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Effects of aloe-emodin on innate immunity, antioxidant and immune cytokines mechanisms in the head kidney leucocytes of *Labeo rohita* against *Aphanomyces invadans*

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ABSTRACT

The effect of aloe-emodin incorporated diets on innate immune response, disease resistance, pro and/or anti-inflammatory cytokine gene transcription in *Labeo rohita* against *Aphanomyces invadans* is reported for the first time. In healthy and infected groups fed with 5 mg aloe-emodin enriched diet the white blood cell (WBC) count increased significantly ($p > 0.05$) after 6th week. In both groups fed with any enriched diet the biochemical parameters such as albumin, globulin, and albumin/globulin ratio did not vary significantly; however with 5 mg aloe-emodin diet the albumin and globulin levels increased significantly ($p > 0.05$) after 6th week. The serum phagocytic activity (PA), respiratory burst activity (RBA), serum complement C3 (CC3), and lysozyme activity (LA) did not increase with any diet between weeks 2 and 4, whereas with 5 mg aloe-emodin diet increased significantly in both groups after 6th week. The pro-inflammatory cytokines such as IL-1β, IL-8, TNF-α, and iNOS significantly modulated the expression in both groups on being fed with 5 mg aloe-emodin incorporation diet on 8th week. Healthy fish fed with any aloe-emodin diet did not suffer mortality. However, the infected fish fed with 1, 5, and 10 mg kg^{-1} aloe-emodin diets registered 5%, 10%, and 15% mortality. The present study indicates that healthy and infected *L. rohita* exhibited enhanced innate immune response, disease resistance, pro and/or anti-inflamatory cytokine gene transcription levels against *A. invadans*.

1. Introduction

Labeo rohita (Hamilton, 1822) generally called as rohu, belonging to *Cyprinidae* family is one of the most very important species among the three Indian major carps (IMCs) and is the most common candidate species used in polyculture in India. It is an Indo-Gangetic riverine inhabitant and successfully bred across northern and central rivers in India, Bangladesh, Pakistan, Myanmar, Nepal, and other parts of Asia as well as Europe [1]. In India, the estimated IMCs production was 4% of global aquaculture production, in which *L. rohita* alone contributed to about 35% (9, 45, 233 mt) [2,3]. At the same time occurrences of infectious pathogens in IMCs culture have increased tremendously through intensification practices in aquaculture. For instance in aquaculture systems in West Bengal, India the epizootic ulcerative syndrome (EUS) formed mainly with *Aphanomyces invadans* as one of the most common diseases has affected about 50% of the yield and resulted in adverse significant socioeconomic impacts among the fish farmers during 1991; when the disease strikes the EUS affects over 90% of the fish trade in India [4,5]. A number of bacterial pathogens pose major risk for *L, rohita* [6]. Similarly, the monogeneans are major problem in IMCs culture affecting the yield of about 42.14% of *Catla catla*, 13.72% of *L. rohita*, and 24.49% of *Cirrihinus mrigala* [7]. To manage the persistent disease problem, the fish farmers traditionally apply large quantity of antibiotics and chemicals to control fish diseases, but it leads to development of drug-resistance strains, bioaccumulation, and causes widespread public health and environmental issues. Hence there is a recent surge of interest in alternative fish disease management using natural immunostimulants including herbals and their active constituents which positively enhance the immunity and afford protection from various pathogens [8–10].

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Aloe vera enriched diet at 2% level has been reported to enhance the growth rate, hematological parameter and immune response in a number of fish species [11–14]. Leucocytes like WBC and lymphocytes elicit immunity inhibiting pathogens [15]. In higher animals like birds also diet supplemented with *A. vera* leaves at 1–10% improved growth and feed efficiency [16–18]. Polysaccharides of *A. vera* act as an immunostimulant exhibiting adjuvant activity and enhancing the release of different kinds of cytokines [19] and stimulate hematopoiesis [20]. Aloe-emodin (1, 8-Dihydroxy-3-(hydroxymethyl)-9, 10-anthraquinone) is abundantly found in *Rheum emodi* and *Aloe vera* [21,22] that promotes anti-tumor activity, including induction of apoptosis through intrinsic (cytochrome c/caspase 9) pathways, immune modulation through extrinsic (TNF-α and FASL) pathways, cell cycle arrest, and cell mobility alterations, which indicate that aloe-emodin harmful effects on mitochondrial membrane permeability or oxidative stress via impairment of reactive oxygen species (ROS) production in various cancer cell lines. It also modulate immune signals by up-regulation and triggering the release of interleukins (ILs), tumor necrosis factor-α (TNF-α), FAS ligand and its associated death domains (TNF-R1 and FAS), p53, cytochrome c, BAX GM-CSF, NF-κB, and growth factors [23,24]. Aloe-emodin also induces NO production and phagocytosis and it activation of caspases-3/7/8/9 in different cancer cell lines [25,26]. *A. vera* is frequently supplemented in diets to enhance immune system as well as expression of immune-related genes in various fishes [27–35]. However, there is no investigation on the effect of feeding with *Rheum emodi* or aloe-emodin in fish. Therefore, the present study was undertaken for the first time to investigate the effect of aloe-emodin incorporated diet on innate immune response, disease resistance, and immune cytokine gene modulation in *L, rohita* against *A. invadans*.

2. Materials and methods

2.1. Preparation of supplementation feed

The basal (control) diet prepared for the present study comprised of fish meal, soybean meal, and wheat meal as protein source, rice bran as carbohydrate source; mustard oil cake as lipid source; in addition tapioca powder was used as binder; a premix of vitamins and minerals was added as source for vitamins and minerals (Table 1). Four supplementation diets were prepared by enriching the basal diet namely: (i) without aloe-emodin (0 mg) and with (ii) 1 mg, (iii) 5 mg, and (iv) 10 mg of aloe-emodin. The ingredients were mixed well and steamed for 20 min in a pressure cooker and made into a soft paste. Then the paste was cooled to room temperature (RT) and the vitamins and minerals premix along with the chosen concentration of aloe-emodin (0, 1, 5, and 10 mg kg^{-1}) were added. The paste pelletized using a hand pelletizer (0.5 mm diameter size), air-dried for 30 min, and kept in an oven at 60 °C until dry. The dried pellets was packed in airtight container and stored in a freezer at −20 °C until used. The proximate composition of the experimental pellet diets were analyzed by standard methods.

2.2. Aphanomyces invadans

Oomycete fungus, *A. invadans* (isolate B99C) was used for challenge study; it was isolated from EUS infected *Cirrhinus reba* obtained from the Department of Aquaculture, Sterling University, Scotland, UK [36]. The motile secondary zoospores suspension was prepared following Harikrishnan et al. [36]. Four aggressively growing mycelium agar pads (3–4 mm diameter) were placed in a petri dish containing glucose peptone yeast (GPY) broth and incubated at 20 °C for 4 days. Afterwards, the nutrient agar was washed out by consecutive transfer through petri dishes containing autoclaved pond water (APW). The mycelium mats were kept overnight at 20 °C and the motile secondary zoospores were collected and then enumerated or adjusted using a Neubarr hemocytometer at 10^4 spores ml^{-1}.

Table 1
Aloe-emodin supplementation experimental pellet diet ingredients and proximate composition.

Ingredients	Aloe-emodin (mg kg^{-1})			
	0	1	5	10
Fishmeal	35.000	35.000	35.000	35.000
Soybean meal	25.000	25.000	25.000	25.000
Mustard oil cake	20.000	20.000	20.000	20.000
Rice bran	6.000	6.000	6.000	6.000
Wheat meal	8.000	8.000	8.000	8.000
Tapioca powder	5.000	4.099	4.095	4.090
Vitamins + minerals premix[a]	1.000	1.000	1.000	1.000
Aloe-emodin	0.000	0.001	0.005	0.010
Total	100	100	100	100
Proximate composition (%)				
Crude protein (%)	40.09	41.11	41.43	40.69
Crude lipid (%)	13.33	13.34	14.27	14.55
Crude carbohydrate (%)	17.45	18.25	17.62	18.36
Crude ash (%)	12.23	12.21	12.28	12.44
Crude fiber (%)	5.14	5.46	5.57	5.77

[a] Composition of vitamin–mineral premix (Emix™ plus, Mumbai, Maharastra, India) (quantity/2.5 kg): vitamin A, 5,500,000 IU; vitamin B2, 2000 mg; vitamin B6, 1000 mg; vitamin B12, 6 mg; vitamin D3, 1,100,000 IU; vitamin E, 750 mg; vitamin K, 1000 mg; choline chloride, 150 g; calcium panthothenate, 2500 mg; Co, 450 mg; Ca, 500 g; Cu, 2000 mg; DL-methionine, 10 g; Fe, 7500 mg; iodine, 1000 mg; L-lysine, 10 g; niacinamide, 10 g; Mn, 27,000 mg; Zn, 5000 mg; P, 300 g; Se, 50 ppm; satawari, 2500 mg; Carrier, quantum sufficient; Lactobacillus, 120 million units, and yeast culture, 3000 crore units.

2.3. Fish

Healthy, *L. rohita* (27.8 ± 1.5 g) were obtained from a commercial fish farm. The fish were transported to the laboratory and disinfected with 5 ppm potassium permanganate ($KMnO_4$) for 5 min. The fish were then acclimatized in 500 l capacity cement tanks with clean bore-well water for two weeks. During the period of acclimation, the fish were provided with basal diet (Table 1) at a rate of 5% of their body weight. Two-third of the water was siphoned out to remove the accumulated wastes and replaced daily.

2.4. Experimental design

After two weeks of acclimatization, the fish were randomly divided into eight group of 25 fish (8 × 25 = 200) as: (1) healthy fish fed basal control diet without aloe-emodin (0 mg kg^{-1}) [H]; the healthy fish fed with diet enriched with (2) 1 mg kg^{-1} [H-1 mg], (3) 5 mg kg^{-1} [H-5 mg], and (4) 10 mg kg^{-1} [H-10 mg] of aloe-emodin; (5) the infected (or challenged) fish fed with basal control diet without aloe-emodin (0 mg kg^{-1}) [I]; the infected fish fed with aloe-emodin enriched diet as: (6) 1 mg kg^{-1} [I-1 mg], (7) 5 mg kg^{-1} [I-5 mg], and (8) 10 mg kg^{-1} [I-10 mg]. All the groups were maintained in three replicates (200 × 3 = 600 fish). Groups 1 to 4 were injected intramuscularly (i.m.) at the dorsal fin with 0.1 ml APW alone and groups 5 to 8 were challenged (injected) i.m. with 0.1 ml APW containing *A. invadans* at 10^4 spores ml^{-1}. The respective pellet diets were provided twice a day at 1000 and 1700 h throughout the experimental period. The water temperature (°C), dissolved oxygen, ammonia concentrations, and pH were measured from 26 to 31, 6.1–7.3 mg l^{-1}, 0.03–0.06 ppm, 7.0 to 8.0 during the experimental period.

2.5. Blood sampling

Six fish were randomly chosen in each experimental tank at the end of weeks 2, 4, 6, and 8 post-challenge with *A. invdans*. The fish were immediately put under anesthesia in 150 ppm buffered MS-222 solution (Sigma-Aldrich, St. Louis, MO, USA). All fish were bled through their

caudal vasculature using a 24-gauge syringe needle. The collected blood was divided and transferred into heparinized and non-heparinized tubes. The non-heparinized tubes with blood samples were placed at RT for 2 h than the sera were separated by centrifugation at 2700 rpm for 10 min and stored at −20 °C until used. The heparinized blood was used immediately for hematological and biochemical study.

2.6. Hematology and biochemical study

The white blood cell (WBC) count was determined by Dacie and Lewis [37]. The serum total protein (TP) concentration and albumin (AB) were determined by citrate buffer and bromocresol green (BCG) dye binding method according to Shaziya and Goyal [38] using total protein and albumin kit (Pars Azmun Company, Iran). The globulin (GB) level was determined by subtracting albumin values from total serum protein whereas the albumin/globulin (A/G) ratio was calculated by diving albumin values by globulin values.

2.7. Isolation of head kidney leukocytes

The head kidney (HK) leukocytes were removed from each fish according to Kamilya et al. [39] after minor modification. The HK leukocytes containing tissues were teasing with forceps in complete RPMI-1640 medium which containing penicillin (100 IU/ml), streptomycin (100 mg/ml), and 10% fetal calf serum (Hi-media, India) for cell suspensions. The cell suspension washed by centrifugation at 1000 rpm for 10 min to collect pellet. The pellet was resuspended in RPMI-1640 medium, carefully layered on top of the leukocyte isolation medium (HiSep, Hi-media, India) after centrifugation at 1500 rpm for 30 min. The leukocytes cells at the HiSep interface medium were transferred into clean tubes and washed twice by centrifugation at 1000 rpm for 10 min. The purified leukocytes were enumerated by using a hemocytometer and the number of leukocytes adjusted to 1×10^6 cells/ml in RPMI-1640 medium. The viability of the leukocytes was determined by trypan blue exclusion assay.

2.8. Immune assays

The phagocytic activity of the blood leukocytes was determined according to Cai et al. [40]. The respiratory burst activity (RBA) of phagocytes was determined by using the nitroblue tetrazolium (NBT, Sigma-Aldrich) assay according to Geng et al. [41]. The serum complement C3 (CC3) level was determined by using a C3 kit (Biocompare, CA, USA) according to Thomas [42]. The serum lysozyme activity (LA) was determined according to Ellis [43].

2.9. Challenge study

A group of 20 fish in each experimental group as mentioned above were maintained separately for cumulative mortality (CM). The fungus culture, challenge study, and the concentration were same as above. The CM (%) was calculated for a period of 30 days as: [(Number of surviving fishes after challenge)/(Number of fish challenged with fungus)] x 100.

2.10. Immune cytokine gene expressions study

The HK leukocytes were isolated from each group (n = 6) as mentioned above, frozen in liquid nitrogen, and stored at −80 °C until used. Total RNA was isolated from HK leukocytes using TRIZOL (Invitrogen, USA) following the manufacturer's guidelines. The concentration and purity of the isolated RNA samples were quantified using a UV spectrophotometer (NanoDrop 2000c, Thermo scientific). The quality of the RNA samples was confirmed by using a 1% agarose gel containing 0.5 μg ml^{-1} ethidium bromide. The RNA was reverse-transcribed to cDNA synthesis by using a SuperScript cDNA synthesis kit (Life Technologies, USA), following the manufacturer's guidelines. The expression of immune genes such as IL-1β, IL-8, IL-10, TNF-α, iNOS, and TGF-β were quantified by real-time PCR (CFX96 Real-Time PCR, Bio-Rad, Laboratories, Inc.) and compared to a housekeeping gene (β-actin) following standard protocols. The primer sequences or design and temperature are described (Table 2). The accuracy of each amplicon verified by melt curve analysis was executed after amplification. All samples with a housekeeping gene were run in parallel in order to normalize cDNA loading. The gene expression results were evaluated by using the $2^{-\Delta\Delta CT}$ method after verifying the primers amplified with an efficiency of approximately 100% [44]. All data from each group were compared to those of the control groups.

Table 2
Real-time PCR primer sequences for the experiment.

Gene name	Primer sequence (5′ to 3′)	Temperature	Accession number
IL-1β	F: ATCTTGGAGAATGTGATCGAAGAG R: GATACGTTTTTGATCCTCAAGTGTGAAG	61.5 °C	AM932525
IL-8	F: GGGTGTAGATCCACGCTGT R: AGGGTGCAGTAGGGTCCA	60.5 °C	HM363518
IL-10	F: AAGGAGGCCAGTGGCTCTGT R: CCTGAAGAAGAGGCTCTGT	61.1 °C	AB010701
TNF-α	F: CCAGGCTTTCACTTCACG R: GCCATAGGAATCGGAGTAG	61.1 °C	FN543477
iNOS	F: GGAGGTACGTCTGCGAGGAGGCT R: CCAGCGCTGCAAACCTATCATCCA	61.1 °C	AM932526
TGF-β	F: ACGCTTTATTCCCAACCAAA R: GAAATCCTTGCTCTGCCTCA	60.5 °C	AF136947
β-actin	F: AGACCACCTTCAACTCCATCATG R: TCCGATCCAGACAGAGTATTTACGC	60.5 °C	AY531753

IL: interleukin, TNF: tumor necrosis factor, iNOS: inducible nitric oxide synthase, TGF: transforming growth factor.

2.11. Statistical analysis

All the data are expressed as arithmetic mean ± stranded error mean (SEM). The data were subjected to one way analysis of variance (ANOVA) and Duncan's Multiple Range Test (SPSS windows 16.0 software) for comparison of means. The significance levels were expressed as *p*-value at 0.05 levels.

3. Results

3.1. Hematology and biochemistry

Dietary aloe-emodin incorporation diets did not significantly ($p < 0.05$) increase the white blood cell (WBC) count. However both the healthy and infected fish fed with 5 mg aloe-emodin incorporated diet had registered significantly ($p > 0.05$) increase in WBC on weeks 6 and 8 when compared to other groups. In infected fish fed with control diet, the WBC level decreased progressively when compared to other groups (Table 3). Both groups fed with dietary aloe-emodin enriched diets had no significant ($p < 0.05$) changes on albumin, globulin, and albumin/globulin ratio during the experimental period, except 5 mg aloe-emodin diet, which had significant ($p > 0.05$) increase in the albumin and globulin on weeks 6 and 8 when compared to other groups (Table 3).

3.2. Immune response

The phagocytic activity (PA) of HK leucocytes did not significantly ($p < 0.05$) enhance in healthy and infected fish fed with any aloe-emodin incorporation diets between weeks 2 and 4. However, it significantly increased in both groups fed with 5 mg aloe-emodin diet on weeks 6 and 8, but the increase was not significant 1 and 10 mg aloe-emodin diet as compared to control (Fig. 1). The respiratory burst

Table 3
Hematological and biochemical parameter of *L. rohita* feeding a graded level (0, 1, 5, and 10 mg kg^{-1}) of aloe-emodin incorporation diets against *A. invadans*.

Indices	Weeks	H-0 mg	I-0 mg	H-1 mg	I-1 mg	H-5 mg	I-5 mg	H-10 mg	I-10 mg
WBC (10^3 ml^{-1})	2	2.17 ± 0.03[a]	2.12 ± 0.03[a]	2.41 ± 0.02[a]	2.33 ± 0.02[a]	2.82 ± 0.03[a]	2.73 ± 0.03[a]	2.48 ± 0.02[a]	2.40 ± 0.03[a]
	4	2.23 ± 0.03[a]	2.20 ± 0.04[a]	2.45 ± 0.03[a]	2.42 ± 0.02[a]	3.45 ± 0.04[b]	2.97 ± 0.03[a]	2.55 ± 0.03[a]	2.45 ± 0.02[a]
	6	2.26 ± 0.04[a]	2.25 ± 0.03[a]	2.54 ± 0.03[a]	2.49 ± 0.03[a]	3.63 ± 0.04[b]	3.41 ± 0.03[b]	2.60 ± 0.03[a]	2.58 ± 0.03[a]
	8	2.30 ± 0.04[a]	2.29 ± 0.04[a]	2.57 ± 0.04[a]	2.53 ± 0.03[a]	3.78 ± 0.04[b]	3.64 ± 0.04[b]	2.74 ± 0.04[a]	2.63 ± 0.03[a]
TP (g dl^{-1})	2	3.26 ± 0.03[a]	3.13 ± 0.03[a]	3.34 ± 0.04[a]	3.31 ± 0.04[a]	3.51 ± 0.03[a]	3.45 ± 0.04[a]	3.45 ± 0.03[a]	3.41 ± 0.04[a]
	4	3.28 ± 0.04[a]	3.09 ± 0.03[a]	3.42 ± 0.03[a]	3.39 ± 0.03[a]	3.78 ± 0.04[a]	3.66 ± 0.05[a]	3.52 ± 0.04[a]	3.49 ± 0.03[a]
	6	3.31 ± 0.04[a]	3.04 ± 0.04[a]	3.53 ± 0.04[a]	3.48 ± 0.03[a]	4.96 ± 0.04[b]	4.88 ± 0.04[b]	3.59 ± 0.04[a]	3.52 ± 0.04[a]
	8	3.32 ± 0.03[a]	3.01 ± 0.03[a]	3.68 ± 0.03[a]	3.52 ± 0.04[a]	5.27 ± 0.05[b]	5.11 ± 0.05[b]	3.63 ± 0.05[a]	3.59 ± 0.04[a]
AB (g dl^{-1})	2	1.28 ± 0.01[a]	1.26 ± 0.01[a]	1.48 ± 0.02[a]	1.42 ± 0.02[a]	1.57 ± 0.03[a]	1.52 ± 0.03[a]	1.43 ± 0.03[a]	1.38 ± 0.02[a]
	4	1.30 ± 0.01[a]	1.24 ± 0.01[a]	1.53 ± 0.02[a]	1.48 ± 0.03[a]	2.17 ± 0.03[a]	2.03 ± 0.03[a]	1.46 ± 0.03[a]	1.43 ± 0.02[a]
	6	1.32 ± 0.02[a]	1.23 ± 0.01[a]	1.61 ± 0.03[a]	1.57 ± 0.02[a]	2.34 ± 0.04[b]	2.25 ± 0.04[b]	1.61 ± 0.04[a]	1.52 ± 0.03[a]
	8	1.35 ± 0.02[a]	1.21 ± 0.02[a]	1.69 ± 0.03[a]	1.62 ± 0.03[a]	2.46 ± 0.05[b]	2.38 ± 0.04[b]	1.67 ± 0.04[a]	1.58 ± 0.03[a]
GB (g dl^{-1})	2	1.43 ± 0.02[a]	1.40 ± 0.01[a]	1.46 ± 0.02[a]	1.43 ± 0.03[a]	1.52 ± 0.03[a]	1.50 ± 0.02[a]	1.48 ± 0.03[a]	1.45 ± 0.02[a]
	4	1.48 ± 0.02[a]	1.34 ± 0.02[a]	1.57 ± 0.03[a]	1.52 ± 0.02[a]	2.33 ± 0.03[b]	2.14 ± 0.03[a]	2.13 ± 0.04[a]	1.97 ± 0.03[a]
	6	1.53 ± 0.01[a]	1.47 ± 0.01[a]	1.72 ± 0.02[a]	1.65 ± 0.03[a]	3.11 ± 0.04[b]	3.05 ± 0.04[b]	3.08 ± 0.04[b]	2.44 ± 0.03[a]
	8	1.56 ± 0.01[a]	1.52 ± 0.02[a]	1.80 ± 0.03[a]	1.73 ± 0.02[a]	3.23 ± 0.04[b]	3.17 ± 0.04[b]	3.14 ± 0.03[b]	2.67 ± 0.04[a]
A/C (g dl^{-1})	2	0.53 ± 0.01[a]	0.51 ± 0.01[a]	0.62 ± 0.02[a]	0.58 ± 0.03[a]	0.83 ± 0.02[a]	0.80 ± 0.02[a]	0.79 ± 0.02[a]	0.82 ± 0.02[a]
	4	0.55 ± 0.01[a]	0.51 ± 0.01[a]	0.65 ± 0.02[a]	0.61 ± 0.03[a]	0.97 ± 0.03[a]	0.95 ± 0.03[a]	0.82 ± 0.02[a]	0.80 ± 0.03[a]
	6	0.57 ± 0.01[a]	0.54 ± 0.02[a]	0.68 ± 0.03[a]	0.64 ± 0.02[a]	1.03 ± 0.04[a]	0.98 ± 0.03[a]	0.86 ± 0.03[a]	0.83 ± 0.02[a]
	8	0.59 ± 0.02[a]	0.57 ± 0.02[a]	0.72 ± 0.03[a]	0.68 ± 0.02[a]	1.26 ± 0.04[a]	1.02 ± 0.03[a]	0.89 ± 0.03[a]	0.86 ± 0.03[a]

WBC: white blood cell, TP: total protein, AB: albumin, GB: globulin, A/G ratio: albumin/globulin ratio.

activity (RBA) did not significantly ($p < 0.05$) vary in fish fed with any enriched diet on weeks 2 and 4 as compared with other groups. The RBA was significantly enhanced in both groups when fed with 5 mg aloe-emodin diet after 6th week; however the increase was not observed with other diet (1 mg or 10 mg of aloe-emodin) on these periods (Fig. 2).

The serum complement C3 (CC3) level did not increase significantly on weeks 2 and 4 when the healthy and infected fish were fed with any aloe-emodin incorporation diet as compared to other groups. However, the CC3 was enhanced significantly with 5 or 10 mg aloe-emodin diets on weeks 6 and 8 (Fig. 3). The serum lysozyme activity (LA) did not vary significantly ($p < 0.05$) in both groups fed with 1 mg or 10 mg aloe-emodin diets during the experiment. Nevertheless, the LA significantly increased in both groups fed with 5 mg aloe-emodin diet during the experiment (Fig. 4).

3.3. Challenge study

A. invadans infected fish fed with basal diet (0 mg kg^{-1} aloe-emodin diet) had shown 65% mortality while the mortality was 5%, 10%, and 15% in post-challenged with *A. invadans* fish fed with 1, 5, and 10 mg kg^{-1} aloe-emodin diets. However, there was no mortality in the healthy fish fed with any aloe-emodin incorporation diets for 30 days (Fig. 5).

3.4. Induction of immune cytokine genes

The induction of immune cytokine gene expression levels were investigated in the head kidney (HK) of healthy and infected fish fed with graded levels (0, 1, 5, and 10 mg kg^{-1}) of aloe-emodin incorporation diets at weeks 2, 4, 6, and 8. The pro-inflammatory cytokines such as IL-1β and IL-8 expression levels was not significant in the healthy and infected fish fed with any dose of aloe-emodin incorporation diet on weeks 2 and 4 as compared to other groups. It was significantly induced with 5 mg aloe-emodin incorporation diet on week 6, but not 1 mg or 10 mg aloe-emodin incorporation diets. The IL-1β expression was significantly up-regulated with 5 mg or 10 mg aloe-emodin incorporation diets whereas the IL-8 was significantly up-regulated with 5 mg aloe-emodin incorporation diet on week 8 as compared to other groups (Fig. 6a and b). However, the anti-inflammatory cytokines such as IL-10

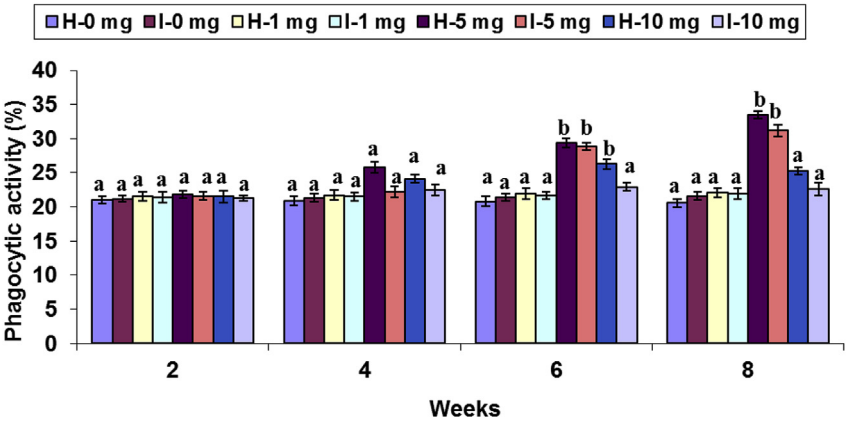

Fig. 1. Serum phagocytic activity (PA) of *L. rohita* ($n = 6$) feeding with a graded level (0, 1, 5, and 10 mg kg^{-1}) of aloe-emodin incorporation diets at weeks 2, 4, 6, and 8. Bars represent the mean ± SEM and different letters represent levels of significant within the groups at $p < 0.05$. Note: H: healthy fish, I: infected fish.

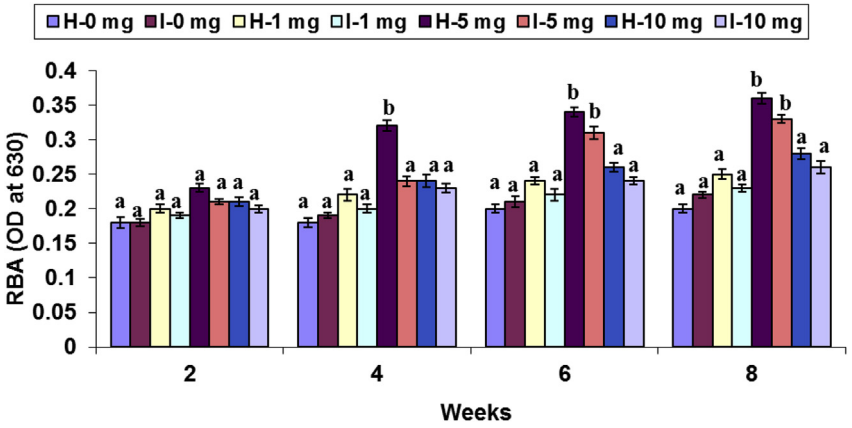

Fig. 2. Serum respiratory burst activity (RBA) of *L. rohita* ($n = 6$) feeding with a graded level (0, 1, 5, and 10 mg kg^{-1}) of aloe-emodin incorporation diets at weeks 2, 4, 6, and 8. Bars represent the mean ± SEM and different letters represent levels of significant within the groups at $p < 0.05$. Note: H: healthy fish, I: infected fish.

and TGF-β observed were down-regulated in both healthy and infected group fed with any aloe-emodin enriched diet during the experiment (Figs. 6c and 7). The other pro-inflammatory cytokines such as TNF-α and iNOS expression levels did not significantly vary in both groups fed with any aloe-emodin incorporation diet during the experiment. However, it was significantly up-regulated after 6th week of feeding in both groups fed with 5 mg aloe-emodin incorporation diet (Figs. 8 and 9).

4. Discussion

In aquaculture administration of natural immunostimulants is a promising practice in prevention and control of fish diseases because it is biocompatible, biodegradable, and provides a safe environment for the host body. The use of natural immunostimulants has offer an eco-friendly prophylactic approach and is a significant potential alternative to chemotherapy and vaccination. It provides increased disease resistance and enhances innate immunity by triggering cytokine gene expression in fish. The immunity and cytokine gene regulation of natural bioactive compounds against disease is not well documented in fish. This study reports on the effects of aloe-emodin, a natural immunostimulant in fish with reference to disease protection and immune cytokine gene transcription in *L. rohita* against *A. invadans* for the first time.

In healthy and infected fish dietary administration of aloe-emodin did not increase the WBC significantly from weeks 2 and 4 while with 5 mg aloe-emodin incorporation diet the WBC count significantly increased after 6th week. A similar increase had been reported with *Aloe vera* enriched diet in various fish [29–33] and with *Azardicha indica* enriched diet in the infected *C. carpio* [45]. The significant increase in the total WBC count comprise different leucocytes like neutrophil, large lymphocytes, monocytes which constitute the primary line of immune defense triggering innate immunity to facilitate the elimination of the pathogen.

The increased level of serum total protein (TP) in moribund fish infected with pathogen indicates the rise in antibody production [46]. The serum proteins contain various humoral components of the innate immune system; a high concentration of serum TP and globulin stimulating the innate immune response in fish against pathogens [29,31,47]. A similar response was observed in this study in both groups with dietary administration of 5 mg aloe-emodin diet after 6th week. As the first line of immune defense, several peptides including lysozymes, antibodies, complement factors and other lytic factors increase in serum which prevents the adherence and colonization of pathogen [48]. Phagocytic activity (PA) initially stimulates the inflammatory reaction before the development of antibody production [49]. Phagocytosis (PA) associated with respiratory burst activity (RBA) is an important indicator of innate immune defense in fish [50]. The RBA produced by phagocytic cells attack the pathogens and hence are a reliable marker in the evaluation of immune defense capacity [50]. The PA and RBA response present a major antibacterial defense mechanism in fish quantified as neutrophils and macrophages which remove the bacteria mainly by the production of reactive oxygen species (ROS) [51,52]. In the present study, the PA and RBA of HK leucocytes did not enhance significantly both groups fed with any aloe-emodin enhriched diet before week 4. It significantly increased in both groups fed with 5 mg aloe-emodin diet after week 6 as compared with 1 mg or 10 mg

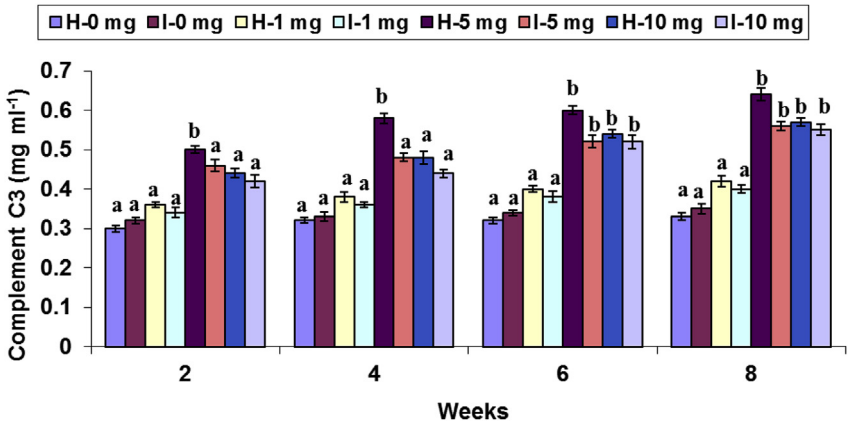

Fig. 3. Serum complement C3 (CC3) level of *L. rohita* ($n = 6$) feeding with a graded level (0, 1, 5, and 10 mg kg^{-1}) of aloe-emodin incorporation diets at weeks 2, 4, 6, and 8. Bars represent the mean ± SEM and different letters represent levels of significant within the groups at $p < 0.05$. Note: H: healthy fish, I: infected fish.

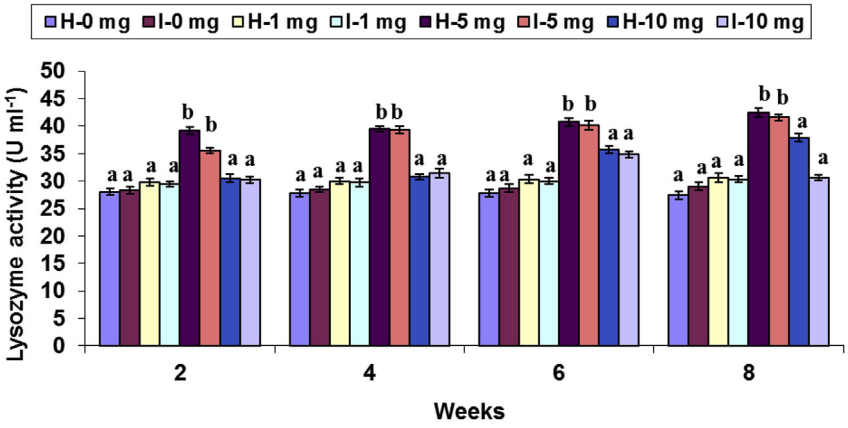

Fig. 4. Serum lysozyme activity (LA) of *L. rohita* ($n = 6$) feeding with a graded level (0, 1, 5, and 10 mg kg^{-1}) of aloe-emodin incorporation diets at weeks 2, 4, 6, and 8. Bars represent the mean ± SEM and different letters represent levels of significant within the groups at $p < 0.05$. Note: H: healthy fish, I: infected fish.

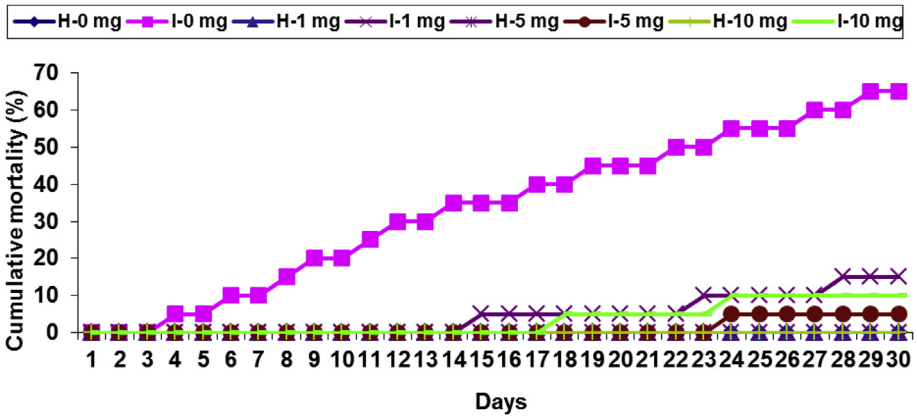

Fig. 5. Cumulative mortality (%) of *L. rohita* ($n = 20$) feeding with a graded level (0, 1, 5, and 10 mg kg^{-1}) of aloe-emodin incorporation diets for 30 days. Note: H: healthy fish, I: infected fish.

aloe-emodin diets. Similarly, dietary administration of polysaccharide fraction of *Chlorophytum borivilianum* enhanced PA and RBA of blood leucocytes in *L. rohita* [53]. In other fish such as *O. mykiss* and *C. carpio* also a similar significant increase PA and RBA of HK leucocytes levels has been reported [29,31].

The complement system is one of the essential constituents of the innate immune defense and plays an important role in alerting and clearing of infectious microorganisms in host [54]. In this study the serum complement C3 (CC3) level did not enhance significantly before week 4 in both groups fed with any enriched diet while the increase was noted when fed with 5 mg or 10 mg aloe-emodin diets after 6th week, indicating time dependence of aloe-emodin to take effect. Our results are in agreement with the reports in *L. rohita* and *Ctenopharyngodon idella* fed with polysaccharide sourced from *C. borivilianum* and *Ficus carica* which significantly enhanced CC3 [55,56]. However, though the complement activation is generally beneficial to fish, its sustained activation could result in unwanted side effects like immune suppression [57]. The present study indicates that increased the serum CC3 may be significantly beneficial in the healthy and infected fish fed with 5 mg or 10 mg aloe-emodin diets after 6th week.

The lysozyme present in the mucus, plasma, and other body fluids of fish plays a significant role in the protection from infectious microorganisms in fish [58]. In the present study the serum lysozyme activity (LA) was not enhanced significantly in healthy and infected fish fed with 1 mg or 10 mg aloe-emodin diets but with 5 mg aloe-emodin diet was significant. In L. *rohita*, *C. idella*, and *C. carpio*, and *A. baerii* a significant increase in serum LA was observed with the dietary administration of polysaccharide from *C. borivilianum* and *F. carica* [31,33,55,56,59]. The LA of HK leucocytes increased significantly in *O. mykiss* fed with *A. vera* enriched diet with medium dose of aloe-emodin for longer durations lead to immunosuppression, but not with low (1 mg) or high (10 mg) doses [29,32].

The present study indicates that the enhancement of serum C3, PA, RBA, CC3, and LA due to aloe-emodin enriched diet significantly enhanced the innate immune response in rohu; however, the enhancement did not manifest during earlier period with low (1 mg) or high (10 mg) doses of aloe-emodin, suggesting that lower level does not work and higher level may depress immunity in rohu as also reported by Giri et al. [53]. Besides, the enhancement of innate immune defense in rohu also provides evidence that aloe-emodin incorporated diet can effectively modulate some pro and/or anti-inflammatory cytokine genes such as IL-1β, Il-8, Il-10, TNF-α, iNOS, and TGF-β. To the best of our knowledge, this is the first evidence about the effect of aloe-emodin on immune cytokine gene induction in fish.

The IL-1β is a pro-inflammatory cytokine gene affects almost all cell type including, TNF [60]. Both IL-1β and TNF-α pro-inflammatory cytokine genes induce inflammatory response. However, the IL-1β plays a significant role in the host response to microorganisms, injury of tissue, and immunological reactions, whereas the TNF-α is crucially responsible for various cellular immune reactions like cell proliferation, differentiation, and stimulation of other cytokines [61]. In the present study there was little induction of pro-inflammatory cytokine gene expression like IL-1β, IL-8, and TNF-α in the healthy and infected fish fed with low (1 mg) or high (10 mg) doses of aloe-emodin incorporation diets. However, the medium dose of 5 mg aloe-emodin enriched diet abundantly induced the pro-inflammatory cytokine gene expression

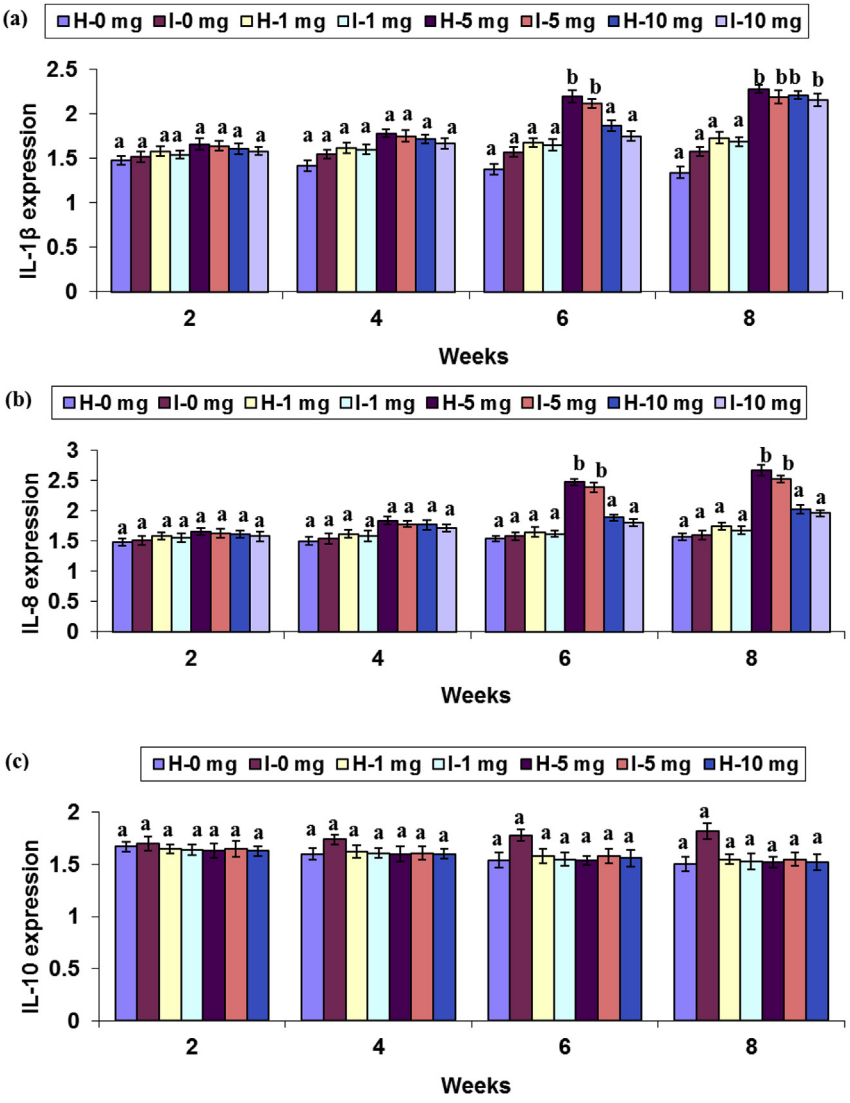

Fig. 6. The relative mRNA expression of (a) IL-1β, (b) IL-8, and (c) IL-10 in the head kidney (HK) leucocytes of healthy and infected *L. rohita* feeding with a graded level (0, 1, 5, and 10 mg kg^{-1}) of aloe-emodin incorporation diets at weeks 2, 4, 6, and 8. Bars represent the mean ± SEM and different letters represent levels of significant within the groups at $p < 0.05$. Note: H: healthy fish, I: infected fish.

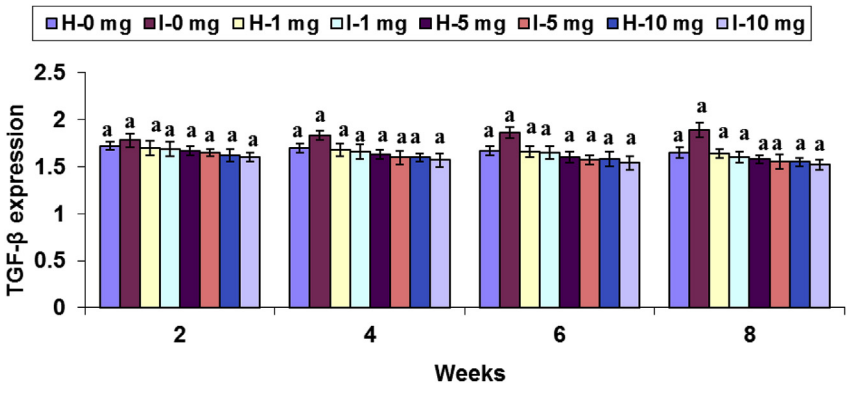

Fig. 7. The relative mRNA expression of TGF-β in the head kidney (HK) leucocytes of healthy and infected *L. rohita* feeding with a graded level (0, 1, 5, and 10 mg kg^{-1}) of aloe-emodin incorporation diets at weeks 2, 4, 6, and 8. Bars represent the mean ± SEM and different letters represent levels of significant within the groups at $p < 0.05$. Note: H: healthy fish, I: infected fish.

after 6th week. Several studies confirm that a host of active compounds present in herbal immunostimulants can significantly induce pro-inflammatory cytokines. Similar results are documented in grass carp fed with polysaccharide of *F. carica* that result in a strong up-regulation of IL-1β and TNF-α gene expression after 3rd week [56]. Similarly, administration of *Spirulina platensis* increased IL-1β and TNF-α gene expression in common carp [62].

In fish the production of iNOS is a well recognized immunoregulatory factor against infectious pathogens [63]. In the present study, the iNOS expression levels did not vary significantly in the earlier period (weeks 2 and 4); however, it was significantly up-regulated after 6 weeks in healthy and infected fish fed with 5 mg aloe-emodin

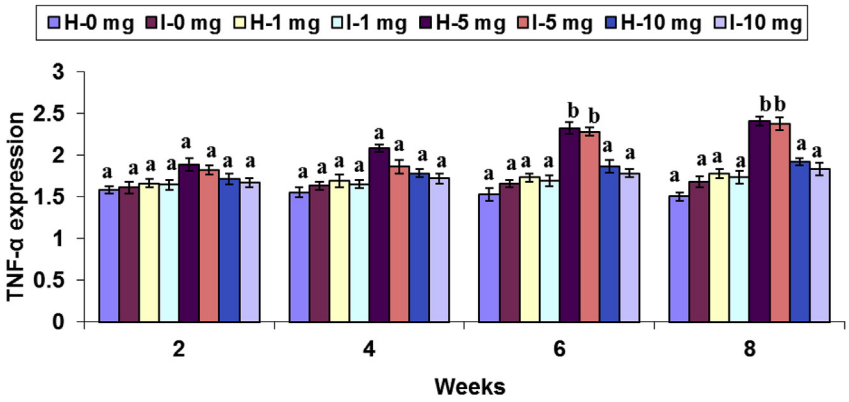

Fig. 8. The relative mRNA expression of TNF-α in the head kidney (HK) leucocytes of healthy and infected *L. rohita* feeding with a graded level (0, 1, 5, and 10 mg kg^{-1}) of aloe-emodin incorporation diets at weeks 2, 4, 6, and 8. Bars represent the mean ± SEM and different letters represent levels of significant within the groups at $p < 0.05$.Note: Note: H: healthy fish, I: infected fish.

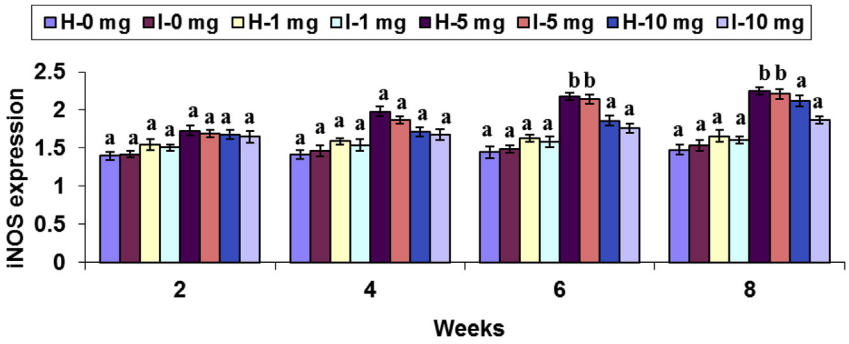

Fig. 9. The relative mRNA expression of iNOS in the head kidney (HK) leucocytes of healthy and infected *L. rohita* feeding with a graded level (0, 1, 5, and 10 mg kg^{-1}) of aloe-emodin incorporation diets at weeks 2, 4, 6, and 8. Bars represent the mean ± SEM and different letters represent levels of significant within the groups at $p < 0.05$. Note: H: healthy fish, I: infected fish.

incorporation diet. The iNOS transcription was up-regulated in the head kidney of common carp fed with *Rehmannia glutinosa* supplementation diet [64]. A recently study also demonstrated that similar results in rohu fed with dietary guava extract significantly up-regulate of iNOS in head kidney, intestine, and hepatopancreas [65]. The induction of IL-1β, IL-8, and iNOS gene expression suggest that may be related to increase the production of reactive-nitrogen intermediates leads to damage pathogens [63] as observed in the present study.

The anti-inflammatory cytokine, IL-10 is a multifunctional cytokine that results in immunosuppression leading to inhibition of cytokine production [65]. The TGF-β anti-inflammatory cytokine usually inhibit the proliferation and differentiation of B and T cell, while provokes pro-inflammatory cytokines, IL-1β, TNF-α, and IFN-γ, and suppression of IL-1β and IL-2 receptors expression. Further, it also directly targets effector of T cells and Treg cells to confirm self-tolerance [66]. In the present study, IL-10 and TGF-β expression was down-regulated in the HK leucocytes of rohu with any diet during the experimental period. The present results are in agreement with *Catla catla* fed with 1.0% *A. aspera* supplemented diet which significantly down-regulated IL-10 expression in HK leucocytes [67]. Similarly, in the common carp fed with *R. glutinosa* incorporated diet lowered the IL-10 and TGF-β expression levels in the kidney, spleen, and intestine [64]. Another recent study also suggest that in rohu fed with diet enriched guava leaves found low IL-10 and TGF-β gene induction [55]. Based on the earlier reports and the present study suggest that such a down-regulation of IL-10 and TGF-β may favor the enhanced pro-inflammatory cytokines expression when healthy and infected rohu were fed with any does of aloe-emodin incorporation diets.

There was no mortality in the healthy fish fed with any aloe-emodin enriched diet; however, the *A. invadans* challenged fish fed without aloe-emodin diet had shown 65% mortality while the mortality was 5%, 10%, and 15% in the infected fish fed with 1, 5, and 10 mg kg^{-1} aloe-emodin diets. The highest protection or survival rate had been reported in the infected common carp, goldfish, and Grass carp after dietary administration of *R. glutinosa*, azadirachtin, and *F. carcica* polysaccharide [56,64,68] and also in *C. carpio* fed with *A. vera* containing rich content of aloe-emodin [31]. This present study provides the first evidence on the influence of aloe-emodin at low levels can influence both innate and humoral immunity in rohu and provoke pro and/or anti-inflammatory cytokine genes expression. Among the different graded levels (0, 1, 5, and 10 mg kg^{-1}) of aloe-emodin incorporation diets, the healthy and infected fish fed with 5 mg kg^{-1} of aloe-emodin incorporation diet effectively modulate innate immunity and up-regulation of pro-inflammatory cytokines (IL-8, IL-1β, TNF-α, and iNOS) and down-regulation of anti-inflammatory cytokines (TGF-β and IL-10) gene expression. However, further detailed studies on the molecular mechanism on disease resistance should be performed in other fish against pathogens with reference to aloe-emodin for aquaculture activity. Further information on the timing and optimum dose of aloe-emodin administration in fish to cope with stress or disease in intensive culture conditions is needed.

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