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Effect of madecassic acid on innate-adaptive immune response and cytokine gene expression in Labeo rohita against Argulus siamensis

Gunapathy Devi[1]; Chellam Balasundaram[2]; Ramasamy Harikrishnan[3]*

[1]Department of Zoology, Nehru Memorial College, Puthanampatti 621 007, Tamil Nadu, India.

[2]Department of Herbal and Environmental Science, Tamil University, Thanjavur 613 005, Tamil Nadu, India.

[3]Department of Zoology, Pachaiyappa's College for Men, Kanchipuram 631 501, Tamil Nadu, India.

Corresponding Author: Ramasamy Harikrishnan

Department of Zoology, Pachaiyappa's College for Men, Kanchipuram 631 501, Tamil Nadu, India.
Email: rhari123@yahoo.com

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Abstract

Madecassic acid is a pentacyclic triterpenoid bioactive compound that possesses anti-inflammatory, anti-diabetic properties altering lipid metabolism, blocking NF-κB activation in macrophages, and can regulate cancer cell line and several immune genes. This is the first study on the effect of madecassic acid on Labeo rohita by dietary feeding against argulosis caused by Argulus siamensis, a crustacean ectoparasite. In healthy and infected L. rohita when fed with 5 mg kg^{-1} of madecassic acid diet the White Blood Cell (WBC) count significantly increased after 4^{th} week; with 10 mg kg^{-1} diet after 6^{th} week and with 1 mg kg^{-1} diet only on 8^{th} week. Serum total protein (TP), Albumin (B), and Globulin (GB) level significantly increased in both groups fed with 5 mg kg^{-1} of madecassic acid enriched diet after 6^{th} week; with other doses the increase was observed only after 6^{th} week. Lysozyme activity increased significantly ($p<0.05$) in both groups treated with any dose of madecassic acid diets, except with 1 mg kg^{-1} diet which manifested only on 6^{th} week. The haemolytic complement activity and lymphokine production index significantly increased in both groups fed with 5 mg kg^{-1} dose of madecassic acid diet on 4^{th} week and all enriched diets after 6^{th} week. The phagocytic activity significantly was enhanced in both groups treated with 5 mg kg^{-1} madecassic acid enriched diet after 6^{th} week. The SOD activity increased significantly in both groups after feeding with 5 mg kg^{-1} diet after 4^{th} week. The IgM production was significantly stimulated in both groups treated with 5 mg kg^{-1} madecassic acid diet on 2^{nd} week; with 5 and 10 mg kg^{-1} doses on 4^{th} week, and all doses on 6^{th} week. TNFα and TLR22 up-regulation in head kidney tissue in both groups treated with 5 mg kg^{-1} of madecassic acid. CC3 and CXCa genes were up-regulated earlier in both groups treated with 5 mg kg^{-1} doses of madecassic acid diet. The lysozyme C, lysozyme G, β2-M, and transferrin genes were significantly up-regulated in both groups fed with 5 mg kg^{-1} dose diet after 6^{th} week. The findings suggest that in rohu the immune system was adversely affected by A. siamensis infection whereas in the infected group oral administration of madecassic acid enriched diet at 5 mg kg^{-1} significantly modulated both the innate-adaptive immune response and immune cytokine genes expression.

Introduction

Indian Major Carps (IMCs) such as rohu (Labeo rohita), catla (Catla catla), and mrigal (Cirrhinus mrigala) are the most popular and economically important culture species in India and other tropical regions of the world [1]. Among, these rohu is the main stay in polyculture of carp production in India which comprised more than 9.45 lakh tonnes during 2006 [2]. The fast growing carp culture under crowed conditions has been facing a severe deterrent problem due to the outbreak of various bacterial, viral, and parasitic diseases [3]. Among these argulus siamensis is a crustacean ectoparasite belonging to the order Arguloida of phylum Arthropoda. Its infection causes argulosis that result in a significant weight loss and sometimes sudden mortality in freshwater fish culture [3]. Several instances of fish mortality associated with argulosis have been reported in fresh water culture ponds in India [4]. The argulosis infected fishes exhibit abnormal behavior like lethargy, irritation, and loss of appetite; the sites of infection often become haemorrhagic and ulcerated, paving the way for opportunistic secondary infections like other parasites, fungal, and bacterial pathogens, further aggravating economic loss in intensive and semi-intensive aquaculture [5]. Rohu is more susceptible to A. siamensis as compared to other IMCs (catla and mrigal) [3,5]. A. siamensis infection has been known to inflict economic loss of over US$ 400/hectare/year in carp polyculture ponds (~7% of production cost) in India [3,5]. Till date, there is no effective preventive or control measure has been developed to control argulosis. Further, no attempt has been made to understand the immune response to A. siamensis in rohu, particularly to understand how the different degrees of parasitic burden influence the survival of host by modulating of innate immune mechanism and immune cytokine related genes regulation.

In recent years identification and characterization of immune related cytokine genes has been investigated during different stages of disease progression. A number of studies have been reported on the expression of immune related cytokine genes in fish against parasites [6-9]. In the head kidneys of Salmo salar during infection with sea lice parasite, Lepeophtheirus salmonis the inflammatory immune cytokine genes such as Interleukin (IL)-1β, tumor necrosis factor-α (TNFα), and Cyclooxygenase-2 (COX-2) up-regulate while down-regulating the Major Histocompatibility Complex of class I gene (MHC class-I) [9]. Similarly, Complement Compound C3 (CC3), MHC I, β-2 Macroglobulin (β-2M), Source of Immunodominant Mhc-Associated Peptides (SIMPs), and Tumor Necrosis Factor (TNF) receptor-associated factor-2 binding protein (T2BP) have been reported to up-regulate in the liver of Ctenopharyngodon idella infected with copepod parasitic, Sinergasilus major [10]. Forlenza et al. [11] had reported up-regulation of C-X-C motif chemokine - a and/or receptor-1 (CXCa and CXCR1), IL-1β, and TNFα in the skin of Cyprinus carpio after exposure to larval/juvenile Argulus japonicus. Similar involvement of TLR22 and TNFα genes are reported in L. salmonis infected rohu [3]; it was suggested that providing evidence of TLR22 and TNFα genes might involve in host body defense system.

Madecassic acid is a pentacyclic triterpenoid bioactive compound first isolated from Centella asiatica [12] that possesses anti-inflammatory, anti-diabetic, altering lipid metabolism, and blocking NF-κB activation in macrophages [13]. Further, it was reported in down-regulates LPS-stimulated expression of iNOS, COX-2, IL-6, TNF-α, and IL-1β in the macrophages and also it was prevents the activation of NF-κB and degradation of IκB kinase [13,15]. The CD^{4+} T cells themselves not only attack cancer cells but they also can help CD^{8+} T cells in attacking cancer cells [15,16]. Madecassic acid modulate CD^{4+} and CD^{8+} T-lymphocytes subpopulations and its ratio and secretion of IFN-γ and IL-4 in tumor-bearing mice [14] indicating that madecassic acid can directly enhance the body's immune defense system to antagonize tumorigenesis. Further, CD^{4+} and CD^{8+} T-lymphocytes secrete multifaceted cytokines like IFN–γ [16,17] that stimulate to increased CD^{4+} and CD^{8+} T-lymphocytes subpopulations, subsequently increasing the amount of IFN-γ and IL-4 production. An increase in the production of IFN-γ and IL-4 may stimulate the Th1-and Th2-mediated immune response and then increase percentage of IFN-γ and IL-4 secretion [16,17] which suggest that madecassic acid has a differential immunostimulation pathway. However, it is unknown whether madecassic acid possesses antioxidant and innate-adaptive immune properties, and the underlying immune cytokine gene mechanism in aquatic organisms.

The CC3, CXCa, lysozyme C & G, TNFα, and transferrin are known to involve in the recognition and presentation of pathogen components such as TLR22 and β2-M examined in the present study. The CC3 is the most prominent complement factor, which plays an important role in the formation of the Membrane Attack Complex (MAC) that helps in destroying pathogens. The TLR play a very important key role in the innate immune system, and form an evolutionary linkage between the innate and adaptive immunity. Lysozyme is a mucolytic enzyme and helps facilitating protection against infectious agents. TNFα is an important pro-inflammatory cytokine, especially useful in the inflammatory response of fish following pathogenic infections. The β2-M is a low molecular protein, plays a critical role in self and non-self-recognition in vertebrates. Transferrin is an important metal ion binding protein, which display antimicrobial properties by restricting iron availability to pathogens. However, there is no clear understanding with regard to immune related cells or components and the involvement of immune cytokine genes as host defense mechanism in L. rohita against A. siamensis. Therefore, the present study was conducted to examine the innate and adaptive immune response and the modulation of immune cytokine genes in the head kidney of rohu against A. siamensis and also to understand how the immune status is being modulated with respect to A. siamensis.

Materials and methods

Preparation of supplementation feed

The formulated control/basal diet comprised were used fish meal, soybean meal, and coconut oil cakes as protein source; sunflower oil as lipid source; wheat, corn flour, and carboxymethyl cellulose as carbohydrate source (**Table 1**). All ingredients were finely pounded and mixed systematically with sterilized water to make in soft dough. It was then transferred into an aluminum container and pressure cooked at 15 psi for 15 min. After cooling of the mixture at Room Temperature (RT), vitamin and mineral pre-mixture, along with the chosen concentrations (0, 1, 5, and 10 mg kg^{-1}) of madecassic acid were mixed thoroughly and used to prepare the four experimental diets namely: (i) without madecassic acid (0 mg) and with (ii) 1 mg, (iii) 5 mg, and (iv) 10 mg of madecassic acid. Pellets were prepared by a hand pelletizer with 2 mm diameter, air dried in an oven at 60 ºC, packed in airtight polythene bags or containers, and labeled properly. The proximate composition of the experimental pellet diets were analyzed by standard methods.

Fish

Healthy fingerlings of L. rohita weighing ~60 g were collected from local fish farm without any clinical symptom of parasitic infection. The fish were transported to the laboratory and disinfected immediately with 5 ppm Potassium Permanganate ($KMnO_4$) for 5 min and then acclimatized in 500 l Fiber Reinforced Plastic (FRP) tanks with continuous aeration for a period of two weeks before starting the experiment. During the acclimatization period two-third water was exchanged to remove debris; the formulated control diet was provided (**Table 1**) at 3% of their body weight at 10.00 and 15.00 h daily. The basic physico-chemical water parameters such as temperature from 28 to 31 ºC, dissolved oxygen at 5.65 ± 0.73 mg L-1, pH at 7.9 ± 0.81, nitrites at 0.016 ± 0.009 mg L-1, and ammonia at 0.109 ± 0.024 mg L-1 were measured and maintain optimal levels systematically once at seven days of interval during the experimental period.

Experimental design

After two weeks of acclimatization, the fish were randomly divided into eight groups of 25 each (8 x 25 = 200) as: (1) healthy fish fed with basal control diet without madecassic acid (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 madecassic acid; (5) the infected (or challenged) fish fed with basal control diet without madecassic acid (0 mg kg^{-1}) [I]; the infected fish fed with madecassic acid 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 x 3 = 600 fish). The groups 1 to 4 were non-infected whereas groups 5 to 8 were infected with 10-25 lice $fish^{-1}$ of A. siamensis. The respective pellet diets were provided twice a day at 1000 and 1700 h throughout the experimental period. The development of A. siamensis infection was observed daily. There was no mortality observed with any of experimental or control tanks, while all fish in the experimental tanks had developed infection after 12 days.

Collection of blood and serum

Six fish were randomly chosen in each experimental tank at the end of weeks 2, 4, 6, and 8 post-infection with A. siamensis. 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 samples were equally 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 ºC until used. The heparinized blood was used immediately for hematological and biochemical study.

Tissue sampling procedure for RNA extraction

After blood sampling, head kidney tissues were aseptically dissected after anaesthetizing the fish with tricaine methanesulfonate (MS222, Sigma) and immediately stored in RNAlater (Ambion, Austin, TX) at -20 ºC for extraction of total RNA.

Determination of biochemical and hematological parameters in blood

White Blood Cell (WBC) count was determined as per the method of Shaw [18]. The different sera samples collected earlier were analysed for Total Protein (TP) following the method of Bradford [19]; Albumin (AB) content according to Doumas et al. [20]; Globulin (GB) content (subtracting albumin from total protein); and the albumin/globulin (A:G) ratio.

Separation of viable leucocytes from blood

The blood was diluted 1:3 (v/v) in Hanks' Balanced Salt Solution (HBSS), layered onto preformed continuous gradients of 51% Percoll (Pharmacia, Uppsala, Sweden) in 0.85% NaCl and centrifuged at 800 xg for 20 min at 4 ºC. The white cell band at the interface of the blood plasma and Percoll layer was harvested with a Pasteur pipette; thus diluted 10-fold with HBSS and recentrifuged at 800 xg for 10 min at 4 ºC to remove residual Percoll. The resulting pellet was again washed twice by resuspending evenly in HBSS with a Pasteur pipette and centrifuging at 800 xg for 10 min at 4 ºC. After the third wash, the concentration and viability of leucocyte cell suspensions were determined in 0.2% trypan blue. The final pellet with more than 95% viability was resuspended in RPMI 1640 and was used to study different cell-mediated immune responses.

Immune assays

Superoxide anion production by phagocytic cells was determined by a slight modification of the method described by Chung and Secombes [21]. The Phagocytosis assay was performed following Siwicki et al. [22] and Park and Jeong [23]. Macrophage Activating Factor (MAF) was determined following the protocol of Graham and Secombes [24] and the supernatants were then collected and analysis for MAF activity according to Steiro et al. [25]. Serum lysozyme activity was measured with the turbidimetric method described by Parry et al. [26]. The immunoglobulin M (IgM) was measured according to Sharma et al [27]. The activity of the alternative complement pathway was assayed using Sheep Red Blood Cells (SRBC) as targets [28].

Extraction of RNA and cDNA synthesis

One hundred milligrams of head kidney tissue stored in RNAlater and utilized for the extraction of total RNA using TRI reagent (Sigma) following the manufacturer's instructions. The resulting RNA was treated with DNase I, RNase-free (Fermentas, USA) followed by inactivation of DNase I according to the manufacturer's instructions. The concentration of the total RNA in the sample was quantified by measuring absorbance at 260 nm. The purity of the samples was also checked by measuring the ratio of OD at 260 nm and OD at 280 nm using Nanodrop ND1000 (Thermo Scientific, USA) with expected values between 1.9 and 2.0. The integrity of RNA was checked by electrophoresis on 1% agarose gel containing 0.5 µg ml^{-1} ethidium bromide at 100V and also screening through RT-PCR using β-actin expression. One microgram of total RNA was used for synthesis of first-strand complementary DNA by reverse transcription using thermocycler (Eppendorf MasterCycler® Gradient, USA) by incubating with 1 µl of random hexamer (Fermentas, USA) (100 µM) at 70 ºC for 5 min. The reaction was cooled at 25 ºC for 10 min to allow primers annealing. Then the following components were added to the reaction in order; 2 µl of 10× RT-buffer (Sigma), 0.70 µl of RNase inhibitor (15U μl^{-1}) (Genei, Bangalore), 2 µl of 100mM dNTPs, 4.3 µl of nuclease-free water and 1 µl of MMLV-RT (200U μl^{-1}, Sigma). The reagents were gently mixed and incubated for 1 h at 42 ºC. The reaction was terminated by heating at 95 ºC for 2 min and cDNAs were stored at −20 ºC till further use. Further, RT-reactions lacking reverse transcriptase (RT minus) but not RNA were also performed to verify that the samples did not contain genomic DNA.

PCR conditions

The constitutively expressed house-keeping gene, β-actin was used both as a positive control and normalization of sample. The primers used for the study were either taken from published papers (**Table 2**) or self-designed using Oligo Primer Analysis Software version 6.71 (Wojciech and Piotr Rychlik, Molecular Biology Insights Inc., USA, 2005) and Primer Premier 5 (version 5.0, Premier Biosoft International, Palo Alto, CA). The sequence and optimum annealing temperature for primers used in the present study are given in **Table 2**. Each PCR was run in standard 50 µl reactions containing 0.5 µl (10 pmol) of each of the primers, 1.0 µl of cDNA, along with 5.0 µl of 10× PCR buffers, 1.0 µl 100mM dNTP, 40.7 µl of nuclease free distilled water and 1.5 units of Taq DNA polymerase (Genei, India). All amplification reactions consisted of an initial denaturation at 95 ºC for 3 min followed by several cycles (number of cycles was optimized to the least number of cycles showing amplification of genes for combination of each tissue and primer (Table 2) of 95 ºC denaturation for 30 s, annealing at respective temperature of each gene for 1 min, and 72 ºC extension for 1 min. The reaction was finished with a 72 ◦C extension period lasting 10 min. The PCR products (8 µl) of each target gene were visualized on a 1% agarose gel stained with ethidium bromide.

Expression analysis

The relative levels of expression of each gene were analyzed by densitometry using AlphaEase®FC Imaging Software (Alpha Innotech Corp., USA). The ratios of immune-related cytokine genes/β-actin products were subsequently calculated after subtraction of the background pixel intensity for each gene of interest and used to assess the differences in expression levels between controls, infected and infected treated fish tissue samples.

Statistical analysis

All the data were analysed by running the General Linear model program available in SAS software. The means were compared using Duncan's multiple range tests to find the difference at the 5% ($p < 0.05$) level.

Results

Serum biochemical and haematological parameters

The healthy and infected fish were fed with 5 mg kg^{-1} of madecassic acid showed increased significant of White Blood Cell (WBC) count after 4th week. However, both groups fed with low dose of 1 mg kg^{-1} of madecassic acid showed WBC count significantly high only on 8th week of sample. On the other hand, the WBC count was observed significant when the healthy fish fed with high dose of 10 mg kg^{-1} of madecassic acid after 6th week. The Total Protein (TP) was observed significant level of the healthy fish fed with low dose of 1 mg kg^{-1} of madecassic acid on weeks 6 and 8, but it was not with the infected fish fed with of the diet. The TP level had been observed in this study significant in both groups fed with medium dose of 5 mg kg^{-1} of madecassic acid after 4th week, whereas both groups treated with high dose (10 mg kg^{-1}) significant TP level only on 8th week. The Albumin (AB) and Globulin (GB) had observed significant level in both groups fed with low dose (1 mg kg^{-1}) and high dose (10 mg kg^{-1}) only on 8th week. It was reported that AB was significant after 4th week and GB after 6th week of both groups fed with medium dose (10 mg kg^{-1}) of madecassic acid. In both groups could not elevate albumin/globulin ratio with any enriched diet during the experiment (**Table 3**).

Table 1: Experimental feed ingredients, composition (g kg-1 feed) and their proximate analysis used in this study.

Ingredients	Madecassic acid (mg kg-1 feed)			
	0 mg kg^{-1}	1 mg kg^{-1}	5 mg kg^{-1}	10 mg kg^{-1}
Fish meal	10.000	10.000	10.000	10.000
Soybean meal	38.000	38.000	38.000	38.000
Coconut oil cake	10.000	10.000	10.000	10.000
Sunflower oil cake	15.000	15.000	15.000	15.000
Wheat flour	14.000	14.999	14.995	14.910
Corn flour	5.000	5.000	5.000	5.000
Sunflower oil	5.000	5.000	5.000	5.000
Carboxymethyl cellulose	1.000	1.000	1.000	1.000
Vitamin + mineral mix[a]	1.000	5.000	5.000	5.000
Madecassic acid	0.000	0.001	0.005	0.010
Proximate composition (dry matter basis, g kg^{-1})				
Organic matter	90.4	91.2	91.6	90.9
Crude protein	34.5	35.3	35.7	34.7
Total carbohydrate	4.88	4.94	5.13	5.02
Ash	8.76	8.81	8.84	8.79

[a]Composition of vitamin mineral mix (EMIX PLUS) (quantity/2.5 kg); Vitamin A, 5,500,000 IU; Vitamin D3, 1,100,000 IU; Vitamin B2, 2000 mg; Vitamin E, 750 mg; Vitamin K, 1000 mg; Vitamin B6, 1000 mg; Vitamin B12, 6 mg; calcium pantothenate, 2500 mg; nicotinamide, 10 g; choline chloride, 150 g; Mn, 27,000 mg; I, 1000 mg; Fe, 7500 mg; Zn, 5000 mg; Cu, 2000 mg; Co, 450 mg; Ca, 500 g; P, 300 g; l-lysine, 10 g; dl-methionine, 10 g; selenium, 50 ppm; satwari, 250 ppm; (Lactobacillus 120 million units and Yeast Culture 3000 crore units).

Table 2: Immune related gene primers size and optimum annealing temperatures for the experiment.

Target gene temperature (º C)	Sequence (5'→3') (bp)	Annealing	Size of amplicon	Accession no.
CC3	F-CACCTGCTAACACCTACATCTC	60.0	106	KF682426
	R-TCTTATTGTCCAACCCAGTGG			
CXCa	F-GGGTGTAGATCACGCTGTC	56.0	102	HM363518
	R-CTTTACAGTGTGGGCTTGGAG			
TNF-α	F-CCAGGCTTTCACTTCAGG	51.6	181	FN543477
	R-GCCATAGGAATCGGAGTAG			
TLR-22-like	F- GCGCTTTCAGGTGGTTTATG	56.0	123	KJ187307
	R- CCTACAACACTGAGGATGAGTC			
Lysozyme C	F-CGCTGTGATGTTGTCCGTATCTTC	52.7	321	EF203085
	R-GTAACTTCCCCAGGTATCC			
Lysozyme G	F-CTTATGCAGGTTGACAAACG	53.5	249	KT184686
	R-GGCAACAACATCACTGGAGTAATC			
β-2M	F-TCCAGTCCCAAGATTCAGGTG	59.7	175	AM774150
	R-TGGTGAGGTGAAACTGCCAG			
Transferrin	F-GGACTACCAGCTGTTGTGCAT	47.5	175	AM709723
	R-GCCACCATCGACTGCAAT			
β-actin	F-GACTTCGAGCAGGAGATGG	55.3	138	AY531753
	R-CAAGAAGGATGGCTGGAACA			

Third Complement Component C3 (CC3); C-X-C Motif Chemokine-A (CXCa); Toll Like Receptor 22 (TLR-22); Tumor Necrosis Factor-α (TNF-α); β2-Microglobulin (B2MG).

Table 3: Immune related gene primers size and optimum annealing temperatures for the experiment.

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('000 cells/ cumm)	2	13.85±0.24^a	13.81±0.22^a	13.98±0.28^a	13.87±0.28^a	15.85±0.30^b	14.91±0.33^a	14.18±0.30^a	14.03±0.30^a
	4	13.91±0.26^a	13.77±0.36^a	14.63±0.34^a	14.63±0.23^a	18.14±0.38^b	17.85±0.35^b	14.36±0.37^a	14.24±0.33^a
	6	13.98±0.32^a	13.72±0.30^a	15.23±0.36^b	14.84±0.31^a	20.37±0.29^b	19.72±0.32^b	15.73±0.42^b	14.38±0.30^a
	8	13.88±0.28^a	13.61±0.25^a	15.78±0.33^b	15.37±0.36^a	21.29±0.35^b	20.54±0.29^b	15.96±0.34^b	14.58±0.36^a
TP (g dl^{-1})	2	1.21±0.15^a	1.19±0.15^a	1.38±0.18^a	1.35±0.15^a	1.69±0.20^a	1.58±0.15^a	1.56±0.20^a	1.48±0.17^a
	4	1.23±0.12^a	1.21±0.17^a	1.84±0.22^a	1.54±0.21^a	2.27±0.24^b	2.13±0.19^b	1.65±0.16^a	1.56±0.19^a
	6	1.25±0.18^a	1.23±0.20^a	2.36±0.24^b	1.94±0.25^a	2.56±0.17^b	2.38±0.23^b	1.79±0.22^a	1.75±0.21^a
	8	1.28±0.16^a	1.25±0.18^a	2.57±0.20^b	1.44±0.23^b	2.96±0.26^b	2.64±0.22^b	2.31±0.25^b	2.21±0.23^b
AB (g dl^{-1})	2	0.710±0.011^a	0.704±0.010^a	0.738±0.015^a	0.725±0.011^a	0.943±0.014^a	0.922±0.012^a	0.713±0.010^a	0.703±0.010^a
	4	0.715±0.013^a	0.696±0.014^a	0.846±0.012^a	0.820±0.014^a	1.382±0.013^b	1.123±0.013^b	0.776±0.014^a	0.754±0.018^a
	6	0.718±0.010^a	0.685±0.010^a	1.236±0.013^b	0.898±0.010^a	1.431±0.014^b	1.339±0.012^b	0.937±0.016^a	0.893±0.013^a
	8	0.722±0.012^a	0.677±0.012^a	1.421±0.014^b	1.367±0.015^b	1.417±0.015^b	1.384±0.016^b	1.253±0.014^b	1.215±0.016^b
GB (g dl^{-1})	2	0.512±0.022^a	0.506±0.020^a	0.521±0.024^a	0.517±0.020^a	0.725±0.019^a	0.711±0.015^a	0.516±0.014^a	0.501±0.016^a
	4	0.518±0.018^a	0.508±0.018^a	0.737±0.021^a	0.725±0.019^a	0.943±0.022^a	0.858±0.020^a	0.721±0.019^a	0.632±0.017^a
	6	0.524±0.020^a	0.512±0.022^a	0.893±0.022^a	0.837±0.025^a	1.246±0.023^b	1.147±0.019^b	0.963±0.021^a	0.749±0.025^a
	8	0.533±0.016^a	0.526±0.027^a	1.252±0.026^b	1.182±0.020^b	1391±0.028^b	1.279±0.018^b	1.258±0.020^b	1.135±0.025^b
A/C (g dl^{-1})	2	1.176±0.032^a	1.146±0.025^a	1.215±0.027^a	1.203±0.029^a	1.325±0.022^a	1.317±0.025^a	1.223±0.025^a	1.214±0.022^a
	4	1.192±0.028^a	1.163±0.021^a	1.235±0.024^a	1.222±0.021^a	1.348±0.027^a	1.325±0.030^a	1.248±0.021^a	1.232±0.027^a
	6	1.247±0.030^a	1.225±0.024^a	1.257±0.030^a	1.238±0.026^a	1.367±0.031^a	1.344±0.028^a	1.276±0.033^a	1.255±0.031^a
	8	1.258±0.026^a	1.323±0.023^a	1.275±0.035^a	1.256±0.024^a	1.398±0.035^a	1.374±0.032^a	1.299±0.030^a	1.271±0.034^a

White Blood Cell (WBC); Total Protein (TP); Albumin (AB); Globulin (GB); A/G ratio: Albumin/Globulin ratio. All data are represented as mean S.D and this values with different superscript letters within a column for a parameter are significantly different ($p < 0.05$).

Immunological response

The changes in Superoxide Anion (SOD) production did not significantly increased in healthy and infected fish fed with any diet; however there was a significant ($p<0.05$) high SOD production observed in both groups fed with medium dose (5 mg kg^{-1}) of madecassic acid after 4th week (**Figure 1**). The Phagocytic Activity (PA) was determined in this study did not significant with any diet during the experiment. However, it was observed significantly high PA of both groups fed with medium dose (5 mg kg^{-1}) of madecassic acid after 6th week (**Figure 2**). The lymphokine production (LP) index did not significant ($p<0.05$) compared with any diet on first week. In both groups fed with medium dose (5 mg kg^{-1}) of madecassic acid significantly increased LP index after 4th week. However, the LP index significantly high in both groups fed with high doses (10 mg kg^{-1}) only on 6th week while low dose (1 mg kg^{-1}) only on 8th week as compared with other group (**Figure 3**). In comparison within groups of lysozyme activity (LA) did not significantly enhanced on 2nd week. A similar result was observed on 4th week of the LA, except both groups treated with medium dose (5 mg kg^{-1}) but did not observed other doses of the period (1 or 10 mg kg^{-1}). A significant ($p<0.05$) high LA was obtained in both groups treated with all doses of madecassic acid, except 1 mg kg^{-1} dose on 6th week (**Figure 4**). The Haemolytic Complement (HC) activity has observed in the present study did not significant in both groups fed with 1 or 10 mg kg^{-1}, but it was shown significant when both groups treated with 5 mg kg^{-1} dose of madecassic acid diet on 4th week. The HC activity had shown significantly high level in all madecassic acid diet groups on 6th and 8th week, except 10 mg kg^{-1} dose of madecassic acid diet on 8th week (**Figure 5**). There was no significant ($p>0.05$) Immunoglobulin M (IgM) production was observed in both groups fed with 1 or 10 mg kg^{-1} doses of madecassic acid diet on 2nd; but it was significant of both group treated with 5 mg kg^{-1} dose of madecassic acid diet of this period. It was observed that IgM level was significant of both groups fed with 5 and 10 mg kg^{-1} doses of madecassic acid diets on 4th week and all doses on 6th week. However, it was observed on 8th week of both groups treated with 1 and 5 mg kg^{-1} doses of madecassic acid diets significantly high IgM protection (**Figure 6**).

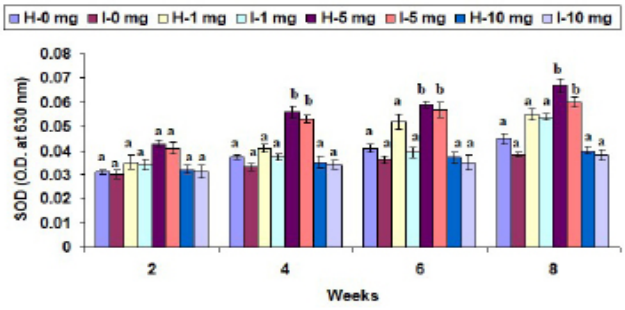

Figure 1: Superoxide anion production (SOD) observed in L. rohita fed with dietary enriched with madecassic acid at 0, 1, 5, and 10 mg kg-1 against A. siamensis. All data are represented as mean S.D and these values with different superscript letters within a column for a parameter are significantly different ($p < 0.05$). H: healthy, I: infected.

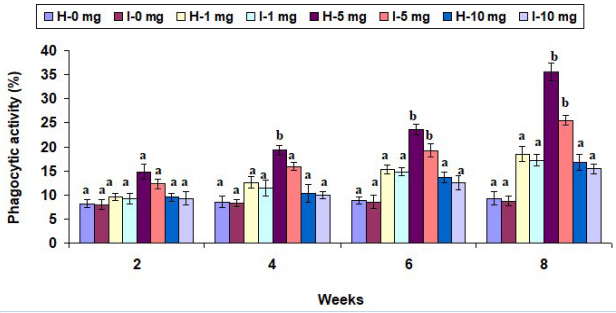

Figure 2: Phagocytic Activity (PA) observed in L. rohita fed with dietary enriched with madecassic acid at 0, 1, 5, and 10 mg kg-1 against A. siamensis. Statistical information is seen in Figure 1. H: healthy, I: infected.

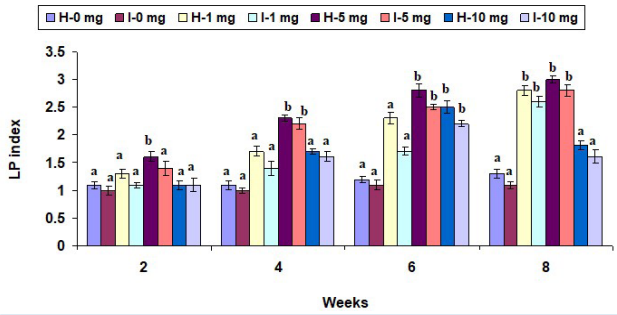

Figure 3: Lymphokine Production (LP) index observed in L. rohita fed with dietary enriched with madecassic acid at 0, 1, 5, and 10 mg kg-1 against A. siamensis. Statistical information is seen in Figure 1. H: healthy, I: infected.

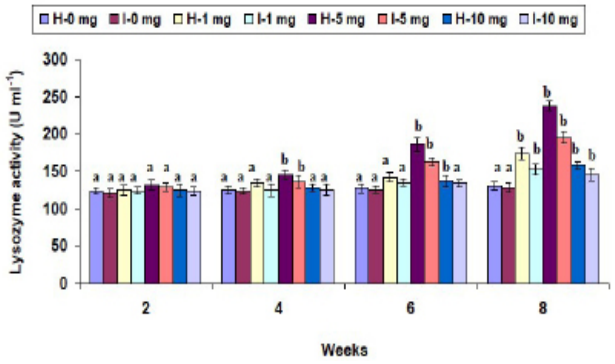

Figure 4: Lysozyme Activity (LA) observed in L. rohita fed with dietary enriched with madecassic acid at 0, 1, 5, and 10 mg kg-1 against A. siamensis. Statistical information is seen in Figure 1. H: healthy, I: infected.

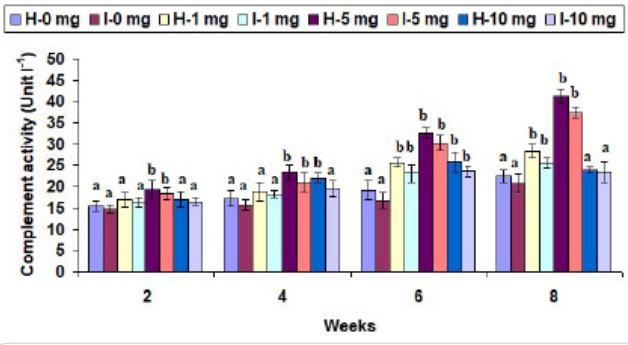

Figure 5: Haemolytic Complement (HC) activity observed in L. rohita fed with dietary enriched with madecassic acid at 0, 1, 5, and 10 mg kg-1 against A. siamensis. Statistical information is seen in Figure 1. H: healthy, I: infected.

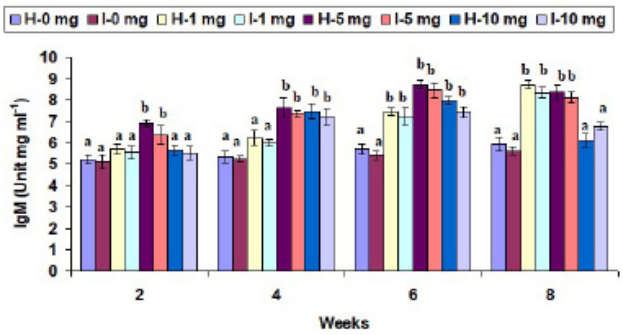

Figure 6: Immunoglobulin M (IgM) level observed in L. rohita fed with dietary enriched with madecassic acid at 0, 1, 5, and 10 mg kg-1 against A. siamensis. Statistical information is seen in Figure 1. H: healthy, I: infected

Immune gene expression

The healthy and infected fish treated with 5 and 10 mg kg^{-1} doses of madecassic acid diets were significantly ($p>0.05$) up-regulate Complement Component 3 (CC3) (**Figure 7(a)**) and C-X-C motif chemokine-a (CXCa) (**Figure 7(b)**) on 2nd and 4th week, but not with 1 mg kg^{-1} dose diet group. It was observed that CC3 and CXCa significantly expressed in both groups fed with all diets on 6th week. However, the CC3 and CXCa expression had observed significantly high level in both groups treated with 1 and 5 mg kg^{-1} doses diets on 8th weeks. The tumor necrosis factor-α (TNF-α) did not significantly expressed with 1 and 10 mg kg^{-1} doses diets on 2nd weeks while both groups treated with 5 and 10 mg kg^{-1} doses diet were significantly expressed on 4th week as compared with other dose of the period. The TNF-α had been reported significantly expressed in high level of both groups treated with all doses diet on 6th week. However, this expression was high level only of the groups treated 1 and 5 mg kg^{-1} doses diet on 8th week as compared with other dose of the diet (**Figure (8a & 8b)**). The Toll Like Receptor 22 (TLR-22) gene had shown in the present study.

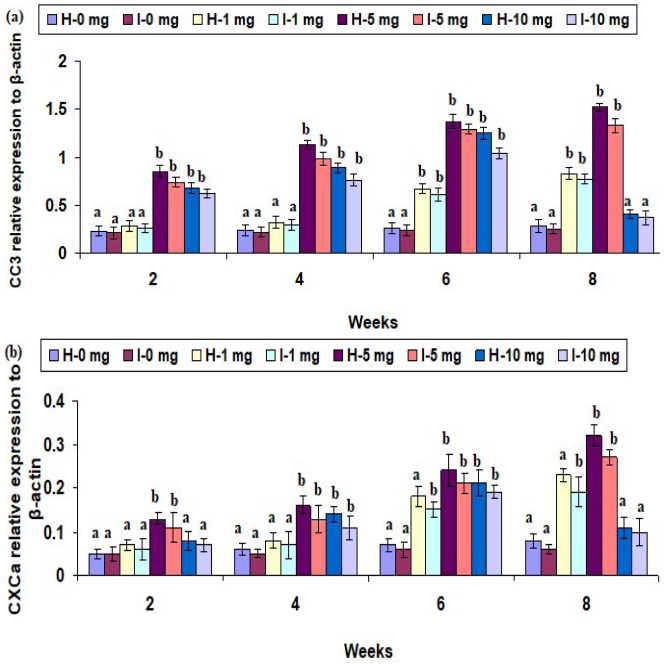

Figure 7: **(a)** Complement component 3 (CC3) and **(b)** C-X-C motif chemokine-a (CXCa) gene expression relative to β-actin observed in *L. rohita* fed with dietary enriched with madecassic acid at 0, 1, 5, and 10 mg kg^{-1} against *A. siamensis*. Statistical information is seen in Figure 1. H: healthy, I: infected.

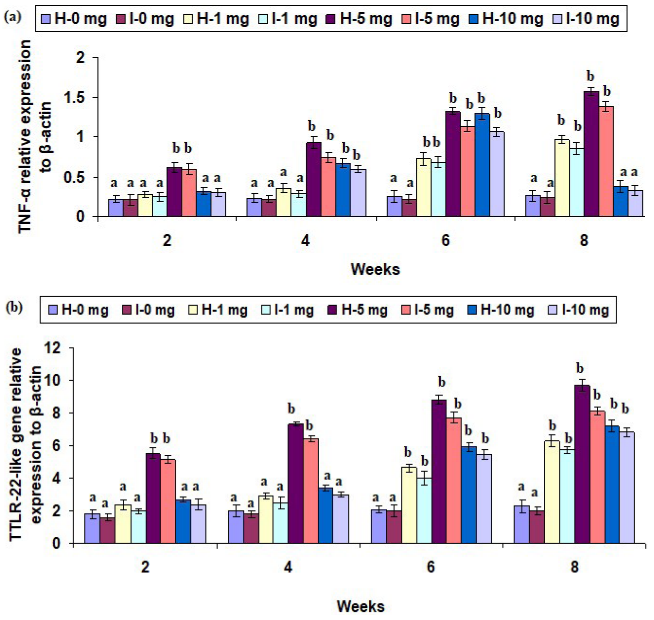

Figure 8: **(a)** Tumor necrosis factor-α (TNF-α) and **(b)** toll like receptor 22 (TLR-22) gene expression relative to β-actin observed in *L. rohita* fed with dietary enriched with madecassic acid at 0, 1, 5, and 10 mg kg^{-1} against *A. siamensis*. Statistical information is seen in Figure 1. H: healthy, I: infected.

The lysozyme C and lysozyme G genes were not significantly up-regulated in both groups fed with any dose of madecassic acid supplementation diets during the experiment. However, these genes were significantly up-regulated in both groups fed with 5 mg kg^{-1} dose diet on 6th and 8th week as compared with other groups (**Figure (9a & 9b)**). Similarly, β2-microglobulin (β2-M) expression was observed did not up-regulated with any doses of the diet in the experiment. On the other hand, this expression was up-regulated in both groups fed with 5 mg kg^{-1} dose diet on 6th and 8th week (**Figure (10a)**). The expression of transferrin gene was up-regulated in both groups treated with 5 mg kg^{-1} dose diet during the experiment whereas fish treated with 1 mg kg^{-1} dose diet expressed only on 6th and 8th week (**Figure (10b)**).

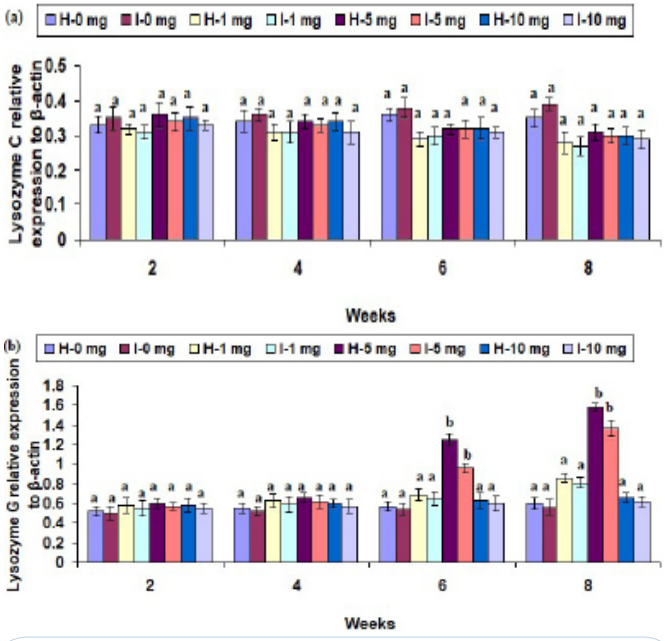

Figure 9: **(a)** Lysozyme C and **(b)** Lysozyme G gene expression relative to β-actin observed in *L. rohita* fed with dietary enriched with madecassic acid at 0, 1, 5, and 10 mg kg^{-1} against *A. siamensis*. Statistical information is seen in Figure 1. H: healthy, I: infected.

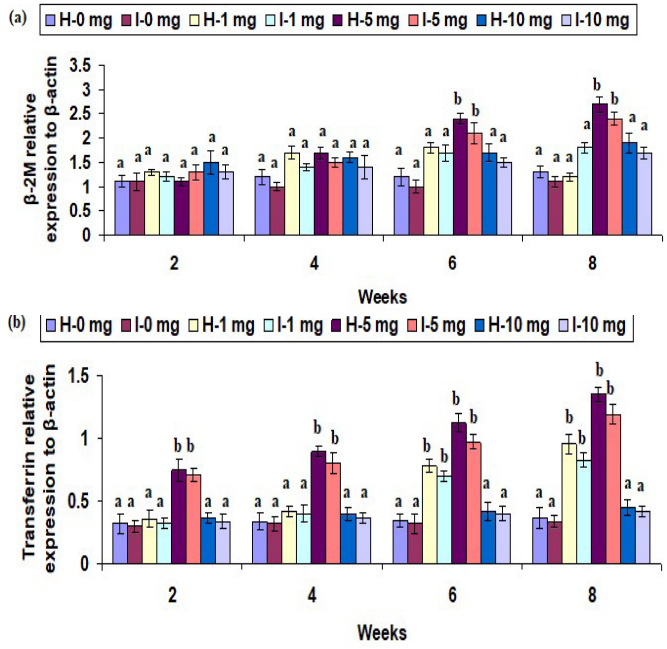

Figure 10: (a) β2-microglobulin (β2-M) and **(b)** transferrin gene expression relative to β-actin observed in *L. rohita* fed with dietary enriched with madecassic acid at 0, 1, 5, and 10 mg kg^{-1} against *A. siamensis*. Statistical information is seen in Figure 1. H: healthy, I: infected.

Discussion

The lysozymes, antibodies, complement, and other lytic peptides are contains in blood serum that act as a first line of defence mechanism where they prevent adherence and colonisation of microorganisms [29]. WBC also called leukocytes which includes many cells as first line of defense when infection strikes and thus essential for good health and protection from diseases. The WBC are generally grouped into phagocytes (or macrophages) and lymphocytes. Leukocytes are move freely in blood and interact with and capture cellular debris, foreign particles, and invading microorganisms. Monocytes help to break down of bacteria; lymphocytes makes antibodies against bacteria, viruses, and other potentially harmful invaders; neutrophils kill and digest of bacteria and fungi; basophils secrete chemicals such as histamine that help control the body's immune response, and eosinophils attack and kill parasites, destroy cancer cells, and help with allergic responses. The WBC was increased in the healthy and infected fish fed with 5 mg kg^{-1} of madecassic acid diet after 4th week, while 10 mg kg^{-1} diet after 6th week, and 1 mg kg^{-1} diet only on 8th week. It was suggested that healthy and infected fish treated with 5 mg kg^{-1} of madecassic acid diet can stimulate to increased WBC count earlier. However, WBC was stimulated in fish treated with low or high doses (1 or 10 mg kg^{-1}) of madecassic acid diet after 6th week. This was confirmed in earlier study in Carassius auratus fed with azadirachtin containing diet, Clarias batrachus fed with emodin enriched diet, and Cirrhina mrigala fed with azadirachtin containing diet could stimulate to increased WBC count against diseases [30-32]. Serum TP, AB, and GB were significantly increased in both healthy and infected groups treated with 5 mg kg^{-1} of madecassic acid diet at earlier; while both groups treated with low or high doses (1 or 10 mg kg^{-1}) of madecassic acid diets increased TP, AB, and GB level very late (after 6th week). It was suggested that fish treated with madecassic acid at medium level (5 mg kg^{-1}) is better to increased TP, AB, and GB level than that of other doses (1 or 10 mg kg^{-1}) of the diet. The albumin:globulin ratio did not changed significantly with any group fed with any doses of madecassic acid enriched diet. Among, the TP containing various peptides including lysozyme and complement factors were maintained at elevated levels upon treated with 5 mg kg^{-1} madecassic acid earlier than that of other doses of the diet. The similar results were previously reported in C. auratus and C. batrachus after feeding with diet containing azadirachtin or emodin compounds [30,32].

Lysozyme activity significantly ($p<0.05$) increased in both groups treated with all doses of madecassic acid diets in this study, except 1 mg kg^{-1} dose only on 6th week. Harikrishnan et al. [32] also reported that serum lysozyme activity increased in C. batrachus after feeding with emodin enriched diet against Aeromonas hydrophila infection. In another study by Harikrishnan et al. [33] reported that lysozyme activity increased significantly increased in C. mrigala treated with azadirachtin, camphor, and curcumin against Aphanomyces invadans. The haemolytic complement activity significantly increased of both groups fed with 5 mg kg^{-1} dose of madecassic acid diet on 4th week and all madecassic acid does diet after 6th week. A better activity was found with 5 mg kg^{-1} dose of madecassic acid diet. An increase in complement activity was reported in C. batrachus and C. mrigala against pathogens [32,33]. Hence, the present study suggested that both groups treated with 5 mg kg^{-1} dose of madecassic acid diet can effectively maintaining the lysozyme activity, complement factors, and other peptides at an elevated level during the experiment. Further, madecassic acid resulted in an increase in serum lysozyme activity and complement factors suggested that contributing the enhancement in the non-specific defence [34]. Such enhancement in the lysozyme and complement components could also be correlated with enhanced phagocytic activity in the present study significantly enhanced in both groups treated with 5 mg kg^{-1} of madecassic acid after 6th week. The phagocytic cells was reported to induced in in C. batrachus and C. mrigala against pathogens [32,33], which may release of lysosomal enzymes, cationic peptides, complement components, and production of reactive oxygen species [35]. Enhancement of phagocytic activity was reported in Cyprinus carpio after treatment with schizophyllan, scleroglucan, and lentinan against Edwardsiella tarda [36].

The enhancement of phagocytosis was suggested due to binding of multivalent β-glucan to its receptors on the phagocytic cells and activated the complement factors via the alternative pathway, which acts as opsonin leading to enhancement of phagocytosis [37]. The SOD activity significantly increased in both groups after feeding with 5 mg kg^{-1} of madecassic acid after 4th week. Since, O_2^- is the first product released during the respiratory burst, it has been accepted as an accurate parameter to quantify the intensity of a respiratory burst [38]. In our study suggested that SOD production was independent of the dosages of madecassic acid used. However, a medium dose of madecassic acid significantly enhanced earlier of SOD production than those of high or low doses of madecassic acid in this study, which probably, at medium dose is adequate to stimulate the functions of phagocytic cells.

The lymphokine production index in both groups fed with medium dose (5 mg kg^{-1}) of madecassic acid significantly increased after 4th week while it was in high dose (10 mg kg^{-1}) only 6th week and low dose (1 mg kg^{-1}) only 8th week. The secretion of macrophage activating factors from the phagocytic cells might have induced the respiratory burst and microbicidal activity helped to maintain a protective state for a longer duration. Furthermore, the IgM production was significant stimulated in both

group treated with 5 mg kg^{-1} dose of madecassic acid diet on 2nd week; 5 and 10 mg kg^{-1} doses diets on 4th week, and all doses diet on 6th week, which suggested that might have maintained the activation of the phagocytic cell population in fish and in turn could have produced increased antibody response against pathogen. Therefore, the present study is clearly suggested that madecassic acid diet at 5 mg kg^{-1} dose has enhanced innate and adapting immune response earlier than that of other doses.

WBC is well known adaptive immune cells, recruited to the site of infection to phagocytose invading virus and present viral antigens to T lymphocytes that directly kill virus-infected cells as well as provide T-cell help to activate B cells which produce antibodies, specific to a virus during the adaptive immune response. Phagocytic cells recognize pathogens on host through several families of Pattern-Recognition Receptors (PRRs) and it distinguish evolutionarily-conserved structures, known as Pathogen-Associated Molecular Patterns (PAMPs). The PRRs are include membrane-bound C-type lectin, TLRs, NOD-like receptors and Retinoic Acid-Inducible Gene 1 (RIG-I)-like receptors families. The viruses enter cells by clathrin-mediated endocytosis are detected by TLRs 3, 7, and 9 present in the endosomes, which all recognize viral nucleic acids. The TLRs are stimulating type I interferons (IFNs) production via the adapter molecules such as TRIF and MyD88 that directly interact with IRF3 and IRF7, respectively. Type 1 IFNs act as autocrine and paracrine signals that up-regulate expression of anti-viral molecules that the IFN receptor is crucial in mediating sensitivity and severity to pathogen infections.

The gene expression study can helped a lot in understanding changes in the levels of immune-related proteins during stress, infection, and nutritional deficiency or administration with supplementation feed to host, which are present in modest amount in the blood or tissue. The expression studies of immune and other related genes in Indian carps have started recently [39-41]; however, the understanding of immune responses to parasitic pathogens in general, particularly in rohu is scare. In this present study is the first of such important immune related genes response to an ectoparasitic infection in rohu, when oral administration with natural bio-compound madecassic acid.

This study was designed keeping in mind of natural field outbreaks where different degrees of infected fish of same species being available in one environment. This experimental infection study indicated that Argulus potentially modulate the host immunity by showing differential expression pattern of immune related cytokine genes in head kidney. It is interesting to note here that majority of immune genes have obtained in this study down-regulation in kidney tissue when the healthy and infected fish treated with high or low doses (1 or 10 mg kg^{-1}) of madecassic acid whereas significantly up-regulation with medium doses (5 mg kg^{-1}) of madecassic acid. A number of studies were confirmed that madecassic acid was up-regulation of immune related gene expression in various cancer cell lines [13-17]. However, till date there was no such immune related gene expression in fish treated with madecassic acid against any pathogens. In the present study revealed that of inflammation-associated gene, TNFα and TLR22 up-regulation in head kidney tissue in both healthy and A. siamensis infected fish treated with madecassic acid. It is interesting to note that TNFα and TLR22 expression changes were high in healthy or A. siamensis infected fish treated with 5 mg kg^{-1} of madecassic acid. However, Chang et al. [42] had reported up-regulation of several immune relevant genes in the liver while down-regulation of few genes in gills of Ctenopharyngodon idella against S. major. Further detailed studies required to establish this hypothesis as the role of TNFα and TLR22 against A. siamensis still remains unclear.

The superfamily of chemokines are including about 40 different small secreted cytokines that direct the movement of immune cells at the sites of inflammation or infection, which play an important roles in resistance to infectious pathogens [43]. Complement is an essential for immune system which involves approximately 35 soluble and membrane bound proteins produce of opsonin molecules, such as anaphylatoxin, that directly killing of pathogens and maintaining homeostasis [44]. Complement component C3 is one of the most among abundant serum proteins play an essential role in classical, alternative, and lectin pathways and interacts with many proteins, including some that participate in or control cell adhesion and cell-to-cell communication [45]. The chemokine, CXCa and the chemokine receptor, CXCR1 are playing an important key role in stimulating the migration of neutrophilic granulocytes at the sites of infection [46]. In the present study, the healthy and infected fish treated with 5 mg kg^{-1} doses of madecassic acid diet was up-regulation earlier of CC3 and CXCa genes than that of 1 or 10 mg kg^{-1} doses of madecassic acid diets. Similarly, an enhancement of CC3 gene expression was evident in the skin and lymphoid organs of O. mykiss against Ichthyophthirius multifiliis [47], in the head kidney, liver, and spleen of common carp against Trypanoplasma borreli [48] and in the liver of C. idella against S. major [39]. In contrast, the present study revealed down-regulation of CC3 of both groups administration with 1 or 10 mg kg^{-1} doses of madecassic acid diets from weeks 2 to 6. These results were strongly correlated in previous study found on lower serum alternative complement activity in rohu against Argulus [49,50]. However, there was no significant difference observed in the expression of CXCa gene in the head kidney of healthy and infected fish treated with 1 or 10 mg kg^{-1} doses of madecassic acid diets in earlier stages of infection, mostly which release is associated with cells involved in inflammation, clearly reveals inflammatory changes in head kidney tissues. Therefore, a detailed histopathological study required of infected fish kidney tissue would possibly strengthen this hypothesis in future. Conversely, Forlenza et al. [51] and Gonzalez et al. [52] had reported that CXCa was up-regulation in the skin of C. carpio against A. japonicas or I. multifiliis.

Lysozyme is a mucolytic enzyme produced by leucocytes particularly monocytes, macrophages and neutrophils and it was very important defence molecule of the innate immune system that plays a role in mediating protection against infectious pathogens. In vertebrates, chicken (c-type) and goose (g)-type lysozyme has been reported, which have divergent properties like amino acid composition, molecular weight, and enzymatic properties [53,54]. The c-type lysozymes are mostly present in phylum Chordata and Arthopoda whereas g-type lysozymes are present in Chordata and some bivalve mollusks. The major function of lysozymes is their contribution to antibacterial defence mechanisms. The g-type of lysozymes are typically contributing to antibacterial defence in various tissues including lymphoid tissue, while c-type of lysozymes high expression in the stomach or gut rather points to a digestive function [55,56]. In this study both groups, the lysozyme C and lysozyme G genes were not significantly up-regulated when fed with any doses of madecassic acid whereas it was significantly up-regulated in both groups fed with 5 mg kg^{-1} dose diet on 6th and 8th week. The changes in expression of various lysozyme components, mainly lysozyme G, might be due to induced localized inflammatory

response, as usually described its major role in antimicrobial defence in fish [55].

The β2-M is a low molecular mass protein (~12 kDa) contains in the serum as free form or cell surface associated form with MHC-I [57] that helps in presenting endogenous peptides derived from proteasomal degradation of phagocytosed viral and bacterial proteins to cytotoxic T cells [58,59]. Thus, further it play an important role along with MHC-I in self or non-self-recognition in vertebrates. Transferrin is widely known as an iron-binding protein and plays an important role to deliver iron from absorption centers in the duodenum and white blood cell macrophages to all tissues. In this study, the β2-M and transferrin genes were up-regulated in both groups fed with 5 mg kg^{-1} dose diet after 6th week, but did with other doses of madecassic acid. Therefore, the present study indicate that madecassic acid at 5 mg kg^{-1} dose is becoming more clear that the cell types, mostly leucocytic series effectively modulate in head kidney and down-regulation with 1 or 10 mg kg^{-1} doses in this major immunocompetent organ might be one of the reasons for poor immune response in fish against A. siamensis. This hypothesis is supported in Salmo salar down regulation of MHC-I gene against Lepeophtheirus salmonis [60].

In conclusion in the present findings demonstrated that the rohu immune system was strongly affected by A. siamensis infection whereas the infected fish after administration with madecassic acid supplementation diet at 5 mg kg^{-1} level significantly modulated in both innate-adaptive immune response and immune cytokine genes expression. Thus was suggested that A. siamensis infected L. rohita after fed with medium dose of madecassic acid prove more beneficial and economical for disease prevention in aquaculture. However, more studies are required, particularly innate-adaptive cellular and molecular mechanisms and to enlighten the mechanisms involving regulation of immune cytokine genes expression in other fish against various parasitic infection before inclusion or recommended of madecassic acid in aquaculture feed supplementation.

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