

Haemato-immunological response of *Labeo rohita* (Hamilton) fingerlings fed leaf extracts and challenged by *Aeromonas hydrophila*

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Abstract

A 35 days feeding trial was conducted to assess the haemato-immunological response of *Labeo rohita* fingerlings fed ethanolic leaf extracts of *Psidium guajava* and *Mangifera indica*, and infected with *Aeromonas hydrophila*. Six iso-nitrogenous (354.6–361.6 g kg⁻¹) purified diets were prepared with graded level of leaf extracts viz., control (basal feed without any extract); TG-5 (5 g kg⁻¹ guava extract); TG-10 (10 g kg⁻¹ guava extract); TM-5 (5 g kg⁻¹ mango extract); TM-10 (10 g kg⁻¹ mango extract); and TGM (5 g kg⁻¹ guava extract + 5 g kg⁻¹ mango extract). Haematological, immunological, biochemical, along with antioxidant enzyme activities were examined after a 35 day-feeding trial and following a 7 day challenge with *A. hydrophila*. The haemoglobin, total leucocyte and erythrocyte counts, respiratory burst activity, lysozyme, total protein, albumin and globulin contents increased significantly ($P < 0.05$) in leaf extracts fed groups compared with the control in pre- and post-challenge conditions. A significant ($P < 0.05$) decrease was observed in SOD (superoxide dismutase) and catalase activities of the treatment groups compared with the higher value in control. The trends in mortality indicated that groups of fish showing significantly elevated haemato-immunological responses had the lowest mortality following challenge with *A. hydrophila*. The results showed that extracts of *P. guajava* and *M. indica* appear to be potential immunostimulant at an inclusion level of 5 g kg⁻¹ in the diet of rohu. But, mixing of both the extract at similar level did not show any synergistic effect, which needs to be tested at its lower level of inclusion.

Keywords: leaf extracts, guava, mango, *Labeo rohita*, non-specific immunity, survival

Introduction

Aquaculture is an important economic activity supplying high quality animal protein to greater number of people worldwide, and the global growth of the industry remains relatively strong with an average annual growth rate of 8.6% (FAO 2014). However, modern farming techniques and recent trend of intensification of aquaculture operation has brought in new challenges that threaten the health of farmed species, leading to growth reduction and immunosuppression, all culminating into massive financial loss and reduced profit margins for farmers (Smith, Brown & Houton 2003; Jha, Pal, Sahu & Mukherjee 2007). Several pathogenic organisms such as bacteria, fungi and viruses have been reported to be principal limiting factors to the growth of aquaculture industry. The most common and frequently encountered bacterial pathogen in tropical climates is *A. hydrophila*, which causes severe damage to carp production (Karunasagar, Rosalind & Karunasagar 1991). It is a deadly pathogen causing stress-related diseases in fish with the common symptoms of ulceration, exophthalmia and abdominal distension (Amin, Abdallah, Elallawy & Ahmed 1985; Rahman, Kusuda & Kawai 1997). The most common approach to treat bacterial diseases and improve growth in cultured system has been through the application of antibiotics and chemotherapeutics agent. However, the extensive use of antibiotics has led to the inevitable emergence of drug-resistant bacterial strains (Wooley, Ritchie & Burnley

2004), and thus a diminution in effectiveness of antibiotics. Moreover, there is added concern about the accumulation of this substance in the environment, their spread up in the food chain, reduced consumer preference for food fish treated with antibiotics, and the concomitant spread of antibiotic-resistant strains to other organisms of human and veterinary importance (Aruna & Chandran 1996). Issues with resistance undoubtedly led to a resurgence of interest in preventive approach initially by the use of vaccine (Austin & Austin 2007). Although use of fish vaccines has become an established and effective method of controlling fish diseases, they are often costly and effective only against the specific pathogen and not against variants that might occur from time to time. Nevertheless, additional means of disease prevention have been researched using nutritional tools such as probiotics, immunostimulants and dietary supplements (e.g. medicinal plants/herbs), to maintain and manage fish health.

Immunostimulants can be described as a substance that enhances the innate immune system by interacting directly with cells of the system, thus activating them. They facilitate the function of phagocytic cells by increasing their bacteria-killing ability, lysozyme activity, stimulate natural killer cells and antibody responses in fish which confer enhanced protection from infectious diseases (Harikrishnan, Balasundaram & Heo 2011) and offer promising alternatives to antibiotics and vaccines (Anderson 1992). Several plant extracts like *Achyranthes aspera* (Rao & Chakrabarti 2005), *Solanum trilobatum* (Divyagnaneswari, Christyapita & Michael 2007), Chinese herbal mixture of *Astragalus membranaceus*, *Polygonum multiflorum*, *Isatis tinctoria* and *Glycyrrhiza glabra* (Yuan, Li, Chen, Sun, Wu, Gong, Tang, Shen & Han 2007) and *Mangifera indica* kernel (Sahu, Das, Mishra, Pradhan & Sarangi 2007) has been studied for their immunostimulatory effect in fish.

Psidium guajava (guava) belongs to the family Myrtaceae, which is a small tree that grows up to 35-feet tall and commonly grown for its fruit in the tropics. It has antibacterial and antioxidative properties (Pachanawan, Phumkhachorn & Pattanachaiakunsopon 2008; Fawole, Sahu, Pal & Lakra 2013). Similarly *Mangifera indica* L., commonly known as mango (Family; Anacardiaceae), is a large evergreen tree of tropical and subtropical regions. Different parts of mango have shown great medicinal potential such as antitumor,

anti-inflammatory, antimicrobial and antioxidant activity (Scartezzini & Speroni 2002; Garrido, Gonzalez, Lemus, Garcia, Lodeiro & Quintero 2004; Fawole *et al.* 2013), as well as immunomodulatory function (Sahu *et al.* 2007).

Guava and mango leaves are abundantly available in the tropical areas and many studies have been conducted on their use but limited work has been done in freshwater fish, especially *Labeo rohita*, a popular carp in South-east Asia. Hence, the aim of this study was to determine the effect of dietary supplementation of guava and mango leaf extracts either alone or in combination on the haemato-immunological response of *L. rohita* challenge by *A. hydrophila*.

Materials and methods

Preparation of extracts

Based on an initial screening of plant leaves extracts for their antioxidant and antibacterial activities (Fawole *et al.* 2013), *P. guajava* and *M. indica* leaf extracts were selected for this study. The leaf extracts were prepared using ethanol solvent following the method described by Harikrishnan, Balasundaram and Heo (2009) with little modification. Twenty five grams of dried leaf powder of *P. guajava* and *M. indica* were dissolved in 250 mL of 95% ethanol in 500 mL conical flask separately. The ethanol-leaf powder mixture was kept for 24 h on an orbital shaker (Orbitek^(R) – Scigenics Biotech, Chennai, India) at 200 rpm. Then, extracts were filtered using muslin cloth to exclude the powder residue. The filtrate collected was centrifuged at 10 000× *g* for 5 min and the solvent was evaporated using rotary vacuum evaporator (IKA^(R)RV10 Basic – Shanghai, China) and stored under refrigerated (4°C) condition prior to use. The percentage yield of extracts was 19.5% and 16.6% for *P. guajava* and *M. indica* dry leaves powder respectively. Extraction was repeated to obtain the required quantity of extracts used for feed preparation.

Fish and maintenance

Fingerlings of *L. rohita* (average weight 10.36 ± 0.04 g), were procured from Hans Aquaculture, Mahad, Raigad District, Maharashtra, India, and transported in a big circular container (500 L) with sufficient aeration to the wet laboratory of

ICAR Central Institute of Fisheries Education, Mumbai. They were carefully transferred to a circular fibre tank (1000 L). The stock was acclimatized under aerated condition for a period of 2 weeks and fed control diet.

Experimental design and feeding

Labeo rohita fingerlings ($n = 180$) were randomly distributed into six distinct experimental groups as control-0% (basal feed without any extract); TG-5 (basal feed +5 g kg⁻¹ guava leaf extract); TG-10 (basal feed +10 g kg⁻¹ guava leaf extract); TM-5 (basal feed +5 g kg⁻¹ mango leaf extract); TM-10 (basal feed +10 g kg⁻¹ mango leaf extract); and TGM (5 g kg⁻¹ guava leaf +5 g kg⁻¹ mango leaf extracts), in triplicate following a complete randomized design. The setup consisted of 18 plastic rectangular tubs (75 L capacity) each containing 10 fish. Fish were fed to satiation, with daily rations divided into two equal parts and fed at 10:00 hours and 18:00 hours under a normal light regime. The water quality parameters were

monitored closely and maintained at optimal levels (temperature 24–28°C, dissolved oxygen 6.1–7.2 ppm, pH 7.5–8.5 and ammonia 0.05–0.09 ppm).

Experimental diets

The experimental diets were made from purified ingredients and the composition is shown in Table 1. Fat free casein and gelatin (Himedia, Mumbai, India) were used as the protein source, whereas cod liver oil and sunflower oil were used as a lipid source, dextrin, starch soluble, cellulose as carbohydrates source and carboxymethylcellulose as binder (Himedia). Ingredients were mixed properly to form dough with the addition of adequate quantity of water. The dough was steam cooked for 5 min in a pressure cooker. Butylated hydroxytoluene, vitamins-mineral mixture along with the graded level of plant extracts (guava and mango) were added after cooling. After incorporation of these ingredients, the dough was mixed properly and pelletized using twin-screw extruder

Table 1 Composition of the experimental diets used during feeding trial (g kg⁻¹)

Ingredients	Control	TG-5	TG-10	TM-5	TM-10	TGM 5 + 5
Casein (fat free) ^a	297	297	297	297	297	297
Gelatin ^a	100	100	100	100	100	100
Dextrin ^a	183	183	183	183	183	183
Starch soluble ^a	215	215	215	215	215	215
Cellulose ^a	100	95	90	95	90	90
Cod liver oil ^b	30	30	30	30	30	30
Sunflower oil ^c	30	30	30	30	30	30
Vitamin-min mix ^d	20	20	20	20	20	20
CMC ^a	15	15	15	15	15	15
Betaine ^a	9	9	9	9	9	9
BHT ^a	1	1	1	1	1	1
Plant extract	–	5	10	5	10	10
Proximate composition of experimental diets (g kg ⁻¹ DM)						
Crude protein	359.3 ± 2.3	354.6 ± 6.1	361.6 ± 2.3	359.3 ± 2.3	357 ± 4.0	354.6 ± 2.3
Ether extract	75.1 ± 2.6	70.5 ± 1.1	74.3 ± 1.6	73.7 ± 2.3	71.9 ± 1.1	78.1 ± 1.6
Crude fibre	19.3 ± 0.9	21.2 ± 1.4	23.3 ± 1.1	19.6 ± 1.5	18.4 ± 1.3	20.6 ± 1.2
Ash	35.1 ± 1.6	32.2 ± 0.3	30.6 ± 0.6	27.2 ± 1.6	32.6 ± 0.6	26.5 ± 2.3
Moisture	102.2 ± 1.1	99.8 ± 3.0	109.5 ± 1.7	107.4 ± 1.3	104.7 ± 1.6	103.6 ± 0.8
Nitrogen free extract	521.2 ± 1.0	521.5 ± 2.6	510.2 ± 1.7	520.2 ± 1.4	520.1 ± 2.2	520.2 ± 2.5

^aHimedia India.

^bUniversal Medicare, Mumbai, India.

^cPurchased from local market, Mumbai, India.

^dComposition of vitamin-mineral mix (Agrimin) (quantity/2.5 kg), Vitamin A, 5 500 000 IU; Vitamin D3, 1 100 000 IU; Vitamin B2, 2000 mg; Vitamin E, 750 mg; Vitamin K, 1000 mg; Vitamin B6, 1000 mg; Vitamin B12, 6 µg; Calcium Pantothenate, 2500 mg; Nicotinamide, 10 g; Choline Chloride, 150 g; Mn, 27 000 mg; I, 1000 mg; Fe, 7500 mg; Zn, 5000 mg; Cu, 2000 mg; Co, 450 L-lysine, 10 g; DL-Methionine, 10 g; Selenium, 50 ppm; Satwari, 2500 mg. BHT, butylated hydroxytoluene.

to get uniform sized pellets, which were spread on a sheet of paper and fan dried. The feed was packed in polythene bag and stored at 4°C until use.

Collection of blood and serum

Blood samples were collected from nine randomly selected fish from each treatment (three from each replicate) after 35 days feeding. The fish were anaesthetized with clove oil (50 µL of clove oil per litre of water) and blood was collected from the caudal vein using a syringe, which was previously rinsed with 2.7% ethylene diamine tetraacetic (EDTA) solution. Collected blood was transferred immediately to a test tube containing EDTA powder (as an anticoagulant) and shaken gently to prevent haemolysis of blood. The blood was used for determination of haemoglobin content, total erythrocyte (RBC), leucocyte counts (WBC) and nitro-blue tetrazolium chloride (NBT) assay. For serum, six fish from each treatment were anaesthetized and blood was collected without anticoagulant in an eppendorf tube and allowed to stand in a tilted position at room temperature for an hour to clot. The sera were collected after centrifugation at $3000 \times g$ for 5 min and kept in -20°C until use. Serum was used for the estimation of immunological and biochemical parameters.

Haematological parameters

Haemoglobin content was determined following the cyanomethemoglobin method (Van Kampen & Zijlstra 1961) using Drabkin solution (Qualigens Fine Chemicals, Division of Glaxo Smithkline Pharmaceutical, Mumbai, India). Total erythrocyte count was done using haemocytometer (Hendrick 1952), and total leucocyte count was determined following the method of Shaw (1930).

Respiratory burst activity

The respiratory burst activity of the phagocytes was quantified by the NBT assay (Secombes 1990) as modified by Stasiak and Baumann (1996). A total of 50 µL of blood was placed into the wells of a U-bottom microtitre plate and incubated at 37°C for 1 h to facilitate adhesion of cells. The supernatant was removed and the wells were washed three times with PBS. After washing, 50 µL of 0.2% NBT was added and incubated for 1 h. The

cells were then fixed with 100% methanol for 2–3 min and again washed thrice with 30% methanol. The plates were then air-dried and 60 µL 2 N KOH and 70 µL DMSO were added into each well to dissolve the formazon blue precipitate formed. The OD of the turquoise blue coloured solution was read at 540 nm in an ELISA reader (Quant; Bio-Tek Instrument, Winooski, VT, USA).

Serum lysozyme activity

Serum lysozyme activity was measured using a colorimetric method by Anderson and Siwicki (1995). The serum sample was diluted with phosphate buffer (pH 7.4) to a final concentration of 0.33 mg serum protein mL^{-1} . In a cuvette, 3 mL of *Micrococcus luteus* (MTCC 106) suspension in phosphate buffer ($A_{450} = 0.5$) was taken, to which 50 µL of diluted serum sample was added. The content of the cuvette was mixed properly for 15 s and measured in a spectrophotometer at 450 nm. The reading of the lysis of the bacteria was immediately recorded at interval of 15, 30 and 270 s. A unit of lysozyme activity was defined as the amount of sample causing a reduction in absorbance of 0.001 min^{-1} and lysozyme activity was expressed as $\text{U min}^{-1} \text{ mg}^{-1}$ protein of serum.

Serum total protein, albumin and globulin

Serum samples were estimated for total protein by the Biuret method (Reinhold 1953) using a diagnostic kit (Total protein and albumin kit; InnolineTM Merck Specialities, Mumbai, India). Albumin was estimated by the bromocresol green binding method (Dumas, Watson & Biggs 1971). The absorbance of the standard and test was measured against a blank in a spectrophotometer at 630 nm. For globulin, albumin was subtracted from serum total protein and albumin:globulin ratio (A/G ratio) was determined.

Determination of antioxidant enzyme activity

For antioxidant enzyme assay, the liver and gill of the fish were carefully removed after decapitation and weighed. The tissue was homogenized with chilled 0.25 M sucrose solution in a plastic tube using a mechanical tissue homogenizer. The tube was continuously kept in ice to avoid heating. The homogenate was centrifuged ($5000 \times g$ for 10 min at 4°C) and supernatant were stored at -20°C

until use. The superoxide dismutase (SOD) activity was assayed according to the method described by Misra and Fridovich (1972) based on the oxidation of epinephrine – adrenochrome by the enzyme, and the catalase enzyme activity was done according to the method described by Takahara, Hamilton, Nell, Kobra, Ogura and Nishimura (1960).

Challenge test with *Aeromonas hydrophila*

Following 35 days of feeding, five fish from each replicate (15 fish in each treatment) were challenged intraperitoneally with 200 µL of *A. hydrophila* (1×10^7 CFU per fish) and maintained for 7 days. The fish were observed regularly for any overt signs of disease including behavioural abnormalities and mortality. Blood from one fish from each replicate (three from each treatment) were used for haematological study and NBT assay. Blood was also drawn from another one fish each for serum collection and stored in -20°C for total protein, albumin and lysozyme determination. All analysis was done in triplicate. Survival at the end of 7 days post infection was calculated using the following formula:

$$\text{Percentage survival(\%)} = \frac{\text{Number of surviving fishes after challenge}}{\text{Number of fishes injected with bacteria}} \times 100$$

Statistical analysis

Data were statistically analysed using one-way analysis of variance (ANOVA – SPSS version 16) and

Duncan's multiple range tests were used to determine the significant differences between the means. Comparisons were made at the 5% probability level.

Result

Haematological parameters

Significant effect of leaf extracts was evidence in all the measured haematological parameters of *L. rohita* fingerlings. The haemoglobin content of the treatment groups was significantly ($P < 0.05$) higher compared to the control group with the highest value recorded in TG-5 and TG-10 groups during pre- and post-challenge conditions (Table 2). However, TG-5 and TG-10 groups showed no significant difference ($P > 0.05$) with the TM-10 and TM-5 groups after challenged with *A. hydrophila*. Similarly, the total erythrocyte count (RBC) value was found to be higher in the treatment groups compared with the control. A significantly ($P < 0.05$) higher total erythrocyte count was recorded in TG-5 group before the challenge, and in TGM group after the challenge.

The leaf extracts in the diet had a significant effect on the total leucocyte count (WBC) during pre- and post-challenge conditions (Table 2). The highest leucocyte count was found in TM-5 and TG-5 groups during pre- and post-challenge condition respectively.

Respiratory burst activity

There was a significant difference ($P < 0.05$) in the respiratory burst activity among the various

Table 2 Pre-challenge and post-challenge haematological parameters of different experimental groups

Treatment	Parameters					
	Haemoglobin (g%)		RBC (10^6 mm^{-3})		WBC (10^3 mm^{-3})	
	Pre-challenge	Post challenge	Pre-challenge	Post challenge	Pre-challenge	Post challenge
Control	4.60 ± 0.20^a	5.73 ± 0.08^a	0.63 ± 0.02^a	0.98 ± 0.00^a	184.13 ± 0.61^a	215.47 ± 0.42^a
TG-5	5.66 ± 0.08^d	6.96 ± 0.04^b	1.05 ± 0.01^d	1.07 ± 0.00^b	231.00 ± 0.84^d	252.57 ± 0.78^d
TG-10	4.83 ± 0.06^{ab}	7.62 ± 0.02^c	0.87 ± 0.02^b	1.09 ± 0.01^b	223.80 ± 0.25^c	239.33 ± 0.37^c
TM-5	5.50 ± 0.05^c	7.60 ± 0.05^c	0.95 ± 0.00^c	1.13 ± 0.00^c	238.77 ± 0.23^d	251.57 ± 0.55^d
TM-10	5.00 ± 0.05^b	6.93 ± 0.08^b	0.97 ± 0.01^c	1.04 ± 0.00^b	218.60 ± 0.30^c	230.57 ± 0.66^b
TGM	5.53 ± 0.06^c	7.56 ± 0.03^c	1.03 ± 0.00^d	1.19 ± 0.00^d	210.47 ± 0.58^b	236.30 ± 0.47^c

Mean values bearing different superscripts in a column under each category differ significantly ($P < 0.05$). Data are expressed as mean $\pm$ SE.

treatment groups (Fig. 1). During pre- and post-challenge condition, the highest respiratory burst activity was observed in TG-5 and TG-10 groups, whereas the least activity was recorded in the control group.

Serum lysozyme activity

The serum lysozyme activity of the treatment groups was significantly ($P < 0.05$) higher than the control group in both pre- and post-challenge conditions (Fig. 2). In pre-challenge condition, the highest serum lysozyme activity was found in TG-5 followed by TM-5 group and the lowest activity was in the control group. Similar trend was also observed in post-challenge condition.

Serum total protein, albumin and globulin

The total protein level increased in all the treatments group compared with the control group (Table 3). Significantly ($P < 0.05$) higher serum total protein level was noticed in TG-5 group in pre-challenge condition, whereas TGM exhibited the highest total protein value in the post-challenge condition. Control group registered the lowest value in both pre- and post-challenge

conditions. Similarly, the albumin and globulin content in the treatment groups were significantly ($P < 0.05$) higher compared with the control. However, the albumin-globulin ratio were found to be significantly ($P < 0.05$) higher in the control compared with the treatment groups in both the pre- and post-challenge conditions (Table 3).

Antioxidant enzyme activity

The activities of SOD enzyme in the liver and gill of the treatment groups varied significantly ($P < 0.05$) compared with the control group (Table 4). The highest SOD value was recorded in the control group compared with the treatment groups in both the conditions. However, there was no significant ($P > 0.05$) variation in the liver of the treatment groups during the pre-challenge period. In case of gill, highest activity was found in control group, which was similar to TG-10 group in both pre- and post-challenge conditions. Similarly, the activity of catalase enzyme in liver and gill of the treatment groups varied significantly ($P < 0.05$) compared with the control group. The treatment groups showed a decrease in catalase enzyme activities with the lowest value recorded

Figure 1 Respiratory burst activity (NBT) of *Labeo rohita* fingerlings fed different experimental diets. Mean values with different superscript under each category differ significantly ($P < 0.05$). Data expressed as Mean $\pm$ SE.

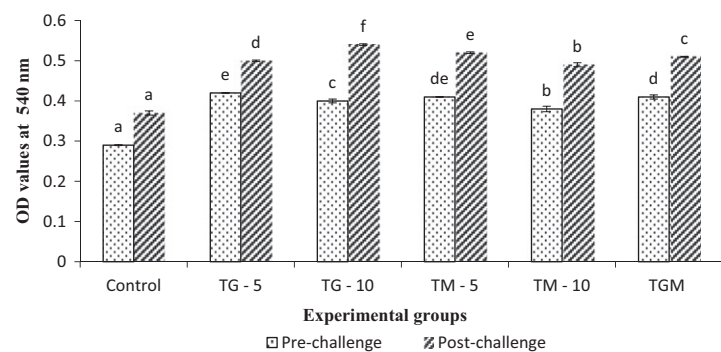


Figure 2 Serum lysozyme activity ($\text{U min}^{-1} \text{mg}^{-1}$ protein of serum) of *Labeo rohita* fingerlings fed different experimental diets. Mean values with different superscript under each category differ significantly ($P < 0.05$). Data expressed as Mean $\pm$ SE.

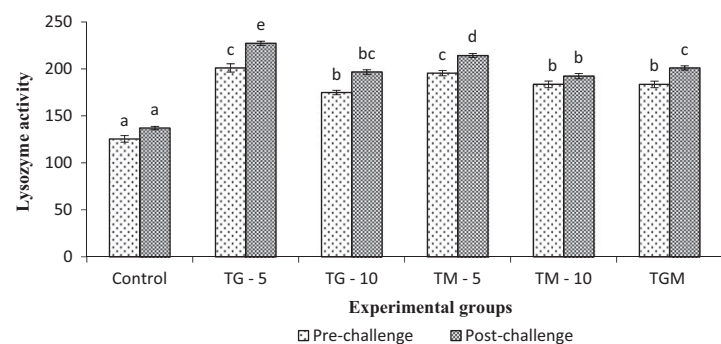


Table 3 Pre-challenge and post-challenge biochemical parameters of different experimental groups

Treatment	Parameters					
	Total protein (g dL ⁻¹)		Albumin (g dL ⁻¹)		Globulin (g dL ⁻¹)	
	Pre-challenge	Post challenge	Pre-challenge	Post challenge	Pre-challenge	Post challenge
Control	1.89 ± 0.02 ^a	2.23 ± 0.11 ^a	1.13 ± 0.00 ^a	0.80 ± 0.03 ^a	0.76 ± 0.02 ^a	1.43 ± 0.07 ^a
TG-5	3.45 ± 0.05 ^e	3.93 ± 0.06 ^c	1.39 ± 0.00 ^d	1.07 ± 0.00 ^b	2.06 ± 0.05 ^d	2.86 ± 0.05 ^d
TG-10	2.75 ± 0.07 ^b	3.63 ± 0.03 ^b	1.39 ± 0.01 ^d	1.03 ± 0.00 ^b	1.36 ± 0.07 ^b	2.60 ± 0.03 ^{bc}
TM-5	3.23 ± 0.02 ^d	3.84 ± 0.03 ^{bc}	1.24 ± 0.00 ^b	1.25 ± 0.01 ^c	1.99 ± 0.02 ^d	2.59 ± 0.04 ^{bc}
TM-10	3.04 ± 0.03 ^c	3.69 ± 0.77 ^b	1.36 ± 0.00 ^c	1.21 ± 0.03 ^c	1.68 ± 0.03 ^c	2.48 ± 0.04 ^b
TGM	2.76 ± 0.05 ^b	4.04 ± 0.04 ^c	1.35 ± 0.00 ^c	1.38 ± 0.00 ^d	1.41 ± 0.04 ^b	2.66 ± 0.04 ^c

Mean values bearing different superscripts in a column under each category differ significantly ($P < 0.05$). Data are expressed as mean ± SE.

in TM-5 and TM-10 groups in liver and gill, respectively, during post-challenge period (Table 4).

Percentage survival

After feeding for 35 days, the fish were challenged with *A. hydrophila* and mortality was observed to be highest in the control group. The highest percentage survival (80%) was in TM-5 followed by TG-5 group (Fig. 3).

Discussion

Evaluation of haemato-immunological parameters are often used as an index of fish health in a number of investigation to detect physiological changes following exposure to stresses like crowding, high temperature, pathogen exposure, etc. (Agrawal & Mahajan 1980; Kumar, Kumar, Pandey, Raman, Prasad, Roy, Kumar & Kumar 2013). In this study, both the leaf extracts investigated had significant effects on the blood parameters of *L. rohita* fingerlings as well as the immune response bio-indicators. From our investigation, it was observed that the haemoglobin content, and RBC count were higher in all the groups fed leaf extracts than the control group in both the pre- and post-challenge conditions. The trend of haemoglobin content value was in consonant with the RBC count. Similar observation was also reported in goldfish (*Carassius auratus*) fed Azadirachtin (Kumar, Raman, Pandey, Mohanty, Kumar & Kumar 2013), and *L. rohita* fingerlings fed *M. indica* kernel (Sahu *et al.* 2007). Leucocytes (WBC) play a significant role in non-specific immunity, and are known as the first line of defence during pathogen invasion (Alberts, Buchan & Altmann 2006). The higher WBC count observed in the treatments group in this study is an indication of the non-specific immune system activation, which is in agreement with the findings of Kumar, Kumar *et al.* (2013) and Sahu *et al.* (2007) in *L. rohita* fingerlings fed I-carrageenan and *M. indica* kernel respectively.

Phagocytosis and pathogen killing ability by phagocytic cells is an important defence mechanism in fish against pathogenic organisms (Neumann, Stafford, Barreda, Ainsworth & Belosevic 2001; Rao, Das, Jyotirmayee & Chakrabarti 2006). The phagocytes in fish are able to produce superoxide anion (O_2^-) and its reactive derivatives

Table 4 Pre-challenge and post-challenge SOD and catalase activity of different experimental groups

Treatment	SOD				Catalase			
	Liver		Gill		Liver		Gill	
	Pre-challenge	Post challenge	Pre-challenge	Post challenge	Pre-challenge	Post challenge	Pre-challenge	Post challenge
Control	30.88 ± 3.46 ^c	36.83 ± 0.81 ^d	36.94 ± 3.36 ^c	39.02 ± 0.24 ^c	4.66 ± 0.39 ^b	12.63 ± 0.90 ^a	1.77 ± 0.04 ^c	4.69 ± 0.11 ^c
TG-5	21.49 ± 0.55 ^a	32.89 ± 0.29 ^c	28.80 ± 0.55 ^{ab}	33.23 ± 0.45 ^a	2.89 ± 0.10 ^a	8.08 ± 0.38 ^{bc}	1.36 ± 0.09 ^{ab}	2.21 ± 0.21 ^{ab}
TG-10	24.15 ± 0.49 ^b	31.39 ± 1.24 ^{bc}	32.02 ± 1.08 ^{bc}	34.92 ± 0.34 ^{bc}	2.55 ± 0.25 ^a	7.05 ± 0.25 ^{ab}	1.55 ± 0.08 ^{bc}	2.79 ± 0.37 ^b
TM-5	21.83 ± 0.30 ^a	28.79 ± 1.85 ^b	27.42 ± 0.58 ^a	33.39 ± 0.58 ^a	2.75 ± 0.09 ^a	4.92 ± 0.22 ^a	0.98 ± 0.07 ^a	2.14 ± 0.15 ^{ab}
TM-10	26.58 ± 0.84 ^b	29.02 ± 0.38 ^b	28.84 ± 2.68 ^{ab}	33.80 ± 0.25 ^{ab}	2.55 ± 0.18 ^a	11.13 ± 0.23 ^{de}	0.97 ± 0.06 ^a	1.97 ± 0.16 ^a
TGM	26.73 ± 1.63 ^b	25.54 ± 0.64 ^a	32.04 ± 0.95 ^{bc}	33.47 ± 0.51 ^a	2.70 ± 0.10 ^a	10.18 ± 0.91 ^{cd}	1.12 ± 0.09 ^{ab}	2.81 ± 0.24 ^b

Mean values bearing different superscripts in a column under each category differ significantly ($P < 0.05$). Data are expressed as mean ± SE.

SOD: $\mu\text{mol mg}^{-1}$ protein min^{-1} at 37°C.

Catalase: $\text{mmol H}_2\text{O}_2$ decomposed min^{-1} mg^{-1} protein at 37°C.

SOD, superoxide dismutase.

during the period of intense oxygen consumption called the respiratory burst (Secombes & Fletcher 1992; Secombes 1996; Sahu *et al.* 2007). It is also considered that these oxygen forms are toxic to the bacterial pathogens (Hardie, Ellis & Secombes 2004), and are generated by phagocytes after stimulation. In this study, the respiratory burst activity of phagocytes was quantified by the NBT assay, which measures the quantity of intracellular superoxide radicals produced by leucocytes (Siwicki & Studnicka 2006). A significantly higher respiratory burst activity was observed in the group fed leaf (*P. guajava* and *M. indica*) extracts, which suggest that the extracts enhanced the formation of reactive oxygen species to fight against the bacteria pathogen and stimulate the non-specific immune function compared with the control. This is consistent with the work of Jian and Wu (2003, 2004), who reported a significantly increased respiratory burst activity in Jian carp and yellow croaker fed for 20 days with 1% and 1.5% of TCM (mixture of *Astragalus*; *Astragalus membranaceus* and *Astragalus sinensis*) extracts. Similarly, feeding of rohu (*L. rohita*) with mango seed kernel increased the superoxide anion production of the blood leucocytes (Sahu *et al.* 2007). Dietary administration of I-carrageenan and azadirachtin was also reported to significantly enhance the respiratory burst activities in *L. rohita* and *C. auratus* respectively (Kumar, Kumar *et al.* 2013; Kumar, Raman *et al.* 2013).

Lysozyme is an important defence molecule of the non-specific immunity and plays a significant role in mediating protection against bacterial invasion (Saurabh & Sahoo 2008). In fish, lysozyme is recognized to be an opsonin, and activates the complement system and phagocytes (Grinde 1989; Saurabh & Sahoo 2008). The ability to disrupt the cell walls of certain pathogens makes lysozyme a natural antagonist to harmful organisms such as bacteria (Basha, Raman, Prasad, Kundan, Ezhil & Saurav Kumar 2013). This study revealed a significant increase in serum lysozyme activity in all the groups fed leaf extracts compared with the control group. This may be due to the ability of the leaf extracts to modulate lysozyme activity of the fish. Enhanced lysozyme activities were also reported in *L. rohita* fed *A. aspera* seed (Rao *et al.* 2006), dietary andrographolide (EC 50%) (Basha *et al.* 2013; and β -glucan (Sahoo & Mukherjee 2001). Higher plasma lysozyme activity was also noticed in Nile tilapia fed Chinese medicinal herbs (*Astragalus*

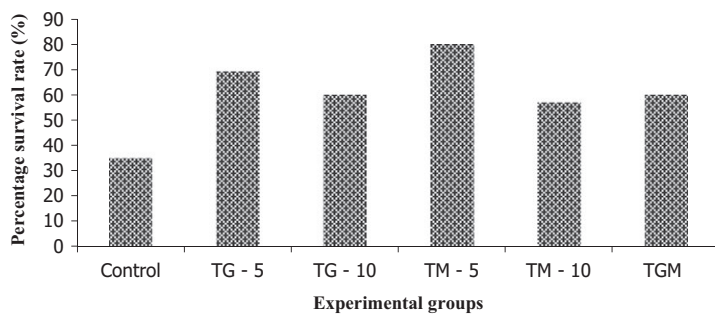


Figure 3 Percentage survival rate of *Labeo rohita* fingerlings challenged with *Aeromonas hydrophila* after fed different experimental diets.

membranaceus and *Lonicera japonica*) alone or in combination after first 7 days of feeding (Ardo, Yin, Xu, Váradi, Szigeti, Jeney & Jeney 2008). It was also observed that the group (TG-5 and TM-5) with maximum lysozyme activity also had the maximum leucocyte count. Therefore, the significantly higher serum lysozyme activities observed in the treatment groups is an indication of the non-specific immune system activation after being fed experimental diet.

The total protein in the serum can be divided into two groups, albumin and globulin. They are the major proteins, which plays an important role in the immune response of fish (Kumar, Sahu, Pal & Kumar 2007). A rise in the total protein, albumin and globulin levels is thought to be associated with a stronger non-specific immune response in fish (Wiegertjes, Stet, Parmentier & Van Muiswinkel 1996). The leaf extracts used in this study significantly enhanced serum total protein, albumin and globulin contents in the treatment groups compared with the control. This is consistent with the findings of Kumar, Raman *et al.* (2013), Basha *et al.* (2013) and Sahu *et al.* (2007) in *L. rohita*. Similarly, a significant increase in total protein, albumin and globulin was recorded in *C. carpio* fed diets containing 0.5% and 1% Chinese herbal medicine (Yuan *et al.* 2007). However, a significantly lower albumin-globulin (A/G) ratio observed in this study indicates the presence of more amounts of globulin in the treatment groups compared with the control. Since gamma fraction makes the largest portion of globulin and are essential for maintaining a healthy immune system in fish, it can be inferred that the treatment diets enhanced the non-specific immune response of *L. rohita* fingerlings. This assertion is supported by increased phagocytic activity, lysozyme, WBC count and relative percentage of survival recorded in this study.

Superoxide dismutase is a class of enzymes that catalyse the dismutation of superoxide into

hydrogen peroxide (H_2O_2), and catalase convert the H_2O_2 to oxygen and water. They are an important antioxidant defence system in nearly all cells and offers a protection against the damaging effects of free radicals induced by oxidative stress. This oxidative stress can alter the antioxidant defence mechanism when fish are exposed to unfavourable condition like high or low temperature, toxicant, pathogen, etc., which may lead to cellular disruption, DNA damage, liver impairment and or immune suppression (Martínez-Alvarez, Morales & Sanz 2005; Patra, Rath, Jena, Rathod & Hrudayanath 2008). However, antioxidant containing compound can quench this harmful effect of free radicals by giving out their own electrons to stop the chain of reaction. Some studies had demonstrated that the antioxidant enzymes can serve as stress and immune – response biomarkers, quantified by the enzyme activity to evaluate the health of animal including fish (Sagstad, Sanden, Haugland, Hansen, Olsvik & Hemre 2007; Tovar-Ramírez, Mazurais, Gatesoupe, Quazuguel, Cahu & Zambonino-Infante 2010; Tancredo, Eduardo, Brum, Acchile, Gabriela, Scheila & Mauricio 2015). They are also said to scavenge free radicals generated by external stimuli such as pathogen, toxicant, etc. (Johnson 2002). In this study, the lower antioxidant enzymes activities recorded in the treatment groups is an indication of the leaf extracts capability to scavenge or neutralize the free radicals generated due to oxidative stress induced by pathogenic organism. This scavenging effects can be attributed to the phenolics, a secondary metabolite present in the leaf extracts which are powerful antioxidant agent (Liu, Qiu, Ding & Yao 2008; Fawole *et al.* 2013). Similar observation was made by Mamdouh and Abdel-Raheim (2003), who observed a significant reduction in SOD activities of rats fed garlic oil.

After challenges with *A. hydrophila*, the highest percentage survival was recorded in the group fed

TM-5 followed by TG-5, and least in the control group. This is due to the enhancement of the non-specific immunity of the fish by the leaf extracts. Our findings reveal that the supplementation of the leaf extracts had a positive influence on the survival of *L. rohita* fingerlings by resisting the *A. hydrophila* infection. Similar results were also reported after feeding *L. rohita* fingerlings with *M. indica* kernel and challenged with *A. hydrophila* (Sahu *et al.* 2007). Basha *et al.* (2013) reported that *L. rohita* fed dietary andrographolide (EC 50%) showed significantly enhanced resistance against *A. hydrophila* infection. Similarly, Pachanawan *et al.* (2008) reported an increased survival in *Oreochromis niloticus* fed *P. guajava* after experimentally infected with *A. hydrophila*.

In conclusion, our study reveals that feeding of leaf extracts of mango (*M. indica*) and guava (*P. guajava*) either alone or in combination to rohu exhibited similar haemato-immunological response and hence survival after challenged with *A. hydrophila*. Although synergistic effect of both the leaf extract was not found at 10 g kg⁻¹ (5 g kg⁻¹ guava extract + 5 g kg⁻¹ mango extract) level of inclusion, but its efficacy at lower level may not be ruled out, which need further research.

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