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A Study on Impact of Natural Immunostimulants on Immune Response of Catla Catla

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Abstract – In recent years, the trend of aquaculture is growing in India at a rapid speed. Fish culturists have to face the common disease found in the fishes these days. Hence, it needs to adopt a good management practice to keep a fish healthier. The demand of natural immunostimulants is among the fish culturists as these stimulants are very useful to prevent the fish diseases.

The natural immunostimulants are supposed to be eco-friendly as they are easily bio-degradable and not harmful for the human and environment. Immunostimulant is generally used in vaccines so as to enhance the immune response. Hence, it consequently improves the ability of the fishes to protect themselves from the diseases.

Fishes were divided into 2 groups one received control diet and another received Plumbago rosea incorporated diet for 14 days. The haematological parameters and serum protein were estimated between control and experiment. There is An increase in TEC (P<0.05) and TLC (P<0.05) in the immunostimulant administered (IS) diet was observed.

Increase in lymphocyte count was noted in immunostimulant incorporated diet. Remarkable increase in Hb from 6.2 to 8g% and serum protein level from 0.4to 0.5g% was observed in experimental fishes. The immunostimulant administered Catla catla when challenged with Aeromonas hydrophila showed a decrease in TEC and an increase in TLC. Increase in lymphocytes was also noted.

Keywords: Immune, Catla Catla, Fish, Plumbago Rosea

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INTRODUCTION

Plumbago rosea is cultivated in gardens in India. It belongs to the plumbaginaceae family. Plumbagin is found in the roots of the plant and it is very useful in controlling the central nervous system. Another benefit of this plant is that it enhances the appetite and strengths the digestion system. Due to its anti-septic property, it is used to cure paralysis by mixing its juice leaves with oil. Therefore, this plant proves to be a natural product for the fishes to protect them from fishes and increasing immune power. In order to check the health status of the fishes, their blood test is examined time to time. With the help of blood cell samples, it can be easier to detect any disease in the fish. The main challenge for this aquaculture is the bacterial infections as the aqua animals like fishes get infected by the bacteria. This suddenly may cause in lowering the digestion and immune system. In some cases, it is also found that the digestion and immune system of the fishes gets lowered due to improper diet. Hence, it is the duty of the aquaculture management persons to keep safe the fishes from the bacteria and feed the proper diets to the fishes so that their health status remains better. It is also found that the antibiotics are not so much effective against bacteria and these are not allowed to be used in the aquaculture system. Hence, the alternative to prevent the fishes from the diseases is to strengthen the fish defence mechanism. Herbal extracts can be used as an option for chemotherapeutic agents in fish culture. It is also found that the plant extracts can be used to enhance the immune power of the fishes. A large number of medicinal plants such as Phyllanthus niruri, Acalypha indica, Azadirachta indica, Piper betle, Mentha piperita, Allium sativum, Astragalus membranaceus, Lonicera japonica, Withania somnifera have immunostimulant activity and are used to enhance the fish immunity thereby preventing mortalities during disease outbreaks.

Aquaculture represents one of the fastest growing food producing sectors. Fish diseases constitute one of the most important problems and challenges fish

culturists. Hence, aqua-culturists are forced to undertake good management practices, so that they can ensure a healthier fish. The use of natural immunostimulants in fish culture for the prevention of disease is a promising new development. Natural immnuostimulants are biocompatible, biodegradable and safe for the environment and human health. Immunostimulant, used in vaccines to amplify the specific immune response or administered as feed additives to modulate non-specific immunity, have been demonstrated to play role in protection against diseases in fish.

Plumbago rosea is a perennial shrub which belongs to the family plumbaginaceae. It is commonly cultivated in gardens throughout India. The root of the plant contains plumbagin which stimulates the central nervous system. The plant is able to enhance the digestive power and promote appetite. It also has antiseptic property. The juice of leaves with oil is used to cure rheumatism and paralysis. It contains plumbagin (2-Methoxy-5-hydroxy-1-4-Napthoquinone), which is a natural napthoquinone possessing various pharmacological activities such antimalarial and antimicrobial. So, the use of plants as a productive system of immunostimulators facilitates a new and safe method of immunization. The regular monitoring of fish blood serves as the diagnostic purpose in establishing the health status of fish. By analyzing blood cell characteristics some clues for diagnosis and prognosis of the disease state may be found. The present study is aimed to assess the efficacy of Plumbago rosea as an immunostimulant and to evaluate the immunostimulant activity in the fish Catla catla, post challenged with Aeromonas hydrophila by analyzing the haematological and serological parameters.

REVIEW OF RELATED LITERATURE

Anderson et al. (2012)[1] described that due to enrichment of protein in fish, the market of aquaculture has increased globally in the past few years and this market is growing at a speed of 3% per year.

D.P. et al. (2012)[2] pointed out that the it is quite common that much of the Catla Catla fishes have low digestive power and weak immune system because of improper diet system. Hence, it is the responsibility of the culturist to feed the good quality of diet to the fishes.

Badrelin et al. (2008)[3] examined some phytochemical pharmacological and Toxicological properties of Ginger and described that Ginger can be much helpful to enhance the immune system and digestive power.

Christy et al. (2009)[4] examined the Eclipta alba leaf aqueous extract and pointed out that these leaves could be used as an excellent agent for the immune system of fishes as it can surely protect them from diseases.

Didry et al. (2011)[5] analyzed the anthroquinone and napthoquinone compounds on oral bacteria Diepharmazie and proved that these compounds can be used to prevent diseases.

Gangauly et al. (2011)[6] inhibited the cellular Immune responses by Tylophora indica and was successful in using it as a tonic for the digestion and immune system.

Gopalakannan et al. (2010)[7] performed an experiment and highlighted the immunomodulatory effects of dietary intake of chitin, chitosan and levamisole on the Immune system of Cyprinus carpio and control of Aeromonas hydrophila infection in ponds.

Hadden et al. (2013)[8] described that the trend of immunostimulants is growing in the aquaculture as it can be used as a natural medicine for empowerment of digestion and immune system of the fishes.

Karuthapandi et al. (2010)[9] examined the immune responses of Tilapia mossambicus administered with rat serum vaccine for Vibrio anguillarium

Likhi et al. (2010)[10] examined the anti malarial napthoquinone from Nepenthes thorelii and found that napthoquinone is an excellent anti-malaria agent which consequently is used to increase the blood cells.

Mishra et al. (2009)[11] performed an experiment for the antibacterial activity of ethanol Andrographis paniculata.

Ortuno et al. (2012)[12] described that the oral administration of yeast, Saccharonmyces cerevisiae increases the cellular innate immune response of giltlead sea bream (Sparus aurata).

Panday et al. (2011)[13] described that the Thiram and Ziram fungicides are used to induce modifications on some haematological variables of fresh water cat fish Heteropneustes fossils.

Benny et al. (2010)[14] described the immunostimulatory behaviour of Musa accuminate peel extracts of Clarias batrachus.

Ramasamy et al. (2010)[15] performed an experiment for the herbal supplementation diets on haematology & innate immunity in gold fish against Aeromonas hydrophila.

Rao et al. (2009)[16] performed an experiment to analyze the impact of Achyranthes aspera on the

immunity and survival of Labeo rohita tainted with Aeromonas hydrophila.

Sahu et al. (2009)[17] performed an experiment to analyze the impact of Magnifera Indica kernel as a feed additive on immunity and resistance of Aeromonas hydrophila is Labeo rohita fingerlings.

Sakai et al. (2013)[18] described the status of fish Immuno stimulant and pointed out that it is used to increase the level of immune power among the fishes.

Shao et al. (2014)[19] performed an experiment to extract the Immune receptors for polysaccharides from the roots of Astragalus membranaceus.

Vanitha et al. (2014)[20] highlighted the immune modulatory and antimicrobial effects of some traditional Chinese medicinal Herbals.

SOURCES OF IMMUNOSTIMULANTS

Immunostimulants are substances that activate the immune system of animals to make them more resistant to microbial infections. The definition has been expanded somewhat to include live organisms or their products that have an impact on the immune system. The use of immunostimulants does not generate a specific response to a certain antigen, but causes an overall response that hastens recognition and elimination of a broad range of infectious agents and foreign substances. Two categories of conserved molecular patterns are recognized by the innate immunity through the various pattern recognition receptors (PRRs): (1) non-self or pathogen-associated molecules; and (2) molecules generated as a result of damage to the host's own tissues signalling danger to the immune system. Pattern recognition is the first step in innate immunity. Immunostimulants, like the presence of infection, are sensed by the various PRRs. So far, 11 types of pattern recognition receptors have been identified in Catla Catla namely, -1,3-glucanase-related proteins, -1,3-glucan-binding proteins, c-type lectins, scavenger receptors, galectins, fibrinogen-related proteins, thioester-containing Down syndrome cell adhesion molecules, serine protease homologs, trans-activation response RNA-binding protein, and Tolllike receptors. Aside from pattern recognition, these PRRs have different binding specificities and effector functions.

Bacterial preparations

Vibrio - is a curved rod-shaped gram-negative bacteria. Vibrio anguillarum is a very efficient vaccine for salmonid fish. The immunostimulant effects of inactivated Vibrio cells have been documented in Catla Catla . The authors reported that injection or immersion of Catla Catla in Vibrio bacterin resulted in reduced mortality suggesting immunostimulation by the ”vaccine” as invertebrates do not have an efficient specific immune response. The immunostimulation is mediated by the PRRs that recognize and bind stimulatory components in bacteria. Vibrio bacterin may therefore act as an immunostimulant since non-specific immune cells such as phagocytic hemocytes are activated. A Vibrio harveyi bacterin was also able to protect P. monodon against WSSV infection.

Live bacteria: probiotics as immunostimulants – A number of studies revealed that the supplementation of probiotic bacteria and commercial probiotics in feed or any sort of inclusion can boost the cellular and hum oral components of the innate immune system in several species of fish and shellfish including salmon IDs and Catla Catla. Immunostimulation by Bacillus S11 bacteria increased phagocytic activity in Penaeus monodon, whereas the administration of Lactobacillus plant arum stimulated phenol oxidase and superoxide dismutase activities leading to enhanced clearance efficiency of Vibrio alginolyticus in Litopenaeus vannamei.

Immunostimulation can strengthen the immune system of farmed aquatic animals and increase their resistance to pathogens during exposure to stress, such as handling, crowding, sampling, transport, vaccination, during reproduction, and also during the larval stages when high levels of mortality occur. Some commercially available immunostimulants are shown in Table.

Table - Some currently available commercial immunostimulants

Name	*Company*	*Active component*
AQUAVAC® Ergosan™	Merck Animal Health	algins and polysaccharides
BZT® PRE-GE	UnitedTech	mannan oligosaccharide and β-Glucan
Vannagen®	Chemoforma Ltd.	nucleotides
Bio-Mos®	Alltech	outer cell wall of *Saccharomyces cerevisiae* yeast
Immustim®	Immudyne, Inc.	B (1,6) branched B (1,3) glucan from yeast
MacroGard®	Biotec-Mackzymal, Norway	B (1,6) branched B (1,3) glucan from yeast
Levamisole	Janssen Pharmaceutica, Belgium	Tetrahydro-6-phenylimidazolthiazole Hydrochloride
VitaStim	Taito Co., Japan	B (1,6) branched B (1,3) glucan from fungi
EcoActiva™	Ecostadt	B (1,6) branched B (1,3) glucan from yeast
Sanoguard® S-PAK	INVE Aquaculture Health	vitamins, nucleotides, immunostimulants

EFFECTS OF IMMUNOSTIMULANTS

Unregulated immune indices such as total hemocyte count, respiratory burst, phenoloxidase activity,

phagocytic activity, agglutination titer, lysozyme and SOD activities, etc. Growth-promoting activity was also found with some immunostimulants. Growth enhancement could result from improved disease resistance due to immunostimulant supplementation. Catla Catla fed with peptidoglycan-supplemented feed also showed better growth and feed conversion rates than those fed a normal diet. Sung et al (1994) demonstrated that black tiger Catla Catla grew faster with glycan immersion which could be attributed to the higher activity of glycan delivered by immersion compared to oral administration.

METHODS AND STRATEGIES IN USING IMMUNOSTIMULANTS

Immunostimulants can be administered through injection, immersion, or oral administration. Injection and immersion require handling of fish/Catla Catla or confining them in a small area during application. However, these methods are laborious, time-consuming, and stressful. Injection method is the most cost-effective method for large fish (>10g). Immersion on the other hand, is not as effective as injection but allows mass immunostimulation and is the most cost-effective method for smaller fish (<5g). Oral immunostimulation is a non-stressful method that can be used with any size of fish but requires a large amount of immunostimulant to provide protection. Furthermore, this method is applicable only for fish fed artificial diet. The outcome of using an immunostimulant is usually determined by the strategy by which it is applied. Continuous use of an immunostimulant may up-regulate the immune system and maintain this status until the immunostimulant is withdrawn, or it may cause adverse effects such as tolerance or immunosuppression. On the other hand, pulse administration (administering immunostimulant-supplemented and non-supplemented diets alternately) oscillate the immune response from arresting level to a heightened response then back to resting, and has been shown to be a better strategy of immunostimulant application.

SOME ISSUES IN THE USE OF IMMUNOSTIMULANTS IN CATLA CATLA

Some immunostimulants may enhance the non-specific immune response in vitro but this does not always result in improved health or increased survival. Some immunostimulants do not show a linear dose/effect relationship; they could be effective at a certain optimum concentration but have no effect or exhibit toxicity at higher concentrations. Consequently, doses determined in vitro cannot be directly extrapolated to large-scale production systems. Hence, these immunostimulants may end up being fed at high doses or for long durations resulting in chronic overstimulation and exhaustion of the immune system. There is lack of unequivocal evidence on the efficacy of some products. Most studies on immune stimulation by microbial polysaccharides claim beneficial effects by improving growth and resistance to pathogen challenge, and/or stimulation of immune responses such as propene oxidase, antibacterial, antioxidant, and agglutination activity, and reactive oxygen production. With particular immunostimulants, some studies reported beneficial effects, whereas no positive effects were found by others. Some immune responses are detrimental. Essentially, the haemocytes perform inflammatory-type reactions such as phagocytosis, haemocyte clumping, production of reactive oxygen metabolites, and the release of microbicidal proteins. Under normal conditions, the inactive form of effector molecules are stored in the hemocytes but are released into the hem lymph through exocytosis and activated upon stimulation by non-self-molecules. In the open circulatory system of crustaceans, immune reactions must be localized to avoid self-damage. Some of the responses and reactions that are potentially self-harming include degranulation of hemocytes, cell clumping and hemacytopenia, cytotoxicity at higher doses, depletion of immune effectors, immune exhaustion by repeated immunostimulation, and high energetic cost of regenerating immune components.

DEFINITIONS AND EXPLANATION TERMS

Catla Catla is a fish with large and broad head, a large protruding lower jaw, and upturned mouth. It has large, greyish scales on its dorsal side and whitish on its belly. It reaches up to 182 cm (6.0 ft) in length and 38.6 kg (85 lb) in weight. Catla Catla is a surface and mid water feeder. Adults feed on zooplankton using large gill rakers, but young ones on both zooplankton and phytoplankton. Catla Catla attains sexual maturity at an average age of two years and an average weight of 2 kg.

Aquaculture

It is one of the most important aqua cultured freshwater species in South Asia. It is grown in polyculture ponds with other carp-like fishes, particularly with the roho labeo and mrigal carp. The reported production numbers have increased sharply during the 2000s, and were in 2012 about 2.8 million tonnes per year.

METHODOLOGY

The experimental fish Catla Catla (125 ± 30g) was collected from fish farm and allowed to acclimatize to laboratory conditions for one week. During acclimatization they was fed with rice bran and groundnut oil cake. Water was renewed daily. During the experimental period the water quality variables temperature (28 ± 1^{o} c) PH (7.4 ± 0.2) salinity (10 ± 2%) and dissolved oxygen (>5 mg^{-1}) was maintained.

The basic diet (Control diet) was prepared by mixing rice bran 10g. Wheat bran 10g, Soya flour 23g, dry fish meal 24g, groundnut oil cake 23g, and Tapioca flour 10g, was made as dough, sterilized in pressure cooker for 30 min, cooled and made in the form of noodles by adding a little amount of sunflower oil. They was shade dried and broken into small desirable pieces and stored. Immunostimulant diet was prepared by using the same propotion by using 2g of Plumbago rosea collected from the local garden.

Experimental design

Experiment 1:

Experiment 1 consists of two groups. One control and one experimental of 15 fishes each. The control group received normal diet and the experimental group received feed formulated with Plumbago rosea powder (IS diet). The fishes were fed with these diets for 14 days and the hematological parameters and serum protein levels were analyzed after 1st, 3rd, 7th & 14th day respectively.

Experiment 2:

Both control and experimental feed fed fishes were subjected to infection with the bacterial Aeromonas hydrophila previously grown in nutrient broth for 24hrs.A dosage of 10-3 and 10-5 (cfu/ml) bacteria were injected intramuscularly and again hematological parameters and serum proteins were studied after 1st , 3 nd, 7th & 14th day.

Haematological and Serological analysis

The blood was collected from the fishes by puncturing the heart by using 1ml insulin syringe. For serological analysis the collected blood were centrifuged at 2500 rpm for 14min. Total erythrocyte count (TEC) and Total leucocyte count (TLC) were carried out using Haemocytometer with improved Neubaurer ruling chamber (Weber & Sons England). Haemoglobin content was estimated by cyanomethomoglobin method(Hemocor-D, crest Biosystems). Blood smears stained with May-Grunewald's Giemsa stain was used for differential leucocyte count The data was analyzed statistically and students 't' test was used to test their significance. For serum protein estimation Gornall's biuret method was followed.

RESULTS

Experiment 1:

The values of TEC, TLC were higher in Immunostimulant incorporated diets and the increase was highly significant (P<0.05) on the 7th & 14th day when compared with standard feed pellets. (Table 1 & 2) DLC could not envisage marked differences however here and there fluctuations were found among the types of cells. (Table 3). Higher percentage of haemoglobin were estimated in immunostimulated fishes, than control fishes (6.2to 8g %). Serum protein level exhibited an increase from 0.4to 0.56g% in IS incorporated fishes (fig 1 & 2).

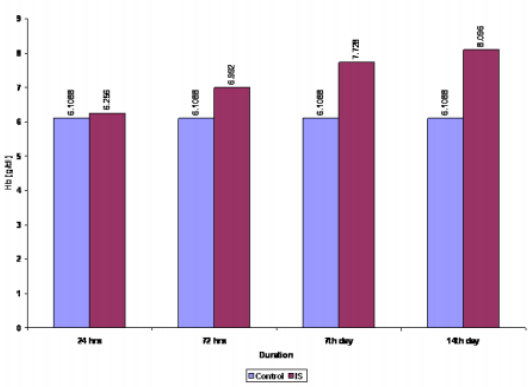

Figure 1: Hb content in C.catla Catla administered with control and IS diet

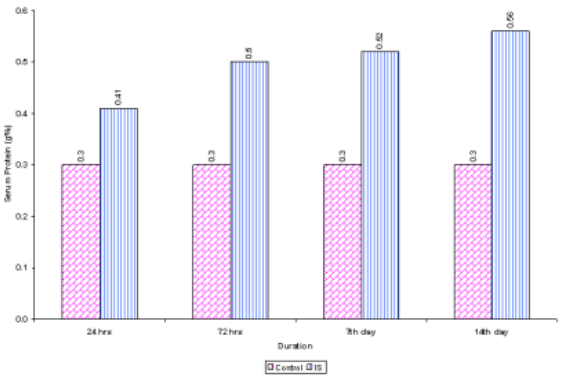

Figure 2: Serum Protein content in C.catla catla administered with control and IS diet

Table 1: TEC (Million cells/mm3) in relation to control and IS diet

Duration Days	Sample	RBC million/cells SD
1	C	0.726±0.5607
	IS	1.3±0.1315*
3	C	0.726±0.5607
	IS	0.796±0.1287*
7	C	0.726±0.5607
	IS	2.222±0.1519*
14	C	0.726±0.5607
	IS	2.456±0.095

P; * significant

Table 2 : TLC (Million cells/mm^3) in relation to control and IS diet.

Duration Days	Sample	WBC million/cells SD
1	C	6810±1125.78
	IS	15110±4027.54*
3	C	9810±1125.78
	IS	14570±654.67
7	C	9810±1125.78
	IS	39200±3520.51
14	C	9810±1125.78
	IS	45860±2810.76

P; * significant

Table 3 : DLC (%) in relation to control and IS diet

Duration Days	Dosage	Lymphocyte %	Monocyte %	Neutrophil %	Eosinophile %	Basophil %
1	C	46	32	11	8	3
	IS	57	15	19	5	4
3	C	52	30	10	5	3
	IS	57	17	21	2	3
7	C	50	18	18	3	11
	IS	60	15	13	7	5
14	C	69	9	13	3	6
	IS	58	20	10	6	6

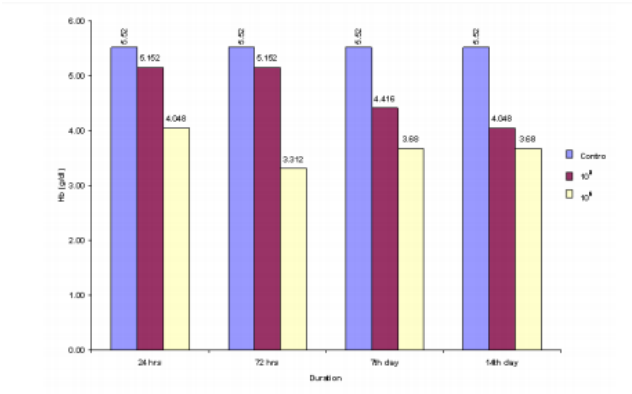

Figure 3: Hb content C.catla catla predministered with IS diet and post challenged with A. hydroph

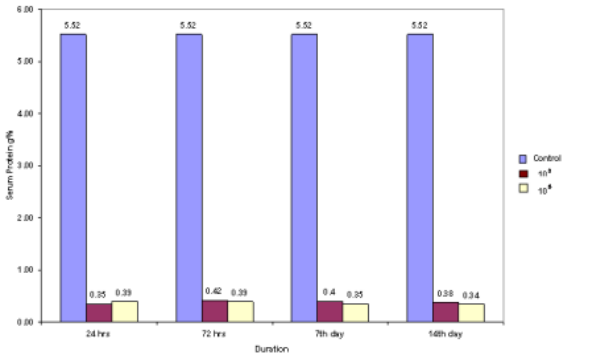

Figure 4: Serum protein C.catla catla preadministered with IS diet and postchallenged with A.hydrophila

Experiment – II

A significant decrease in TEC was noticed in IS fed fishes when infected with A.hydrophila at a dosage of both 10-3 & 10-5 cfu/ml. A significant increase in TLC was observed on 14th day (P<0.05) in both 10-3 & 10-5 cfu/ml in the IS incorporated fishes (Table 4 & 5). Lymphocytes and neutrophils showed an increase in C.catla catla fed with IS incorporated diet (Table 6) Hb content and serum protein level got decreased in experimentally infectedfishes in both 103 and 10-5 cfu/ml. (Fig 3 & 4).

Table 4: TEC (Million cells/mm3) pre administered with IS diet in catla catla and post challenged with Aero monas hydrophila

Duration Days	Dosage (cfu/ml)	RBC million/cells SD
1	C	0.65±0.030
	10^{-3}	0.61±0.0172*
	10^{-5}	0.56±0.030*
3	C	0.65±0.030
	10^{-3}	0.60±0.0172*
	10^{-5}	0.53±0.0215*
7	C	0.65±0.030
	10^{-3}	0.572±0.007*
	10^{-5}	0.506±0.014*
14	C	0.65±0.030
	10^{-3}	0.522±0.030*
	10^{-5}	0.50±0.026*

P; * significant

Table 5: TLC (Cells/mm3) pre administered with IS diet in catla catla and post challenged with Aero monas hydrophila

Duration Days	Dosage (cfu/ml)	RBC million/cells SD
1	C	8310±208.33
	10^{-3}	10680±235.80*
	10^{-5}	9040±241.79*
3	C	8310±208.33
	10^{-3}	11810±226.72*
	10^{-5}	11020±310.81
7	C	8310±208.33
	10^{-3}	13270±248.19
	10^{-5}	10350±202.42
14	C	8310±208.33
	10^{-3}	14680±211.19*
	10^{-5}	11930±128.84*

P; * significant

Table 6 : DLC (%)pre administered with IS diet in catla catla and post challenged with Aeromonas hydrophila

Duration Days	Dosage (cfu/ml)	Lymphocyte %	Monocyte %	Neutrophil %	Eosinophil %	Basophil %
1	C	36	32	11	8	3
	10^{-3}	46	18	30	4	2
	10^{-5}	44	20	19	9	7
3	C	46	32	11	8	3
	10^{-3}	52	11	28	5	4
	10^{-5}	55	12	18	9	6
7	C	46	32	11	8	3
	10^{-3}	56	12	22	5	5
	10^{-5}	59	13	20	4	4
14	C	46	32	11	8	3
	10^{-3}	55	28	12	3	2
	10^{-5}	60	15	10	9	6

SIGNIFICANCE OF THE STUDY

Dietary supplementation of immunostimulant can improve animal health and considerably reduce the

management costs. There is a possibility of using L. acidissima as a feed additive to considerably reduce the antibiotic/or disinfectant in intensive aquaculture farms. It is found that therapeutic potential of the L. acidissima fruit as dietary supplement would enhance the innate immunity of fish as it has reduced the mortality of C. catla catla after challenge with A. hydrophila.

Present analysis will highlight new understanding into immunostimulatory capacity of the L. acidissima incorporated in fish diet to avert disease outbreaks and improve the economic growth in the aquaculture industry. However, it remains for further work to validate the Limonia acidissima Fruit diet as immunostimulant using molecular analysis with special reference to immune gene expression patterns of C. catla catla.

CONCLUSION

From the above results, it is clear that Plumbago rosea acts as a potent immunostimulant, since it induces the blood parameters in the experimental fish catla catla. Recent studies revealed that the herbal extracts have a potential application as an immunostimulant. Medicinal plant is the unique source of various types of compounds having diverse chemical structure. Post challenge studies with Aeromonas hydrophila also provide positive immune potential of Plumbago rosea which enhance the non-specific immunity of the fish. Based on the results it is appropriate to conclude that the plant extract of Plumbago rosea may act as a potent Immunostimulant in fish.

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