



Full length article

Ameliorating effects of dietary mixture of *Withania somnifera* root extract and vitamin C in *Labeo rohita* against low pH and waterborne iron stressesC. Laltnanmawia^a, Ratan Kumar Saha^a, Himadri Saha^{a,*}, Pradyut Biswas^b^a Department of Aquatic Health and Environment, College of Fisheries, CAU, Lembucherra, Agartala, Tripura, 799210, India^b Department of Aquaculture, College of Fisheries, CAU, Lembucherra, Agartala, Tripura, 799210, India

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ABSTRACT

The present study was conducted to elucidate the possible effect of a combination of *Withania somnifera* (Ashwagandha) root extract and dietary L-ascorbic acid (vitamin C) on the immune responses and survivability in *Labeo rohita* fingerlings against low pH and waterborne iron toxicity. Four diets were prepared containing 0% (control), 0.01% (T1), 0.1% (T2) and 1.0% (T3) of a combination of *Withania somnifera* (Ashwagandha) root extract and dietary L-ascorbic acid (vitamin C) at the rate of 1:1 (w/w). Fishes were fed for 60 days to find out the best dose and duration of feeding by examining different haematological (haemoglobin, haematocrit, total RBC count, total WBC count, MCV, MCH and MCHC), immunological (NBT, lysozyme and total immunoglobulin activity) and biochemical (glucose, total protein, ALP, SGPT and SGOT level) parameters on 7, 15, 30 and 60 days of feeding. The best response was observed in fish fed with diet T3 within 15 days. The result showed a significant ($p < 0.05$) increase in haemoglobin, haematocrit, total RBC count, total WBC count, NBT, lysozyme activity, total immunoglobulin and total protein whereas a significant ($p < 0.05$) decrease in glucose, ALP, SGPT and SGOT level compared to control. Challenged study against waterborne iron (6.02 mg l^{-1}) and low pH (4.2–4.5) after feeding the fish for 15 days with diet T3 showed significantly ($P < 0.05$) higher protection than control. The study concludes that inclusion of 1.0% *Withania somnifera* (Ashwagandha) root extract and dietary L-ascorbic acid (vitamin C) combination in diet have a stimulatory effect on immune response and reduces the effect of multiple stresses (i.e., low pH and waterborne iron toxicity) in *L. rohita* fingerlings.

1. Introduction

In polluted or contaminated water fishes are continuously exposed to several environmental stressors. Among several stressors low water pH and heavy metal (such as iron) toxicity become one of the primary concerned in aquaculture especially in North Eastern parts of India, where the soil and water are highly acidic with excess iron due to high content of ferrous ions and high rainfall [1–4]. In the North Eastern region of India, natural springs and dug wells are the only cost-effective and viable means of fulfilling the needs of freshwater for aquaculture. The iron content in water is relatively high and groundwater in almost all states contain iron above the permissible limit. The pH of natural water is also found to be acidic [5], which may be a reasonable cause for slow growth and low production of cultured fish species in the region [1–4].

Water pH and iron play an important role in the maintenance and physiological well-being of aquatic animals. Exposure to low water pH level results in acute or chronic stress [6]. This lead to the impairment

in the ionic and acid-base regulatory mechanisms, ammonia excretion, mucus secretion at the gills [7,8]. Iron in water is present as soluble ferrous iron (Fe^{2+}) or insoluble ferric iron (Fe^{3+}) [9]. Iron is a vital micronutrient for life being involved in cellular respiration and oxygen transfer [10–13]. However, excess iron is toxic, it can precipitate and accumulate in or around the gills forming flocs resulting in clogging and damage of gills, reduce oxygen uptake and impairment of ionic regulation [14,15]. The deposition of iron flocs on to the gill epithelium was reported by several authors [16–18]. At cellular level iron can participate in Fenton's reaction leading to the formation of free radicals called as reactive oxygen species (ROS). The free radicals are highly toxic, affecting the enzymatic activity of antioxidants, causes peroxidation of membrane lipid, modification of nucleic acid and result in tissue injury and cell death [19]. The availability of ferrous iron was found to increase with a decrease in pH, which is considered to be more toxic [20].

In the current context, synthetic chemotherapeutic agents are increasingly used for minimizing environmental stress, as well as

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preventing and curing of the different aquatic disease outbreak. However, these chemicals are very corrosive in nature and resulting in an accumulation of its residues in the aquatic ecosystem that has a detrimental effect on fish health. The chemotherapeutic agents like antibiotics can also lead to the development of drug resistance pathogenic microorganisms, thus raising food safety concerns. There is a high pressure on aquaculture to reduce the use of synthetic antibiotics and therapeutic chemicals because of the risk caused to humans and to the environment.

In recent years, nutritional dietary supplements termed as nutraceuticals were used to fight against exclusive sort of stresses and diseases. The use of nutraceuticals in fisheries for health management, value addition, and stress mitigation becomes a rising vicinity of aquaculture research [21]. Nutraceuticals are food, or parts of food, that provide medical or health benefits, including the prevention and treatment of disease [22]. Nutraceuticals can act as therapeutic agents for the protection and prevention of stress or disease in fish. It acts as anti-stress, antimicrobial agents, promotes various activities including immunomodulation, immunostimulation, antioxidation and growth without causing any harmful effect to the environment, to the fish and to the consumer [23].

Study on plants, plant extracts or natural plant compounds having medicinal value as a nutritional dietary supplement become a field of research as a potential alternative to synthetic chemicals for the stimulation of immune responses and disease resistance in fish [23]. In regard to this North Eastern region has a rich source of biodiversity which includes the high potential of naturally occurring medicinal plants. Various medicinal plants found in this region can be used for nutraceutical and pharmaceutical preparation [24]. Medicinal plants like *Withania somnifera* (Ashwagandha), *Andrographis pauculata* (Kalmegh), etc., which is found abundantly in this region can be a suitable choice for study. *Withania somnifera* (Ashwagandha) also known as Indian ginseng has been known to possess different health benefit acting as an immunostimulant, antioxidant, antistress, anti-inflammatory and immunomodulatory [25,26]. The active compounds such as sitoindosides VII-X and Withaferin-A have been shown to have significant anti-stress activity [26,27]. Besides herbs or herbal products, a dietary supplement like vitamins, minerals, amino acid, essential fatty acid, etc. have the competency to amend health condition, stress tolerance, growth, immunological response and disease resistance in fish [28]. Vitamin C is an essential nutrient for growth and maintenance [29]. It is also an important antioxidant, scavenging free radicals and improving stress tolerance by reducing the toxic effect of pollution [30–32]. Studies on a combination of nutraceutical especially herbs and vitamins for mitigating stress is scanty. Besides, no study has been reported the use of a combination of these nutraceuticals in fish as a means of mitigating environmental stress such as waterborne iron and low pH toxicity. So, keeping in mind the harmful effect of waterborne iron and low pH toxicity, the constraint and harmful effect of synthetic chemotherapeutic agents while the availability, cost-effectiveness and the efficacy of nutraceuticals in promoting growth, enhancing the immune system against different stressors, the present study was conducted.

2. Materials and method

2.1. Collection and maintenance of experimental animal

Labeo rohita fingerlings (average length 10.38 ± 1.02 cm and weight 11.8 ± 2.4 g) were collected from Battala fish market, Agartala, Tripura. The fish were well acclimatized in a 500-litre circular fiber reinforced plastic (FRP) tanks for 25 days before the experiment. Adequate aeration with sponge filter was provided and fed with extruded floating pellets at the rate of 3% of their body weight twice a day.

2.2. Collection and selection of nutraceuticals

Roots of *Withania somnifera* (Ashwagandha) were procured from Agartala, Tripura market and dietary L-ascorbic acid (vitamin-C) from Hi-Media Pvt. Ltd. Mumbai, India. Selection of nutraceutical combination was based on our earlier preliminary *in-vitro* study (Results under communication, unpublished).

2.3. Preparation of extract

The extract was prepared from *Withania somnifera* (Ashwagandha) root. First, the roots of ashwagandha were washed with clean water, dried and chopped into smaller pieces. The dried sample was then ground into fine pieces and soaked in alcohol at the ratio of 1:10. The mixture was filtered using Whatman filter (No.1) paper after constant mixing in a rotary shaker for 48 h and centrifuged at 5000 rpm for 10 min. The supernatant was collected and kept in an amber bottle at 4 °C. The solvent was then evaporated in a rotary evaporator until it becomes semi-solid and finally dried in a freeze drier for 24 h. The extract was then kept at –20 °C until further use [33].

2.4. Preparation of nutraceutical diets

Four practical diets (control, diet T1, diet T2 and diet T3) were prepared by incorporating dietary nutraceutical combination (*W. somnifera* root extract and vitamin C @ 1:1 w/w) at the rate of 0.0%, 0.01%, 0.1% and 1.0%, respectively. All the ingredients in the required quantity (Table 1) were mixed with water to make the dough. Cooking was done in an autoclave at 120 °C and 15 psi for 2 min. The completely cooled dough was mixed with vitamin-mineral mixture and nutraceutical combination as per the treatments. The dough was pressed through hand pelletizer to get uniform size pellet. The pellets were dried at room temperature for 48 h. After drying, the pellets were packed in polythene bags sealed and labelled according to the different doses of treatment for further use. Proximate composition of the experimental diets was analyzed following AOAC [34] (Table 1).

2.5. Experimental design and feeding diet

The experiment was set up in 500L capacity FRP tanks. There were 4 experimental groups (control, T1, T2, and T3) with three replicates each. Well acclimatized *Labeo rohita* fingerlings (average length of 10.74 ± 2.15 cm and weight of 12.02 ± 3.68 g) were randomly distributed in 12 tanks (30 fish per tank) and fed with experimental diets at the rate of 3% of their body weight twice a day at 10:00 a.m. and 3:00 p.m., respectively. The control group diet was devoid of nutraceutical while the remaining groups T1, T2 and T3 were fed with feed containing 0.01%, 0.1% and 1.0% of nutraceutical combination. Adequate aeration was provided, siphoning of waste materials was done daily and 50% of the water was exchanged twice a week. The experiment was conducted for 60 days and sampling for various haemato-immuno-biochemical parameters was done on the 7th, 15th, 30th and 60th day of feeding trials. For each sampling, 5 fishes were randomly selected from each tank. Water quality parameters were routinely analyzed throughout the experiment.

2.6. Collection of blood, plasma and serum

Fishes were anaesthetized with clove oil (Merck, Germany) at the rate of 50 µl per litre of water. The blood was collected from randomly selected 5 fish from each tank and drawn from the caudal vein using 1.0 ml hypo-dermal syringe and 24 gauge needles. The collected blood was immediately transferred into two vials, where one vial was coated with thin layer of anticoagulant EDTA (for blood and plasma collection) and the other one without EDTA (for serum collection). From the pooled blood samples, about 500 µl of samples were used for the

Table 1
Ingredients and proximate composition of control and experimental diets.

The composition of control and experimental diets (100 g).				
Ingredients	Quantity (in g)			
	Control diet	Diet T1 (0.01%)	Diet T2 (0.1%)	Diet T3 (1.0%)
Casein	37	37	37	37
Gelatin	8	8	8	8
Dextrin	30	30	30	30
Cellulose	9.7	9.69	9.6	8.7
CMC	2	2	2	2
Vegetable oil	3.5	3.5	3.5	3.5
Betaine	0.2	0.2	0.2	0.2
BHT	0.1	0.1	0.1	0.1
Cod liver oil	3.5	3.5	3.5	3.5
Vitamin-mineral premix ^a	6	6	6	6
Nutraceutical	0	0.01	0.1	1.0
Total	100	100	100	100
Proximate composition of control and experimental diets; Values are means ± SEM, n = 3.				
Moisture (%)	3.71 ± 0.28	4.16 ± 0.01	3.94 ± 0.07	4.01 ± 0.09
Ash (%)	14.93 ± 0.01	14.96 ± 0.03	14.32 ± 0.22	14.87 ± 0.01
Protein (%)	35.2 ± 0.03	35.55 ± 0.24	35.9 ± 0.36	35.61 ± 0.33
Lipid (%)	7.95 ± 0.03	7.98 ± 0.01	7.69 ± 0.24	7.96 ± 0.02
Carbohydrate (%)	41.92 ± 0.02	41.51 ± 0.09	41.09 ± 0.27	41.56 ± 0.12

^a Composition of vitamin-mineral premix (PREEMIX PLUS) (quantity 2.5 kg⁻¹): Vitamin A, 5500000 IU; Vitamin D3, 1100000 IU; Vitamin B2, 2000 mg; Vitamin E, 750 mg; Vitamin K, 1000 mg; Vitamin B6, 1000 mg; Vitamin B12, 6 mcg; 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 mg; L-lysine, 10 g; DL- Methionine, 10 g; Selenium, 50 mg l⁻¹.

collection of plasma by centrifuging at 3500 rpm for 15 min at 4 °C. Serum was collected after keeping the tube in a slanting position for about 2 h. Both serum and plasma were then stored at –20 °C until use.

2.7. Determination of haematological parameters

Haematological parameters like haemoglobin (Hb), haematocrit (Hct) or packed cell volume (PCV), total erythrocyte count or RBC count and total leucocyte count or WBC count were measured using the standard method as described by Schaperclaus [35]. RBC and WBC were counted in haemocytometer (Improved Neubauer Weber Scientific Ltd). Packed cell volume (PCV) was determined by haematocrit reader after centrifuging heparinised capillary tubes filled with blood for 5 min at 5000 x g and estimation of hemoglobin (Hb) in blood was done by using Sahli Haemometer (Marinefield, Germany). Mean corpuscular volume (MCV), Mean corpuscular haemoglobin (MCH) and Mean corpuscular haemoglobin concentration (MCHC) were calculated using the following formula [36]:

$$MCV = \frac{\text{Haematocrit or PCV (\%)}}{\text{RBC count (EC in million mm}^{-3}\text{)}} \times 10$$

$$MCH = \frac{\text{Haemoglobin (g/dl)}}{\text{RBC count (EC in million mm}^{-3}\text{)}} \times 10$$

$$MCHC = \frac{\text{Haemoglobin (g/dl)}}{\text{Haematocrit or PCV (\%)}} \times 100$$

2.8. Determination of immunological parameters

2.8.1. Nitroblue tetrazolium (NBT) assay

NBT assay was carried out following the modified Anderson and Siwicki [37] method. In a centrifuge tube, 0.1 ml blood and an equal amount of 0.2% NBT solution were added. The tube was incubated for 30 min at room temperature after mixing properly. 1.0 ml N, N-dimethyl formamide (DMF) was taken out in another tube and 0.05 ml of the NBT blood cell suspension was added. The tube was then centrifuged at 3000 rpm for 5 min. The supernatant was siphoned out and the sample absorbance was read on the spectrophotometer at 540 nm.

2.8.2. Lysozyme activity

Estimation of lysozyme activity was carried out following Anderson and Siwicki [37] with a slight modification. In 0.1 ml of plasma, a suspension of 0.9 ml *Micrococcus lysodeikticus* (Sigma, St Louis, MO, USA) (0.75 mg ml⁻¹ in phosphate buffered saline, pH 6.2) was added. After proper mixing the absorbance was measured in spectrophotometer (450 nm) at 1 min intervals for 10 min. Change of absorbance was calculated.

2.8.3. Total immunoglobulin

Total immunoglobulin was estimated following modified Anderson and Siwicki [37] method. 0.1 ml of 12% polyethylene glycol was added in a plastic serum vial containing 0.1 ml of plasma. Under constant mixing, the sample mixture was incubated at room temperature for 2 h. The sample was then centrifuged at 7000 rpm for 10 min to get clear supernatant and immunoglobulin content in the sample was determined with the help of total protein analysis kit. Protein reading from the supernatant was the amount of protein taken out by absorption to polyethylene glycol.

Total immunoglobulin (mg ml⁻¹) = Total protein in individual samples plasma - Total protein taken out by absorption to polyethylene glycol.

2.9. Biochemical parameters

Different biochemical parameters like glucose, total protein, serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT) and alkaline phosphatase (ALP) were estimated following standard kits (as per the manufacturer's instructions; Medsource Ozone Biomedicals Pvt. Ltd., India).

2.10. Challenge experiment

Based on the finding of first experiment *Labeo rohita* fingerlings (average length of 11.24 ± 1.02 cm and weight of 14.4 ± 3.68 g) were fed with experimental diet T3 (containing 1.0% of a combination of *W. somnifera* root extract and dietary L-ascorbic acid) and control diet for 15 days. After feeding, the fish were challenged against waterborne iron (6.05 mg l⁻¹) and low pH (4.2–4.7) in a uniform size rectangular

glass aquarium of 2'X1'X1'. The concentration of iron was decided based on the previous study by Debnath et al. [13] and the pH range was decided based on the study of Khan et al. [38]. The range was also decided based on the level of iron and pH prevailing in the region [39]. The iron water was prepared using dried ferrous sulphate ($\text{FeSO}_4 \cdot x\text{H}_2\text{O}$) and pH was adjusted using hydrochloric acid (HCl) and sodium hydroxide (NaOH), the control water contains 0.035 mg l^{-1} of iron (Fe^{2+}) with a pH of 7.5–8.0. There were 8 treatment groups with 2 replicates each. 10 fishes were kept in each of the treatment aquarium containing 30L of water. Iron water was exchanged and pH was adjusted daily. Mortality was observed for 10 days and relative percentage survival (RPS) was calculated as follows:

Relative percentage survival (RPS) = (Number of surviving fish after challenge/Number of fish challenged with waterborne iron and low pH) X 100 [40].

2.11. Statistical analysis

Statistical analysis of data was performed using SPSS (SPSS Inc., Chicago IL, USA). Results are presented as a mean \pm standard error. Comparisons of the mean values were determined by One-way ANOVA and Duncan's test. The probability level of 0.05 is used to find out the significance in all cases.

3. Result

3.1. Haematological parameters

The haematological responses of *Labeo rohita* fingerlings fed with nutraceutical combination (*Withania somnifera* (Ashwagandha) root extract and dietary L-ascorbic acid (vitamin C)) based diets are presented in Table 2. The Haemoglobin (Hb) content in T1, T2 and T3 were higher in all the sampling days and significantly ($p < 0.05$) higher in 7th, 15th and 60th day of sampling compared to control. The highest value was observed in T3 on 15th day. PCV of T1, T2 and T3 were higher in all the sampling days except at 30th day where T1 was highest compared to control. T3 was significantly ($p < 0.05$) higher than all the other treatment groups at day 7th, 15th and 60th. All the treatment groups had higher EC or RBC in all the sampling days compared to control. The value increases in all the treatment groups except for T2 at 7th day. In comparison to all the groups T3 was significantly ($p < 0.05$) higher. The total leucocyte count (LC) or WBC in all the treatment groups were significantly ($p < 0.05$) higher than control at 15th, 30th and 60th day of sampling. However, LC was highest in T1 at 7th day and significantly differs from the group. MCV of T2 and T3 were

Table 2
Effect of nutraceutical combination on the haematological parameters of *Labeo rohita*.

Sampling days	Diet	Hb (g%)	PCV (%)	Total RBC ($\times 10^6/\text{mm}^3$)	Total WBC ($\times 10^4/\text{mm}^3$)	MCV (μ^3)	MCH (pg)	MCHC (%)
7 th Day	Control	3.97 \pm 0.09 ^d	18 \pm 0.58 ^c	1.4 \pm 0.05 ^c	5.37 \pm 0.06 ^b	128.6 \pm 1.93 ^c	28.36 \pm 0.65 ^c	22.07 \pm 0.74
	Diet T1	4.37 \pm 0.07 ^c	20.67 \pm 0.88 ^b	1.57 \pm 0.04 ^{ab}	6.73 \pm 0.13 ^a	131.25 \pm 3.13 ^c	27.76 \pm 0.27 ^c	21.19 \pm 0.72
	Diet T2	5.07 \pm 0.13 ^b	22.33 \pm 0.33 ^b	1.53 \pm 0.02 ^b	5.27 \pm 0.02 ^b	146.01 \pm 2.54 ^b	33.14 \pm 1.23 ^b	22.69 \pm 0.53
	Diet T3	6.2 \pm 0.12 ^a	26 \pm 0.58 ^a	1.65 \pm 0.03 ^a	5.53 \pm 0.09 ^b	157.61 \pm 2.91 ^a	37.59 \pm 0.63 ^a	23.85 \pm 0.09
15 th Day	Control	5.4 \pm 0.1 ^c	20.67 \pm 0.67 ^c	1.48 \pm 0.11 ^b	3.15 \pm 0.08 ^c	140.2 \pm 6.47 ^{ab}	36.78 \pm 2.73 ^a	26.17 \pm 0.73 ^a
	Diet T1	5.77 \pm 0.03 ^b	22.33 \pm 0.33 ^b	1.52 \pm 0.04 ^b	4.85 \pm 0.03 ^a	146.89 \pm 5.18 ^a	37.91 \pm 0.95 ^a	25.83 \pm 0.33 ^{ab}
	Diet T2	5.77 \pm 0.03 ^b	23.33 \pm 0.33 ^b	2.14 \pm 0.01 ^a	4.43 \pm 0.17 ^b	109.21 \pm 1.64 ^c	26.99 \pm 0.32 ^b	24.72 \pm 0.3 ^{ab}
	Diet T3	6.7 \pm 0.1 ^a	28.33 \pm 0.33 ^a	2.15 \pm 0.02 ^a	4.87 \pm 0.07 ^a	131.78 \pm 1.17 ^b	31.17 \pm 0.63 ^b	23.66 \pm 0.62 ^b
30 th Day	Control	5.1 \pm 0.26	21.33 \pm 1.33	1.56 \pm 0.1	5.48 \pm 0.16 ^b	136.6 \pm 2.97 ^{ab}	32.69 \pm 0.86	23.97 \pm 1.01
	Diet T1	5.4 \pm 0.42	25.67 \pm 0.33	1.65 \pm 0.15	6.25 \pm 0.13 ^a	157.99 \pm 11.95 ^a	32.86 \pm 0.47	21.02 \pm 1.43
	Diet T2	5.47 \pm 0.48	22.33 \pm 1.45	1.62 \pm 0.04	6.2 \pm 0.08 ^a	137.59 \pm 5.53 ^{ab}	33.65 \pm 2.14	24.41 \pm 0.6
	Diet T3	5.63 \pm 0.07	23.33 \pm 0.33	1.89 \pm 0.05	6.35 \pm 0.09 ^a	123.76 \pm 1.73 ^b	29.89 \pm 0.55	24.15 \pm 0.32
60 th Day	Control	4.57 \pm 0.09 ^c	19.33 \pm 0.67 ^b	1.62 \pm 0.05 ^b	4.37 \pm 0.07 ^c	119.58 \pm 0.45 ^a	28.28 \pm 0.44 ^a	23.65 \pm 0.42
	Diet T1	4.8 \pm 0.12 ^{bc}	20.67 \pm 1.76 ^b	1.78 \pm 0.14 ^b	5.3 \pm 0.12 ^b	116.31 \pm 2.94 ^a	27.26 \pm 1.56 ^a	23.46 \pm 1.39
	Diet T2	5 \pm 0.1 ^b	21.67 \pm 1.67 ^{ab}	1.84 \pm 0.15 ^b	5.58 \pm 0.1 ^{ab}	117.66 \pm 1.86 ^a	27.39 \pm 1.61 ^a	23.27 \pm 1.23
	Diet T3	5.43 \pm 0.15 ^a	25.33 \pm 0.33 ^a	2.49 \pm 0.12 ^a	5.85 \pm 0.08 ^a	102.05 \pm 3.42 ^b	21.86 \pm 0.42 ^b	21.44 \pm 0.33

Values are means \pm SEM, n = 3 per treatment group.

Means in a column without a common superscript letter differ ($P < 0.05$) as analyzed by one-way ANOVA and the DUNCAN test.

Table 3
Effect of nutraceutical combination on the immunological parameters of *Labeo rohita*.

Sampling days	Diet	NBT (OD at 540 nm)	Lysozyme (U min^{-1})	Total immunoglobulin (g dl^{-1})
7 th Day	Control	0.79 \pm 0.04 ^c	3.84 \pm 0.13 ^d	0.74 \pm 0.07 ^b
	Diet T1	0.83 \pm 0.01 ^{bc}	4.38 \pm 0.07 ^c	3.59 \pm 0.43 ^a
	Diet T2	0.88 \pm 0.02 ^{ab}	5.52 \pm 0.15 ^b	2.79 \pm 0.4 ^a
	Diet T3	0.91 \pm 0.01 ^a	7.16 \pm 0.07 ^a	1.54 \pm 0.11 ^b
15 th Day	Control	0.68 \pm 0.03 ^c	6.29 \pm 0.13 ^c	2.47 \pm 0.39 ^b
	Diet T1	0.78 \pm 0.01 ^b	6.36 \pm 0.06 ^c	2.69 \pm 0.06 ^b
	Diet T2	0.81 \pm 0.01 ^b	6.8 \pm 0.03 ^b	3.61 \pm 0.28 ^a
	Diet T3	0.92 \pm 0.01 ^a	7.35 \pm 0.14 ^a	3.93 \pm 0.03 ^a
30 th Day	Control	0.8 \pm 0.04 ^b	4.1 \pm 0.2 ^c	1.57 \pm 0.12 ^d
	Diet T1	0.83 \pm 0.03 ^b	5.36 \pm 0.18 ^b	2.24 \pm 0.08 ^c
	Diet T2	0.95 \pm 0.02 ^a	5.3 \pm 0.32 ^b	2.72 \pm 0.14 ^b
	Diet T3	0.85 \pm 0.02 ^b	6.84 \pm 0.05 ^a	3.37 \pm 0.13 ^a
60 th Day	Control	0.77 \pm 0.05 ^b	4.67 \pm 0.27	1.72 \pm 0.1 ^c
	Diet T1	0.83 \pm 0.06 ^b	4.71 \pm 0.27	3.29 \pm 0.22 ^a
	Diet T2	0.97 \pm 0.03 ^{ab}	4.29 \pm 0.09	2.13 \pm 0.23 ^{bc}
	Diet T3	1.07 \pm 0.1 ^a	5.19 \pm 0.08	2.66 \pm 0.1 ^b

Values are means \pm SEM, n = 3 per treatment group.

Means in a column without a common superscript letter differ ($P < 0.05$) as analyzed by one-way ANOVA and the DUNCAN test.

significantly higher ($p < 0.05$) than control at 7th day and significantly lower ($p < 0.05$) in T3 at 60th day. The MCV value first increases and gradually decrease in sampling days. A significant ($p < 0.05$) higher MCH value was found in T2 and T3 at 7th day whereas a significantly ($p < 0.05$) lower value was found in T2 and T3 at 15th day and in T3 at 60th day of sampling compared to control. However, there was no significant difference in the entire group at 30th day of sampling. No significant difference was found in all the treatment groups at different sampling days except that T3 was significantly ($p < 0.05$) lower than control at 15th day.

3.2. Immunological parameters

The immunological responses of *Labeo rohita* fingerlings fed with nutraceutical combination (*Withania somnifera* (Ashwagandha) root extract and dietary L-ascorbic acid (vitamin C)) based diets are presented in Table 3. NBT value of T3 was significantly ($p < 0.05$) higher than control at 7th, 15th and 60th day whereas at 30th day T2 was significantly ($p < 0.05$) higher than control. At 7th and 30th day the lysozyme in T1, T2 and T3 were significantly ($p < 0.05$) higher than control whereas T2 and T3 at 15th day. However, no significance

Table 4
Effect of nutraceutical combination on the biochemical parameters of *Labeo rohita*.

Sampling days	Diet	Glucose (mg l ⁻¹)	Total protein (g dl ⁻¹)	ALP (IU l ⁻¹)	GPT (IU l ⁻¹)	GOT (IU l ⁻¹)
7 th Day	Control	86.7 ± 1.24 ^a	2.8 ± 0.04 ^d	21.75 ± 1.62 ^a	16.04 ± 1 ^a	25.87 ± 0.79 ^a
	Diet T1	84.59 ± 0.33 ^a	6.69 ± 0.15 ^a	21.44 ± 2.14 ^a	13.94 ± 0.43 ^b	24.19 ± 0.75 ^a
	Diet T2	79.32 ± 0.04 ^b	6.02 ± 0.18 ^b	15.62 ± 2.12 ^{ab}	15.65 ± 0.2 ^{ab}	25.41 ± 1.36 ^a
	Diet T3	74.61 ± 0.33 ^c	4.58 ± 0.16 ^c	12.87 ± 1.4 ^b	8.95 ± 0.45 ^c	20.5 ± 0.56 ^b
15 th Day	Control	115.73 ± 1.97 ^a	5.17 ± 0.08 ^b	20.22 ± 0.53 ^a	19.53 ± 1.11 ^a	22.31 ± 1.4 ^a
	Diet T1	107.78 ± 0.41 ^b	5.24 ± 0.18 ^b	15.01 ± 1.71 ^b	15.52 ± 1.4 ^b	14.81 ± 0.26 ^b
	Diet T2	106.11 ± 0.41 ^{bc}	6.04 ± 0.12 ^a	13.17 ± 0.81 ^b	15.39 ± 0.85 ^b	14.29 ± 1.02 ^{bc}
	Diet T3	103.55 ± 0.62 ^c	6.35 ± 0.09 ^a	6.43 ± 1.4 ^c	14.1 ± 0.91 ^b	11.77 ± 0.17 ^c
30 th Day	Control	100.32 ± 3.82	4.1 ± 0.2 ^b	13.48 ± 1.62	12.09 ± 1.27	30.13 ± 2.27
	Diet T1	100.16 ± 4.37	5.23 ± 0.36 ^a	12.25 ± 0.81	14.32 ± 1.56	25.48 ± 2.48
	Diet T2	100.97 ± 1.65	5.48 ± 0.08 ^a	15.32 ± 3.41	14.68 ± 1.48	29.36 ± 0.76
	Diet T3	100.2 ± 5.25	5.9 ± 0.02 ^a	14.09 ± 1.34	15.13 ± 2.2	26.45 ± 0.95
60 th Day	Control	91.21 ± 5.24	4.51 ± 0.21 ^c	12.87 ± 1.4 ^b	15.07 ± 2.38	30.2 ± 2.36 ^a
	Diet T1	98.66 ± 4.46	6.45 ± 0.16 ^a	17.46 ± 1.06 ^a	13.52 ± 1.52	14.61 ± 1.04 ^c
	Diet T2	81.27 ± 1.5	5.02 ± 0.1 ^c	19.3 ± 0.53 ^a	15.26 ± 1.46	20.95 ± 0.51 ^b
	Diet T3	88.72 ± 4.36	5.57 ± 0.17 ^b	16.24 ± 1.71 ^{ab}	13.71 ± 1.34	17.4 ± 0.86 ^{bc}

Values are means ± SEM, n = 3 per treatment group.

Means in a column without a common superscript letter differ ($P < 0.05$) as analyzed by one-way ANOVA and the DUNCAN test.

difference was found in all the treatment groups on 60th day. Total immunoglobulin (TIg) of all the treatment groups T1, T2 and T3 were higher as compared to the control in all the sampling days. T1 and T2 were significantly ($p < 0.05$) higher than control at 7th day, T2 and T3 at 15th day and T1 and T3 at 60th day of samplings. T1, T2 and T3 were significantly ($p < 0.05$) higher than control at 30th day of sampling.

3.3. Biochemical parameters

The biochemical responses of *Labeo rohita* fingerlings fed with nutraceutical combination (*Withania somnifera* (Ashwagandha) root extract and dietary L-ascorbic acid (vitamin C)) based diets are presented in Table 4. There is a significant ($p < 0.05$) decrease in blood glucose level in all the treatment groups at 7th and 15th day as compared to the control. However, no significant difference was found in all the other groups at 30th and 60th day. The total protein was higher in all the treatments group than control at all sampling days. A significant ($p < 0.05$) difference was seen in T1, T2 and T3 at 7th day, T2 and T3 at 15th day, T1, T2 and T3 at 30th day and in T1 and T3 at the 60th day of sampling as compared to the control. Alkaline phosphatase (ALP) of T3 at day 7 and T1, T2 and T3 at day 15 were significantly ($p < 0.05$) lower than control while T1 and T2 were significantly ($p < 0.05$) higher than control at 60th day. However, no significant difference was found in treatment groups on the 30th day of sampling. The GPT of T1 and T3 were significantly ($p < 0.05$) lower than the control at 7th day while T1, T2 and T3 were significantly ($p < 0.05$) lower than the control at 15th day. No significant difference was found on 30th and 60th day in all the groups. However, a significant ($p < 0.05$) difference was found among the treatment groups at 7th day but all the treatment were same in 15th day. All the treatment groups had lower GOT value in all the sampling days compared to control. On 7th day, T3 was significantly ($p < 0.05$) lower than the control. At 15th day and 60th day T1, T2 and T3 were significantly ($p < 0.05$) lower than control. No significant difference was found on 30th day.

Table 5
Relative percentage survival (RPS) of *Labeo rohita* fingerlings after challenge study.

	No stress	Low pH (4.4–4.7)	Waterborne iron 6.05mg l ⁻¹	Low pH + Waterborne iron
Control fish	100 ± 0 ^a	50 ± 10 ^b	85 ± 5 ^a	0 ± 0 ^b
Supplemented fish	100 ± 0 ^a	95 ± 5 ^a	95 ± 5 ^a	65 ± 5 ^a

Values are means ± SEM, n = 2 per treatment group.

Means in a column without a common superscript letter differ ($P < 0.05$) as analyzed by one-way ANOVA and the DUNCAN test.

3.4. Challenge study

Challenge study against waterborne iron and low pH toxicity shows that *Labeo rohita* fingerlings fed with diet T3 for 15 days have higher resistance and are more tolerance to stressor provided in all the treatment groups (except in the group without stress) compared to fish fed with control feed for the same duration. Supplemented fish shows significantly ($P < 0.05$) higher survivability when exposed to low pH and multiple stressors (waterborne iron and low pH) compared to control fish. Supplemented fish also shows higher survival rate under exposure to waterborne iron than control fish. High mortality was found in control fish exposed to low pH, and 100% mortality under multiple stressors (waterborne iron and low pH). The relative percentage survival (RPS) of *Labeo rohita* fingerlings in different experimental groups after 10 days are given in Table 5 and Fig. 1.

4. Discussion

Studies on nutraceutical for health management in aquaculture become a major field of research in recent years [21]. According to De Felice nutraceuticals not only provide nutrients necessary for growth and maintenance but also have the medicinal effect [41]. The present study also witnesses the effect of *Withania somnifera* (Ashwagandha) root extract and dietary L-ascorbic acid (vitamin C) combination on the haematological, immunological and biochemical parameters of *Labeo rohita* fingerlings.

4.1. Haematological parameters

Haematological parameters are good indicators of the physiological status of animals [42] and are widely used to measure an index of health status in a number of fish species [43,44]. The present haematological study shows an increase in the haemoglobin content, haematocrit value, RBC and WBC count with an increase in the concentration of diets compared to control at all sampling days. A significantly higher

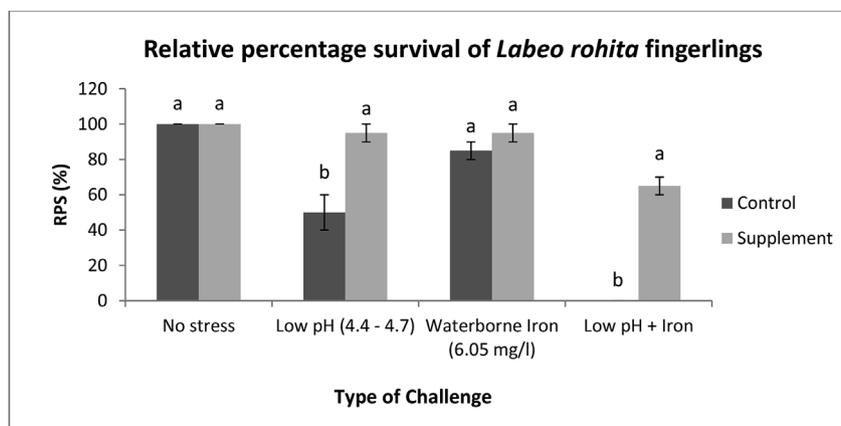


Fig. 1. Relative percentage survival of *Labeo rohita* fingerlings after challenge study. Means without a common superscript letter differ ($P < 0.05$) as analyzed by one-way ANOVA and the DUNCAN test.

value was found in fish fed with a diet containing 1.0% of nutraceutical combination. The present result supports the finding of Khara et al. [45] in Caspian brown trout supplemented with α -tocopherol (vitamin E) and ascorbic acid (vitamin C) combination. The same trend was also observed by Sharma et al. [46] in *Labeo rohita* fed with *Withania somnifera* root powder. The increase in haemoglobin content, haematocrit value and RBC may be attributed to vitamin C in its role in releasing liver ferritin, deposits within reticuloendothelial cells and its utilization for erythropoiesis [47,48] or may be due to the stimulating effect of *Withania somnifera* on the haematopoietic cells [49]. The antioxidant properties of vitamin C [50] and *W. somnifera* [26] also have a role in protecting the cell membrane including red blood cells against oxidative damages [51]. WBC plays an important role in the innate immunity, acting as the first line of defence against invading pathogens [52]. The increase in WBC count in the present study indicates the effect of nutraceutical combination in stimulating the immune system. The nutraceutical combination, *W. somnifera* and vitamin C are known to be a potent immunostimulant and their immunostimulatory effects were also documented in fish species such as brown trout [45], gillhead seabream [53,54] with vitamin C supplemented and in *L. rohita* [25] with *W. somnifera* supplemented. The red cell indices are useful in elucidating the etiology and morphological classification of anemias [55]. MCV, MCH and MCHC in the present study showed minor changes within the group at various sampling days. A significant difference was found in T2 and T3 at 7th day in MCV and MCH showing increasing value in the group compared to control whereas T3 was significantly lower at 15th day in MCHC and at 60th day in MCV and MCH. There was no significant difference in the entire other groups. Similar responses were also reported by Esmaili and Khara [56] with an increase in MCV and MCH in rainbow trout and by Sharifzadeh et al. [57] with no significant difference in MCV, MCH and MCHC in common carp fed with dietary nutraceutical combinations. The present result suggested that increase in MCV and MCH may be attributed to the protective ability of the nutraceutical combination because of their antioxidant property which prevents the cell by scavenging free radicals, thus preventing premature destruction of RBC (erythrocyte fragility) in the presence of stress [58,59]. However, among the other groups, no significant difference was found which suggested that the nutraceutical combination in the diet did not have a negative impact on these parameters.

4.2. Immunological parameters

The respiratory burst activity of fish fed with a diet containing 1.0% of *Withania somnifera* (Ashwagandha) root extract and dietary L-ascorbic acid (vitamin C) combination show a significant increase in 7th, 15th and 60th day of sampling. The respiratory burst, quantified by Nitroblue Tetrazolium (NBT) assay measures the quantity of

intracellular superoxide radical produced by leukocytes [60,61]. NBT activity is an indicator of oxygen-dependent bactericidal activities [62] and increase in respiratory burst activity can be correlated with increased bacterial pathogen killing activity of phagocytes [63]. The increase in respiratory burst activity was also reported by Sharma et al. [25] in *Labeo rohita* fingerlings fed with *W. somnifera* root powder, a similar response was also observed by Gupta et al. [64] in *Cyprinus carpio* fed with dietary microbial levan. Serum lysozyme activity also shows a significant increase in all the supplemented group at all sampling days except on 60th day. Fish supplemented with diet T3 shows the highest activity. The present observation was similar to the finding of Kumar et al. [65] in *L. rohita* fed with *Mikania cordata* leaf powder. Lysozyme is a humoral component of the non-specific defense mechanism that causes lysis of the bacterial cell wall [66]. Enhancement in lysozyme activity could be attributed to the increasing number of white blood cells producing lysozyme and stimulation of mucosal cells in different areas of the body including the skin and gastrointestinal tract [67]. These suggest that the nutraceutical combination has immunoboostring property in *L. rohita* fingerlings. Elevated lysozyme activity was also observed by Khara et al. [45] in Caspian Brown Trout, *Salmo Trutta Caspius* juveniles fed with α -tocopherol (vitamin E) and ascorbic acid (Vitamin C) combination. Immunoglobulin is a protein constituting antibody that specifically binds to alien substances such as viruses and bacteria, preventing infection. The total immunoglobulin in the present study also showed a significant increasing trend in the treatment group at 15th and 30th day of sampling. Similar responses were also reported by Sharma et al. [25] in *L. rohita* fed with *W. somnifera* root powder and also by Khara et al. [45] in Caspian Brown Trout, *Salmo Trutta Caspius* juveniles fed with α -tocopherol (vitamin E) and ascorbic acid (Vitamin C) combination. Changes in the immunological parameters and its enhancement as a result of supplementation of nutraceutical combination in the present study may be possibly due to, the ability of vitamin C in modulating immune function through modulation of redox-sensitive cell signaling pathways [68] or, the immunostimulatory and the protective effect of antioxidant present in vitamin C and *W. somnifera* by directly protecting important cell structural components against oxidative damage induced by free radicals [68–70]. Thus, the present results suggested that the nutraceutical combination has the ability to enhance some non-specific immune parameters, such as lysozyme activity, immunoglobulin, and respiratory burst activity.

4.3. Biochemical parameters

Glucose has a major role in the bioenergetics of animals [71] and as an indicator of stress [72]. The present study showed a significant decrease in glucose level at 7th and 15th day of sampling which shared a

similar result with Asimi and Sahu [73] in *Labeo rohita* fed with clove and cardamom extracts. No significant variation was found in the group at 30th and 60th day of sampling, which was similar to the study done by Das et al. [74] in *L. rohita* fed with *Ocimum sanctum* extract. Glucose level is one of the most common stress indicators and an increase in its level indicates stress in fish [75–77]. Since, the glucose level in the present study was less than or almost similar level with control, the study suggests the ameliorating effect of nutraceutical combination because of their antioxidant property and showed that supplementation of the selected diets does not impart stress on fish when fed for 30 days. Total serum protein was increased in all supplemented group compared to control at all sampling days. The value within the group varies with a significant difference in T3 at all sampling days. The increase in total serum protein was also documented by Kumar et al. [78] in goldfish and Gupta et al. [64] in *Cyprinus carpio* fed with nutraceuticals. Protein plays an important role in the maintenance and regulation of the immune system [79]. A strong innate immunity is correlated with the increase in total serum protein [80]. This suggests the finding that nutraceutical combination fed in the diet of *L. rohita* fingerlings has the ability to raise the immunity with an increase in protein. The serum enzyme, alkaline phosphatase (ALP), glutamic pyruvic transaminase (GPT) and glutamic oxaloacetic transaminase (GOT) showed a decrease in the supplemented group compared to the control on the 7th and 15th day. Fish fed with the diet containing 1.0% of nutraceutical combination was found to have a significantly lower value as compared to control. Alkaline phosphatase, a zinc-containing metalloenzyme, plays an important role in phosphorus metabolism. A significant decrease in the activity of ALP in blood serum was observed by Metwally [81] in tilapia (*Oreochromis niloticus*) fed with garlic (*Allium sativum*). GPT and GOT actively take part in transamination, serving the changing energy demands of the organisms in various adaptive situations [82]. Thereby, plays an important role in the assessment of liver damage [83] or in the diagnosis of disease or disorder. GPT and GOT enzymes are present in the cytosol of the hepatocytes. GPT is present primarily in liver whereas GOT is found in all body tissues especially heart, liver, skeletal muscle and in the mitochondria. Both the enzymes are specific to liver and increased activity of GPT and GOT in serum, in absence of acute necrosis or ischaemia of other organs such as myocardium, suggests liver cell damage and leaching of these enzymes in blood [78]. In contrast, the present study showed a significant decrease in these enzymes which shared similar results with the study done by Asimi and Sahu [73] in *L. rohita* fed with 0.5% of clove and cardamom extracts. A similar response was also observed by Tahmasebi-Kohyani et al. [84] in rainbow trout (*Oncorhynchus mykiss*) fed dietary nucleotides with a fall in ALP, GPT and GOT level as compared to control. The decline in serum enzyme ALP, GPT and GOT in treated fish in this study may be due to the fall in the rate of synthesis of glycogen, resulting from the low metabolic demands [85] and a decrease in metabolic transport [86] due to the action of antioxidant activity of nutraceutical combination. The preventive effect of the nutraceutical combination (*W. somnifera* and vitamin C) induced biochemical changes indicates its anti-stress activity.

4.4. Challenge study

The survival rate of *Labeo rohita* fingerlings fed with supplemented diet T3 containing 1.0% of *Withania somnifera* (Ashwagandha) root extract and dietary L-ascorbic acid (vitamin C) for 15 days were higher than the fish fed with control diet for the same period. Challenged study against waterborne iron, low pH and multiple (waterborne iron and low pH) stresses for 10 days showed that, exposure to single or multiple stresses have a negative impact on fish (whether supplemented or control fish) resulting in mortality compared to a group with no stress having 100% survival rate. The mortality rate was highest in the control group challenged against multiple stresses with 100% mortality. This result can be correlated with the increase in ferrous iron (Fe²⁺) at low

pH than ferric iron (Fe³⁺), which was considered to be more toxic to fish and has the ability to precipitate on the fish gills [14]. Mortality due to waterborne iron toxicity was also documented by Gonzalez et al. [87] in brook charr *Salvelinus fontinalis* (Mitchill) and Debnath et al. [13] in fry of Indian major carps. Mortality of fish at low water pH was also documented by Zahangir et al. [6] in zebrafish. However, compared to the fish fed with control diet the supplemented fish showed a higher survival rate in all the treatment groups. The higher survival rate in the supplemented group can be attributed to the ameliorative effect of nutraceutical combination. Vitamin C is a good antioxidant, able to scavenge free radicals and reduce metal toxicity [30–32,88] whereas *Withania somnifera* (ashwagandha) is known to possess anti-stress activity [27]. The increasing tolerance of supplemented fish against the stresses can be the effect of the combination of these two nutraceuticals. Akhtar et al. [89] also showed that, supplementation of dietary tryptophan augments growth, lower energy demand and helps in mitigating thermal and salinity stress in *L. rohita*. However, further research will be needed to know the active principle compound and its mechanisms responsible for making the fish tolerance to the provided stresses.

In conclusion, the inclusion of 1.0% of a combination of *Withania somnifera* (Ashwagandha) root extract and dietary L-ascorbic acid (vitamin C) in the diet has an immunostimulatory effect and feeding for 15 days can increase the anti-stress activity by showing higher survival rate against multiple stresses (waterborne iron and low pH toxicity) in *Labeo rohita* fingerlings.

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