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 *Averonii* 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 fish community including *Cirrhinus mrigala*, *Labeo rohita*, *Labeo buccula* and *Catla catla*. 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.

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.
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.

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 mm. The tip of the pipette was touched 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

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

Depth of fluid - 0.1 mm

Description 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)
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 centrifuged 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 – cikoletteau 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:

\[
\text{The amount of Protein in mg / g} = \frac{\text{OD of the test sample} \times \text{the amount of BSA in OD of the standard BSA solution}}{\text{the standard}}
\]

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 in 5 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;

\[
\text{The amount of lipid in mg / g} = \frac{\text{OD of the test sample} \times \text{the amount of cholesterol in OD of the standard solution}}{\text{the standard}}
\]

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 diet against Aeromonas hydrophila. In control groups showed 60% mortality 10g of died fed groups was 20% mortality and 30g of feed 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 (10⁶ 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 lost of skin layer was observed. The RBCs count in the control groups was found to be 5.83±0.57 x10⁶ cells/ml. The plant extract treated fishes showed the RBCs 6.10±0.63 x10⁶ cells /ml (10g) 6.18 ±0.32 x10⁶ cells /ml (20g) and 6.23±0.23 x10⁶ 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±10.16×10³ 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±0.43×10³ cells /ml in the initial day (oday) and 6.72 ±0.64×10³ 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 (×10⁶ cells /ml) of C.catla intraperitoneally injected with 0.1ml of 10⁶ CFU / ml of Aeromonas hydrophila and treated with different concentrations of leaf extract of Aegle marmelos.

| Dose (mg) | Days after administration |
|----------|--------------------------|
| Normal fish | 7 | 14 | 21 | 28 | 35 |
| Control | 0 | 5.83±0.57 | 5.92±0.62 | 6.10±0.23 | 6.33±0.12 | 6.54±0.19 |
| (Aeromonas hydrophila treated fish) | 5.9±0.08 | 5.42±0.28 | 5.26±0.16 | 5.08±1.00 | 4.92±0.15 |
| Experimental fish | 10 | 6.10±0.63 | 6.21±0.05 | 6.46±0.42 | 6.67±0.09 | 6.82±0.39 |
| (A. hydrophila + A. paniculata treated) | 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 |
Table 2: Total WBC count (<10⁵ cells /ml) of C. catla intraperitoneally injected with 0.1ml of 10⁵ 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 | 5.65 ± 0.27               | 5.83 ± 0.34              | 6.08 ± 0.13              | 6.28 ± 0.09              | 6.42 ± 0.42              |
| Control (Aeromonas hydrophilatreated fish) | 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. paniculatreated) | 5.90 ± 0.53               | 6.10 ± 0.19              | 6.36 ± 0.72              | 6.51 ± 0.13              | 6.62 ± 0.05              |
|           | 5.93 ± 0.07               | 6.18 ± 0.62              | 6.23 ± 0.16              | 6.45 ± 0.53              | 6.58 ± 0.08              |
|           | 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 Aegle marmelos

| Value     | Control (Aeromonas hydrophilatreated 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 compared with control and other experimental groups. [22], observed the similar results in Oreochromis niloticus fed with Allium cepa and chloromphenical. The increased concentrations showed significant elevation in the plasma glucose, protein and lipid content respectively.

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