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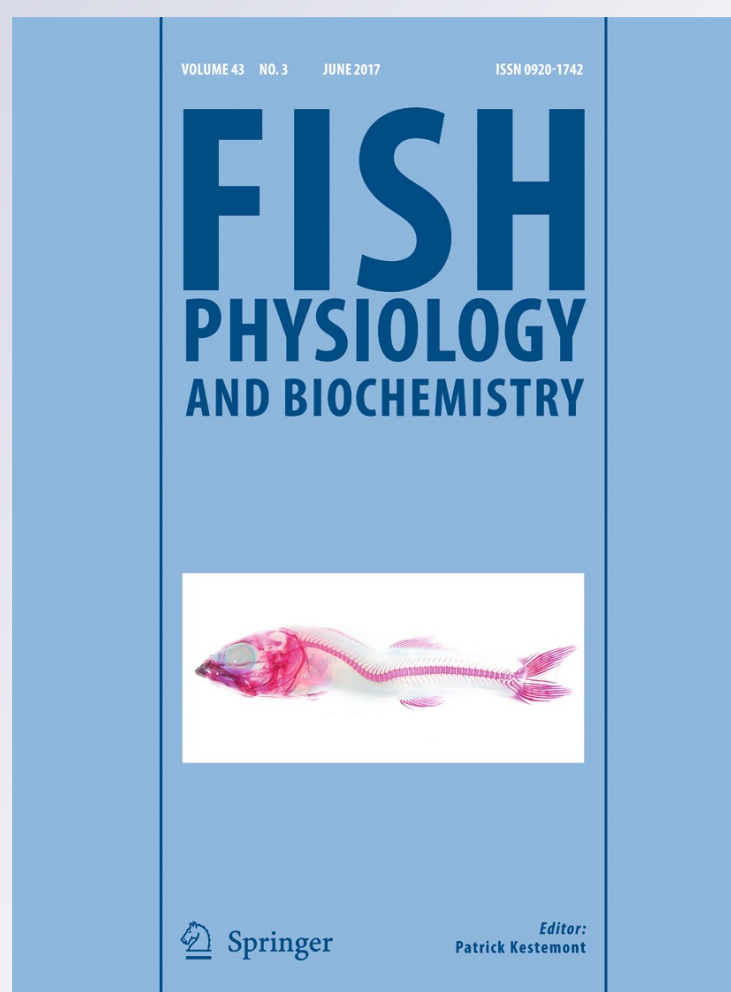
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Haemato-biochemical, non-specific immunity, antioxidant capacity and histopathological changes in *Labeo rohita* fingerlings fed rubber protein isolate

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Abstract A 60-day feeding trial was conducted to evaluate the haemato-biochemical, innate immune response, antioxidant capacity and histopathological changes in *Labeo rohita* fingerlings fed rubber protein isolates (RPI). One hundred and eighty fingerlings (average weight 4.45 ± 0.01 g) were distributed into five experimental groups in triplicate and fed with isonitrogenous and isocaloric diets. Soybean protein isolate (SPI) served as the reference diet (Control), and the treatment diets were formulated as RPI₂₅, RPI₅₀, RPI₇₅ and RPI₁₀₀ replacing 25, 50, 75 and 100% of SPI protein, respectively. The growth performance indices like final body weight (9.54–10.27 g), net weight gain (5.09–5.84 g), metabolic growth rate (4.54–5.02) and feed efficiency ratio (0.60–0.65) among the various groups were not significantly different ($P > 0.05$). All the haematological parameters, except red blood cells, showed no significant differences compared with the control group ($P > 0.05$). The immuno-biochemical parameters like

albumin, globulin, total immunoglobulin, respiratory burst and lysozyme activities among the various groups did not differ significantly ($P > 0.05$). The stress enzyme such as superoxide dismutase (SOD), catalase (CAT) and oxidative stress marker malondialdehyde (MDA) showed no significant difference ($P > 0.05$). Histopathological examination of the liver revealed no marked changes. In summary, the results showed that RPI was well utilised by the fish and its inclusion did not generate any oxidative-induced stress, thus, RPI may be suggested as a potential replacement for SPI in fish diets without any detrimental effects. Hence, protein isolation offers a unique opportunity for the utilisation of rubber seed meal.

Keywords Protein isolate · Haematology · Innate immunity · Oxidative enzyme · *Labeo rohita*

Introduction

Soybean meal is one of the most researched and widely accepted plant protein sources in commercial aquafeed production. The nutritional quality in terms of amino acid composition, digestibility and acceptability has made it an ingredient of choice by feed manufacturers for animal feed production and is considered as a standard protein to which other plant ingredients are compared (Cromwell 1999; Yue and Zhou 2008). However, the rapid expansion of aquaculture enterprises and the increasing demand by human and other animal production sectors have pushed up the price for this ingredient.

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Hence, there is a need to search for more alternative plant protein ingredients especially those that do not compete with human food security.

Rubber seed meal (RSM) is the main by-product of rubber seeds after oil extraction. The seed meal contains about 21–25% crude protein (Sharma et al. 2014; Fawole et al. 2016a) and high essential amino acids especially isoleucine, leucine, threonine and valine (Aigbodion and Bakare 2005; Eka et al. 2010). However, despite the relatively good protein and amino acid composition, the inclusion of the meal in animal feed is limited due to the presence of cyanogenic glycosides, which on hydrolysis liberate hydrogen cyanide (HCN). Cyanide is a potent cytotoxic agent and rapidly acting asphyxiant that has the potential to inhibit cytochrome oxidase enzyme, thus hindering ATP production via electron transport chain (Egekeze and Oehme 1980; Makkar et al. 2007). Its affinity towards the haem component of the haemoglobin and red pigment in the blood (Isom and Way 1974) ultimately affects the erythropoiesis processes, red blood cell formation and oxygen-carrying capacity of the blood (Sharma et al. 2014). Feeding high level of HCN to the animal could result in growth depression, amino acid depletion in the body, decreased protein synthesis, impaired metabolism and suppression of the immune system (Vitharauge Mallika et al. 1991; Ukebor et al. 2007; Deng et al. 2015). Rubber seed meal has been tested as an alternative protein source ingredient in different fish species such as *Cyprinus carpio* (Suprayudi et al. 2015), *Labeo rohita* (Sharma et al. 2014) and tilapia (Alegbeleye et al. 2004; Deng et al. 2015), with little success due to HCN toxicity. Several methods have been used to reduce the level of this toxic factor in RSM such as heat treatment and storage (Gandhi et al. 1990; Sharma et al. 2014), fermentation (Okafor and Anyanwu 2006), sun-drying, soaking, boiling etc. The use of the above methods had shown that feeding RSM beyond 30% to fish could cause depression in growth and feed efficiency, decline in haematological indices and immune suppression (Sharma et al. 2014; Deng et al. 2015). Nevertheless, protein extraction method has proven to overcome many of these detrimental effects.

Protein isolates are good alternative protein sources for fish owing to their nutritional values as characterised by high protein and amino acid digestibility, low anti-nutritional content and consistent quality (Dersjant-Li 2002; Fawole et al. 2016a). Protein isolate from soybean, pea and canola meal are commercially available

and used in aquafeed, but no previously published data or information are available on protein isolate from rubber seed meal and its effect on fish health and growth. Haemato-biochemical parameter is a fundamental tool used in several studies to assess the nutritional and health condition of the animal in response to different diets (Akpodiete and Ologbodo 1998; Jahanbakhshi et al. 2013). Hence, the present study aim was to evaluate the effects of rubber protein isolate on the haemato-biochemical parameters, innate immune response, liver antioxidant capacity and histopathological changes in *L. rohita* fingerlings.

Materials and methods

Rubber protein isolate production

The rubber kernel meal (RKM) used for the production of protein isolate (RPI) was obtained from Tripura (22°56' N–24°32' N, 90°09' E–92°20' E), India, dehulled, powdered, and defatted with hexane three times. Protein isolate was prepared following the method of Saetae and Suntornsuk (2011) with little modifications. Briefly, RKM was dispersed in distilled water at a ratio of 1:15 (w/v). The pH of the solution was adjusted to 12 with 1 N NaOH and stirred for 2 h at room temperature. The insoluble fibre was removed by centrifugation at 7000 rpm for 20 min, and the supernatant was collected and adjusted to pH 4 with 1 N HCl to precipitate the protein. The precipitated protein was centrifuged and washed twice with distilled water to obtain the protein isolate. The wet protein isolate was lyophilised (Scanvac™ cool safe 100-9 pro), and the dried sample was analysed for proximate composition and used in feed preparation.

Estimation of anti-nutritional factors

Phytic acid

For phytic acid extraction, 0.5 g of dried sample was weighed and transferred to a 125-mL Erlenmeyer flask and extracted with 10 mL of 3.5% HCl. The samples were constantly shaken in an orbital shaker (ORBITEK®; Scigenics, India) for 1 h at 200 rpm, followed by centrifugation at 5000 rpm for 10 min, and the supernatant was collected for analysis (Makkar et al. 2007). The phytic acid in the extract was

determined according to the colorimetric assay described by Gao et al. (2007), with slight modification. Exactly 0.1 mL of sample extract was diluted to 3 mL with distilled water and then thoroughly mixed with 1 mL of Wade Reagent (0.03% $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ + 0.3% sulphosalicylic acid). The mixture was centrifuged at 5000 rpm for 10 min after standing for 30 min, and the absorbance of supernatant was immediately measured at 500 nm against distilled water. The phytic acid content was calculated from the calibration curve of phytic acid standard and expressed as milligrammes of phytic acid (PA) per gramme of sample (mg g^{-1}). All the extractions were carried out in triplicates.

Hydrogen cyanide (HCN)

Cyanide content of the sample was estimated by the method of AOAC (2000). Finely powdered sample of 10 g was taken and added with 200 mL of distilled water and allowed to stand at room temperature for 2 h. It is then steam distilled, and about 150–170 mL of the distillate was collected in 15 mL of NaOH (0.5 g in 20 mL distilled water). The solution was made up to 250 mL with distilled water. An aliquot of about 10 mL was taken, and 8 mL of 6 N ammonium hydroxide (NH_4OH) and 2 mL of 5% potassium iodide were added and titrated against 0.02 N silver nitrate (AgNO_3) until permanent turbidity appears. For easy recognition of the end point of titration, a black background was used. Analysis was done in triplicates and cyanide content was determined using the formula given below.

$$1 \text{ mL of } 0.02 \text{ N AgNO}_3 = 1.08 \text{ mg HCN}$$

Feed formulation and experimental animal

Five isonitrogenous ($326.2 \pm 1.3 \text{ CP kg}^{-1}$) and isocaloric ($18.47 \pm 0.08 \text{ MJ kg}^{-1}$) experimental diets were formulated for the trial. The soybean protein isolate (SPI) served as the reference diet (Control). The diets were prepared as 0, 25, 50, 75 or 100% (designated as Control, RPI_{25} , RPI_{50} , RPI_{75} and RPI_{100} , respectively) of SPI protein replaced with RPI on equivalent protein basis. Other ingredients and vitamin–mineral mix were also included in equal proportion, except wheat flour, as shown in Table 1. All the ingredients were milled and mixed thoroughly to form a homogenous blend, and

hand pelletised using a 2-mm die. The pellets were air dried and stored at -20°C until use.

Labeo rohita (rohu) fingerlings obtained from Srushti Aquaculture, Sudhagad, Raigad District, Maharashtra, India, and acclimatised to the laboratory condition in the Division of Fish Nutrition, Biochemistry and Physiology, ICAR–Central Institute of Fisheries Education, Mumbai, were used for the experiment. One hundred and eighty rohu fingerlings were randomly distributed in 15 plastic rectangular tubs (75 L capacity) each containing 12 fish (average weight $4.45 \pm 0.01 \text{ g}$) per tank, in triplicate. Fish were hand fed with their respective diets to apparent satiation twice daily at 10:00 h and 18:00 h under normal light regime. The fish in each tub were batch weighed every 2 weeks over a period of 60 days. The fish were starved 24 h before weighing. Water quality parameters were monitored and maintained between pH 7.5–8.1, DO 6.3–7.5 mg L^{-1} , temperature 25–29 $^\circ\text{C}$ and ammonia 0.05–0.09 ppm.

Growth performance indices

Growth was assessed by measuring the final body weight (FBW), net weight gain (NWG), metabolic growth rate (MGR) and feed efficiency ratio (FER). Calculations were made using the following formula: $\text{NWG (g fish)} = \text{final body weight (g)} - \text{initial body weight (g)}$; $\text{MGR (g kg}^{-0.8} \text{ day}^{-1}) = (\text{net weight gain in g}) / [\{(\text{initial body weight in g}/1000)^{0.8} + (\text{final body weight in g}/1000)^{0.8}\} / 2] / \text{feeding days}$; $\text{FER} = \text{weight gain (g)} / \text{dry feed given (g)}$.

Fish sampling for blood and serum collection

Blood samples were collected from randomly selected fish (six fish per treatment) after a 60-day feeding trial. The fish were anaesthetised with clove oil (50 μL of clove oil per litre of water) and blood 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 an EDTA-coated tube. The blood was used for the determination of haemoglobin (Hb) content, total erythrocyte (RBC) and leucocyte counts (WBC), and nitroblue tetrazolium (NBT) assay. Another fresh blood was collected (four from each replicate) from the caudal vein without anticoagulant into a dried Eppendorf tube and allowed to stand in a slanted position at room temperature. The sera were centrifuged at 5000 rpm for 10 min

Table 1 Feed and proximate composition of the experimental diets (DM basis) fed to *Labeo rohita* fingerlings

Ingredients (g kg ⁻¹)	C	RPI ₂₅	RPI ₅₀	RPI ₇₅	RPI ₁₀₀
Soy protein isolate ^a	200	150	100	50	0
Rubber protein isolate ^b	0	47	93	139	187
Ground nut cake ^c	126	126	126	126	126
Fish meal ^c	50	50	50	50	50
Rice bran ^c	240	240	240	240	240
Wheat flour	290	293	297	301	303
Sunflower oil/cod liver oil(1:1) ^d	60	60	60	60	60
Dicalcium phosphate ^e	10	10	10	10	10
Vitamin–mineral mix	10	10	10	10	10
Choline chloride ^e	2	2	2	2	2
Butylated hydroxytoluene ^e	2	2	2	2	2
Carboxymethyl cellulose ^e	10	10	10	10	10
Total	1000	1000	1000	1000	1000
Proximate composition (g kg ⁻¹ DM)					
Dry matter	921	921	918	917	921
Crude protein	329	329	323.8	323.8	325.5
Crude lipid	62.5	62.5	65.0	65.0	62.5
Ash	84.6	79.6	79.6	75.0	75.0
Crude fibre	65.0	70.0	70.0	70.0	70.0
NFE [*]	459	459	462	466	462
Gross energy (MJ kg ⁻¹) [†]	18.4	18.4	18.4	18.5	18.6
Cyanide (mg HCN kg ⁻¹) ^{††}	0.00	1.27	2.51	3.75	5.05
Phytic acid (g kg ⁻¹) ^{††}	2.86	2.87	2.86	2.86	2.88

^a Soy Growth™ Medicamen organics limited, India; 84.5% crude protein, 3.5% crude lipid, 4% ash

^b RPI; 90.8% crude protein, 1.5% crude lipid, 2% ash

^c Purchased from local animal feed ingredient dealer, Mumbai, India

^d Universal Medicare Private Limited, Mumbai, India

^e Himedia Pvt., Mumbai, India

*NFE (nitrogen-free extract) = 1000 – (crude protein + crude lipid + crude fibre + ash)

[†] Calculated gross energy (MJ kg⁻¹) = [23.9 × CP (g kg⁻¹) + 39.8 × CL (g kg⁻¹) + 17.6 × NFE (g kg⁻¹)]/1000

^{††} Calculated based on cyanide and phytic acid concentration in RPI (27 mg HCN kg⁻¹, 15.4 g kg⁻¹) and SPI (14.3 g kg⁻¹)

Composition of vitamin–mineral mix (PRE-EMIX PLUS) (quantity kg⁻¹): vitamin A, 5,500,000 IU; vitamin B, 2000 mg; vitamin D3, 1,100,000 IU; vitamin E, 750 mg; vitamin K, 1000 mg; vitamin B6, 1000 mg; vitamin B1, 2.6 µg; vitamin C, 100 mg; calcium pantothenate, 2500 mg; nicotinamide, 10 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, 125 mg

in a cooling centrifuge (REMI CPR-24, India), transferred into a dry Eppendorf tube and kept in –20 °C until use. The serum was used for the estimation of immunological and biochemical parameters.

Haematological parameters

Haemoglobin (Hb) content was determined following the cyanomethemoglobin method (Van Kampen and

Zijlstra 1961) using Drabkin's fluid (Qualigens, India). Haematocrit was determined following standard method and the percentage of the packed cell volume (% PCV) was determined. Total erythrocyte count (RBC) was done using a haemocytometer (Hendrick 1952), and total leucocyte count (WBC) was determined following the method of Shaw (1930). The haematological indices such as mean cell volume (MCV), mean cell haemoglobin (MCH) and mean cell haemoglobin

concentration (MCHC) were calculated from the values of Hct, Hb and RBC as described by Haney et al. (1992).

Serum protein biochemistry

Serum total protein and albumin were determined using a commercial kit (Erba® Diagnostic Mannheim; Transasia Bio-medicals Ltd., Solan, HP, India). Globulin content was calculated by subtracting albumin from total protein and the albumin/globulin ratio (A/G ratio) determined.

Immunological parameters

Total immunoglobulin (Ig) in the serum

Total immunoglobulin concentration was determined following the method described by Anderson and Siwicki (1995) by mixing 50 µL of serum with 50 µL 12% polyethylene glycol (PEG) solution (SRL® Mumbai, India). The mixture was incubated at 25 °C for 2 h under constant mixing in order to precipitate the immunoglobulin molecules. The precipitate was removed by centrifugation (5000 rpm, 4 °C, 15 min) and the supernatant collected and measured. The serum total protein was measured using a kit (Erba® Diagnostic Mannheim; Transasia Bio-medicals Ltd., Solan, HP, India). Total immunoglobulin concentration was determined using the formula below:

$$\text{Total Ig (mg mL}^{-1}\text{)} = \text{total serum protein} - \text{PEG absorbed protein}$$

Serum lysozyme activity

Serum lysozyme activity was based on the lysis of lysozyme sensitive *Micrococcus lysodeikticus* as described by Anderson and Siwicki (1995). In a cuvette, 50 µL of serum was added to 2 mL of *M. lysodeikticus* (Sigma, India) suspension in phosphate buffer (pH 7.3). 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 intervals 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 units (U) per minute.

Respiratory burst activity

The respiratory burst activity of the phagocytes was quantified by the NBT (nitroblue tetrazolium) assay (Secombes 1990). 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, 100 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 70% methanol. The plate was then air dried and 120 µL 2 N potassium hydroxide and 140 µL dimethyl sulphoxide were added into each well to dissolve the formazan blue precipitate formed. The OD of the turquoise blue coloured solution was read at 620 nm in a microplate reader (Quant; Bio-Tek Instrument, Winooski, VT, USA).

Biochemical test

Serum samples were used for the determination of alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), total bilirubin (TBR), phosphorus and calcium using a diagnostic kits (Erba® Diagnostic Mannheim; Transasia Bio-medicals Ltd., Solan, HP, India).

Antioxidant enzyme and oxidative stress marker

Liver tissues were dissected out from the sacrificed fish and homogenised in cold 250 mM sucrose solution using a Teflon-coated mechanical homogeniser (MICCRA D-9; ART Prozess and Labortechnik, Germany). The tubes were continuously kept in ice to avoid heating. The homogenate was centrifuged (5000 rpm for 10 min at 4 °C) and the supernatant stored at –80 °C until analysis. Using the supernatant, 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 (CAT) enzyme activity was done according to the method described by Takahara et al. (1960). The malondialdehyde (MDA) content in the liver was measured by TBA (thio-barbituric acid) method as described by Sharma and Krishnamurthy (1968). Protein contents of the tissue were determined according to Bradford (1976), adapted to microplate in order to express enzyme activities as a function of the protein content. Bovine serum albumin was used as the protein standard.

Histopathological study

Liver samples were taken from each treatments as well as control and fixed in 10% neutral formalin fixative for 24 h, dehydrated and embedded in paraffin. The serial sections of 5 μm thickness were prepared and stained with haematoxylin and eosin. Histological sections were observed under an Olympus FSX 100 phase contrast microscope equipped with a canon EOS 500D digital camera.

Statistical analysis

All the data were statistically analysed by statistics package IBM SPSS (version 20), and one-way ANOVA with Duncan's multiple range test was used to determine if the results of the treatments were significantly different from the control. The significance level was made at $P < 0.05$. Data are given as mean $\pm$ standard error of the mean.

Results

Growth performance

The growth performance indices like final body weight (9.54–10.27 g), net weight gain (5.09–5.84 g), metabolic growth rate (4.54–5.02) and feed efficiency ratio (0.60–0.65) among the experimental groups were not significantly different ($P > 0.05$) (Table 2). The fortnightly body weight increments of different groups are shown in Fig. 1.

Haematological changes

The haematological parameters of *L. rohita* fingerlings fed different experimental diets are given in Table 3. The

lowest RBC value was recorded in the group fed RPI₅₀ ($1.92 \pm 0.02 \times 10^6 \text{ mm}^{-3}$) diet compared to other groups ($P < 0.05$), while Hb, WBC, Hct, MCV, MCH and MCHC values showed no significant differences among the various groups ($P > 0.05$).

Serum protein biochemistry

The control ($2.43 \pm 0.02 \text{ g dL}^{-1}$) group registered the highest serum total protein, which was similar to the RPI₂₅ ($2.30 \pm 0.17 \text{ g dL}^{-1}$) and RPI₇₅ ($2.16 \pm 0.03 \text{ g dL}^{-1}$) groups, but differed significantly from other groups ($P < 0.05$). There were no significant differences in the albumin, globulin and A/G ratio values of the RPI-fed groups compared to the control ($P > 0.05$) (Table 4).

Innate immune parameters

The serum total immunoglobulin (TIM), respiratory burst activity of the phagocyte (NBT assay) and serum lysozyme activity among the treatment groups showed no significant differences compared to the control group ($P > 0.05$) (Table 4).

Serum biochemical test

The results of the serum biochemical test such as TBR, ALP, ALT, LDH, phosphorus and calcium are given in Table 5. ALP and LDH activities among the various groups showed no significant differences ($P > 0.05$). Highest TBR concentration was observed in RPI₇₅ ($1.17 \pm 0.13 \text{ mg dL}^{-1}$) group, which was similar ($P > 0.05$) to the control ($0.90 \pm 0.08 \text{ mg dL}^{-1}$) and RPI₂₅ ($0.85 \pm 0.05 \text{ mg dL}^{-1}$), but differ significantly from RPI₅₀ and RPI₁₀₀ ($P < 0.05$). A significantly higher

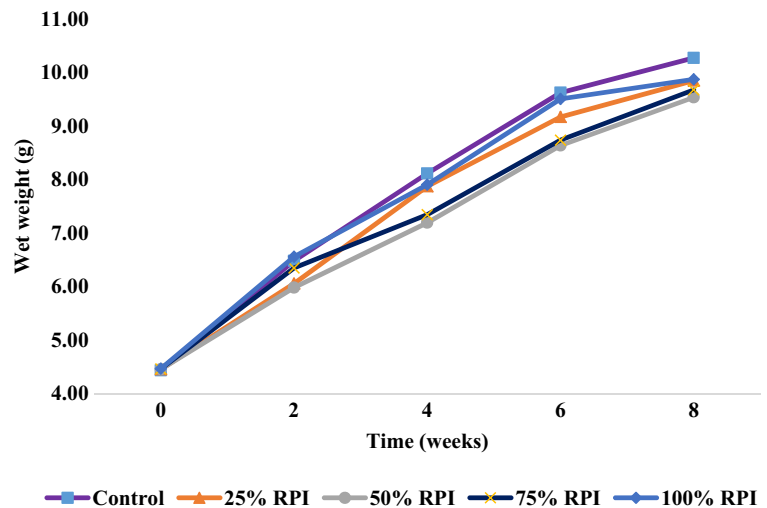
Table 2 Growth performance indices of *Labeo rohita* fingerlings fed different experimental diet

Treatments	IBW	FBW	NWG	MGR	FER
Control	4.43 ± 0.02	10.27 ± 0.22	5.84 ± 0.19	5.02 ± 0.10	0.65 ± 0.01
RPI ₂₅	4.45 ± 0.03	9.85 ± 0.09	5.39 ± 0.09	4.73 ± 0.06	0.62 ± 0.01
RPI ₅₀	4.45 ± 0.01	9.54 ± 0.19	5.09 ± 0.19	4.54 ± 0.12	0.61 ± 0.04
RPI ₇₅	4.46 ± 0.01	9.68 ± 0.19	5.22 ± 0.19	4.62 ± 0.12	0.61 ± 0.03
RPI ₁₀₀	4.46 ± 0.01	9.87 ± 0.31	5.42 ± 0.31	4.74 ± 0.19	0.60 ± 0.02
<i>P</i> value	0.784	0.221	0.186	0.160	0.688

Mean values with different superscripts in the same column differ significantly ($P < 0.05$). Data are expressed as mean $\pm$ SE, $n = 3$ (each replicate was stocked with 12 fish)

IBW initial body weight, FBW final body weight, NWG net weight gain (g), MGR metabolic growth rate (g), FER feed efficiency ratio

Fig. 1 Body weight gain (g) of *Labeo rohita* fingerlings fed different experimental diets



ALT activity was recorded in RPI₅₀ and control compared to the lower value found in RPI₂₅ group ($P < 0.05$). The level of calcium in the serum was significantly higher in RPI₇₅ compared to other groups ($P < 0.05$), but no significant differences were detected in the serum phosphorus level among the various experimental groups ($P > 0.05$) (Table 5).

Antioxidant enzyme and oxidative status

No significant differences were observed in the liver SOD and CAT activities, and MDA contents among the various groups ($P > 0.05$) (Table 6).

Histopathology

The histopathological examination of the liver revealed round hepatic nuclei with dispersed chromatin and

prominent nucleoli, and granular cytoplasm in the control group (Fig. 2). RPI₂₅ and RPI₅₀ showed no discernible changes compared to the control. However, RPI₇₅ group showed a diffusely distributed cytoplasmic vacuolation of the hepatocyte, but no further pathological changes could be observed. The cytoplasmic vacuolation was found to be reduced in the RPI₁₀₀ fed fish compared to the RPI₇₅ group.

Discussion

As the search for alternative plant protein ingredients intensifies, it is of great importance that such ingredients should not only support the growth of the cultured organism but also ensure that the immunity of the fish is not compromised (Kiron 2012).

Table 3 Effects of different experimental diets on haematological parameters of *Labeo rohita* fingerlings

Treatments	Hb (g dL ⁻¹)	RBC (10 ⁶ cells mm ⁻³)	WBC (10 ³ cells mm ⁻³)	Hct (%)	MCV (fL)	MCH (pg)	MCHC (g dL ⁻¹)
Control	7.63 ± 0.09	2.24 ± 0.04 ^{ab}	36.37 ± 2.46	36.33 ± 4.32	161.90 ± 17.40	34.07 ± 0.92	21.57 ± 2.35
RPI ₂₅	7.93 ± 0.70	2.27 ± 0.05 ^a	36.67 ± 1.27	35.17 ± 4.24	154.67 ± 15.78	34.93 ± 2.38	22.73 ± 0.83
RPI ₅₀	5.97 ± 0.03	1.92 ± 0.02 ^d	34.67 ± 1.89	26.83 ± 2.27	139.80 ± 10.44	31.17 ± 0.19	22.53 ± 1.69
RPI ₇₅	6.67 ± 1.09	2.10 ± 0.05 ^c	32.93 ± 2.72	27.90 ± 2.15	132.53 ± 7.26	31.83 ± 5.46	24.37 ± 5.04
RPI ₁₀₀	6.57 ± 0.09	2.14 ± 0.02 ^{bc}	34.53 ± 1.62	28.43 ± 0.72	133.13 ± 4.28	30.73 ± 0.66	23.13 ± 0.28
<i>P</i> value	0.181	<0.001	0.708	0.154	0.370	0.760	0.959

Mean values with different superscripts in the same column differ significantly ($P < 0.05$). Data are expressed as mean ± SE, $n = 3$

Hb haemoglobin, *RBC* red blood cell, *WBC* white blood cell, *Hct* haematocrit, *MCV* mean cell volume, *MCH* mean cell haemoglobin, *MCHC* mean cell haemoglobin concentration

Table 4 Immuno-biochemical parameters of *Labeo rohita* fingerlings fed different experimental diets

Treatments	Total protein (g dL ⁻¹)	Albumin (g dL ⁻¹)	Globulin (g dL ⁻¹)	A/G ratio	Total immunoglobulin (mg mL ⁻¹)	NBT	Lysozyme (U min ⁻¹)
Control	2.43 ± 0.02 ^a	1.07 ± 0.09	1.35 ± 0.07	0.80 ± 0.11	1.92 ± 0.10	0.54 ± 0.06	6.46 ± 2.12
RPI ₂₅	2.30 ± 0.17 ^{ab}	1.00 ± 0.05	1.29 ± 0.20	0.83 ± 0.18	1.69 ± 0.21	0.40 ± 0.06	8.11 ± 2.86
RPI ₅₀	1.98 ± 0.05 ^c	0.94 ± 0.02	1.04 ± 0.04	0.91 ± 0.04	1.52 ± 0.07	0.35 ± 0.01	5.25 ± 0.54
RPI ₇₅	2.16 ± 0.03 ^{abc}	0.98 ± 0.05	1.18 ± 0.02	0.83 ± 0.06	1.67 ± 0.06	0.33 ± 0.07	8.69 ± 2.32
RPI ₁₀₀	2.11 ± 0.06 ^{bc}	1.03 ± 0.04	1.08 ± 0.09	0.97 ± 0.12	1.59 ± 0.06	0.39 ± 0.03	5.78 ± 2.74
<i>P</i> value	0.033	0.519	0.219	0.802	0.222	0.116	0.785

Mean values with different superscripts in the same column differ significantly ($P < 0.05$). Data are expressed as mean ± SE, $n = 6$

A/G ratio albumin/globulin ratio, NBT nitroblue tetrazolium

Growth performance

The present study showed that dietary RPI inclusion in replacement for SPI in the diets of *L. rohita* did not cause any detrimental effects on the growth performance indices. The net weight gain, metabolic growth rate and feed efficiency ratio were found to show no differences statistically compared to the control. This signifies that RPI was better utilised when compared to detoxified rubber seed meal fed to rohu, common carp and tilapia (Sharma et al. 2014; Suprayudi et al. 2015; Deng et al. 2015) where a reduction in growth and nutrient utilisation were noted at higher inclusion levels. The reason for the growth depression observed in those findings may not be unconnected with the deleterious effect of cyanide, which apart from damaging vital organs also causes depletion of body amino acid when ingested (Deng et al. 2015). Documented literature had shown that high cyanide-containing diets may cause growth depression in animal through sulphur amino acid metabolism, as well as interference with iodine uptake, thereby leading to amino acid imbalance and hence

reduced protein turnover (Okolie and Osagie 1999; Okafor and Anyanwu 2006). Consistent with this study, canola protein isolate was reported to replace fish meal totally in rainbow trout (Slawski et al. 2013) without any adverse effect on growth and nutrient utilisation. Similarly, in our previous study, rohu fingerlings fed with rubber protein isolate showed comparable growth performance, nutrient utilisation and metabolic enzyme activities with the soybean protein isolate fed group (Fawole et al. 2016a).

Haematological parameter

Haematological indices have been used in several studies to assess the nutritional and or pathological condition of an animal in response to different diets (Akpodiete and Ologbodo 1998; Jahanbakhshi et al. 2013), and are considered as indicators of animal health status. Previous findings reported that cyanide, a degraded product of cyanogenic glycoside, has a greater affinity towards the haem component of the haemoglobin and erythrocyte (Isom and Way 1974), thus affecting the

Table 5 Effects of different experimental diets on total bilirubin (mg dL⁻¹), alkaline phosphatase (ALP, U L⁻¹), alanine transaminase (ALT, U L⁻¹), lactate dehydrogenase (LDH, U 100 mL⁻¹),

phosphorus (mg dL⁻¹) and calcium (mg dL⁻¹) levels in the serum of *Labeo rohita* fingerlings

Treatments	Total bilirubin	ALP	ALT	LDH	Phosphorus	Calcium
Control	0.90 ± 0.08 ^{ab}	14.74 ± 3.69	92.28 ± 5.95 ^a	86.35 ± 5.69	6.30 ± 0.23	15.09 ± 2.30 ^b
RPI ₂₅	0.85 ± 0.05 ^{ab}	18.43 ± 3.69	67.52 ± 3.90 ^b	90.66 ± 5.01	8.47 ± 0.97	14.17 ± 1.57 ^b
RPI ₅₀	0.58 ± 0.15 ^b	20.27 ± 4.88	99.03 ± 2.25 ^a	83.83 ± 4.30	7.91 ± 0.65	12.88 ± 0.09 ^b
RPI ₇₅	1.17 ± 0.13 ^a	18.43 ± 3.69	85.53 ± 8.12 ^{ab}	108.47 ± 8.37	6.77 ± 0.13	21.70 ± 0.20 ^a
RPI ₁₀₀	0.71 ± 0.13 ^b	16.58 ± 3.19	83.27 ± 5.95 ^{ab}	77.35 ± 12.48	6.55 ± 0.27	18.49 ± 2.59 ^{ab}
<i>P</i> value	0.040	0.874	0.026	0.132	0.076	0.025

Mean values with different superscripts in the same column differ significantly ($P < 0.05$). Data are expressed as mean ± SE, $n = 6$

Table 6 Antioxidant enzymes and oxidative stress marker in *Labeo rohita* fingerlings fed different experimental diets

Treatments	SOD (liver)	CAT (liver)	MDA (liver)
Control	16.20 ± 0.05	18.42 ± 3.21	1.57 ± 0.31
RPI ₂₅	17.11 ± 0.60	15.63 ± 2.35	1.49 ± 0.12
RPI ₅₀	18.50 ± 1.60	14.96 ± 0.86	1.58 ± 0.49
RPI ₇₅	14.36 ± 1.37	13.96 ± 3.05	1.16 ± 0.48
RPI ₁₀₀	20.23 ± 3.70	16.11 ± 1.46	1.38 ± 0.26
<i>P</i> value	0.318	0.740	0.913

Mean values with different superscripts in the same column differ significantly ($P < 0.05$). Data are expressed as mean ± SE, $n = 3$. SOD superoxide dismutase ($\mu\text{mol mg protein}^{-1} \text{ min}^{-1}$ at 37 °C), CAT catalase ($\text{mmol H}_2\text{O}_2$ decomposed $\text{min}^{-1} \text{ mg protein}^{-1}$ at 37 °C), MDA malondialdehyde ($\text{nmol MDA mg protein}^{-1}$)

erythropoiesis processes, red blood cell formation and oxygen-carrying capacity of the blood (Sharma et al. 2014). In the present study, all the haematological parameters except RBC showed no significant differences between the control and the treatment groups, suggesting that the erythropoiesis processes were not affected after feeding RPI to rohu. This can be attributed to the low level of cyanide ($1.27\text{--}5.05 \text{ mg kg}^{-1}$) present in the diets. Supporting this study, Sharma et al. (2014) observed that feeding detoxified rubber kernel meal containing a low level of cyanide ($<39 \text{ mg kg}^{-1}$ diet) to *L. rohita* fingerlings caused no adverse effect on the Hb, PCV and RBC contents. Although a lower RBC value was recorded in the RPI₅₀ compared to the control and other treatment groups, the value obtained was still within the normal range ($1.1\text{--}2.2 \times 10^6 \text{ mm}^{-3}$) reported for carp (Ghittino 1983; Kumar et al. 2010). The MCV, MCH and MCHC play a significant role in the diagnosis of anaemia in most animals and are known to be indicative of the erythrocyte status and oxygen-carrying capacity of the blood in fish (Houston 1997). These parameters showed no differences among the fish in the present study, thus revealing that the oxygen-carrying capability of the blood for various metabolic functions were not hampered. According to Suprayudi et al. (2015), chronic exposure of fish to hydrogen cyanide through diets could elicit stress conditions, thereby suppressing fish immunity and increased susceptibility to infection. However, feeding RPI to rohu in this study did not cause any appreciable stress to the fish irrespective of the inclusion levels as indicated by the MDA content, and the immune system was not suppressed. Several authors have reported that Hb and Hct assays are good

bio-indicators of general health condition of fish (NRC 1993), and the values recorded among the various dietary groups in this study were within the normal range for fish (Sun et al. 1995; Kumar et al. 2010; Akinleye et al. 2012). WBC count is another important haematological parameter that plays a significant role in the non-specific immunity of fish, and the current study showed that compared to the control, feeding RPI-based diet elicited no adverse effect on WBC count. Therefore, the results obtained from this study suggest that the fish were in good nutritional and healthy condition, and the tested ingredient (RPI) had no detrimental effect on *L. rohita* fingerlings.

Serum biochemistry and immunological response

Serum total protein is another important parameter showing the nutritional and health status in fish (Martinez 1976) and comprises albumin and globulin, whose functions in the immune response of fish have been documented (Kumar et al. 2010; Makkar et al. 2011; Fawole et al. 2016b). Okafor and Anyanwu (2006) observed no significant changes in the blood total protein and albumin contents of an albino rat fed detoxified rubber seed mash containing $9.25 \text{ mg HCN kg}^{-1}$ diet. A similar result was recorded in the present study, where serum total protein was found to be similar between the control, RPI₂₅ and RPI₇₅ groups. The significantly lower total protein value observed in RPI₅₀ may be due to increased catabolism of proteins and inhibition of certain enzymes as reported by Canli (1996). A similar reduction in total protein has been reported in common carp fed detoxified *Jatropha* kernel meal (DJKM) (Ja50 and Ja75) compared to groups fed soybean meal and fish meal (Kumar et al. 2010). The authors attributed this to impaired protein metabolism in the liver. Nonetheless, the insignificant differences in the globulin contents of the control and RPI-fed groups is an indication that the immune system was not suppressed after feeding RPI, as globulin values are usually associated with the immunological responses in fish. Similar findings were reported by Latif et al. (2015) and Kumar et al. (2012) in common carp fed detoxified *Jatropha* protein isolate and DJKM diets, respectively.

Suppression of the innate immune capacity due to high inclusion of rubber seed meal has been reported in many fish species (Suprayudi et al. 2015; Sharma et al. 2014; Deng et al. 2015), and these were attributed to high concentration of cyanide in those diets.

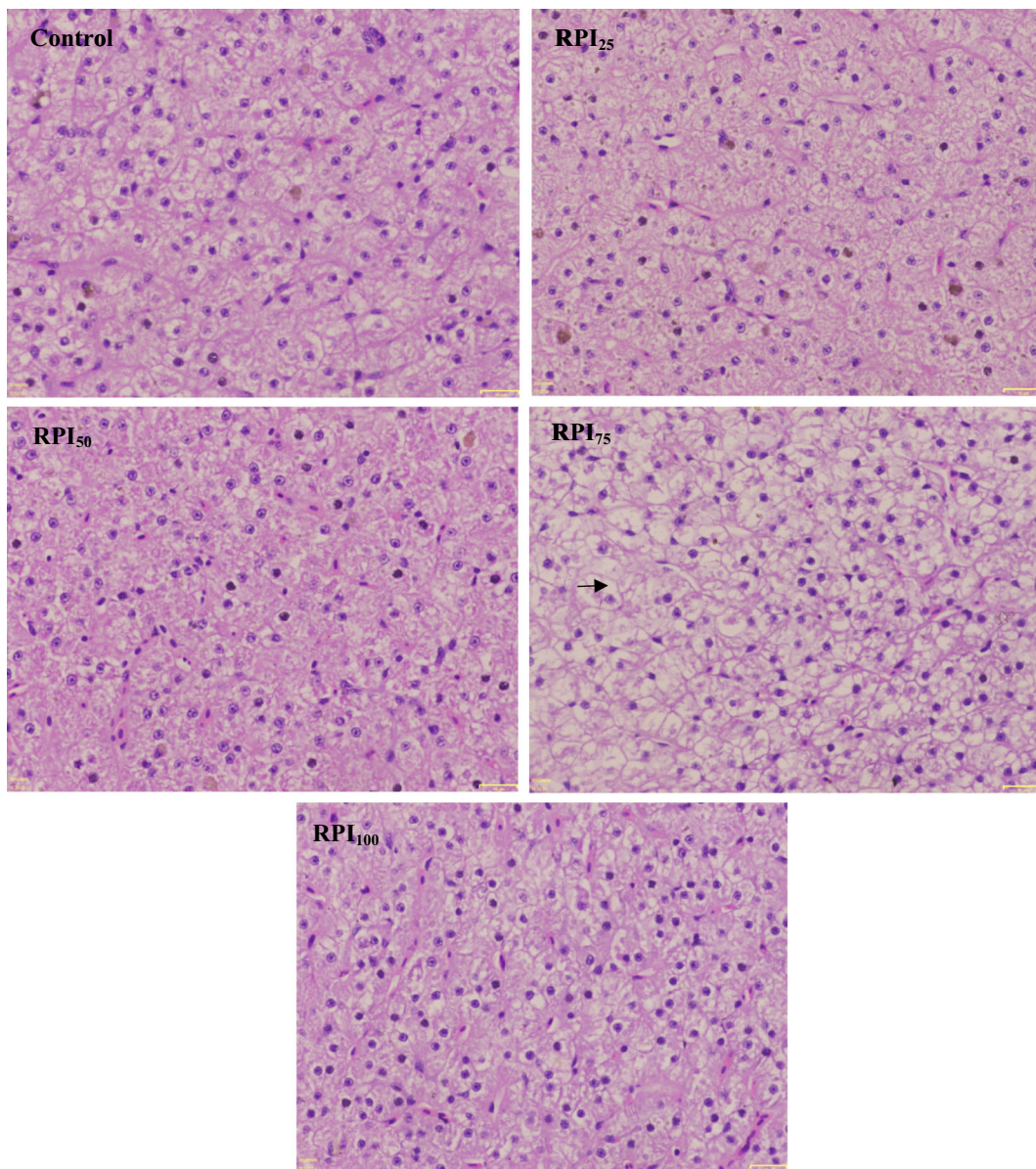


Fig. 2 The histopathological examination of the liver revealed round hepatic nuclei with dispersed chromatin and prominent nucleoli, and granular cytoplasm in the control group. RPI₂₅ and RPI₅₀ showed no discernible change compared to the control. However, RPI₇₅ group showed a diffusely distributed cytoplasmic

vacuolation of the hepatocyte (→), but no further pathological changes could be observed. The cytoplasmic vacuolation was found to be reduced in the RPI₁₀₀-fed fish compared to the RPI₇₅ group. Scale: 16 μ m

However, in the present study, the effects of RPI was examined on both the humoral (serum lysozyme and Ig) and cellular (respiratory burst) innate immunity parameters, and no significant differences were found among the various dietary groups. This suggests that the innate immune system of rohu fed RPI diets were not altered or compromised, and the reason may be

due to the low level of cyanide present in the diets. The result of this study is in accord with the findings of Bransden et al. (2001), who observed no significant changes in the lysozyme activity and plasma total immunoglobulin of Atlantic salmon (*Salmo salar*) fed plant protein-based diets in replacement for fish meal.

Biochemical test

Alanine aminotransferase (ALT) enzyme are found abundantly in the cytosol of the hepatocyte where they help in catalysing the transfer of amino groups to form hepatic metabolite, oxaloacetate (Price and Alberti 1979). Alkaline phosphatase (ALP) enzyme is also present in the hepatocyte. However, in the case of hepatocellular injury, intrahepatic infiltrative diseases of the liver, and bile duct obstruction, ALT and ALP often leak into the blood and rise dramatically in acute condition (Racicot et al. 1975; Goel et al. 1984). Thus, a rise in the serum level of ALT and ALP gives information on the damage to liver cells. Previous findings revealed that exposing fish to hydrogen cyanide could lead to increased level activity of ALT, AST and ALP in the plasma and causes hepatic degeneration (Dixon and Leduc 1981; Sadati et al. 2013; Deng et al. 2015). In the present study, the levels of ALT and ALP were found to be similar in all the treatment groups and comparable to the control, indicating that feeding RPI diets containing low level of cyanide to rohu may not have acute toxic effects on the vital organ, and this was further supported by the non-remarkable histopathological changes observed in the liver. Feeding rats with a diet compounded from oven-dried mashed seeds containing 9.25 mg HCN kg⁻¹ caused no changes in the plasma AST, ALT and ALP (Okafor and Anyanwu 2006). Contrarily, Deng et al. (2015) observed significant changes in the plasma ALT levels in hybrid tilapia (*Oreochromis niloticus* × *O. aureus*) when SBM was replaced at rates higher than 30% by rubber seed meal (RSM). The reason was attributed to the detrimental effect of oxidative stress triggered by the high level of cyanide in the diet. Also, the levels of serum ALT, AST, ALP and LDH in common carp exposed to potassium cyanide (0.1–0.2 mg L⁻¹) were found to increase significantly compared to the control (Sadati et al. 2013). However, such effects were recorded in this study, and the cyanide levels in the current experimental diets were below the range reported not harmful in various studies such as *L. rohita* (<39 mg kg⁻¹ diet), hybrid tilapia (<25.1 mg kg⁻¹ diet) and rats (9.25 mg HCN kg⁻¹ diet). The total bilirubin (TBR) levels were found to be similar among the RPI-fed groups and comparable to the control. This is indicative of normal liver function as TBR is often used as an indicator of liver dysfunction (Tietz 1986), hence, suggesting that RPI inclusion in replacement for SPI causes no alteration in the liver function and that the fish are

healthy. The phosphorus levels in the serum of RPI-fed fish and the control were found to be similar to the value reported for common carp fed plant protein-based diets supplemented with phytase (Kumar et al. 2010; Makkar et al. 2011). This indicates that processing rubber kernel meal to protein isolate can help in releasing phosphorus from bounded phosphorus-phytate, thereby making it available for various metabolic functions. The higher serum calcium levels recorded in RPI₇₅ and RPI₁₀₀ were similar to those reported in hybrid tilapia, where calcium levels were found to increase significantly with increasing dietary RSM levels (Deng et al. 2015).

Antioxidant enzyme and oxidative status

Superoxide dismutase (SOD) and catalase (CAT) are important antioxidant defence systems in nearly all cells and offer a protection against the damaging effects of free radicals induced by oxidative stress (Fawole et al. 2016b). This oxidative stress can alter the antioxidant defence mechanism when fish are exposed to an unfavourable conditions like toxin, extreme temperature, toxicant, pathogen etc., thus causing cellular disruption, DNA damage, liver impairment and/or immune suppression (Martinez-Alvarez et al. 2005; Patra et al. 2008). On the other hand, MDA, the end-product of lipid peroxidation, is one of the most commonly used biomarkers of oxidative stress (Nwani et al. 2010). Studies had revealed that high cyanide intake causes increased lipid peroxidation and free radical production (Daya et al. 2000). Similarly, feeding dietary RSM containing a high level of cyanide (>31.8 mg kg⁻¹) to hybrid tilapia elevated the MDA level in the serum, indicating oxidative stress (Deng et al. 2015). However, in the current study, the inclusion of RPI in the diets of rohu did not cause any stress or trigger oxidative insult to the fish as indicated by the similar MDA contents among the experimental groups. The insignificant differences observed in the SOD activity among the various groups were in consonance with the findings of Deng et al. (2015). Further, it may be mentioned that the haemato-biochemical and innate immune parameters studied were in line with the activities of hepatic antioxidant enzymes and MDA levels.

Histopathology changes

Nayak et al. (1996) stated that non-lipid cytoplasmic vacuolation of liver cells following exposure to low doses of toxins are often due to excessive accumulation of

glycogen. This is in accordance with an earlier report by Bannasch (1968) who pointed out that glycogen accumulation is often mistaken for the hydropic degeneration of the hepatocyte when examined under the light microscope. Nayak et al. (1996) further stressed that many of the vacuolated hepatocytes often seen in liver injury are actually cells which have adapted and altered to resist further damage rather than cells undergoing hydropic degeneration. Therefore, mild cytoplasmic vacuolation noticed in the liver of fish fed RPI₇₅ could be the result of the adaptive response of the hepatic cells (cellular adaptation of protective nature). Further, as the results of the liver function test in the present study are in the normal range and the hepatic stress marker enzyme activity showed no sign of oxidative insults compared to the control, it can be inferred that the liver is in a healthy condition and can tolerate cyanide level up to 5.05 mg kg⁻¹ diet.

Conclusion

This study shows that rubber protein isolate can be used in the diet of *L. rohita* with no adverse effects on growth, haemato-biochemical indices, innate immunity, antioxidant status and liver histology. Further, the level of hydrogen cyanide in RPI and its inclusion in the diet did not generate any oxidative-induced stress; thus, RPI may be suggested as a potential replacement for SPI in fish diets. Hence, protein isolation offers a unique opportunity for the utilisation of rubber seed meal for aquafeed development. Nonetheless, further study is recommended in other fish species to explore the potential of this product (RPI) for commercial use.

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