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Research Article

Modulation of Immune Response in Edible Fish against *Aeromonas hydrophila*

Abstract

The present study was to obtain a basic knowledge of the hematology of *Catla catla* Post challenged with *Aeromonas hydrophila*. Fish were fed diets representing different supplementation levels of *Aegle marmelos* fruit extract. The various concentration of fruit extract were 10mg, 20mg and 30mg per 100g for each diet of fish. Supplementation of experimental feed after seven days collected from serum in treated fish to haematological parameters and biochemical parameters were analysed between control and experiment. The results of challenge test suggest that the fishes fed with 30mg *Aegle marmelos* diet had better immunostimulatory activity compared to the control group. Thus, our finding confirms our concentration that *Aegle marmelos* is a growth promoter and immunostimulant.

Introduction

Aquaculture is one of the important sectors contributing significantly in the Indian economy. Fish culturists are encouraged towards intensification of culture system to increase production and profit. In such practice of fish and shrimp farming, disease becomes major threats. Disease is one of the most important constraints of fish production both in culture system, as well as in wild condition [1]. For the last twenty years, the problem of microbial diseases has emerged as a major constraint to aquaculture industry. Increased disease occurrences have resulted due to the transfer of pathogenic organisms among cultured species of fish and shrimp, between different countries without proper quarantine measures. Due to this, the fish industry in India as well as other Southeast Asian countries has suffered significant economic losses [2]. Fish production is decreased due to the occurrence of disease caused by different pathogens in aquaculture. Aquaculture has been a tradition in several parts of Asia and according to FAO statistics, over 80% of fish produced by aquaculture come from Asia, where the production was 31.07 million metric tons valued at $ 38.855 billion [4]. *Aeromonas hydrophila* is a gram negative motile bacterium. The ulcerative disease is mostly caused by gram negative bacterium. *Aeromonas hydrophila* is pathogenic not only to fishes but also to amphibian, reptiles and mammals including man [5]. *Aeromonas* sp. is a ubiquitous inhabitant of aquatic ecosystems such as, freshwater, coastal water and sewage. These bacteria are usually microbiota as well as primary or secondary pathogens of fish and amphibians. Some motile species of *Aeromonas*, such as, *Aeromonas caviae*, *A.hydrophila* and *A.veronii* are opportunistic pathogens of humans. Among the species belonging to *Aeromonas* genus, one of the most important is *A. salmonicida*, a fish pathogen which causes a common disease among salmonids, named furunculosis or ulcerative furunculosis [6]. The Indian major carps, *Catla catla, Labeo rohita* and *Cirrhinus mrigala* are the most important commercial fishes in India with a maximum market demand and acceptability as food by the consumers due to their taste and flesh. They contribute about 67% of total freshwater fish production [7]. *Catla catla* and *Labeo rohita* contribute a major portion to the freshwater fish production in South India. The Indian major carp *Catla catla* mainly inhabits in rivers. It can also be easily cultured in ponds and lakes *Catla* is non-predatory and its feeding is restricted of the *Catla* enables its safe introduction into fish community including *Rohu, Mrigal*, etc. despite its reputations as a vegetarian species [8]. *Aegle marmelos*, a plant indigenous to India has been used by the inhabitants of the Indian subcontinent for over 5000 years. The leaves, bark, roots, fruits and seeds are used extensively in the Indian traditional system of medicine the Ayurveda and in various folk medicine to treat myriad ailments. Bael fruits are of dietary use and the fruit pulp is used to prepare delicacies like murabba, puddings and juice. Bael fruits are also used in the treatment of chronic diarrhea, dysentery, and peptic ulcers, as a laxative and to recuperate from respiratory affections in various folk medicines [9]. Hence, the present study has been carried out the Haematological studies on disease induced Indian major carp; Catla catla (L) fed with *Aegle marmelos* formulated diet.

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Materials and Methods

Alive and activity fishes (12± 1g) were collected from High-tech fish farm, Madurai, Tamil Nadu, India. The fishes were maintained in non-chlorinated at 20 day. The ground nut oil cake, fish meal and rice bran, tapioca, soybean, were mixed and sterilized. And then add a multivitamin tablet. The above mixed foods were added with different concentrations (1.0g, 1.5g and 2.0g) of plant extract prepared using shoxlet apparatus. These extract Melia azedarach extract used for experimental fishes and without plant extract diet for control fish. The food was made into small pellets. 0.1 ml of 10 CFU/ ml of Aeromonas hydrophila was injected intraperitoneally both for control and experimental. In every seven days following physiological studies such as

Survival and mortality

The survival and mortality rate was calculated by dividing the number of fish died to the total number of fish.

$$\text{Survival rate} = \frac{\text{Number of fish died}}{\text{Total number of fish}} \times 100$$

Erythrocyte count

Erythrocytes were counted by the method of Rusia and Sood [10] using haemocytometer.

Principle: The blood specimen is diluted with red cell diluting fluid which does not remove the white cells but allow red cells to be counted in a known volume of fluid. Finally, the number of cells in undiluted blood is calculated and reported as the number of red cells per cubic millimeter of whole blood.

Procedure: Blood was drawn in a clean RBC pipette up to its 0.5 mark. The tip of the pipette was wiped clean and dipped vertically into the red cell diluting fluid, which was then gently sucked up to mark 101. Then the tip of the pipette was closed with a finger and the contents were mixed thoroughly by shaking the pipette at right angles to its long axis. The red bead in the bulb helps for proper mixing of blood with the diluting fluid. The counting chamber of the haemocytometer was washed with distilled water, covered with a clean special cover glass and focused under a compound microscope. The ruled area of the haemocytometer was located clearly. Then the first drop of the fluid in the pipette was discarded by holding the pipette at 450 nm. The tip of the pipette was touched between the cover slip and the counting chamber and the diluted blood was applied by blowing. The blood was drawn into the chamber was left as such for 3 minutes to allow the cells to settle down.

Counting: The slide was first examined under low power and then under high power magnification. The counting chamber of the haemocytometer has a central heavy ruled area of 1 sq. mm. This central area is RBC counting chamber. It is divided into 25 squares and each square is sub-divided into 16 small squares. For the erythrocyte count, the cells falling with in and those touching the right and upper margin of the four corner squares and the central square (8.0 small squares) were counted. The total number of erythrocytes per cubic millimeter of whole blood was then calculated.

Calculation

$$\text{Erythrocytes} = \frac{\text{No. of erythrocyte X Dilution counted}}{\text{No. of Area counted X Depth of fluid Dilution}} (\text{million / cu.mm of blood})$$

Dilution - 200

Area counted - 5 X 0.04 = 0.2

Square mm Depth of fluid - 0.1 mm

Leucocyte count

Leucocytes were counted by the method of Rusia and sood [10] using haemocytometer.

Principle: Blood is diluted with acid solution which removes the red cells by haemolysis and also accentuates the nuclei of the white cells, thus the counting of the white cells become easy. Counting is done with a microscope under low power and knowing the volume of fluid examined and the dilution of the blood, the number of white cells per cubic millimeter in undiluted whole blood is calculated.

Procedure: Blood was drawn up to the 0.5 mark using a clean WBC pipette. Then the pipette was immediately kept in a watch glass containing WBC diluting fluid and it was drawn up to mark, taking care that no air bubbles included. The contents were mixed well by rotating the pipette between the palms of the hands. The white bead in the pipette helps for proper mixing of blood with the diluting fluid. The diluted blood was allowed to stand as such for 3 minutes for haemolysis of red cells to occur. Again the contents were mixed by rotating the pipette. After discarding the first few drops of diluted blood the counting chamber of the haemocytometer was charged with the fluid making sure that no air bubble were trapped between the cover slip and the chamber. The cells were allowed to settle down for a minute.

Counting: For the counting of leucocytes, the slide was examined under low power magnification of microscope. The neubaur's counting chamber is divided into two counting area which are ruled. Each counting chamber is divided into a total ruled area of 9 sq.mm. The area of each square is 1 sq.mm area of the 4 corner slide was used for the counting of leucocytes. The cells falling within the four corners square were counted and the total number of leucocytes per cubic millimeter of whole blood was calculated.

Calculation

$$\text{Leucocytes} = \frac{\text{No. of leucocytes X Dilution method}}{\text{No. of Area counted X Depth of fluid}} (1000 / \text{cu.mm of blood})$$

Dilution - 20

Area counted - 4 X 1 = 4 quare.mm

Depth of fluid - 0.1 mm

Estimation of Protein: The amount of protein present in the muscle tissue was determined colorimetrically following Lowry et al. (1951).

A standard solution of protein (Bovine serum albumin)

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at a concentration of 0.2mg/ml was prepared. One ml of the standard solution was taken in a test tube. 10 mg of muscles was isolated and homogenized with a mortar and pestle by adding 5 ml of 10% TCA and certifuged at 3000 rpm for 15 minutes. Then the precipitate was dissolved in 1 ml of 0.1N NaOH solution taken in the test tube. A blank was also prepared with 1 ml of distilled water.

To the test tube, 5.5 ml of reagent (50 ml of reagent A + 1 ml of reagent B) Reagent A-2% sodium carbonate in .1N NaOH reagent B-0.5% copper sulphate in solution in 1 % sodium potassium tartarate (freshly prepared).

After 15 minutes 0.5 ml of folin – cicolteau reagent was added. Blue color was developed and optical density was measured in photoelectric colorimeter at red filter after 20 minutes. The amount of protein in 10 mg of tissue was calculated by using the formula.

$$\text{The amount of Protein in 10mg of tissue } (\text{mg}/\text{g}) = \frac{\text{OD of the test sample} \times \text{the amount of BSA in}}{\text{OD of the standard BSA solution the Standard}}$$

Estimation of lipid: The amount of lipid present in the muscle tissue was determined calorimetrically following modified method of Bragdon (1951).

A standard solution of lipid was as taken in a test tube and to this 3 ml of 2 % potassium dichromate (in conc, sulphuric acid) and 3ml of distilled water were added. The optical density was measured in photo electric colorimeter at red filter.

10 mg of muscle was homogenized with a mortar and pestle in 5 ml of Bloor's mixture (Ether and Ethanol in 2:1 ratio) then the homogenate was centrifuged at 2500 rpm for 5 minutes. The supernatant was collected in a test tube. The pellet was washed with 10 ml of Bloor's mixture and centrifuged at 3000 rpm for 10 minutes. The supernatant were pooled and evaporated to dryness in a boiling water bath. The residue was dissolved in 1 ml of chloroform. A blank with 1 ml of chloroform was also prepared to the test tubes 5 ml of 2% potassium dichromate was added and mixed well.

Then O.D. of the sample was read in colorimeter using red filter. The amount of lipid present in 10 mg of muscle was estimated by using the following formula;

$$\text{The amount of lipid in 10mg of tissue mg}/\text{g} = \frac{\text{OD of the test sample} \times \text{the amount of cholesterol in}}{\text{OD of the standard solution the Standard}}$$

Results and Discussion

In this study the cumulative percentage of Mortality, RBCs, and WBCs, were studied in disease induced Catla catla using different concentration of *Aegle marmelos* formulated died against *Aeromonas hydrophila.* In control groups showed 60% mortality 10g of died fed groups was 20% mortality and 30g of fed diet no mortality in experimental groups. Similar result were observed by [11], reported that *Mikania cordata* leaf powder significantly increased non-specific immunity and decreased mortality in *C. catla* experimentally infected with *Aphanomyces invadans*. The *M. cordata* leaf powder supplemented diet showed significantly ($p<0.05$) high disease resistance against *A. invadans* infection when compared with control group [12]. The highest percentage survival was recorded in 20ppm (71.06%) followed by 10ppm (60.95%) and 30ppm (49.84%) groups. reported that the experimental groups of *C. carpio* administered with different dose of Cannon-ball tree, *Couroupita guianensis* plant extract treated fishes showed no mortality and 100% survival. This is due to the immunostimulant potential of plant extract. Also [13], reported that *A. hydrophila* (10^6 CFU/ml) injected fishes showed 89.47 % mortality and severe lesions and wound were noticed in the infected portions. The injured tails appeared reddish in colour and loss of skin layer was observed. The RBCs count in the control groups was found to be 5.83± 0.57 $x10^6$ cells/ml. The plant extract treated fishes showed the RBCs 6.10±0.63 $x10^6$ cells /ml (10g) 6.18 ±0.32 $x10^6$ cells /ml (20g) and 6.23±0.23$x10^6$ cells /ml (30g) in the initial day (0 day) (Table 1). The RBCs count was increased with increasing concentration of plant extract formulated diet in different day of treatment [7,14,21, 28,35]. Similarly result are also observed by the [14], reported that WBC and RBC counts were higher in *Labeo rohita* fingerlings fed *Mangifera indica* kernel when compared to control [15], reported that fish fed with herbs had significantly higher WBC and RBC counts compared to the control. [16], reported that mixed herbal extract supplementation diets the altered haematological parameters and triggered the innate immune system of goldfish against *A.hydrophila* infection. Studied that the serum protein, albumin, globulin, WBC, RBC and haemoglobin content were enhanced in fish fed herbal diets (*Solanum trilobatum* and *Ocimum sanctum*) against *Aeromonas* hydrophila [17]. In the present study the WBCs count was varied from both experimental and control fishes. The WBCs count in the control fishes showed 5.32±10.16×10^3 cells /ml and the plant extract formulated diet treated fishes showed maximum number of WBCs was observed. In 30g plant extract formulated diet found to be 5.95±0.43×10^3 cells /ml in the initial day (0day) and 6.72 ±0.64×10^3 cells /ml (35 day). Similar results were observed by Innocent et al. [18]. The WBCs count was increased with increasing concentrations of leaf extract of *Plumbago rosea* formulated diet treated with disease induced *Catla catla.* White blood cells afford protection against infectious agent caused by microbial and chemical factors. [19], reported the herbal diets could increase the hemoglobin content, WBC and RBC counts of fish in experimental groups compared to control group. In agreement with the finding, reported that WBC and RBC counts were higher in *Labeo rohita* fingerlings fed *Magnifera*

Table 1: Total RBC count (×10^6 cells /ml) of C.catla intraperitoneally injected with 0.1ml of 10^5 CFU / ml of *Aeromonas hydrophila* and treated with different concentrations of leaf extract of *Aegle marmelos.*

	Dose (mg)	Days after administration				
		7	14	21	28	35
Normal fish	0	5.83 ± 0.57	5.92 ± 0.62	6.10 ± 0.23	6.33 ± 0.12	6.54 ± 0.19
Control (*Aeromonasn hydrophila* treated fish)	0	5.9 ± 0.08	5.42 ± 0.28	5.26 ± 0.16	5.08 ± 1.00	4.92 ± 0.15
Experimental fish(*A. hydrophila* + *A. paniculata* treated)	10	6.10 ± 0.63	6.21 ± 0.05	6.46 ± 0.42	6.67 ± 0.09	6.82 ± 0.39
	20	6.18 ± 0.32	6.37 ± 0.53	6.68 ± 0.21	6.85 ± 0.62	6.98 ± 0.13
	30	6.23 ± 0.23	6.42 ± 0.62	6.83 ± 0.82	6.93 ± 0.08	7.18 ± 0.02

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Table 2: Total WBC count (×10³ cells /ml) of C.catlaintraperitoneally injected with 0.1ml of 10⁵ CFU / ml of *Aeromonas hydrophila*and treated with different concentrations of leaf extract of *Aeglemarmelos*

	Dose (mg)	Days after administration				
		7	14	21	28	35
Normal fish	0	5.65 ± 0.27	5.83 ± 0.34	6.08 ± 0.13	6.28 ± 0.09	6.42 ± 0.42
Control (*Aeromonashydrophila*treated fish)	0	5.32 ± 0.16	4.92 ± 0.28	4.80 ± 0.16	4.62 ± 1.00	4.43 ± 0.15
Experimental fish(*A.hydrophila + A.paniculata*treated)	10	5.90 ± 0.53	6.10 ± 0.19	6.36 ± 0.72	6.51 ± 0.13	6.62 ± 0.05
	20	5.93 ± 0.07	6.18 ± 0.62	6.23 ± 0.16	6.45 ± 0.53	6.58 ± 0.08
	30	5.95 ± 0.43	6.22 ± 0.63	6.47 ± 0.23	6.62 ± 0.09	6.72 ± 0.64

Table 3: Biochemical parameters of C.catla intraperitoneally injected with 0.1ml of 10⁵ CFU / ml of *Aeromonas hydrophila*and treated with different concentrations of leaf extract of *Aeglemarmelos*.

value	control	Control (Aeromonas hydrophila treated fish)	Aegle marmelos/100g diet		
			10 mg	20 mg	30 mg
Initial	20.62±0.20	19.32 ± 1.00	21.80 ± 0.43	22.72 ± 0.06*	23.87 ± 0.53*
Final	28.14±0.80	18.60 ± 0.57	29.23 ± 0.98*	31.33 ± 0.67	**33.7 ± 0.55***
Initial	0.69±0.63*	0.53 ± 0.72	0.74 ± 0.16*	0.87 ± 0.29*	0.97 ± 0.33*

indica kernel when compared to control. [20], reported that *Aloe barbadensis* formulated diet showed significant increase in white blood cells (WBC), lymphocytes and neutrophils after 21 days of feeding, while the highest monocyte counts among extract concentrations for this diet were shown after 15 days of feeding. [21], reported that *Andrographis paniculata* formulated diet treated fishes (*C.carpio*) showed white blood cells when compared to control. The protein level of control group was observed in minimum value as (20.62±0.20) & maximum level was observed as (23.87±0.53). The protein values of *Aegle marmelos* treated fishes was found to be 33.57±0.55 when compared with control and other experimental groups. [22], observed the similar results in *Oreochrombis niloticus* fed with *Allium cepa* and *chloromphenical*. The increased concentratios showed significant elevation in the plasma glucose, protein and lipid content respectively.

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