ISSN: 2347-5129
IJFAS 2015; 2(4): 218-223

www.fisheriesjournal.com
Received: 20-12-2014
Accepted: 25-01-2015

Srinivasan Ponnuraj
Aquaculture Research Lab, Department of Biotechnology, Karpagam University, Coimbatore – 641 021, Tamil Nadu, India.

Manimekalai Kanagarajan
Aquaculture Research Lab, Department of Biotechnology, Karpagam University, Coimbatore – 641 021, Tamil Nadu, India.

Dineshbabu Jaganathan
Aquaculture Research Lab, Department of Biotechnology, Karpagam University, Coimbatore – 641 021, Tamil Nadu, India.

Teepica Priya Darsini Deivamarudachalam
Aquaculture Research Lab, Department of Biotechnology, Karpagam University, Coimbatore – 641 021, Tamil Nadu, India.

Correspondence
Srinivasan Ponnuraj
Aquaculture Research Lab, Department of Biotechnology, Karpagam University, Coimbatore – 641 021, Tamil Nadu, India.

Expression analysis of IL-10 and IFN-γ genes in head kidney of *Catla catla* (Ham.) fed with *Limonia acidissima* L

Srinivasan Ponnuraj, Manimekalai Kanagarajan, Dineshbabu Jaganathan, Teepica Priya Darsini Deivamarudachalam

Abstract
Immunostimulants are the most important elements for the control of fish diseases in fresh water aquaculture. Study on the functional mechanism of immunostimulants in fish is vital for elucidating their medicinal property. The effect of dietary supplementation of *Limonia acidissima* on the immune gene expression of *Catla catla* was studied. Catla (5 ± 5.5 g) were fed with experimental diets containing 1.5 (T_1), 3 (T_2) and 6% (T_3) Limonia fruit (LF) powder and control diet (T_0) for different experimental feeding periods 30, 60, 90, and 120 days. Following each feeding period fish were immunized with heat-killed *Aeromonas hydrophila*. Tissue samples were collected from kidney after 10 days of immunization. Five fish were sacrificed for tissue collection and gene expression analysis was performed for innate immune genes such as IL-10 and IFN-γ with β- actin as control. There were up-regulations of IL-10 and IFN-γ gene expressions in kidney compared to control. Significantly ($p \leq 0.05$) higher expressions of IL-10 and IFN- γ were observed in T_2 diet fed fish compared during 30, 60, and 90 days feeding period compared to other groups. This study revealed tissue specific gene expression pattern in catla kidney.

Keywords: Catla, *L. acidissima*, Immunostimulant, Gene expression.

1. Introduction
Disease outbreaks and indiscriminate use of antibiotics in aquaculture has increased the percentage of antibiotic resistant microorganisms leading to economic loss [1]. Immunostimulants are used as an alternative to antibiotics for enhancing the immunity of fish against the infectious disease. The use of plant compounds as herbal immunostimulants has been increasing rapidly in aquaculture to avert the overuse of hazardous antibiotics. *Limonia acidissima* L. belongs to family Rutaceae and is a traditional medicinal plant with therapeutic properties. The Limonia fruit had been studied for its rich antioxidant, medicinal properties and growth promoting properties in carps [2, 3]. Elucidation of fish immune system is important for the development of alternative treatment and disease prevention strategies [4]. Cytokine genes play an important role in regulating the immune system of fish. Recently, many cytokine and immune-related genes have been identified and characterized in fish, which has supported in numerous studies on the expression of these genes during pathogen exposure [5, 6, 7].
Innate immune response plays a vital role in the first line defence in fish against pathogen infection. This system consists of a large number of cells involved in body fluids. Administration of immunostimulants such as *Spirulina* sp. enhanced the expression tumour necrosis factor –alpha (TNFα) and interleukin one beta (IL-1β) genes in *Cyprinus carpio* [8]. Among different cytokine genes interleukin 10 (IL-10) is known for its significant anti-inflammatory properties and enables the organism to respond to infectious agent, inducing an inflammatory cascade along with other defensive responses [9, 10]. Interferon gamma (INF-γ) is a key cytokine which regulates innate and adaptive immunity against viral and intracellular bacterial infections. IFN-γ stimulates macrophage-mediated phagocytosis, regulates pro-inflammatory cytokines and induce proteins that bind to iron and create bacteriostatic environment by limiting the availability of iron to the pathogens [11, 12]. Constitutive expression of IFN- γ genes were demonstrated in *Cyprinus carpio* in several immune organs, cell types and could be accounted for the regulation by immunostimulants [13].
Catla catla is an Indian major carp of high commercial value and farmed in a polyculture system. The fish is prone to diseases such as aeromoniasis, edwardsielosis etc. Modulation of

immune system by immunostimulants in Indian major carp, especially in catla is still unclear. The present investigation was undertaken to study the impact of dietary supplementation of Limonia fruit on the expression pattern of some immune related genes such as IL-10 and IFN-γ in the kidney tissue of catla.

2. Materials and methods

2.1 Experimental setup and feeding of fish

Catla catla (5.0 ± 5.5 g) fingerlings were obtained from Induced carp spawning and seed rearing centre, Tamil Nadu Fisheries Development Corporation (TNFDC, Govt. of India), Aliyar, Tamil Nadu, India and where randomly distributed in twelve tanks (50 fish per 100 L). Fish were acclimatized for 10 days. Fish were fed with experimental diets containing 1.5% (T_1), 3% (T_2) and 6% (T_3) *Limonia acidissima* fruit (LF) powder and control diet (T_0) at the rate of 5% body weight (Table 1). The feed was rationed in two portions and was given at 9.30 am and 4.30 pm. Water temperature and pH ranged from 27 to 30 °C and 7.2 to 8.0, respectively. The experimental feeding trial was carried out for 30, 60, 90 and 120 days feeding period.

Table 1: Composition of control and experimental diets

Materials	Formulation (g/100 g on dry matter basis)			
	Control (T_0)	T_1	T_2	T_3
Rice bran	45	45	45	45
Groundnut oil cake	20	20	20	20
Maize	10	10	10	10
Finger millet	10	10	10	10
Pearl millet	10	10	10	10
Agrimin forte*	5	5	5	5
Limonia acidissima fruit	0	1.5	3.0	6.0
Crude Protein	38.83	39.23	39.14	39.21
Crude Lipid	11.47	11.83	11.85	11.9
Crude fiber	2.23	2.06	2.07	2.13
Ash	9.3	9.52	9.6	9.3
Moisture	6.3	6.05	6.48	6.2

Abbreviations: T_0 : Control feed ; T_1 : feed mixed with 1.5% *L. acidissima* ; T_2 : feed mixed with 3% *L. acidissima* ; T_3 : feed mixed with 6% *L. acidissima.*

*Vitamin and Minerals pre mix, Vit-C free ; Nutritional value (g or IU kg^{-1}) : Vitamin A - 700000 IU; Vitamin D_3 - 140000 IU; Vitamin E - 500 mg; Vitamin B12 - 1000 mcg; Folic Acid - 100 mg; Nicotinamide - 1000 mg; Copper - 1200 mg; Cobalt - 150 mg; Iron - 1500 mg; Zinc - 3000 mg; Iodine - 325 mg; Selenium - 10 mg; Magnesium - 6000 mg; Manganese - 1500 mg; Potassium - 100 mg; Calcium - 27 mg; Phosphorus - 13 mg; Sulphur - 0.72 mg; Fluorine - 300 mg.

[a] Moisture is expressed as percentage of fresh weight; crude protein, crude lipid, and ash are expressed as percentages of dry matter. Each datum is a mean from three separate determinations.

2.2 Culture of pathogen and Immunization of fish

Aeromonas hydrophila MTCC 1739 (IMTECH, Chandigarh) was cultured in Tryptone Soya Broth (TSB) (Himedia, India) for 24 h at 37 °C. The culture was then centrifuged at 3000 × g for 10 min. The supernatant was discarded and the pellet was resuspended in phosphate buffered saline (PBS, pH 7.4 ±0.1). The absorbance of the bacterial suspension was adjusted to 0.5 at 450 nm using Micro plate Reader (Biotek, Model No: ELX 800, Synergy, HT, NY, USA) and was kept in water bath at 65 °C for 2 h. Sterility was confirmed by the absence of bacterial growth on Tryptone Soya Agar, and this bacterial suspension was used for immunization of fish.

After each feeding trial, 10 fish were anaesthetized with clove oil (Himedia, India) and were given intra peritoneal injection with 1 ml of heat killed *A. hydrophila* (1×10^6 CFU) for immunization. Then the fish were released in to the individual culture tanks maintained for post immunization process.

2.3 Immune-related gene expression study

2.3.1 RNA isolation and cDNA synthesis

Total RNA was extracted using 50-100 mg of kidney tissue using TRI reagent (Sigma, U.S.A) as per manufacturer's protocol. The RNA concentration in the sample was quantified by measuring the absorbance at 260 nm using Bio photometer Plus (Eppendorf, India). The purity of the samples was analyzed by measuring the ratio of OD 260 nm/OD 280 nm. The samples having OD ratio of 1.8 - 2.0 were taken for the cDNA synthesis.

Total RNA (2 µg) was used for first strand cDNA synthesis using Revert aid first strand cDNA synthesis kit (Thermo scientific, U.S.A) in thermo cycler (Eppendorf, Master cycler Pro S, U.S.A). RNA was incubated with 1 µl of random hexamer (20 µM) at 65 °C for 5 min followed by 4 °C for 5 min for primer annealing. After this, the following components were added to the reaction in order: 4 µl of 5X MMLV-RT buffer, 1 µl of Ribolock RNase inhibitor (20 U/µl), 2 µl 10 mM

dNTPs, 1 µl of RT enzyme, 5 µl DEPC water. The reagents were gently mixed and incubated in the order, 5 min at 25 °C, 1 h at 42 °C, and 10 min at 70 °C. The synthesized cDNA was stored at 4 °C for further use.

2.4 Gene expression analysis

The amplification of IL-10 and IFN-γ genes were determined. β-actin was used as a positive control and for sample normalization. Each PCR reaction consisted of 12.5 µl nuclease free water, 2 µl 10X PCR buffer (Kcl^+, $MgCl^-$), 0.5 µl 10 mM dNTPs, 1.5 µl 25 mM $MgCl_2$, 0.5 µl (10 pmol) of each forward and reverse primers, followed by 0.5 µl (0.05 U/µl) of Taq DNA polymerase and 2 µl cDNA template.

The amplification profile was followed in order: Initial denaturation at 95 °C for 5 min followed by 30 cycles consisting of denaturation at 95 °C for 30 s, at an optimum primer annealing temperature for 45 s (Table 2) and extension at 72 °C for 45 s, followed by final extension at 72 °C for 10 min. The final PCR products were analyzed by electrophoresis on 1% agarose gel. The relative levels of expression of each gene were analyzed using Image J software version 1.49 (NIH, U.S.A).

Table 2: Primers used in this study and their annealing temperature and sizes of PCR amplicons.

Target gene	Primer	Primer sequence (5′ to3′)	Optimum annealing temperature (℃)	PCR amplicon size (bp)
IL-10	IL-10 FP	ACC TGC ACA TTG CTA CAG CA	59	235
	IL-10 RP	CCC GCT TGA GAT CCT GAA AT		
IFN-γ	IFN-γ FP	AAG GGT TCC TGC TCT TGT CA	54	210
	IFN-γ RP	GCC ATT TTT CAC CTC GAC TG		
β-Actin	β-Actin FP	GGT CAT CAC CAT TGG CAA TG	60	N.S
	β-Actin RP	CAG GGA TGT GAT CTC CTT CT		

2.5 Statistical analysis

The ratio of immune-related gene/beta actin product was further calculated after subtraction of the background pixel intensity for each gene of interest and mean value was calculated from three samples. Data were analyzed using paired sample T-test. Significance level was considered at $p \leq 0.05$.

3. Results and Discussion

The immune response of LF supplemented diet fed fish after immunization with heat killed *A. hydrophila*, was assessed by measuring the expression of genes encoding IL-10 (anti-inflammatory cytokine) and INF-γ (pro-inflammatory cytokine). Densitometry quantification of these immune genes relative to β-actin was compared among kidney tissues of control and experimental diet fed catla following exposure with heat killed pathogen.

In kidney tissues, the IL-10 expression was up-regulated in *L. acisissima* fruit supplemented diet fed groups (9-32%) as compared to control group (Figure 1) over the entire feeding period. Significantly ($p \leq 0.05$) higher IL-10 expression was observed in fish fed with 1.5% (T_1) LF supplemented diet fed for 30 days, whereas 3% (T2) LF supplemented diet fed fish showed significant ($p \leq 0.05$) IL-10 expression during 30, 60, and 90 days. However there IL-10 expression was insignificant ($p \leq 0.05$) among the experimental groups during 120 days feeding period. The expression of INF- γ was up-regulated (9-21.5 %) in LF supplement diet fed catla compared to control group during the entire feeding scheme. Interestingly, the IFN-γ expression was significant ($p \leq 0.05$) in 3% (T_2) LF supplemented diet fed fish during 30, 60 and 90 days feeding period (Figure 2). The IFN-γ expression in experimental groups were insignificant ($p \geq 0.05$) during 120 days of feeding period as compared to β-actin control. Moreover, it was observed that the expression of IFN- γ relatively decreased in all groups after 60 days feeding period.

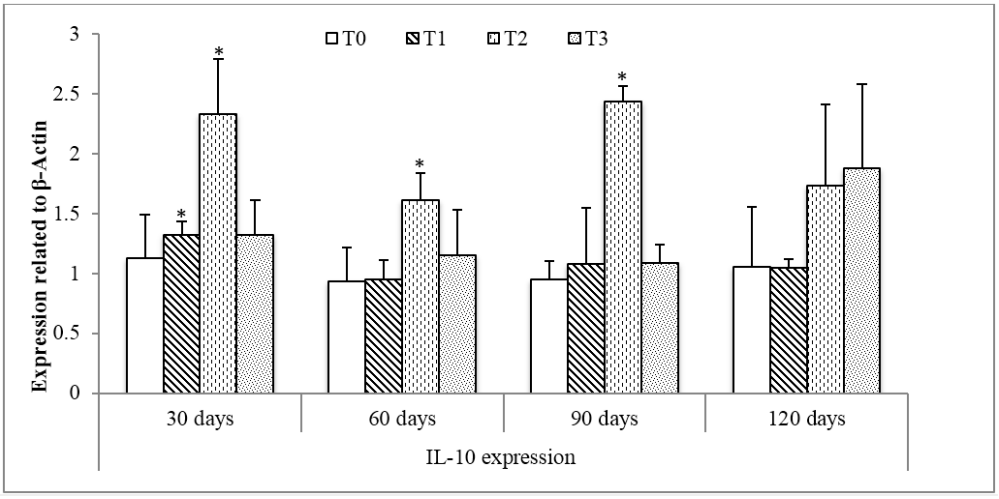

Fig 1: Expression pattern of IL-10 gene relative to β-actin in the kidney of *Catla catla*. Values are expressed as mean ± S.E. Values denoted with superscripts, differ significantly (* $p \leq 0.05$) with that of control (n=3).

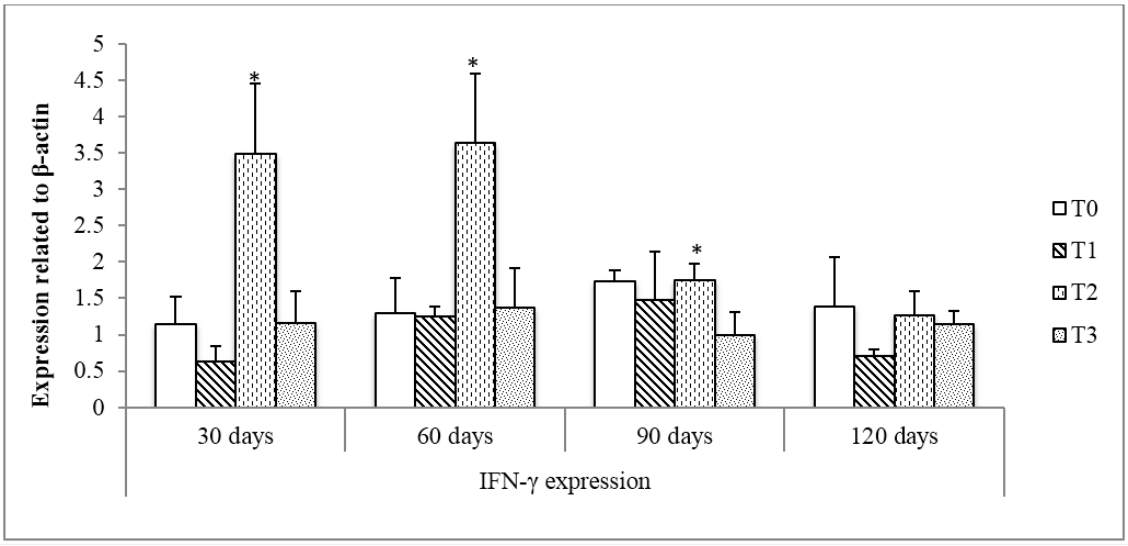

Fig 2: Expression pattern of IFN-γ gene relative to β-actin in the kidney of *Catla catla*. Values are expressed as mean ± S.E. Values denoted with superscripts, differ significantly (* $p \leq 0.05$) with that of control (n=3).

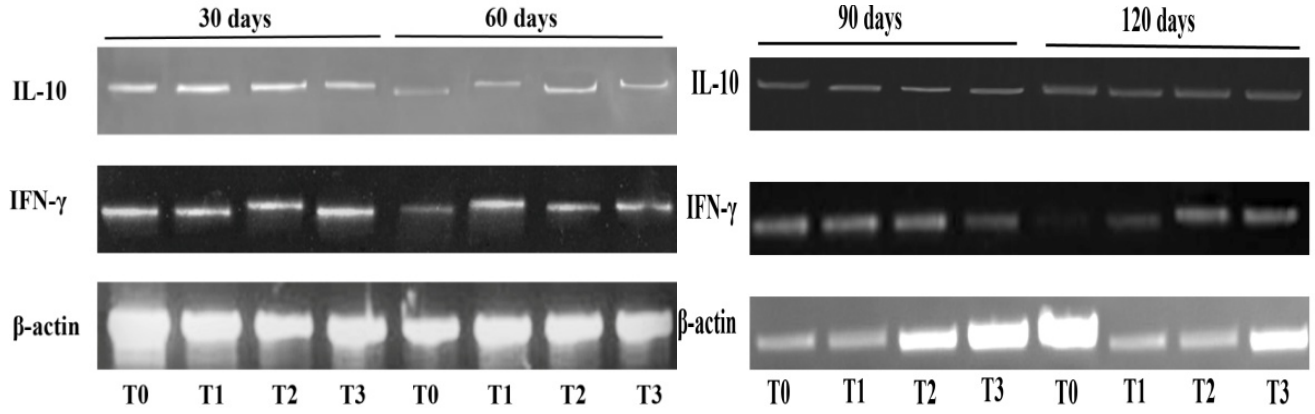

Fig 3: Expression pattern of immune genes in *Catla catla* fed with *L. acidissima* fruit (LF) supplemented diets. T_0 (Control diet), T_1 (1.5% LF), T_2 (3% LF), and T_3 (6% LF).

Our study has revealed the effect of dietary supplementation of *L. acidissima* fruit on the immunity of catla against *A. hydrophila*. The changes in the immune system following the infection were also indicated by the up- or down- regulation of immune related genes. However, expression studies of immune related gene in Indian major carps and understanding of immune responses to bacterial infections is at a nascent phase. The present study, measured the tissue specific immune gene expression pattern of catla, exposed to heat killed *A. hydrophila* after an experimental feeding trial (30-120 days) with LF supplemented diet. The cytokine IL-10 exhibits anti-inflammatory function and are regulated by multiple immune factors [14]. The primary function of IL-10 is to negate the pro-inflammatory cytokines (IL-1β, TNFα and IFN-γ) response and prevent tissue damage [15].

In the present study, IL-10 expression was up-regulated in the kidney tissues of experimental group fish of different feeding period, which may be attributed to the prolonged immunostimulatory property of *L. acidissima* on fish immunity. The expression patterns of IL-10 vary extensively among different species of fish. Increased expression levels of IL-10 was recorded for fish fed with different immunostimulants, which were correlated to the transcription occurring in innate immune cells such as macrophages [16, 17]. Pinto *et al*. [18], reported up-regulated expression of IL-10 in sea bass (*Dicentrarchus labrax*) after artificial infection with UV-killed *Photobacterium damselae*. In addition, IL-10 up-regulation was observed in Atlantic cod (*Gadus morhua*) following LPS treatment [19]. In rohu, IL-10 mRNA transcripts in gill, liver and kidney showed up-regulation after *A. hydrophila* infection in treated fish as compared to control. Research on the mechanism of catla IL-10 induction revealed that, blocking nuclear factor (NF)-kB-signalling with BAY11-7082 in catla kidney cell culture suppressed IL-10 induction by LPS. This may elucidate the role of catla IL-10 as an anti-inflammatory cytokine and its induction via NF-kB signal pathway [20].

Interferons (IFNs) modulate innate and adaptive immune response by multifactorial transcriptional regulation. IFN-γ belongs to type II IFN family and is produced by Natural killer cells and T lymphocytes in response to viral, bacterial and parasite antigens [22]. Rainbow trout and carp IFN–γ have several functional properties in common with mammalian IFN –γ, including the ability to enhance respiratory burst activity, nitric oxide production, and phagocytosis of bacteria in macrophages [23-27]. Mulder *et al* [28] observed up-regulation of IFN-γ in rainbow trout (*Oncorhynchus mykiss*) intestine tissue following *A. salmonicida* exposure. Ellingsen *et al*. [29] observed up-regulation of IFN- γ and IL-10 in head kidney of Atlantic cod (*Gadus morhua*) after intra peritoneal injection with *Francisella noatunensis*. Up-regulation of pro-inflammatory IFN-γ gene expression in LF supplemented diet

fed fish suggests that the immunity of experimental group fish was enhanced as compared to control group. It has been well established that interferon expressions are widely associated with anti-viral infections, but recent expression studies had revealed that bacterial components such as lipopolysaccharides (LPS) and peptidoglycan (PGN) were effective inducers of IFN-γ in common carp [24].

Furnes *et al*. [30] observed an up-regulated expression pattern of IFN- γ in cod infected with formalin-killed *Vibrio anguillarum*. In Atlantic salmon IFN- γ was up-regulated against bacterial derived compounds such as CpG [31]. Considering together, it may be hypothesized that INF-γ expression in the current study could be activated by *A. hydrophila* virulence factors. However, in our study the relative expressions of IL-10 and IFN-γ varied among experimental groups of different feeding period. According to Swain *et al*. [20] the expression of IL-10 an IFN-γ were inversely related in catla exposed to *A. hydrophila*. This may be correlated with differential expression pattern involving high IL-10 and relatively IFN-γ among the experimental groups of catla as compared to control. Further, our findings elucidate the anti-inflammatory property of IL-10. In the present study, gene expression varied with experimental groups and time duration of feeding trial. Significant gene expression was observed in T_1 and T_2 during early days of feeding period (30-90 days) whereas at 120 days the expressions were insignificant in all experimental groups. Studies have shown that the effects of immunostimulants are not does dependent, and over dosage may suppress immune response. Conversely, Immunostimulants influence the non-specific immune system over a short duration of time and prolonged administration may prove ineffective based on dose [32, 33]. Highest expression of IL-10 and IFN-γ was observed in 3% LF supplemented diet fed fish. In addition, the immunostimulatory property of test diets corroborate with enhanced growth and some innate immune parameters of fresh water fishes as evident from our previous studies [2, 3, 34].

4. Conclusion

Our study provided the first information concerning *L. acidissima* fruit induced gene expression in fish. The LF supplementation in diets influenced the immune gene expression and up-regulated the expression of IL-10 and IFN-γ in head kidney of catla following pathogen exposure. These results will be an added proof for understanding the function of important immune genes in response to different immunostimulants, probiotics, vaccines and disease resistance against various fish pathogens. However it remains for further study to determine the effect of LF diet supplementation on immune genes of other fish tissues such as liver, spleen, gills and intestine which is being focused in our laboratory.

5. Acknowledgement

Authors would like to thank Department of Science and Technology, Science and Engineering Research Board (DST-SERB, Govt. of India), New Delhi, India, for financial support in the form of project (Ref. No. SR/FT/LS-128/2012. Dated 18.12.2012). Authors gratefully acknowledged the facilities provided by Karpagam University, Coimbatore, Tamil Nadu, India. The support provided by TNFDC (Govt. of India), Aliyar, Tamil Nadu, India is gratefully acknowledged.

6. References

1. Kaleeswaran B, Ilavenil S, Ravikumar S. Dietary supplementation with *Cynodon dactylon* (L.) enhances innate immunity and disease resistance of Indian major carp, Catla catla (Ham.). Fish and Shellfish Immunology 2011; 31:953-62.
2. Teepica PDD, Maheshu V, Vishnupriya M, Nishaa S, Sasikumar JM. Antioxidant potential and amino acid analysis of underutilized tropical fruit *Limonia acidissima* L. Free Radicals and Antioxidants 2013; 3(S):62-69.
3. Teepica PDD, Maheshu V, Srinivasan P, Dineshbabu J, Castro J, Sasikumar JM. Growth efficacy and feed utilization of fresh water fishes *Cirrhinus mrigala* (Ham.) and *Cyprinus carpio* L. fed with *Limonia acidissima* L. Research Journal of Recent Science 2014; 3:127-131.
4. Aoki T, Ikuo H. Immune relevant genes of Japanese flounder, *Paralichthys olivaceus*. Comparative Biochemistry and Physiology Part D Genomics Proteomics 2006; 1:115-21.
5. Lindenstrom T, Secombes CJ, Buchmann K. Expression of immune response genes in rainbow trout skin induced by *Gyrodactylus derjavini* infections. Veterinary Immunology and Immunopathology 2004; 97:137-48.
6. Purcell MK, Kurath G, Garver KA, Herwig RP, Winton JR. Quantitative expression profiling of immune response genes in rainbow trout following infectious haematopoietic necrosis virus (IHNV) infection or DNA vaccination. Fish and Shellfish Immunology 2004; 17:447-62.
7. Sigh J, Lindenstrom T, Buchmann K. The parasitic ciliate *Ichthyophthirius multifiliis* induces expression of immune relevant genes in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Journal of Fish Diseases 2004; 27:409-17.
8. Watanuki H, Ota K, Tassakka ACM, Kato T, Masahiro S. Immunostimulant effects of dietary *Spirulina platensis* on carp, *Cyprinus carpio*. Aquaculture 2006; 258:157-63.
9. Moore KW, de Waal RL, Coffman RL, O'Grra A. Interleukin-10 and Interleukin-10 receptor. Annual Review of Immunology 2001; 19:683-765.
10. Magnadottir B. Innate immunity of fish (overview). Fish and Shellfish Immunology 2006; 20:137-51.
11. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. Journal of Leukocyte Biology 2004; 75(2):163–89.
12. Rosenzweig SD, Holland SM. Defects in the interferon-gamma and interleukin-12 pathways. Immunological Reviews 2005; 203:38–47.
13. Milev-Milovanovic I, Long S, Wilson M, Bengten E, Miller NW, Chinchar VG. Identification and expression analysis of interferon gamma genes in channel catfish. Immunogenetics 2006; 58(1):70–80.
14. Savan R, Igawa D, Sakai M. Cloning, characterization and expression analysis of interleukin-10 from the common carp, *Cyprinus carpio* L. European Journal of Biochemistry 2003; 270:4647-54.
15. Moore KW, de Waal RL, Coffman RL, O' Grra A. Interleukin-10 and Interleukin-10 receptor. Annual Review of Immunology 2001; 19:683-765.
16. Akira S, Hemmi H. Recognition of pathogen-associated molecular patterns by TLR family. Immunology Letters 2003; 85:85-95.
17. Pengal RA, Ganesan LP, Wei G, Fang HQ, Ostrowski MC, Tridandapani S. Lipopolysaccharide-induced production of interleukin-10 is promoted by the serine/threonine kinase Akt. Molecular Immunology

2006; 43:1557-64.
18. Pinto RD, Nascimento DS, Reis MIR, Do vale A, Dos Santos NM. Molecular characterization, 3D modelling and expression analysis of sea bass (*Dicentrarchus labrax* L.) interleukin-10. Molecular Immunology 2007; 44:2066-75.
19. Seppola M, Larsen AN, Steiro K, Robertsen B, Jensen I. Characterization and expression analysis of the interleukin genes, IL-1β, IL-8 and IL-10, in Atlantic cod (*Gadus morhua* L.). Molecular Immunology 2008; 45:887-97.
20. Swain B, Basu M, Samanta M. Cloning of interleukin-10 gene in the Indian major carp, *Labeo rohita* (Hamilton 1822) and its functional characterization following Aeromonas hydrophila infection. Indian Journal of Fisheries 2011; 58:39-47.
21. Swain B, Samanta M, Basu M, Panda P, Sahoo BR, Maiti NK *et al*. Molecular characterization, inductive expression and mechanism of interleukin-10 gene induction in the Indian major carp, catla (*Catla catla*). Aquaculture Research 2012; 43:897-907.
22. Robertsen B. The interferon system of teleost fish. Fish and Shellfish Immunology. 2006; 20(2):172-91.
23. Zou J, Carrington A, Collet B, Dijkstra JM, Yoshiura Y, Bols N, *et al*. Identification and bioactivities of IFN-gamma in rainbow trout *Oncorhynchus mykiss*: the first Th1-type cytokine characterized functionally in fish. The Journal of Immunology 2005; 15:175(4): 2484-94.
24. Stolte EH, Savelkoul HF, Wiegertjes G, Flik G, Lidy Verburg-van Kemenade BM. Differential expression of two interferon-gamma genes in common carp (*Cyprinus carpio* L.). Developmental and Comparative Immunology 2008; 32(12):1467-81.
25. Grayfer L, Garcia EG, Belosevic M. Comparison of macrophage antimicrobial responses induced by type II interferons of the goldfish (*Carassius auratus* L.). The Journal Biological Chemisty. 2010; 30:285(31):23537-47.
26. Arts JA, Tijhaar EJ, Chadzinska M, Savelkoul HF, Verburg-van Kemenade BM. Functional analysis of carp interferon-gamma: evolutionary conservation of classical phagocyte activation. Fish and Shellfish Immunology 2010; 29(5):793-802.
27. Grayfer L, Belosevic M. Molecular characterization, expression and functional analysis of goldfish (*Carassius auratus* L.) interferon gamma. Developmental and Comparative Immunology 2009; 33:235–246.
28. Mulder IE, Wadsworth S, Secombes CJ. Cytokine expression in the intestine of rainbow trout (*Oncorhynchus mykiss*) during infection with *Aeromonas salmonicida*. Fish and Shellfish Immunology 2007; 23(4):747-59.
29. Ellingsen T, Inami M, Gjessing MC, Van Nieuwenhove K, Larsen R, Seppola M *et al*. *Francisella noatunensis* in Atlantic cod (*Gadus morhua* L.); waterborne transmission and immune responses. Fish and Shellfish Immunology 2011; 31(2):326-33.
30. Furnes C, Seppola M, Robertsen B. Molecular characterisation and expression analysis of interferon gamma in Atlantic cod (*Gadus morhua*). Fish and Shellfish Immunology 2008; 26:285–292.
31. Strandskog G, Skjaeveland I, Ellingsen T, Jørgensen JB. Double stranded RNA and CpG DNA induced immune responses in Atlantic salmon: comparison and synergies. Vaccine 2008; 26:4704– 4715.
32. Lopez N, Cuzon G, Gaxiola G, Taboada G, Valenzuela M, *et al*. Physiological, nutritional and immunological role of dietary β 1-3 glucan and ascorbic acid 2-monophosphate in *Litopenaeus vannamei* juveniles. Aquaculture 2003; 224: 223-243.
33. Barman D, Nen P, Mandal SC, Kumar V. Aquaculture Health Management: A new Approach. Journal of Marine Science Research and Development 2013; 3: 139.
34. Srinivasan P, Maheshu V, Sasikumar JM, Castro J, Teepica PDD. Dietary Supplementation of *Limonia Acidissima* L. Fruit Enhances the Immunological Parameters of *Cyprinus carpio* L. International Journal of Pharmacology Biological Sciences 2014; 8(2):51-57.