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Differences in Haematological Parameters in Normal, Infected and Immune-Primed Fingerlings of Red Tilapia (*Oreochromis mossambicus x Oreochromis niloticus*)

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ABSTRACT : Infectious diseases are always a major problem causing heavy loss to the fish farmers. The fingerlings of red tilapia (*Oreochromis mossambicus x Oreochromis niloticus*) were fed with extracts prepared either by acid hydrolysing process or ethanolic extracts from oyster (*Saccostrea cucullata*; Born, 1778) and seaweed (Sargassum baccularia; Mertens). These extracts were mixed at different concentrations in commercial diet and fed to the fingerlings till 15 days. All groups of fishes fed with experimental and normal diets were challenged by a gram-negative bacterium (*Aeromonas hydrophila*) to see the effects of pathogen. Fishes fed with experimental feed consisting of ethanolic extract of oyster showed better survival as compared to other experimental feeds. Maximum total leucocytes ($22 \pm 43 \times 10^3$ cell/mm^3) and erythrocytes ($32 \pm 33 \times 10^6$ cells/mm^3) were recorded in fishes fed with ethanolic extract of oyster ($P < 0.05$) which were similar to fishes of control group. Significant ($P < 0.05$) changes were also observed in the haematological parameters such as; lymphocyte, neutrophil, monocyte and basophil cells in feed incorporated with ethanolic extract of oyster. Similarly, higher values of albumin; amylase; total cholesterol; GGT; glucose; phospholipids; triglycerides; total serum protein; lactase, LDH and lipase in the infected fish were observed as compared to the control group ($p < 0.05$). Fishes fed with ethanolic extract showed relatively much closer values as observed in control group. Our results strongly suggest that ethanolic extract of oyster (*S. cucullata*) could combat the microbial infection by stimulating the non-specific immune response effectively at fingerlings stage of red tilapia.

Keywords : Haematological parameters normal, infected, immune-primed fingerlings, red tilapia.

INTRODUCTION

Intensification of culture systems has resulted outbreak of many diseases causing very high economic losses especially to tilapia industry in recent years. The cultivated fishes in enclosure and cages become more susceptible not only to pathogenic but also to the opportunistic bacteria. Although development of disease resistant strains of fish has broad appeal to fish culture but this area is less explored as compared to the development of new vaccine and antibiotics. Generally vaccination enhances specific immune responses of the fish which has been proved to be one of the most effective methods for controlling different pathogenic diseases in commercially important fishes (Andro *et al.* 2008). However, there have always been several limitations in using vaccines in fishes as a single vaccine is effective against only one type of pathogen (Murrey *et al.* 2003); Gopalakannan and Arul (2006). Secondly immunization of young fish by vaccines is a difficult exercise in hatchery practices (Kaattari and Piganelli 1997). Additionally, use of these chemicals always creates problems with emergence of drug resistance strains of bacteria which further resulted in severe toxicity and considerable accumulation of these chemicals both in fish and in environment (Citarasu *et al.* 2002). Sagdic and Ozcan 2003). It is also true that for many pathogens like *Aeromonas hydrophila*, no effective vaccine has been developed so far due to their heterogeneity.

The use of natural products, like plant extracts in controlling fish diseases is new and emerging field which needs further researches to find out the most effective measures to replace chemotherapy (Sivaram 2004). A number of plant extracts have been screened and used with encouraging results in controlling bacterial and viral diseases in fishes. Pachanawan *et al.* (2008) have tested extracts prepared from fourteen herbs against *A. hydrophila* infection in tilapia (*Oreochromis niloticus*). They found that the ethanolic extract of *Psidium guajava* showed the highest antimicrobial activity. Increased survival and resistance to White Spot Syndrome Virus (WSSV) infection in black tiger shrimp, *Penaeus monodon* feeding with immune-stimulant herbal supplemented diets has successfully been demonstrated by Citarasu *et al.* (2006). In a similar manner, dietary supplementation of Achyranthes aspera seed has been found to stimulate the immunity and enhancing resistance against *Aeromonas hydrophila* infection in fingerlings of *Labeo rohita* (Rap *et al.* 2006). Considering all these problems and above mentioned constraints, an attempt thus was made primarily to incorporate different extracts of seaweed and oyster in commercial feed with ultimate aim to produce disease resistant progeny of red tilapia with enhanced immunity and better survival.

MATERIAL AND METHODS

Extracts from seaweed and oyster were prepared following solvent extraction and hydrolyzing processes as described by Chatterji *et al.* (2002). For experimental feed, pellets of commercial feed (Highashi) were ground to make a uniform powder. The freeze dried seaweed and oysters extracts (ethanolic and hydrolysate) were mixed thoroughly in this powder of commercial feed at the ratios of 2.5, 5 and 7.5% seaweed and oyster hydrolysates and 2.5% ethanolic extracts. The extract incorporated feeds were re-pelletized using a table top pelletizer.

The bacterial isolate (*A. hydrophila*) maintained in the laboratory was scaled up in nutrient broth from fresh agar petri-plates following standard protocol. After incubation at 37°C for 24 hours, the culture was harvested by centrifugation (5000 rpm for 3 min) and re-suspended in PBS.

Fingerlings of red tilapia (10-15 g) were divided into three groups consisting of 20 healthy individuals in each group. The first group (Group-A) was treated as control group where fishes were fed two times with the normal diet (without extracts) at the rate of 6% of the body weight till the completion of the experiment. Fishes of this group were injected with only 100 µl PBS, intramuscularly, using a 1 ml insulin syringe on the day 15 of the culture period. In the second group (Group-B), fishes were injected intramuscularly with 100 µl of bacterial suspension on day 15 and these fishes were allowed to feed commercial feed (Highashi) two times in a day till the completion of the experiment. In the third group (Group-C- experimental or immune-primed group), the fingerlings of red tilapia were initially fed with experimental feed (6% of biomass at two times in a day) for 15 days and then 100 µl of bacterial suspension was injected intramuscularly on day 15. Fishes of control and immune-primed groups were fed with commercial feeds throughout the experimental period. All fishes were maintained in 100 l fiber tanks with continuous aeration at room temperature (27-30ºC) in the hatchery for 90 days with daily exchange of freshwater. All these experiments were conducted in triplicates. The fingerlings were checked twice daily for the appearance of clinical sign of diseases and mortality, if any. The dead animals were removed immediately and data recorded to assess the survival rates among each group separately.

After 30th day of the experiment when fishes started showing about 60% mortality in infected group, blood samples from all groups of fishes were withdrawn from the lateral line just near to the caudal peduncle of the fish with the help of a sterile needle fitted with a 2 ml hypodermal syringe. All blood samples were subjected for thin blood smear and stained with corresponding diluting fluids for leucocytes and erythrocyte counts and Wright's staining for differential blood cells counts. For leucocytes and erythrocyte counts, 20 µl blood of fish was mixed with 3980 µl of corresponding diluting fluids (200 µl of glacial acetic acid mixed in 10 ml distilled water for leucocytes and 3 g sodium citrate mixed with 1 ml formalin and making the solution to 100 ml for erythrocyte counts). The cell counting was performed using a Neubauer's cells counting chamber.

All slides with freshly prepared smears and air dried completely were placed in methanol for exactly 30 seconds and then Wright stain was poured for duration of 3 minutes. Immediately after 3 minutes, Wright stain was removed with the help of a pipette and oxidizing solution containing Wright Stain and Wright Stain Buffer mixture was put replacing Wright stain from the slides. The slides were then allowed to stand for 6 minutes and observed for appearance of metallic sheen on top of the slides. The slides were placed in Wright Stain Buffer for 1.5 minutes followed by air drying of the slides. The slides were examined with immersion oil using an advanced research microscope (ARM), Nikon Eclipse 80*i* (USA) under 100 X magnifications and micro-photographs were taken for further analysis.

After proper Wright staining, the poly-morphonuclear neutrophil cells were looked with dark blue nuclei, reddish lilac granulates and pale pink cytoplasm. Similarly, the eosinophil cells were with blue nuclei, red to orange-red granules and blue cytoplasm whereas basophil cells with purple to dark blue nuclei and dark purple granules. The lymphocytes and monocyte cells were with dark purple nuclei and sky blue cytoplasm. Number of different cells in one microscopic field was counted independently and the same procedure was repeated for 20 times. The average number of each blood cell were then calculated accordingly.

The blood samples from all groups of fishes were withdrawn separately from the lateral line just near to the caudal peduncle of the fish with the help of a sterile needle fitted with a 2 ml hypodermal syringe. The blood was then immediately transferred into 2 ml eppendorf micro test tubes (Eppendorf Biopur). The micro test tubes were then allowed to stand for 1 hour at 37°C allowing the blood to clot. The samples were further left at 4°C overnight to allow the clot to contract. This was followed by losing the clot from the sides of the micro tube with the help of a glass pasteur carefully. Utmost care was taken for not allowing the red cells to lyse. The serum was then separated by centrifugation at 4000 rpm for 20 minutes at 4°C temperature. The serum was then gently pipetted off into another clean micro test tubes using a glass pasteur. The tubes were then appropriately labeled and stored at –20°C for further analysis.

Biochemical parameters such as albumin, amylase, total cholesterol, gamma-glutamyl transferase, glucose, phospholipids, triglycerides, total serum protein, lactase and lactase dehyrogenase in control, infected and immune-primed fish were analyzed using a 902 Automatic analyzer (Hitachi Boehringer Mannheim Japan). Detailed steps for preparation of samples and biochemical analysis were followed according to the Technical manual of Roche specifically prepared for Automatic Serum Analyzer (Roche Diagnostic GmbH, Technical manual, 2007: 1-800-4282336).

All data collected for different groups of fishes were analyzed by running the General Linear model program available in SAS software. Differences between means were compared using Duncan's multiple range tests to find the significance at 5% ($p < 0.05$) level.

RESULTS

Survival of the fishes till 90th day was observed in fishes fed with different concentrations of hydrolysate (2.5, 5 and 7.5%) prepared from seaweed and oyster and among infected fishes by *A. hydrophila.* In control (normal) group, the survival was 85% at 90th day of the experimental period. However, the infected fishes started showing mortality from the day 20 after the introduction of the infection and on day 90, only 15% survival was observed (Table 1). The survival of fishes fed with seaweed hydrolysate at the ratios of 5 and 7.5% also showed mortality on 50th day onward and till the day 90, the survival was only 35 and 40% respectively (Table 1). This confirmed that though seaweed hydrolysate showed some activity but it was not very significant ($p > 0.05$). The oyster hydrolysate (7.5%) relatively showed better activity as compared to seaweed. The feed incorporated with 7.5% oyster hydrolysate showed survival rate of 50% on the day 90 (Table 1).

The feed incorporated with 2.5% of both seaweed and oyster ethanolic extracts showed better survival in experimental groups. The survival of fishes fed with ethanolic seaweed extract showed 55% survival at the end of 90th day of experiment whereas ethanolic oyster extract showed 90% survival during the period of the experiment. In fishes of control group, the survival was 85% till the completion of the experiment (Fig. 1). The infected fishes showed high mortality starting from day 30 and on 90th day, only 15% survival was observed in this group (Fig. 1).

Table 1: Survival data of healthy (control), infected by *A. hydrophila* and immune-primed fishes.

Survival (%)		*Days*									
		0	10	20	30	40	50	60	70	80	90
Groups Control		100	100	100	90	90	90	90	85	85	85
Infected Fishes		100	100	40	25	20	15	15	15	15	15
Seaweed	2.5% (HL)	100	100	75	60	40	30	30	25	25	25
	5.0% (HL)	100	100	80	65	50	50	40	35	35	35
	7.5% (HL)	100	100	80	70	60	50	45	40	40	40
Oyster	2.5% (HL)	100	100	90	80	45	45	40	35	25	25
	5.0% (HL)	100	100	95	80	60	60	50	50	45	45
	7.5% (HL)	100	100	95	95	90	80	60	60	55	50

[HL = Hydrolysate]

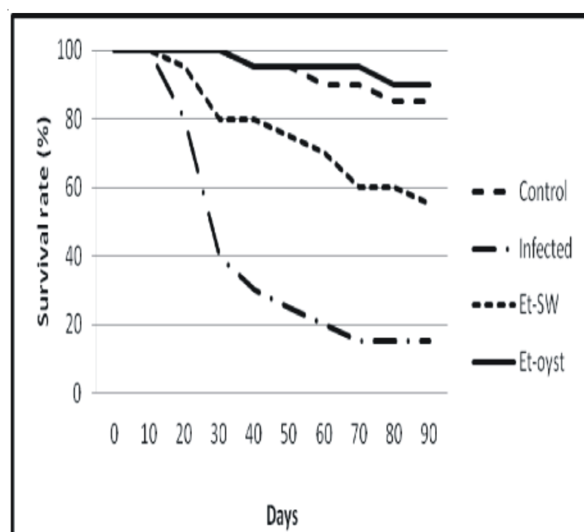

Fig. 1. Survival data of fishes in different groups after 90th days of experiment.

[ET-SW = Ethanol seaweed; ET-oyst = Ethanol oyster]

Table 2 : Total leucocytes and erythrocyte counts in the blood of healthy (control), infected with *A. hydrophila* and immune-primed fish.

Groups		*Total Leucocyte count* (× 10^3 *cells/mm*^{3})	*Total Erythrocyte count* (× 10^6 *cells/mm*^{3})
Control		24 ± 3	38 ± 3
Infected Fishes		18 ± 9	20 ± 8
	2.5% (HL)	18 ± 4	22 ± 6
	5.0% (HL)	20 ± 5	23 ± 4
Seaweed	7.5% (HL)	21 ± 3	22 ± 4
	2.5% (HL)	18 ± 3	22 ± 7
	5.0% (HL)	19 ± 2	23 ± 5
Oyster	7.5% (HL)	20 ± 4	24 ± 4

[HL = Hydrolysate]

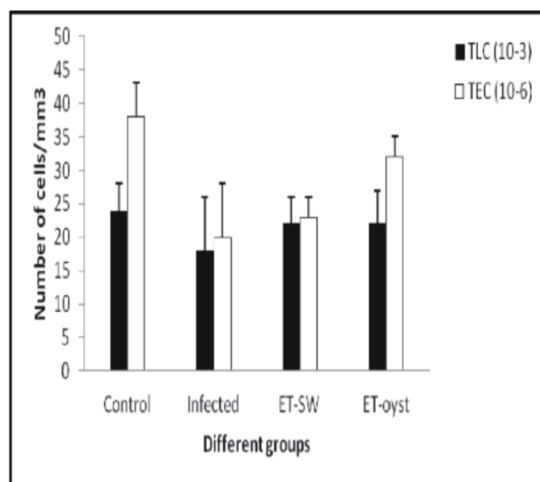

Fig. 2. Total leucocytes and erythrocytes counts in control, infected and immune-primed fishes.

[(ET-SW = Ethanol Seaweed, ET-oyst = Ethanol Oyster; TLC = Total leucocytes count ($\times 10^3$); TEC = Total erythrocytes counts ($\times 10^6$)]

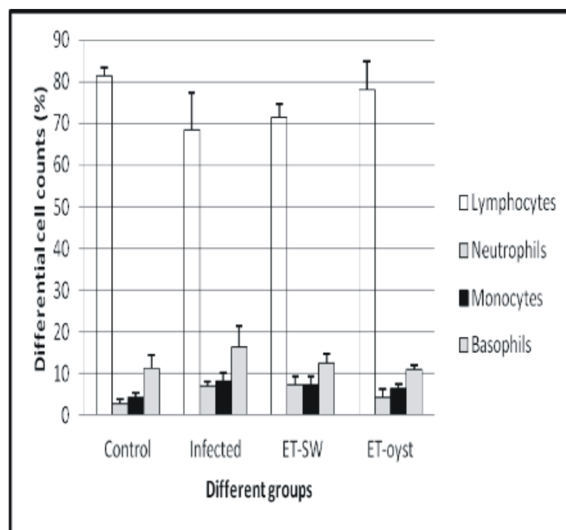

Fig. 3. Percentages of lymphocytes, neutrophils, monocytes and basophils counts in control, infected and immune-primed fishes.

(ET-SW = Ethanol Seaweed, ET-oyst = Ethanol Qyster)

The total leucocytes and erythrocytes counts in the blood of control, infected and immune-primed fishes fed with different concentrations of experimental feeds consisting of seaweed and oyster hydrolysate (2.5, 5.0 and 7.5%) are summarized in Table 2. In this experiment it was again evident that both seaweed and oyster hydrolysates were working on concentration depending manner where significant increase ($p < 0.05$) in leucocyte counts were observed in the experimental feed incorporated with 7.5% hydrolysate prepared from both the organisms (Table 2). Similarly the erythrocyte counts were also significantly higher ($p < 0.05$) in fishes fed with feed incorporated with 7.5% of the hydrolysates of both the organisms. These values were very close to fishes of control groups. Fishes fed with feed incorporated with 2.5 and 5% of hydrolysate did not show much significant increase in cell counts in experimental groups ($p > 0.05$). In infected fishes, the values of leucocytes ($18 \pm 9 \times 10^3$ cells/mm^3) and erythrocytes ($20 \pm 8 \times 10^6$ cells/mm^3) were significantly low ($p < 0.05$) as compared to fishes of control groups with $24 \pm 3 \times 10^3$ cell/mm^3 (leucocytes) and $38 \pm 3 \times 10^6$ cells/mm^3 (erythrocytes) (Table 2).

In fishes fed with 2.5% of seaweed and oyster ethanolic extracts, a maximum total leucocytes and erythrocytes counts were recorded in fishes fed with ethanolic extract of oyster ($p < 0.05$). The leucocytes and erythrocytes were; $22 \pm 43 \times 10^3$ cells/mm^3 and $32 \pm 33 \times 10^6$ cells/mm^3 respectively (Fig. 2). These values were much closed to fishes of control groups as evident in Fig. 2. The present results clearly indicated that the ethanolic extract of oyster was significantly effective ($p < 0.05$) in marinating the blood profile of the immune-primed fishes in terms of leucocytes and erythrocytes counts.

The percentages of lymphocytes, neutrophils, monocytes and basophils were evaluated in control, infected and immune-primed fishes fed with 2.5, 5 and 7.5% of seaweed and oyster hydrolysates. The percentage of lymphocytes decreased significantly ($P < 0.05$) with an increase in the percentages of neutrophil, monocytes and basophils ($P < 0.05$) in the infected fishes as compared to control fishes. Fishes fed with 2.5% of hydrolysates from both the species did not show much variation as compared to infected fishes ($P > 0.05$) (Table 3).

Table 3 : Immunological parameters of healthy (control), infected with *A. hydrophila* and immune-primed fish.

Groups		*Lymphocytes (%)*	*Neutrophil %*	*Monocytes %*	*Basophils %*
Control		81.42 ± 2	2.85 ± 1	4.28 ± 1	11.42 ± 3
Infected Fishes		65.36 ± 9	8.14 ± 3	9.16 ± 3	17.36 ± 5
	2.5% (HL)	68.75 ± 7	8.33 ± 3	6.25 ± 2	15.62 ± 2
Seaweed	5.0% (HL)	71.08 ± 5	3.61 ± 2	8.43 ± 3	16.83 ± 3
	7.5% (HL)	71.27 ± 3	4.25 ± 3	8.51 ± 2	14.89 ± 2
	2.5% (HL)	70.00 ± 8	7.00 ± 5	11.00 ± 2	11.00 ± 2
Oyster	5.0% (HL)	68.36 ± 5	7.14 ± 3	8.16 ± 3	16.36 ± 3
	7.5% (HL)	71.57 ± 5	7.36 ± 4	7.36 ± 2	12.63 ± 2

[HL = Hydrolysate)

The efficiency of feed incorporated with 2.5% ethanolic extracts of seaweed and oyster was further evaluated on control, infected and fishes fed with experimental feed. The results clearly showed that the percentages of lymphocytes, neutrophils, monocytes and basophils did not change much after challenging fishes by A. hydrophila which further confirmed that fishes fed with ethanolic extract of oyster showed relatively higher immunity as compared to other extracts (Fig. 3).

There was a significant increase ($P < 0.05$) in albumin; amylase; total cholesterol; GGT; glucose; phospholipids; triglycerides; total serum protein; lactase, LDH and lipase in infected fish as compared to the control group (Table 4). Similarly, fishes fed with 2.5, 5 and 7.5% of hydrolysate extracts also showed relatively higher values as compared to control group (Table 4).

In another experiment, 2.5% of ethanolic extracts prepared from seaweed and oyster were used to compare the efficacy of these feeds using different immunological parameters. Similar higher values of albumin; amylase; total cholesterol; GGT; glucose; phospholipids; triglycerides; total serum protein; lactase, LDH and lipase in the infected fish were observed as compared to the control group ($p < 0.05$). Fishes fed with ethanolic extract showed relatively much closer values as observed in control group (Table 5). These results thus clearly indicated that a feed incorporated with ethanolic extract of oyster could combat the microbial infection by stimulating the immune response in red tilapia.

DISCUSSION

Fish diseases are a great threat to economic viability of any aquaculture practices. In advanced aquaculture practices, several antibiotics, vaccines and chemotherapeutic agents as well as some immune-stimulants have been used to prevent the spread of viral, bacterial, parasitic and fungal diseases (Priya *et al.* 2004); Kumar *et al.* 2005). Immunity in fish unlike any other vertebrates plays an important role in protecting the fish against many types of pathogens. In fish, immunity can either be non-specific type which is an innate defensive mechanism, or an acquired specific immunity. One of the most promising methods of controlling diseases in aquaculture is strengthening the defense mechanisms of fish through prophylactic administration of

Table 4 : Different immunological parameters of healthy (control), infected with *A. hydrophila* and immune-primed fishes (blood serum analysis) fed with different experimental feeds.

Groups	*Alb (g/l) b*	*Amyl (U/l)*	*Chol. (mmol/1)*	*GGT (mmol/1)*	*Glu (mmol/1)*	*Phos (mmol/1)*	*Trig (mmol/1)*	*T. Prot (g/l)*	*Lact (mmol/1)*	*LDH (U/l)*	*Lipase (U/l)*
Control	8.7	<3	2.80	<3	3.2	3.34	1.43 1.43	33.9 9	0.80	1482	7.8
Infected Fishes	20.3	>3	7.98	>3	4.8	7.99	15.43	54.4	3.36	7288	23.8
Seaweed 2.5% (HL)	15.4	>3	5.18	<3	4.1	6.60	9.84	51.3	2.82	4875	23.8
5.0% (HL)	13,5	<3	4.88	<3	3.8	6.34	5.18	46.9	1.43	3521	15.0
7.5% (HL)	12,2	<3	3.07	<3	3.7	6.00	4.24	44.5	1.29	2621	11.0
2.5% (HL)	14.3	>3	4.69	>3	3.9	5.60	7.82	54.4	3.36	5950	10.1
5.0% (HL)	10.8	<3	4.84	<3	3.8	4.44	5.53	46.9	1.32	2577	11.4
7.5% (HL)	11.5	<3	3.07	<3	3.6	5.52	2.10	40.8	1.29	1552	16.2

[HL = Hydrolysate; Alb = Albumin; Amyl = Amylase; Chol = total cholesterol; GGT = Gamma-glutamyl transferase; Glu = Glucose; Phos = Phospholipids; Trig = triglycerides; T. Prot = Total serum protein; Lact = Lactase; LDH = Lactase dehyrogenase]

Table 5 : Different immunological parameters of healthy (control), infected with *A. hydrophila* and immune-primed fishes fed with 2.5% of hydrolysates and ethanolic extracts prepared from seaweed and oyster.

Groups	*Alb (g/l) b*	*Amyl (Ul)*	*Chol. (mmol/1)*	*GGT (mmol/1)*	*Glu (mmol/1)*	*Phos (mmol/1)*	*Trig (mmol/1)*	*T. Prot (g/l)*	*Lact (mmol/1)*	*LDH (U/l)*	*Lipase (U/l)*
Control	8.9	<3	2.85	<3	3.4	3.44	1.46	34.9	0.80	1582	8.2
Infected Fishes	21.4	>3	8.28	>3	5.8	8.19	16.42	53.4	3.24	8288	24.2
ET-SW (2.5%)	12.8	<3	3.86	<3	4.4	5.22	3.40	41.8	1.18	2125	9.2
ET-oyst (2.5%)	9.6	<3	3.02	<3	3.6	3.24	1.40	34.5	0.85	1550	7.7

(ET-SW = Ethanol Seaweed; ET-oyst = Ethanol Oyster; Alb = Albumin; Amyl = Amylase; Chol = total cholesterol; GGT = Gamma-glutamyl transferase; Glu = Glucose; Phos = Phospholipids; Trig = triglycerides; T. Prot = Total serum protein; Lact = Lactase; LDH = Lactase dehyrogenase]

immune-stimulants (Robertsen 1999). Several plant materials/ products such as *Eclipta alba* (Christybapita *et al.* 2007), *Aloe vera* (Kim *et al.* 1999), *Ocimum sanctum*(Logambal 2000), *Viscum album*, *Urtica dioica* and *Zingiber officinale* (Dugenci 2003), *Solanum trilobatum* (Divyagnaneswari 2007), *Astragalus radix* (Yin *et al.*, 2006), *Scutellari radix* and *Achyranthes aspera* (Vasudeva and Chakrabarti 2005*a*, 2005*b*) have been reported to enhance the immunity of fish.

The principal objective of this present study was to evaluate the effect of extracts prepared from seaweed and oyster which was added to commercial diet to examine the immunological, serum biochemical and blood parameters of red tilapia challenged against A. hydrophila infection. The results of the present study clearly indicated that dietary oyster ethanolic extract supplementation could significantly ($p < 0.05$) enhance the resistance in red tilapia against A. hydrophila infection. We assumed that the survival of fishes fed with ethanolic extract of oyster might be responsible in probably enhancing the non-specific immune system in red tilapia. Sahu *et al.* have also reported higher survival rates in *Labeo rohita* fed with the diets containing *Magnifera indica* kernel (a kind of herb) and challenging the fish with *A. hydrophila. Tilapia* (*Oreochromis niloticus*) fed with two Chinese medicinal herbs and challenging fishes with *A. hydrophila* showed similar results with a high survival rates as observed by Ardo *et al.* (2008). The survival rates in tilapia (*Oreochromis niloticus*) fed with diets containing either dry leaf powder of Psidium guajava or ethanol extract of leaf *P. guajava* plant and then challenging the fish with *A. hydrophila* showed much higher survival Sahu *et al.* (2007).

In the present investigation, commercial feed incorporated with ethanolic extracts prepared from seaweed and oyster showed better activities. Relatively, highest activity was recorded in the ethanolic extract of oyster in immune-primed fishes fed with this feed and showed comparatively increased hemoglobin content, WBC and RBC counts. In agreement with the present findings, WBC and RBC counts were also found higher in *Labeo rohita* fingerlings fed with *Magnifera indica* kernel as compared to fish of the control group as observed by Sahu *et al.* (2007). Similar observations have been made by Gopalakannan and Arul (2006) who reported an increase in WBC count after feeding the common carp with immune-stimulants like chitin. A study conducted in detail on long-term administration of different dosages of levamisole on growth, immune response and disease resistance against *Aeromonas hydrophila* and Edwardsiella tarda in *Labeo rohita* fingerlings by Misra *et al.* 25. As observed by other investigators, fishes administered with low and medium dosages of levamisole were showing higher WBC count significantly ($P < 0.05$) compared to control group. These researchers have also observed that fishes for all the assay days except on 42nd day when the group of fish was fed with highest dose of 500 mg/kg diet, a significant increase in WBC count was noticed as compared to control fish.

In the present study, the percentage of lymphocytes was significantly low ($P < 005$) with higher percentages of neutrophil, monocytes and basophils in the infected fishes as compared to fishes of control group. However, fishes fed with ethanolic extract of oyster for 90 days did not show much change in percentages of lymphocytes, neutrophils, monocytes and basophils which were recorded same as compared to control group. This further confirmed that fishes fed with ethanolic extract of oyster acquired significant immunity where introduction of infection through *A. hydrophila* did not affect the differential cell counts. Rudneva (1997) observed that the overall number of leucocytes varies in number from species to species for example, the normal range of lymphocytes in the salmonid and Rainbow Trout (*Oncorhynchus mykiss*), is between 7.8 and 20.9×10^3 cells/mm^3.

It is generally believed that the immunization of fishes at early stages requires only a short duration of treatment that reduces the cost of treatment by other known methods, considerably. The immune system of fingerlings of red tilapia fed with ethanolic extract of oyster could have been activated as it was evident by the increased percentages of leucocytes which are considered to be the main scavenger cells in defence system (Mulero *et al.* 1998); Kumari and Sahoo 2006). A similar result was reported by Chandran *et al.* (2006) in Catla catla, *Labeo rohita* and *Cirrhinus mrigala* where fishes were immune-primed against *A. hydrophila*. The total leucocyte count in the normal catfish was found to be 21×10^3 cells/mm^3 whereas in the bacteria injected catfish it 20×10^3 cells/mm^3 at 24 hrs post infection and 18×10^3 cells/mm^3 at 96 hrs post infection. In the present study, for differential leucocyte count, the lymphocyte percentage was higher followed by monocytes and basophils. A higher percentage of lymphocyte (78.32 + 5%) was observed in the present study. However studies carried out on perch, the percentage of lymphocyte was $99.1 \pm 0.99\%$ as compared to low percentage ($84.2 \pm 5.08\%$) in rainbow trout (Kumari and Sahoo 2006). Monocytes have been reported to be sporadic in carp, Tench, European catfish, rainbow trout, bream and perch. Nonetheless, in the present study, a significant number of monocytes (11.5 ± 1.15) were recorded. Granulocytes were mostly represented by neutrophils. Siwicki (1989) further observed increased leucocyte count till 12 weeks after feeding with levamisole for 15 days. The administration of 2.5% ethanolic extract could significantly increased the phagocytic cells in engulfing bacteria which could have been measured in terms of phagocytic ratio. However, due to limited facilities and time constraint, this particular aspect was not addressed in the present study.

Fishes injected with *A. hydrophila* revealed significant changes ($p < 0.05$) in the biochemical parameters of the

blood plasma such as albumin; amylase; total cholesterol; GGT; glucose; phospholipids; triglycerides; total serum protein; lactase, LDH and lipase. Glucose level was found to increase significantly (5.8 mmol/l) in infected fishes. The total protein and lactase levels were also found to increase in bacteria injected fish. Maximum increase in LDH and Lipase was recorded in fishes challenged with bacteria. Similar observations have been made in several other fishes (Chandran *et al.* 2002). Rehulka (1998) reported that *Aeromonas* induced ulcerous dermatitis in rainbow trout *Oncorhynchus mykiss* which has been found to result in an increase in total protein and cholesterol levels in the plasma as observed in the present study. On the contrary, Waagbo *et al.* (1988) reported that the level of protein, albumin, triglycerides and cholesterol were all significantly reduced in infected fish as compared to control fish.

The short dietary administration of ethanolic extract did not result any disturbances in physiological functions such as metabolism as indicated by the reduced glucose level, the secondary response to any stressor, throughout 15 days of immune-modulation trial period. However feeding red tilapia fingerlings with supplemented diets containing oyster extracts enhanced total plasma protein, albumin and globulin values in immune-primed groups. Similar observations have been made in *C. carpio* where feeding fishes with diets containing herbal plant extracts enhanced the total plasma protein, albumin and globulin values in treatment groups (Rudheva, 1997). Sahu *et al.* (2007) also reported that serum protein, albumin and globulin levels in *L. rohita* fingerlings fed with *Magnifera indica* kernel were higher than control. Rao *et al.* (2006) found exactly the same results after feeding rohu fingerlings (*Labeo rohita*) with Achyranthes aspera seeds. As the serum proteins include various humoral elements of the non-specific immune system, high concentrations of total serum protein, albumin and globulin might be due to the enhancement of non-specific immune response of fishes fed with ethanolic extract of oyster.

The results of the present study clearly demonstrated that fishes fed with ethanolic extract have shown significant decrease in the value of plasma glucose. This probably is due to the capability of oyster extract to reduce the effects of stressors caused by pathogen. It has also been demonstrated that glucose level increases in the infected or stressed fishes to ward off the infection or stress (Citarasu, 2006). Similar to present study other researchers have also found similar results such as in *Labeo rohita* fingerlings (Mishra *et al.*, 2009) and black tiger shrimp, *Penaeus monodon* where glucose levels were significantly reduced after feeding the fish with herbal immune-stimulant diets.

Sustainable aquaculture depends on perfect balance between growth and health condition of the fish. The use of antibiotics and chemotherapeutics to combat fish diseases has the risk of generating resistant pathogens, bioaccumulation and environmental pollution. Dietary administration of ethanolic oyster extract possessed immune-modulatory properties which showed maximum efficacy independently on each parameter in the present study. Fishes fed with feed incorporated with oyster extract raised WBC count and other biochemical blood plasma parameters as observed in control group. Further trials are warranted to ascertain the cellular and molecular mechanisms of the effect of oyster extract with reducing duration of administration period.

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