 ± 5.11^{b}	182.31 ± 1.10^{b}	170.99 ± 4.22^{ab}	180.10 ± 2.82^{b}

Note: Data are mean values of three replicates expressed as mean ± standard error of mean (M ± SE). Significant differences are indicated where values have different superscript letters in the same row compared to the control group; where WGR = weight gain ratio, SGR = specific growth rate, FCR = feed conversion ratio; PER = Protein efficiency ratio.

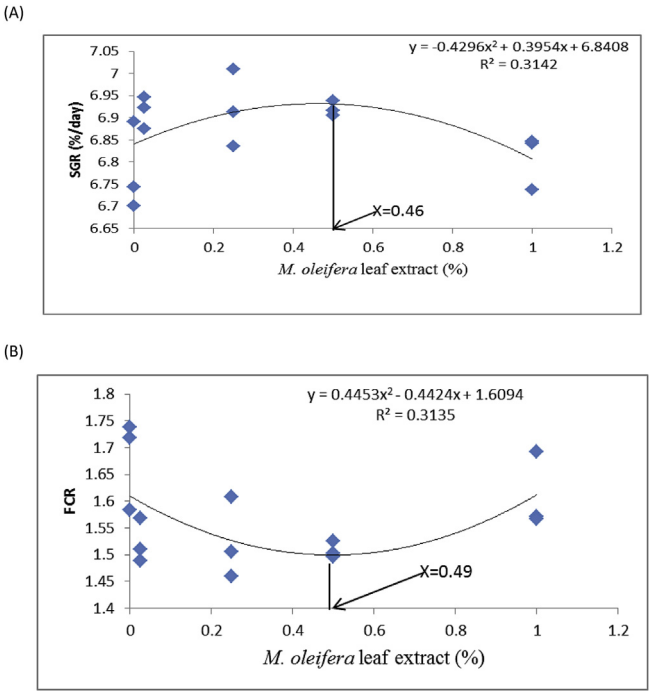

Fig. 1. The optimum level of *M. oleifera* leaf extract supplementation based on second order polynomial analysis on specific growth rate (A) and feed conversion ratio (B) of *M. rosenbergii*.
Note: Data are mean values of three replicates expressed as mean ± SE.

shrimps fed with the control group. The higher final weight, WG, SGR as well as PER was recorded in the 0.25–0.5% *M.oleifera* extract group with the lower being recorded in the control group. Based on a second order regression analysis the optimum level at which the lowest feed conversion ratio (FCR) and highest specific growth rates (SGR) were achieved was determined to be at an inclusion level of between 0.46 and 0.49% *M. oleifera* leaf extract (see Fig. 1).

3.2. Effects of M. oleifera leaf extract on haemolymph ALT and AST

The ALT and AST activity in haemolymph are presented in Fig. 2. Before stress, the ALT and AST activities in the group of 0.5%, 1.0% *M.oleifera* leaf extract group was significantly reduced in comparison with the control group ($P < 0.05$, Fig. 2A and B). After stress, the levels of AST in the group of 0.25% *M.oleifera* leaf extract increased significantly above the control group ($P < 0.05$, Fig. 2B). However, there was no significant difference in the pre-challenge and post challenge levels ($P > 0.05$).

3.3. Effects of M. oleifera leaf extract on haemolymph total protein and albumin

The haemolymph total protein and albumin are shown in Fig. 3. There was no significant differences in the haemolymph total protein and albumin between the control group and treatment group before or after stress ($P > 0.05$, Fig. 3). However, the concentration of TP in the control group after challenge was significantly lower than the pre-challenge level ($P < 0.05$, Fig. 3A).

3.4. Effects of M. oleifera leaf extract on antioxidant indices

The haemolymph catalase, superoxide dismutase and glutathione peroxidase are shown in Fig. 4. There was a significant increase in the haemolymph catalase of the 0.5% *M.oleifera* extract group compared with both the control and 1.0% *M.oleifera* extract groups before stress

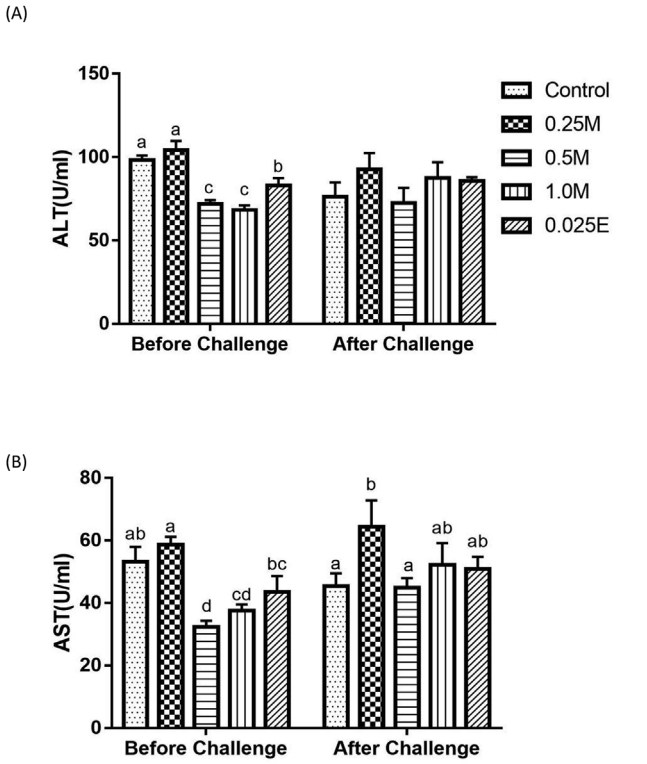

Fig. 2. Effects of *M.oleifera* leaf extract on haemolymph alanine aminotransferase (A) and aspartate aminotransferase (B) of *M. rosenbergii* before and after ammonia stress.
Note: Data are mean values of nine replicates expressed as mean ± SE. Data with different superscripts show significant differences ($P < 0.05$) among the different groups.

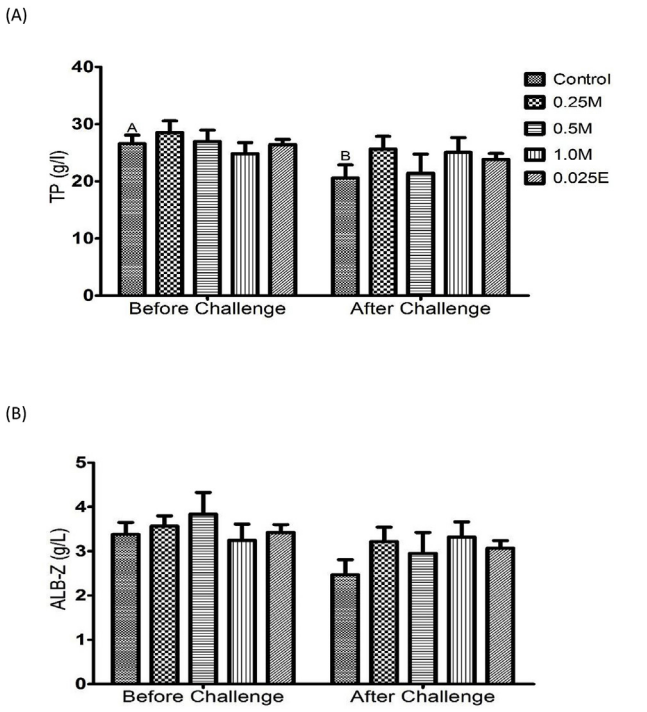

Fig. 3. Effects of *M.oleifera* leaf extract on haemolymph total protein (A) and albumin (B) of *M. rosenbergii* before and after ammonia stress.
Note: Data are mean values of nine replicates expressed as mean ± SE. The different capitalized letters (A and B) indicated a significant difference ($P < 0.05$) between the initial stage and after infection with the same group.

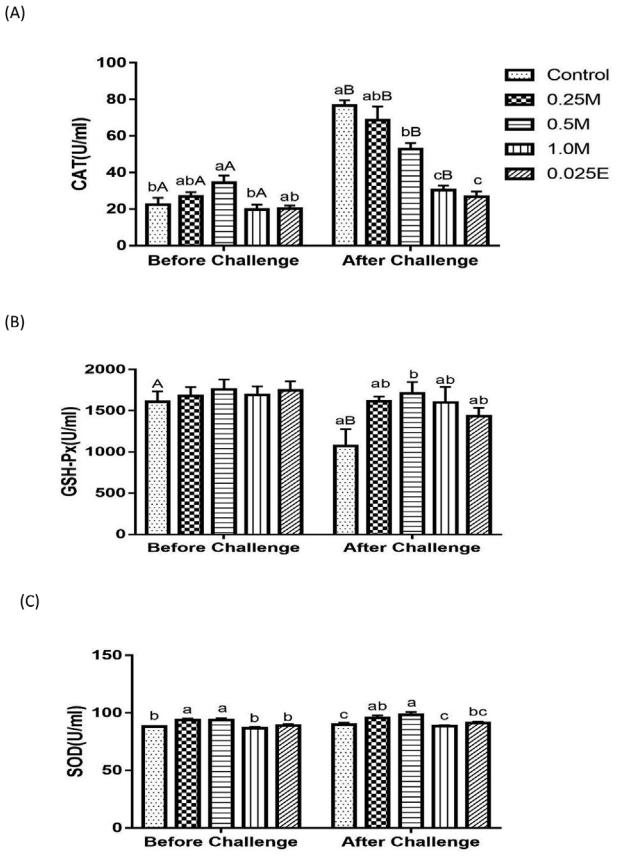

Fig. 4. Effects of *M.oleifera* leaf extract on haemolymph catalase (A), superoxide dismutase (B) and glutathione peroxidase (C) of *M. rosenbergii* before and after ammonia stress.
Note: Data are mean values of nine replicates expressed as mean ± SE. Legends are the same as in Fig. 3.

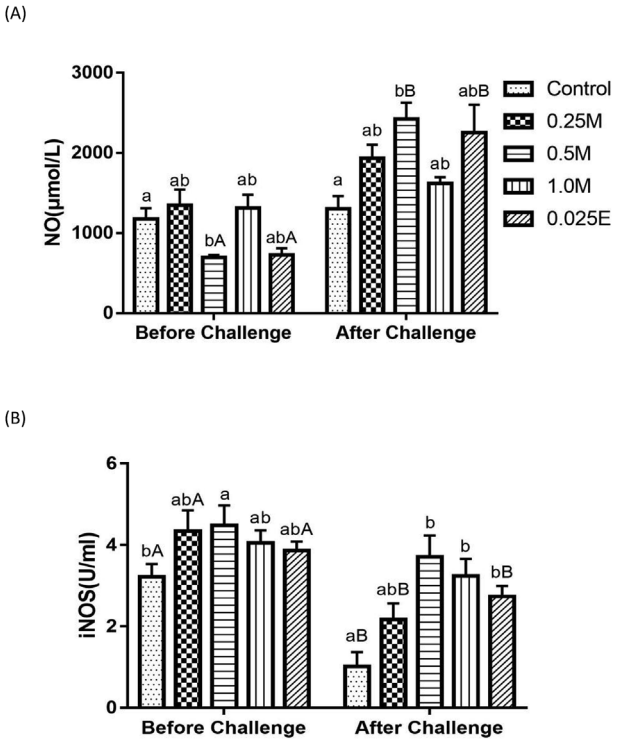

Fig. 5. Effects of *M.oleifera* leaf extract on nitric oxide (A) and inducible nitric oxide synthase (B) of *M. rosenbergii* before and after ammonia stress.
Note: Data are mean values of nine replicates expressed as mean ± SE. Legends are the same as in Fig. 3.

($P < 0.05$, Fig. 4A). After stress, the group of 0.5%, 1.0% *M.oleifera* extract and 0.025% enrofloxacin significantly decreased in the haemolymph catalase compared with the group of control ($P < 0.05$, Fig. 4A). In addition, the activities of catalase in the control group, 0.25%, 0.5%, 1% *M.oleifera* extract after challenge was significantly higher than the pre-challenge level ($P < 0.05$, Fig. 4A).

Fig. 5B indicates that SOD activities showed a gradual rise from the control group up the 0.5% inclusion. The groups of 0.25%, 0.5% *M.oleifera* extract were significantly higher than that of the control group ($P < 0.05$). After stress, the group of 0.25%, 0.5% *M.oleifera* extract significantly increased in the haemolymph SOD activity compared with the group of the control, 1.0% *M.oleifera* extract and 0.025% enrofloxacin ($P < 0.05$, Fig. 4B). There was no significant difference between the pre and post-challenge activities ($P > 0.05$).

In comparison with the control group, haemolymph GSH-Px content showed a continuous increase trend from the control to the 1.0% group without any significant differences (Fig. 4C). After stress, the group of 0.5% *M.oleifera* extract significantly improved in the haemolymph GSH-Px activity compared with the group of control ($P < 0.05$, Fig. 4C). In addition, the GSH-Px content in the control group after challenge was significantly lower than the pre-challenge level ($P < 0.05$, Fig. 4C).

3.5. Effects of M. oleifera leaf extract on the haemolymph NO and iNOS

The haemolymph NO and iNOS are shown in Fig. 5. The haemolymph NO content before stress was significantly reduced in the 0.5% group in comparison with the control ($P < 0.05$, Fig. 5A). After stress, the group of 0.5% *M.oleifera* extract significantly improved in the haemolymph NO content compared to the group of control ($P < 0.05$, Fig. 5A). Furthermore, the NO levels showed an increasing trend with 0.5% *M.oleifera* extract and 0.025% enrofloxacin group being significantly increased post-challenge in comparison with their pre-challenge levels ($P < 0.05$, Fig. 5A).

The haemolymph iNOS content in the group of 0.5% *M.oleifera* extract was higher than that of the control group before stress ($P < 0.05$, Fig. 5B). After stress, the iNOS content in the control group was significantly lower than the group of 0.5%, 1.0% *M.oleifera* extract and 0.025% enrofloxacin ($P > 0.05$, Fig. 5B). Additionally there was a general reduction in all the iNOS contents compared to the pre-infection levels with the control, 0.25% *M.oleifera* extract and the 0.025% enrofloxacin group being significantly reduced post challenge ($P < 0.05$).

3.6. Effects of M. oleifera leaf extract on relative expression of peroxiredoxin 5 and HSP 70

The relative expression of HSP 70, peroxiredoxin 5 and Relish genes are shown in Fig. 6. Peroxiredoxin 5 expression did not show any significant differences before stress ($P < 0.05$, Fig. 6A). After stress, the 0.025% enrofloxacin group improved the peroxiredoxin 5 expression level compared with the group of 0.25% *M.oleifera* extract ($P < 0.05$, Fig. 6A). There was a reduction in the post-challenge levels of peroxiredoxin 5 compared the pre-challenge levels with all the groups with the exception of the 1.0% group ($P < 0.05$, Fig. 6A).

Fig. 6B indicates expression of HSP 70 genes before stress showed a significant reduction in the 0.5% group in comparison with the control group ($P < 0.05$, Fig. 6B). After stress, the expression of HSP 70 genes in the group of 0.25%, 0.5% *M.oleifera* extract was significantly higher than that of the control group ($P < 0.05$, Fig. 6B). After stress, the control as well as the 0.025% enrofloxacin groups were significantly reduced in comparison to their pre-challenge levels ($P < 0.05$) whereas the 0.5% group was significantly increased in comparison to the pre-challenge levels ($P < 0.05$, Fig. 6B).

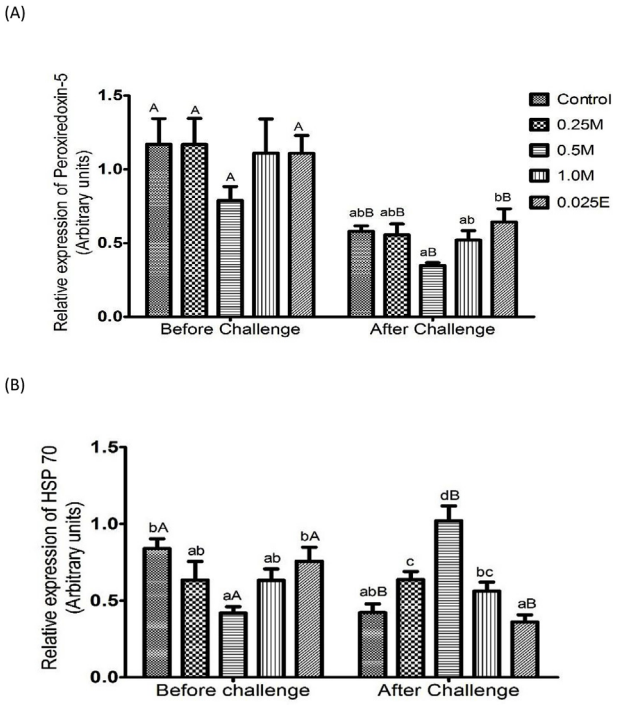

Fig. 6. Effects of *M.oleifera* leaf extract on relative expression of peroxiredoxin 5 (A) and HSP 70 (B) of *M. rosenbergii* before and after ammonia stress.
Note: Data are mean values of nine replicates expressed as mean ± SE. Legends are the same as in Fig. 3.

3.7. Effects of M. oleifera leaf extract on relative expression of NFκBI-α, IMD and relish genes

The relative expression of NFκBI-α, IMD and Relish genes are shown in Fig. 7. IMD expression in the haepatopancreas before stress exhibited a significant reduction in the 0.5% and 1.0% *M.oleifera* extract groups in comparison with the control group ($P < 0.05$, Fig. 7A). After stress, the 0.025% enrofloxacin group improved the IMD expression compared with the control group ($P < 0.05$). The IMD expression levels in all the groups with the exception of the 0.025% enrofloxacin group were lower than pre-challenge levels ($P < 0.05$, Fig. 7A).

The expression of NFκBI-α before stress showed an increasing pattern from the control group to the supplemented groups (Fig. 7B) with the 0.025% enrofloxacin group being significantly reduced in comparison with the 0.25% *M.oleifera* extract group. In contrast, after stress, all the treatment groups reduced with respect to the expression of NFκBI-α compared with the control group ($P < 0.05$, Fig. 7B). Additionally the levels of NFκBI-α in the group of 0.25%, 0.5% and the 1.0% *M.oleifera* extract was higher than pre-challenge levels ($P < 0.05$, Fig. 7B).

Before stress, expression of relish genes was generally in a decreasing trend with the 0.5%, 1.0% as well as the 0.025% enrofloxacin groups being significantly reduced in comparison with the 0.25% *M.oleifera* extract group ($P < 0.05$, Fig. 7C). After stress, the group of 0.25%, 1% *M.oleifera* extract reduced expression of relish genes compared with the group of control ($P < 0.05$, Fig. 7C). There was a decreasing trend of relish levels all the groups with the exception of the 0.025% enrofloxacin group being reduced significantly post-challenge ($P < 0.05$, Fig. 7C).

3.8. Effects of M. oleifera on the mortality after V. anguillarum challenge

The current results showed that the highest mortality in a period of 96 h in the challenge occurred in the control group (53%) with the lowest mortality being in the 0.5% group (27%), thereby exerting higher bacterial activity against *V.anguillarum*. Also, the group of

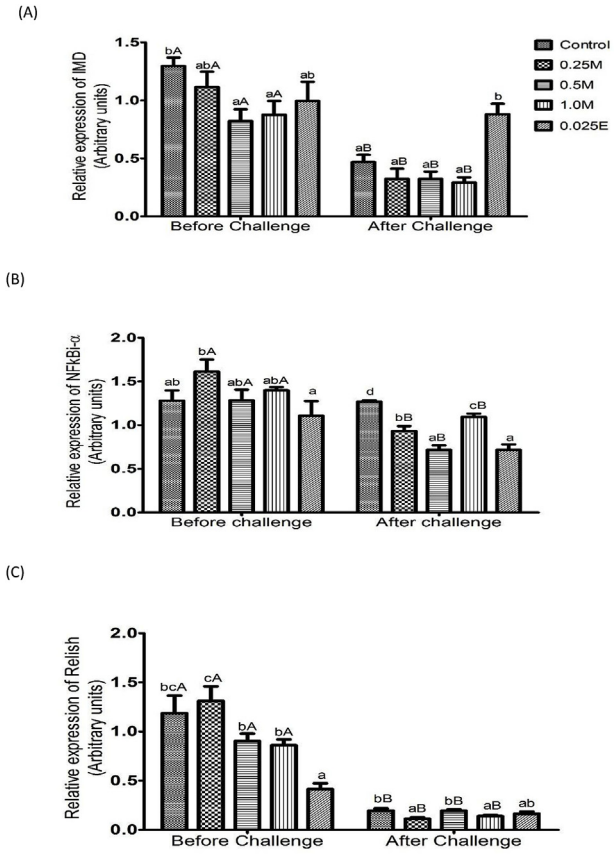

Fig. 7. Effects of *M.oleifera* leaf extract on relative expression of IMD (A), NFκBI-α(B) and Relish (B) of *M. rosenbergii* before and after ammonia stress.
Note: Data are mean values of nine replicates expressed as mean ± SE. Legends are the same as in Fig. 3.

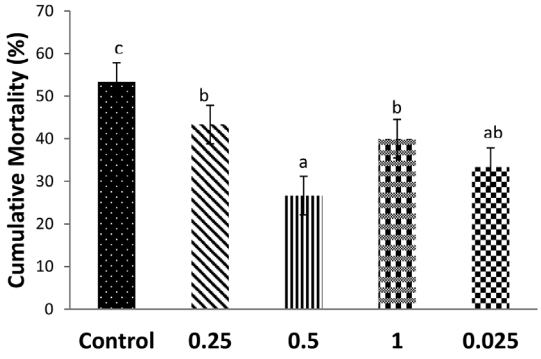

Fig. 8. Effects of *M.oleifera* leaf extract on cumulative mortality of *M. rosenbergii* of challenge with *V.anguillarum*.
Note: Data with different superscripts show significant differences ($P < 0.05$) among the different groups.

0.25%, 0.5%, 1% *M.oleifera* extract and 0.025% enrofloxacin significantly reduced in the mortality compared to the group of the control ($P < 0.05$, Fig. 8). This therefore indicated that better protection can be offered by *M. oleifera* extract in an unfriendly *V. anguillarum* challenge.

4. Discussion

4.1. Effects of M. oleifera leaf extract on growth performance of M. rosenbergii

The present study demonstrated that the freshwater prawns fed with

supplemented 0.25%–0.5% *M. oleifera* leaf extract performed better in terms of growth and feed utilization compared with the control group. It was observed in this experiment that the prawns fed 0.5% *M. oleifera* leaf extract produced the highest final weight, specific growth rate and protein efficiency ratio in comparison with the control. Also the lowest feed conversion ratio was produced by the prawns fed with 0.5% *M. oleifera* leaf extract, which was significantly different compared to the control. This results were similar to the *Penaeus monodon* fed with a combination of herbal products [50]. A higher feed efficiency ratio and weight gain was achieved in *Litopenaeus vannamei* with the herbal product ginger [51]. A significant improvement in growth performance as well as nutrient utilization was achieved at an inclusion level of 0.5%, 2.5% *Aloe vera* extract in common carp [52] and 0.1%, 1% *Aloe vera* extract in rainbow trout [53]. Also in agreement with the results of tilapia fingerlings fed with *M.oleifera* flower extract [54]. The improvement in growth and feed utilization parameters could be attributed to improvement in the activities of digestive enzymes in the shrimps which ultimately play a very important role when it comes feed intake, digestion and overall growth [55,56].Also, improved physiological functions as a result of increased energy flows resulting from incorporation of herbs in diets have been achieved [57]. Based on a second order regression analysis of feed conversion ratio (FCR) and specific growth rates (SGR), the optimum level was determined to be at an inclusion level of between 0.46 and 0.49% *M.oleifera* leaf extract for *M.rosenbergii.* On the contrary, application of high quantity of *Moringa oleifera* seed led to adverse growth of fish due to the presence of toxic substances or anti-nutritional factors [26,27]. The reasons may be related to the differences in aquatic species, fish size, and the part of *M.oleifera* used in the feed formulation. It needs to be studied further.

4.2. Effects of M. oleifera leaf extract on haemolymph biochemical parameters of M. rosenbergii

It can be concluded that there is a minor damage to liver when there is an elevation of ALT, AST activities in the serum or extracellular fluid [58]. Results of the current experiment indicated ALT, AST activities of 0.5%, 1.0% *M.oleifera* leaf extract group was significantly reduced in comparison with the control group before stress. The exposure of *Sparus aurata* and *Cyprinus carpio* to heavy metals led to an elevation in the levels of blood ALT and AST activities [59,60]. In the same light, Korean rockfish and Nile tilapia (*Oreochromis niloticus*) exposed to cypermethrin experienced an increase in the levels of ALT and AST in the serum [61,62]. The decrease in the levels of AST, ALT of 0.5%–1% *M.oleifera* leaf extract could be attributed to the protective capabilities of the herb. The haemolymph protein content can be used in determining the health status of prawns [63,64]. The results of this current experiment showed that there was general reduction in level of haemolymph total protein the pre-challenge compared to the level post-challenge in the control. This agrees with works which concluded that the levels of total protein in the serum decreased with an exposure of *O. niloticus* [65] and *Rhamdia quelen* [66] to Cu as well as cypermethrin. Also the lower albumin levels were observed in Jian carp after challenging with *Aeromonas hydrophila* [67]. In this study, findings indicated there was a decreasing trend of albumin in the pre-challenge levels compared to the post-challenge levels. Our results suggest that the dose of 0.5%, 1.0% *M.oleifera* extract has potential to alleviate the oxidative damage of liver in *M. rosenbergii* as indicated by lower levels with regards to indicators such as AST and ALT after challenge.

4.3. Effects of M. oleifera leaf extract on antioxidant parameters of M. rosenbergii

The stress response might also increase free radical contents, which may lead to an imbalance between lipid peroxides and the antioxidant system [68]. For example, upon challenging blunt snout bream (*Megalobrama amblycephala*) under high ammonia stress for a period of 3, 6 and 24 h, fructooligosaccharide caused an increase in the activities of CAT activities beyond that of the control [69]. Liu et al. (2014) [70] stated that 0.1–0.2% anthraquinone extract increased activities of hepatic catalase and superoxide dismutase before stress or 6, 12, 24h after high temperature stress in *M. rosenbergii* compared to the control. Xie et al. (2018) [71] concluded that supplementation of *Forsythia suspensa* extract in the diet of *Penaeus monodon* caused an elevation in the activities of GSH-Px above that of the control after challenging with *Vibrio parahaemolyticus*. Consistent with these studies, our study showed that the group of 0.5% *M. oleifera* leaf extract improved the CAT, SOD and GSH-Px activity before ammonia stress compared to the control.

Furthermore, CAT activities were higher in all the supplemented groups compared with pre-challenge activities and GSH-Px was lower in the control than pre-challenge activity. Oxidative stress under the high ammonia stress was further determined by peroxiredoxins gene experession, which can help in mitigating organisms from the harmful oxidative stress [72–74]. The current results indicated that there was a reduction in the post-challenge levels of peroxiredoxin 5 expression compared the pre-challenge levels with all the groups with the exception of the 1.0% *M.oleifera* extract group. In addition, there was decrease level of trend about peroxiredoxin 5 expression in the group of 0.5% *M.oleifera* leaf extract compared with the control group.

Immune regulation, antiviral, antibacterial, anti-malignant tumor preventions are some of the properties that are exhibited by nitric oxide in organisms [75]. The enzyme that is responsible for the production of nitric oxide is inducible nitric oxide synthase (iNOS). The control group in comparison with the 0.5% group had reduced levels of haemolymph nitric oxide levels before stress. After stress, the group of 0.5% *M.oleifera* extract significantly improved in the haemolymph NO content and iNOS activity compared to the group of control. Similar to results of the present study, Vitamin C and emodin supplementation caused an increase in the levels of nitric oxide above the control upon challenging Wuchang bream (*M.amblycephala*) under high temperature for a duration of 2 and 12 h [76]. Moreover, anthraquinones extracted from *Rheum officinale* Bail at a supplementation rate of 0.1, 0.2 and 0.4% led to an increase in the levels of nitric oxide above the control upon challenging *M. rosenbergii* for a period of 2–6 weeks [77]. Taken together, our results suggest that the dose of 0.5% *M.oleifera* extract reduces the potential for oxidative damage following ammonia stress in *M. rosenbergii* and the mechanism through which this occurs is possibly through the enhancement of phagocytic cells which are essential cellular components in shrimps and play very vital roles in their innate immunity.

4.4. Effects of M. oleifera leaf extract on immune related gene expressions

Immune protection, as well as increased tolerance to many different kinds of stresses are involved in by some related immune genes such as HSP 70, IMD, NFκBI-α, and Relish. Lipid peroxides under high ammonia stress may trigger HSP expression [18]. The elevation of HSP 70 contributed to modulate cellular anti-stress responses and to play key roles in protecting organisms against heat stress [78,79]. Wan et al. (2014) [80] found that there was an elevation in the levels of HSP 70 above the control in Vitamin C supplemented diet for juvenile *M. amblycephala* under pH stress. Liu et al. (2012) [81] found that 60 m/kg emodin and 0.1% anthraquinone extract in the diet could enhance the levels of liver HSP70 gene expression 2, 6h after high temperature stress for juvenile *M. amblycephala*. Results of the current study indicated the group of 0.25%, 0.5% *M.oleifera* extract significantly improved expressions of HSP 70 post-challenge.

IMD, Toll, and JAK/STAT are considered as the three signaling pathways that are involved in the innate immune responses in shrimps [82]. IMD could resist to Gram-negative bacteria but has normal response to fungi and Gram-positive bacteria [83]. In this study, there were general reductions in the post-challenge expressions of IMD with all the *M.oleifera* leaf extract groups compared to the pre-challenge

level. And the group of 0.5%, 1.0% *M.oleifera* extract significantly reduced expressions of IMD before stress. This differed from conclusions reached by Ningning et al. (2014) [84] who stated that *Fenneropenaeus chinensis* exposed to both vibrio and micrococcus injection for a period of 1 h recorded higher expressions of IMD in comparison with their control. Wang et al.(2009) [85] studied that IMD mRNA was expressed in most tissues and is induced in hepatopancreas and hemocytes after immune challenge. It was possible that the dose of 0.5%, 1.0% *M.oleifera* extract could keep at lower levels the liver expressions of IMD of *M. rosenbergii* so that the body might not have to activate the IMD signaling pathway in order to resist the pathogenic infection and have the potential for improving the immunity of *M. rosenbergii.* It needs further study.

In immunity as well as inflammation in organisms, NF-κB proteins are highly involved [86,87]. One member of a family of cellular proteins that function to inhibit the NF-κB transcription factor is the NF kappa B inhibitor alpha (NFκBI-α) [67]. From the current study, there was a general reduction in the expression of NFκBI-α with all the *M.oleifera* leaf extract group compared with the control after stress. This indicated lower expression of the NFκBI-α in the supplemented *M.oleifera* leaf extract diets meant higher probability of NF-κB being released into the nucleus to bind to specific protein helping to ward off the effects of the ammonia stress. The results were in agreement with conclusion reached by Qiu et al. (2014) [88] which stated that after challenging *Litopenaeus vannamei* with White Spot Syndrome Virus (WSSV), there was a significant increase in the expression of Dorsal as well as Relish which form part of the NF-κB family proteins. In the IMD signal pathway, Relish is one of the NF-κB transcription factors. In order to activate the production of antimicrobial peptides, there has to be the involvement of Relish [89]. The outcome of the current work showed the group of 0.25%, 1.0% *M.oleifera* extract significantly reduced expressions of Relish after ammonia stress compared to the control. Similarly, Duan et al. (2015) [90] concluded that there was a down-regulation in the expression of Relish genes in the haemocytes of the River prawn upon challenge with ammonia-N. However, contrary to the current finding, upon challenging *M. rosenbergii* [91] and *P. fucata* [92] with *V. anguillarum*, there was an elevated expression of Relish genes in comparison with the control. It needs further study.

4.5. Effects of M. oleifera on mortality after V. anguillarum challenge

V. anguillarum, a Gram-negative bacterium is a major important pathogen in aquaculture [93]. Several experiments have proved the positive effects of medicinal plants in helping aquatic species resist several pathogenic organisms. After infection with *Edwardsiella tarda*, dietary inclusion of green tea [94], *Undaria* [95], anthraquinone extract [40] resulted in an improvement in the survival of *Paralichthys olivaceus* fish or *M. rosenbergii.* The results showed that the highest mortality in a period of 96 h in the challenge occurred in the control group (53%) compared with the lowest mortality in the 0.5% *M. oleifera* extract group (27%), thereby exerting higher anti-bacteria ability against *V. anguillarum*. Also, all the other supplemented groups performed better in terms of survival against the effects of the bacteria. This therefore indicated that better protection can be offered by 0.5% *M. oleifera* extract in an unfriendly *V.anguillarum* environment.

5. Conclusion

In short, these results can be correlated with the increased haemolymph CAT, SOD and iNOS and the decreased haemolymph aspartate aminotransferase, alanine aminotransferase, NO, hepatopancreas HSP70 and IMD expression levels in the group treated with the supplement of 0.5% *M. oleifera* leaf extract. After ammonia stress, the group of 0.5% *M. oleifera* leaf extract also could improve the haemolymph SOD, glutathione peroxidase, NO, iNOS, hepatopancreas HSP70 expression level and reduce haemolymph CAT, hepatopancreas peroxiredoxin 5 and NFκBI-α expression levels compared with the control group. So the supplement of 0.25–0.5% *M.oleifera* leaf extract enhanced non-specific immunity, antioxidative capacities, enhanced resistance to high ammonia stress, protected freshwater prawn against *V. anguillarum* infection, and promoted growth performance and feed utilization of *M.rosenbergii*.

Acknowledgements

This work was supported by China Agriculture Research System-48 (CARS-48), and the Three New Projects of Fishery in Jiangsu province (D2017-04). The authors gratefully acknowledge the postgraduate students of the Fish Disease and Nutrition Department, Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences, Wuxi City, the Popular Republic of China for their assistance during the sampling period.

References

[1] Ü. Acar, O.S. Kesbiç, S. Yılmaz, N. Gültepe, A. Türker, Evaluation of the effects of essential oil extracted from sweet orange peel (Citrus sinensis) on growth rate of tilapia (Oreochromis mossambicus) and possible disease resistance against Streptococcus iniae, Aquaculture 437 (2015) 282–286.
[2] W.Q. Cai, S.F. Li, J.Y. Ma, Diseases resistance of Nile tilapia (Oreochromis niloticus), blue tilapia (Oreochromis aureus) and their hybrid (female Nile tilapia- male blue tilapia) to Aeromonas sobria, Aquaculture 299 (2004) 79–87.
[3] A. Rico, T.M. Phu, K. Satapornvanit, J. Min, A.M. Shahabuddin, P.J.G. Henriksson, F.J. Murray, D.C. Little, A. Dalsgaard, P.J. Van den Brink, Use of veterinary medicines, feed additives and probiotics in four major internationally traded aquaculture species farmed in Asia, Aquaculture 412–413 (2013) 231–243.
[4] H. Sørum, Farming of Atlantic salmon—an experience from Norway, Acta Vet. Scand. Suppl. 93 (2000) 129–134.
[5] F.J. Angulo, P.M. Griffin, Changes in antimicrobial resistance in Salmonella enterica serovar Typhimurium, Emerg. Infect. Dis. 6 (2000) 436–438.
[6] O.E. Heuer, H. Kruse, K. Grave, P. Collignon, I. Karunasagar, F.J. Angulo, Human health consequences of use of antimicrobial agents in aquaculture, Clin. Infect. Dis. 49 (2009) 1248–1253.
[7] J.I.R. Castanon, History of the use of antibiotic as growth promoters in european poultry feeds, Poultry Sci. 11 (2007) 2466–2471.
[8] A. Roque, A. Molina-Aja, C. Bola´n-Mej´ıa, B. Gomez-Gil, In vitro susceptibility to 15 antibiotics of vibrios isolated from penaeid shrimps in Northwestern Mexico, Int. J. Antimicrob. Agents 17 (2001) 383–387.
[9] I. Poher, G. Blanc, S. Loussoouarn, Pharmacokinetics of oxolinic acid in seabass, Dicentrarchus Ž.labrax L., 1758, after a single rapid intravascular injection, J. Vet. Pharmacol. Ther. 20 (1997) 267–275.
[10] D.A. Stoffregen, G.A. Wooster, P.S. Bustos, P.R. Bowser, J.G. Babish, Multiple route and dose pharmacokinetics of enrofloxacin in juvenile Atlantic salmon, J. Vet. Pharmacol. Ther. 20 (1997) 111–123.
[11] L. Intorre, S. Cecchini, S. Bertini, A.M. Cognetti Varriale, G. Soldani, G. Mengozzi, Pharmacokinetics of enrofloxacin in the sea bass (Dicentrarchus labrax), Aquaculture 182 (2000) (2000) 49–59.
[12] S.C. Ji, G.S. Jeong, G.S. Im, S.W. Lee, J.H. Yoo, K. Takii, Dietary medicinal herbs improve growth performance, fatty acid utilization, and stress recovery of Japanese flounder, Fish. Sci. 73 (2007) 70–76.
[13] B.K.H. Tan, J. Vanitha, Immunomodulatory and antimicrobial effect of some traditional Chinese medicinal herbs, Curr. Med. Chem. 11 (2004) 1423–1430.
[14] A.A. Adedapo, O.O. Falayi, A.A. Oyagbemi, Evaluation of the analgesic, anti-inflammatory, anti-oxidant, phytochemical and toxicological properties of the methanolic leaf extract of commercially processed M. oleifera in some laboratory animals, J. Basic Clin. Physiol. Pharmacol. 26 (2015) 491–499.
[15] C. Ramachandran, K.V. Peter, P.K. Gopalakrishnan, Drumstick (M. oleifera, A multipurpose Indian vegetable, Econ. Bot. 3 (1980) 276–283.
[16] V. Pakade, E. Cukrowska, L. Chimuka, Metal and flavonol contents of M. oleifera grown in South Africa, South Afr. J. Sci. 109 (2013) 1–7.
[17] S. Mutheeswaran, P. Pandikumar, M. Chellappandian, S. Ignacimuthu, Documentation and quantitative analysis of the local knowledge on medicinal plants among traditional Siddha healers in Virudhunagar district of Tamil Nadu, India, J. Ethnopharmacol. 137 (2011) 523–533.
[18] A. Leone, A. Spada, A. Battezzati, A. Schiraldi, J. Aristil, S. Bertoli, Cultivation, genetic, ethnopharmacology, phytochemistry and pharmacology of M. oleifera Leaves: an Overview, Int. J. Mol. Sci. 16 (2015) 12791–12835.
[19] G.A. Asare, B. Gyan, K. Bugyei, S. Adjei, R. Mahama, Addo, L. Otu-Nyarko, E.K. Wiredu, A. Nyarko, Toxicity potentials of the nutraceutical M. oleifera at supra-supplementation levels, J. Ethnopharmacol. 139 (2012) 265–272.
[20] J.O. Popoola, O.O. Obembe, Local knowledge, use pattern and geographical distribution of M. oleifera Lam. (Moringaceae) in Nigeria, J. Ethnopharmacol. 150 (2013) 682–691.
[21] S.G. Mahajan, R.G. Mali, A.A. Mehta, Effect of moringa oleifera Lam. seed extract on toluene diisocyanate-induced immune-mediated inflammatory responses in rats, J. Immunotoxicol. 4 (2007) 85–96.

[22] K.N.V. Rao, V. Gopalakrishnan, V. Loganathan, Anti-inflammatory activity of moringa oleifera Lam, Ancient Sci. Life 18 (1999) 195–198.
[23] N. Richter, P. Siddhuraju, K. Becker, Evaluation of nutritional quality of moringa (moringa oleifera Lam.) leaves as an alternative pollen source for nile tilapia (oreochromis niloticus), Aquaculture 217 (2003) 599–611.
[24] I. Bbole, C. Mumba, N. Mupenda, A.S. Kefi, Analysis of growth performance and haematological parameters of Oreochromis niloticus fed on a varying diet of M. oleifera Lam. leaf meal as an additive protein source, Int. J. Fish. Aquac. 8 (2016) 105–111.
[25] R.S.N. Brilhante, J.A. Sales, C.M. de Souza Sampaio, F.G. Barbosa, M.D.A.N. Paiva, G.M. de Melo Guedes, D.D.S.C.M. Castelo, Vibrio spp. from Macrobrachium amazonicum prawn farming are inhibited by Moringa oleifera extracts, Asian Pac. J. Trop. Med. 8 (2015) 919–922.
[26] E. Dongmeza, P. Siddhuraju, G. Francis, K. Becker, Effects of dehydrated methanol extracts of moringa (Moringa oleifera, Lam.) leaves and three of its fractions on growth performance and feed nutrient assimilation in nile tilapia (oreochromis niloticus), Aquaculture 261 (2006) 407–422.
[27] C. Kavitha, M. Ramesh, S.S. Kumaran, S.A. Lakshmi, Toxicity of moringa oleifera seed extract on some hematological and biochemical profiles in a freshwater fish, cyprinus carpio, Exp. Toxicol. Pathol. 64 (2012) 681–687.
[28] Z.X. Liang, R. Liu, D.P. Zhao, L.L. Wang, M.Z. Sun, M.Q. Wang, L.S. Song, Ammonia exposure induces oxidative stress, endoplasmic reticulum stress and apoptosis in hepatopancreas of pacific white shrimp (Litopenaeus vannamei), Fish Shellfish Immunol. 54 (2016) 23–528.
[29] M.M. Hegazi, Z.I. Attia, O.A. Ashour, Oxidative stress and antioxidant enzymes in liver and white muscle of Nile tilapia juveniles in chronic ammonia exposure, Aquat. Toxicol. 99 (2010) 118–125.
[30] H. Ren, J. Li, P. Liu, Z. Liang, J. Wu, Transcript profiles of mitochondrial and cytoplasmic manganese superoxide dismutases in Exopalaemon carinicauda under ammonia stress, Chin. J. Oceanol. Limnol. 33 (2015) 714–724.
[31] C.H. Cheng, F.F. Yang, R.Z. Ling, S.A. Liao, Y.T. Miao, C.X. Ye, Effects of ammonia exposure on apoptosis, oxidative stress and immune response in pufferfish (Takifugu obscurus), Aquat. Toxicol. 164 (2015) 61–71.
[32] M. Li, N. Yu, J.G. Qin, E. Li, Z. Du, L. Chen, Effects of ammonia stress, dietary linseed oil and Edwardsiella ictaluri challenge on juvenile darkbarbel catfish Pelteobagrus vachelli, Fish Shellfish Immunol. 38 (2014) 158–165.
[33] Z.L. Ding, Y. Kong, Y. Zhang, J. Li, F. Cao, J. Zhou, J.Y. Ye, Effect of feeding frequency on growth, body composition, antioxidant status and mRNA expression of immunodependent genes before or after ammonia-n stress in juvenile oriental river prawn, macrobrachium nipponense, Fish Shellfish Immunol. 68 (2017) 428–434.
[34] E. Nugroho, L. Emmawati, Giant Freshwater Prawn Culture in Indonesia, SEAFDEC, Bangkok, 2004, pp. 14–17.
[35] E. Nugroho, K. Sugama, M. Maskur, Genetic improvement of M. rosenbergii in Indonesia, Aquacult. Res. 36 (2005) 739–745.
[36] B. M, New Nair CM, Global scale of freshwater prawn farming, Aquacult. Res. 43 (2012) 960–969.
[37] F.A.O. FishStatJ, Fisheries and Aquaculture Department, Statistics and Information 957 Service FishStatJ: Universal Software for Fishery Statistical Time Series. Version 3.03, (2017).
[38] K. Ranjeet, B.M. Kurup, Management strategies associating batch-graded and size-graded post larvae can reduce heterogeneous individual growth in M. rosenbergii (de Man), Aquacult. Res. 33 (2002) 1221–1231.
[39] A. Gupta, H.S. Sehgal, G.K, Sehgal Growth and caracass composition of giant freshwater prawn, Macrobrachium rosenbergii (De Man), fed different iso-nitrogenous and isocaloric diets, Aquacult. Res. 38 (2007) 1355–1363.
[40] Y.T. Su, B. Liu, Q.L. Zhou, Y.J. He, J. Xie, P. Xu, Influence of anthraquinones extracted from rheum officinale bail promoting Macrobrachium rosenbergii's resistance to vibrio anguillarum, J. Fish. China 32 (2008) 455–463 (In Chinese).
[41] V. Kumaresan, R. Palanisamy, M. Pasupuleti, J. Arockiaraj, Impacts of environmental and biological stressors on immune system of Macrobrachium rosenbergii, Rev. Aquacult. 9 (2017) 283–307.
[42] L. Solórzano, Determination of ammonia in natural waters by the phenolhypochlorite method, Limnol. Oceanogr. 14 (1969) 799–801.
[43] J. Rodriguez, V. Boul, E. Mialhe, E. Bachere, Characterization of shrimp haemocytes and plasma components by monoclonal anti-bodies, J. Cell Sci. 108 (1995) 1043–1050.
[44] C. Rice-Evans, N.J. Miller, Total antioxidant status in plasma and body fluids, Methods Enzymol. 234 (1994) 279–293.
[45] H.T. Wang, A.Q. Yao, L. He, X.Y. Xie, Measurement of glutathione peroxidase (GSH-Px) in the blood of Cyprinus carpio, J. Hubei Agric. College 1 (1999) 28–30 (In Chinese).
[46] R. Xiang, D.N. Wang, The improvement of the method spectrophotometer on lipid peroxides thiobarbituric acid, Prog. Biochem. Biophys. l7 (1990) 241–242 (In Chinese).
[47] H.Y. Zhu, G.J. Wang, D.G. Yu, J. Xie, Changes in nitric oxide level and nitric oxide synthase and sensitivity to Vibrio parahaemolyticus in serum of white leg shrimp exposed to sudden changes in water temperature, J. Dalian Fish. Univ. 1 (2006) 46–50 (In Chinese).
[48] Yanru Shi, Study on Sequences Analysis and Function of IMD and Relish in IMD Signal Pathway in Macrobrachium Rosenbergii [D], Nanjing Normal University, 2016.
[49] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2-\Delta\Delta CT$ method, Methods 25 (2001) 402–408.
[50] M.N. Chandran, S. Moovendhan, A.M. Suganya, A. Tamilselvi, Bebin, G. Immanuel, A. Palavesam, Influence of polyherbal formulation (AquaImmu) as a potential growth promotor and immunomodulator in shrimp Penaeus monodon, Aquaculture Reports 4 (2016) 143–149.
[51] Y.P. Chang, C.H. Liu, C.C. Wu, C.M. Chiang, J.L. Lian, S.L. Hsieh, Dietary administration of zingerone to enhance growth, non-specific immune response, and resistance to Vibrio alginolyticus in Pacific white shrimp (Litopenaeus vannamei) juveniles, Fish Shellfish Immunol. 32 (2011) 284–290.
[52] M. Mahdavi, A. Hajimoradloo, R. Ghorbani, Effects of Aloe vera extract on growth parameter of common carp (Cyprinus carp), World J. Med. Sci. 9 (2013) 55–60.
[53] M. Heidarieh, R.A. Mirvaghefi, A. Sepahil, N. Sheikhzadel, A.M. Shahbazfar, M. Akbari, Effects of dietary Aloe vera on growth performance, skin and gastro-intestinal morphology in rainbow trout (Oncorhynchus mykiss), Turk. J. Fish. Aquat. Sci. 3 (2013) 367–373.
[54] E.W. Tekle, N.P. Sahu, Immunomodulatory and growth responses of Nile Tilapia fingerlings to dietary extract of Moringa flower, IJSRP 5 (2015) 285–296.
[55] R. Shanthi, P.S. Bhavan, S. Radhakrishnan, Influence of medicinal herbs, (Andrographis paniculata, Cissus quadrangu- laris and Eclipta alba) on growth, digestive enzymes, biochem- ical constituents and protein profile of the freshwater prawn Macrobrachium rosenbergii, Elixir BioTechnology 42 (2012) 6478–6484.
[56] S. Radhakrishnan, P.S. Bhavan, C. Seenivasan, T. Muralisankar, R. Shanthi, Effect of native medicinal herbs (Alteranthera sessius, Eclipta alba and Cissus qudrangularis) on growth performance, digestive enzymes and biochemical constituents of the monsoon river prawn Macrobrachium malcolmsonii, Aquacult. Nutr. 21 (2015) 496–506.
[57] P.S. Bhavan, N. Manickam, S. Radhakrishnan, Influence of herbal greens, Murraya koenigii, Coriandrum sativum and Menthe arvensis on growth performance of the freshwater prawn Macrobrachium rosenbergii post larvae, Res. J. Biotech. 7 (2012) 149–157.
[58] V. Palanivelu, K. Vijayavel, S. Ezhilarasibalasubramanian, M.P. Balasubramanian, Influence of insecticidal derivative (Cartap Hydrochloride) from the marine polychaete on certain enzyme systems of the freshwater fish Oreochromis mossambicus, J. Environ. Biol. 26 (2005) 191–196.
[59] A. Vaglio, C. Landriscina, Changes in liver enzyme activity in the teleost Sparus aurata in response to cadmium intoxication, Ecotoxicol. Environ. Saf. 43 (1999) 111–116.
[60] V. Karan, S. Vitorovic, V. Tutundzic, V. Poleksic, Functional enzymes activity and gill histology of carp after copper sulfate exposure and recovery, Ecotoxol Environ Saf 40 (1998) 49–55.
[61] J.H. Jee, F. Masroor, J.C. Kang, Responses of cypermethrin-induced stress in haematological parameters of Korean rockfish, Sebastes schlegeli (Hilgendorf), Aquatic Research 36 (2005) 898–905.
[62] Özgür Fırat, T.A. Yüzereroğlu, G. Gök, Özge Fırat, F. Kargin, Y. Kötemen, A comparative study on the effects of a pesticide (cypermethrin) and two metals (copper, lead) to serum biochemistry of nile tilapia, oreochromis niloticus, Fish Physiol. Biochem. 37 (2011) 657–666.
[63] J.L. Yang, H.C. Chen, Effects of gallium on common carp (Cyprinus carpio): acute test, serum biochemistry, and erythrocyte morphology, Chemosphere 53 (2003) 877–882.
[64] E. Bachère, E. Mialhe, J. Rodriguez, Identification of defense effectors in the haemolymph of Crustaceans with particular reference to the shrimp Penaeus japonicus (Bate): prospects and applications, Fish Shellfish Immunol. 5 (1997) 597–612.
[65] M. Oner, G. Atli, M. Canli, Changes in serum biochemical parameters of freshwater fish Oreochromis niloticus following prolonged metal (Ag, Cd, Cr, Cu, Zn) exposures, Environ. Toxicol. Chem. 27 (2008) 360–366.
[66] A. Borges, L.V. Scotti, D.R. Siqueira, R. Zanini, F. Amaral, D.F. Jurinitz, G.F. Wassermann, Changes in hematological and serum biochemical values in jundia' Rhamdia quelen due to sub-lethal toxicity of cypermethrin, Chemosphere 69 (2007) 920–926.
[67] X.Y. Yuan, W.B. Liu, C. Liang, C.X. Sun, Y.F. Xue, Z.D. Wan, G.Z. Jiang, Effects of partial replacement of fish meal by yeast hydrolysate on complement system and stress resistance in juvenile Jian carp (Cyprinus carpio var. Jian), Fish Shellfish Immunol. 67 (2017) 312–321.
[68] S. Akhtar, A. Shoaib, N. Akhtar, R. Mehmood, Separate and combined effects of Macrophomina phaseolina and copper on growth, physiology and antioxidative Enzymes in Vigna mungo L, J. Animal Plant Sci. 26 (2016) 1339–1345.
[69] C.N. Zhang, X.F. Li, H.Y. Tian, D.D. Zhang, G.Z. Jiang, K.L. Lu, G.X. Liu, W.B. Liu, Effects of fructooligosaccharide on immune response, antioxidant capability and HSP70 and HSP90 expressions of blunt snout bream (Megalobrama amblycephala) under high ammonia stress, Fish Physiol. Biochem. 41 (2015) 203–217.
[70] B. Liu, P. Xu, J. Xie, X.P. Ge, S.L. Xia, C.Y. Song, Q.L. Zhou, L.H. Miao, M.C. Ren, L.K. Pan, R.L. Chen, Effects of emodin and vitamin E on the growth and crowding stress of Wuchang bream (Megalobrama amblycephala), Fish Shellfish Immunol. 40 (2014) 595–602.
[71] J.J. Xie, X. Chen, T.Y. Guo, S.W. Xie, H.H. Fang, Z.L. Li, Y.M. Zhang, L.X. Tian, Y.J. Liu, J. Niu, Dietary values of Forsythia suspensa extract in Penaeus monodon under normal rearing and Vibrio parahaemolyticus 3HP (VP3HP) challenge conditions: effect on growth, intestinal barrier function, immune response and immune related gene expression, Fish Shellfish Immunol. 75 (2018) 316–326.
[72] J. Aguirre, M.R. Momberg, D. Hewitt, W. Hansberg, Reactive oxygen species and development in microbial eukaryotes, Trends Microbiol. 13 (2005) 111–118.
[73] I. Tetsuro, W. Eiji, Y. Toru, Novel roles of peroxiredoxins in inflammation, cancer and innate immunity, J. Clin. Biochem. Nutr. 50 (2012) 91–105.
[74] Y. Valero, F.J. Martínez-Morcillo, M.A. Esteban, E. Chaves-Pozo, A. Cuesta, Fish peroxiredoxins and their role in immunity, Biology 4 (2015) 860–880.
[75] L. Villamil, J. Gomez-Leon, M. Gomez-Chiarri, Role of nitric oxide in the defenses of Crassostrea virginica to experimental infection with the protozoan parasite Perkinsus marinus, Dev. Comp. Immunol. 31 (2007) 968–977.
[76] J.H. Ming, J. Xie, P. Xu, X.P. Ge, W.B. Liu, J.Y. Ye, Effects of emodin and vitamin C

on growth performance, biochemical parameters and two HSP70s mRNA expression of Wuchang bream (Megalobrama amblycephala Yih) under high temperature stress, Fish Shellfish Immunol. 3 (2012) 651–661.
[77] B. Liu, J. Xie, X.P. Ge, P. Xu, A.M. Wang, Y.J. He, Q.L. Zhou, L.K. Pan, R.L. Chen, Effects of anthraquinone extract from Rheum officinale Bail on the growth performance and physiological responses of M. rosenbergii under high temperature stress, Fish Shellfish Immunol. 29 (2010) 49–57.
[78] Z.Z. Zheng, J. Miao, M. Zhao, M. Tang, A.E. Yeo, H. Yu, J. Zhang, N.S. Xia, Role of heat-shock protein 90 in hepatitis E virus capsid trafficking, J. Gen. Virol. 91 (2010) 1728–1736.
[79] M.E. Feder, G.E. Hofmann, Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology, Annu. Rev. Physiol. 61 (1999) 243–282.
[80] J.J. Wan, X.P. Ge, B. Liu, J. Xie, S.L. Cui, M. Zhou, S.L. Xia, R.L. Chen, Effect of dietary vitamin C on non-specific immunity and mRNA expression of three heat shock proteins (HSPs) in juvenile Megalobrama amblycephala under pH stress, Aquaculture 434 (2014) 325–333.
[81] B. Liu, J. Xie, X.P. Ge, P. Xu, L.H. Miao, Q.L. Zhou, L.K. Pan, R.L. Chen, Comparison study of the effects of anthraquinone extract and emodin from rheum officinale bail on the physiological response, disease resistance of megalobrama amblycephala under high temperature stress, Turk. J. Fish. Aquat. Sci. 12 (2012) 905–916.
[82] F.H. Li, J.H. Xiang, Recent advances in researches on the innate immunity of shrimp in China, Dev. Comp. Immunol. 39 (2013) 11–26.
[83] B. Lemaitre, E. Kromer-Metzger, L. Michaut, E. Nicolas, M. Meister, P. Georgel, J.M. Reichhart, J.A. Hoffmann, A recessive mutation, immune deficiency (imd), defines two distinct control pathways in the Drosophila host defense, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 9465–9946.
[84] N.N. Feng, D.D. Wang, R. Wen, F.H. Li, Functional analysis on immune deficiency (IMD) homolog gene in Chinese shrimp Fenneropenaeus chinensis, Mol. Biol. Rep. 41 (2014) 1437–1444.
[85] P.H. Wang, Z.H. Gu, X.D. Huang, B.D. Liu, X.X. Deng, H.S. Ai, et al., An immune deficiency homolog from the white shrimp, Litopenaeus vannamei, activates antimicrobial peptide genes, Mol. Immunol. 46 (2009) 1897–1904.
[86] S. Ghosh, M. Karin, Missing pieces in the NF-kappa B puzzle, Cell 109 (2002) S81–S96.
[87] M.S. Hayden, S. Ghosh, Shared principles in NF-kappaB signaling, Cell 132 (2008) 344–362.
[88] W. Qiu, S. Zhang, Y.G. Chen, P.H. Wang, X.P. Xu, C.Z. Li, Y.H. Chen, W.Z. Fan, H. Ya, S.P. Weng, S. FrancisChan, J.H. He, Litopenaeus vannamei NF-κB is required for WSSV replication, Dev. Comp. Immunol. 45 (2014) 156–162.
[89] M. Hedengren, B. Åsling, M.S. Dushay, I. Ando, S. Ekengren, M. Wihlborg, D. Hultmark, Relish, a center facotr in the control of humoral but not celluar immunity in Drosophila, Mol. Cell 4 (1999) 827–837.
[90] Y. Duan, J. Li, Z. Zhang, J. Li, Q. Ge, P. Liu, The role of oncoprotein NM23 gene from Exopalaemon carinicauda is response to pathogens challenge and ammonia-N stress, Fish Shellfish Immunol. 47 (2015) 1067–1074.
[91] Y.R. Shi, M. Jin, F.T. Ma, Y. Huang, X. Huan, J.L. Feng, L.L. Zhao, Y.H. Chen, Q. Ren, Involvement of Relish gene from M. rosenbergii in the expression of antimicrobial peptides, Dev. Comp. Immunol. 52 (2015) 236–244.
[92] X.D. Huang, W.G. Liu, Y.Y. Guan, Y. Shi, Q. Wang, M. Zhao, S.Z. Wu, M.X. He, Molecular cloning and characterization of class I NF-κB transcription factor from pearl oyster (Pinctada fucata), Fish Shellfish Immunol. 33 (2012) 659–666.
[93] M.E. Hickey, J.L. Lee, A comprehensive review of Vibrio (Listonella) anguillarum: ecology, pathology and prevention, Rev. Aquacult. 10 (2017) 585–610.
[94] S.H. Cho, S.M. Lee, B.H. Park, S.C. Ji, M. Kwon, Y. Kim, J.H. Lee, S. Park, H.K. Han, Effect of dietary inclusion of various sources of green tea on immune system and challenging test of juvenile olive flounder Paralichthys olivaceus, J. Aquacult. 19 (2006) 84–89.
[95] M. Kwon, S.U. Park, J.D. Bang, B. Cho, S. Lee, S. Park, The effects of supplementary diets on the water temperature stress in olive flounder, Paralichthys olivaceus, J. Fish Pathol. 16 (2003) 183–191.