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Effects of Dietary Detoxified *Jatropha curcas* Protein Isolate on Some Physiological Parameters, Intestine, and Liver Morphology of *Labeo rohita* Fingerlings

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Abstract

The rising cost of aquafeed ingredients as global aquaculture expands has led researchers to look for other ingredient sources that are not in conflict with human food. In this regard, this study examined the possibility of incorporating detoxified jatropha protein isolate (JP) into the diet of *Labeo rohita* (rohu) and the effect on the haematology, innate immunity, and organ integrity. A total of 216 fish (3.25 ± 0.02 g/fish) were randomly distributed in 15 tanks and fed for 60 days. The control diet was formulated with soy protein isolate (SP) and gradually replaced with JP at 25%, 50%, 75%, and 100%. The results showed that the red blood cell (RBC) and haemoglobin (Hb) were not significantly influenced ($p > 0.05$). White blood cell (WBC) counts registered a significant overall effect and followed a quadratic trend ($p = 0.001$), while the haematocrit value showed overall significant effects ($p < 0.05$) but no linear or quadratic trends were observed ($p > 0.05$). Serum lysozyme activity increased with increasing JP level ($p = 0.002$), but the total immunoglobulin value was significantly higher in fish fed JP 100. The respiratory burst activity, malondialdehyde (MDA) content, superoxide dismutase, and catalase enzyme activities among the various groups did not differ significantly ($p > 0.05$). Compared to the control, the photomicrograph of the mid-intestine and liver of fish fed a JP-based diet showed no inflammatory or degenerative changes. In summary, the substitution of SP in the diet with JP improved the innate immunity of *L. rohita* without any negative impact on the blood profile or the histoarchitectural structure of the liver.

Introduction

Aquaculture has been regarded as one of the fastest growing industries in the animal food-producing sector and currently contributes almost 53% of the food fish and shellfish directly consumed by humans (FAO, 2020). This growth has been expedited by the availability of high-quality aquafeed in which fishmeal (FM) is the chief protein source ingredient. However, competition for this highly sourced ingredient from the poultry and other livestock industries, increased demand, a decline in wild fish catches, and uncertainty

and constraint in global FM production (Shamna et al., 2015; Fawole et al., 2016), have resulted in an unprecedented increase in the price of the ingredients, thus negatively impacting farmers' returns. To sustain the growth of the aquaculture industry, significant efforts have been made to use plant-based ingredients such as soybean meal (SBM), canola meal (CM), corn gluten (CG), etc., to reduce the heavy reliance on FM without compromising the nutritional quality of the feed, and SBM has emerged as the most promising alternative among the plant-based proteins studied (Storebakken et al., 2000; Fawole et al., 2017; Vikas et al., 2020; Hossein et al., 2021). However, with increasing pressure on the utilization of SBM in human and livestock diets coupled with the high quantity needed to support the projected increase in aquafeed demand, SBM may not be in adequate supply to support the ever-increasing global aquaculture industry. As a result, it is of great importance to investigate alternative protein sources that are not in competition with humans for food and are economically advantageous in lowering aquafeed costs (Saha and Ghosh, 2013; Fawole et al., 2018, 2020; Okomoda et al., 2021).

Jatropha curcas is an oil-bearing tree with potential for sustainable clean energy production (i.e. biofuel), and has been planted on more than 1, 000 000 hectares of land worldwide (Edrisi et al., 2015; Moniruzzaman et al., 2017). The yields potential of jatropha was reported to vary between 5-12 t/ha (Ofori-Boateng and Lee, 2011), and about 70-75% oilseed cake can be produced from undecorticated seeds after mechanical oil extraction (Siang, 2009). The by-products of *J. curcas* (i.e. seed cake or kernel meal) are protein-rich non-edible feed ingredients with relatively good amino acid compositions comparable with soybean meal and fishmeal (Makkar et al., 1998; Fawole et al., 2018, Musa et al., 2018). The protein content of the seed cake and kernel meal is typically between 27-32% and 57.8-63.8% (Makkar and Becker, 2009; Shamna et al., 2015; Phulia et al., 2017; Fawole et al., 2018), respectively. However, its direct utilization in animal feeds is impeded by the presence of toxic components such as phorbol esters and other antinutritional factors. (Makkar et al., 2008; Shamna et al., 2015; Okomoda et al., 2021; Phulia et al., 2021). Nonetheless, further processing could be applied to enhance the nutritional quality of *J. curcas* by-products and reduce the concentration of the toxic components.

The jatropha protein isolate is a purified form of protein containing more than 85% protein (Nepal et al., 2018; Fawole et al., 2018). Although a few reports are available on jatropha protein isolate (JP) in fish (Kumar et al., 2012; Latif et al., 2015; Nepal et al., 2018; Fawole et al., 2018; Zhao et al., 2018), there is no study on the effect of jatropha protein isolate on the antioxidant capacity and health status of *L. rohita*. Hence, this study emphasizes the dietary impacts of detoxified jatropha protein isolate, hereafter referred to as JP, on the haematology, lysozyme activity, total immunoglobulin, total cholesterol, triglyceride content, antioxidant enzyme activity, and the histomorphological assessment of the liver and intestine of *Labeo rohita*, the most preferred and commercially dominant Indian major carp in Southeast Asia.

Materials and Methods

Diets Formulation and Chemical Analysis

The jatropha seed cake used to produce the protein isolate was purchased from A1 Oil, Borivali, Mumbai, India, processed into the protein isolate, and detoxified according to the method described by Fawole et al. (2018). The soy protein isolate (SP) produced by Medicamen Organics, India, was used in the trial as a reference ingredient. Five diets were designed to be

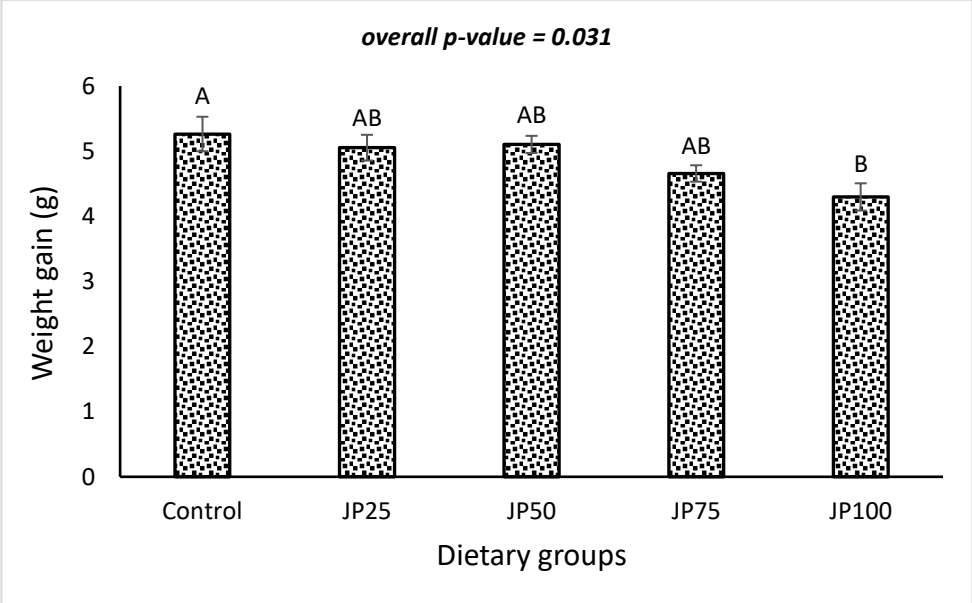


Figure 1. Weight gain of *Labeo rohita* fed detoxified Jatropha protein isolate. Mean ± SE value with different superscript differ significantly ($p<0.05$).

isonitrogenous (311 g of crude protein/kg diet), and the diets were prepared to replace SP with JP at 0% (control), 25% (JP 25), 50% (JP 50), 75% (JP 75), and 100% (JP 100), corresponding to dietary inclusion levels of 0 g Kg^{-1}, 44 g Kg^{-1}, 87 g Kg^{-1}, 130 g Kg^{-1} and 174 g Kg^{-1}, respectively. Other ingredients were incorporated in equal proportion in all diets (except wheat flour), thoroughly mixed, and water was added to form dough. The dough was processed into a pellet using a hand pelletizer (2 mm diameter die), air dried and stored at -20 ^{0}C until use. Feed and fish tissue were analyzed for chemical composition according to the standard procedure of the Association of Official Analytical Chemists (AOAC 1995).

Experimental Animal and Design

A 60-day trial was carried out on the fingerlings of rohu (*L. rohita*) purchased from a private farm (Srushti Aquaculture, Maharashtra, India) after 2 weeks of acclimatization to the experimental tanks. Twelve rohu (3.25g ± 0.02) fingerlings were randomly stocked in each of the 15 experimental tanks (75 L capacity), and each of the five diets was assigned to three replicate tanks per dietary group. The fish were intensively managed in the experimental tank using the static renewal method, and 30% of the water was replaced every 2 days. The faeces were siphoned out daily in the morning before feeding. A central compressor system supplies air to the experiment tank using an aeration stone. The fish were fed by hand to satiation and no feed rejection was observed during the trial. The fish were fed at 10:00 h and 18:00 h. The fish in each experimental tank were weighed in bulk at the start and every two weeks throughout the trial period to monitor the fish response to diet. The water quality parameters were monitored for dissolved oxygen (6.9 mg/L ± 0.38), temperature (27.9°C ± 0.82), pH (7.7 ± 0.42), ammonia (0.08 ppm ± 0.03), and were all within acceptable levels for rohu.

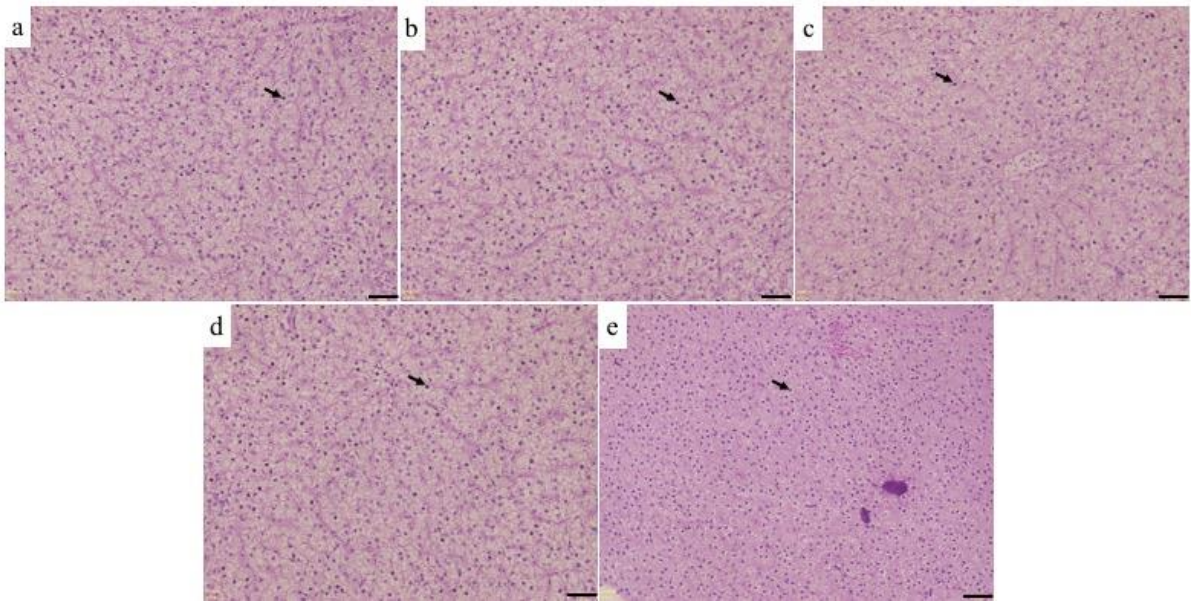


Figure 2. Light micrograph of the liver structure of rohu, *Labeo rohita* fed Control (a), JP 25% (b), JP 50% (c), JP 75% (d), and JP 100% (e) diets, showing hepatic cells and their nuclei (arrows). Light microscopic staining: Haematoxylin and Eosin (Scale bar = 32 µm).

Table 2. Haematological and some innate immunity parameters of rohu fingerlings fed graded levels of detoxified jatropha protein isolate substituted for soy protein isolate.

	Dietary groups					Polynomial contrast			
Haematology	Control	JP 25	JP 50	JP 75	JP 100	SEM	Overall	Linear	Quadratic
Haematocrit (%)	17.53	16.43	15.03	20.10	11.97	1.527	0.037*	0.153	0.213
Haemoglobin (g dl^{-1})	4.87	4.97	5.80	5.63	4.00	0.577	0.264	0.572	0.065
RBC (10^6 cells mm^{-3})	1.16	1.14	1.28	1.34	0.91	0.226	0.718	0.690	0.316
WBC (10^4 cells mm^{-3})	11.26	15.18	13.06	14.79	10.06	0.769	0.003*	0.280	0.001*
Serum immune parameters									
Total immunoglobulin (mg mL^{-1})	1.80	2.08	1.90	1.54	2.21	0.109	0.002*	0.419	0.150
Respiratory burst activity	0.39	0.36	0.39	0.38	0.38	0.029	0.958	0.961	0.831
Lysozyme (U mL^{-1})	8.91	9.98	10.38	12.23	13.30	0.588	0.002*	<0.001*	0.524

Mean values with different superscripts in the same row differ significantly (*P < 0.05*). SEM - Standard error of the mean (n=3). JP, detoxified jatropha protein isolate, RBC, red blood cell; WBC, white blood cell.

Fish Sampling for Blood Parameters, Respiratory Burst Activity, and Serum Biochemistry

For the samplings, fish were euthanised with 50µl of clove oil L^{-1} of deionized water. A total of nine fish per group were randomly removed (n=3 per tank), and blood was collected from the *vena caudalis* using an EDTA-rinsed hypodermic syringe. The blood sample was immediately placed in an EDTA bottle for haematological study and the respiratory burst activity of the phagocytes was quantified using the method of Secombes (1990). Fresh blood was collected from another four fish per replicate into a dried plain eppendorf tube and allowed to stand in a tilted position at room temperature for a few minutes. The samples were centrifuged at 3000 x g in a cooling centrifuge for 10 min, transferred to another plane tube, and stored at -20 ^{o}C for the biochemical assay. The yellow straw sera samples were assayed for the determination of total protein, cholesterol, triglyceride, phosphorus, and glucose using a commercial diagnostics kit (Erba® Diagnostic, India). The sacrificed fish from each replicate tank were carefully dissected for liver collection and homogenized in a cold 250mM sucrose solution. The liver homogenate was centrifuged (5000 x g for 10 min at 4°C) and the supernatant was carefully collected and stored at -80 °C for the analysis of antioxidant enzyme activity.

Serum total immunoglobulin and lysozyme activity

Total immunoglobulin level in the serum was quantified according to the protocol of Anderson and Siwicki (1995) as described by Fawole et al. (2017), while the lysozyme activity was measured based on lysozyme sensitivity to *Micrococcus lysodeikticus* (Sigma®, India) (Anderson and Siwicki, 1995). The bacteria lysis reading was observed at intervals of 15, 30, and 270 s on a UV-Spectrophotometer at 450nm.

Histological assessment

At the end of the trial, liver and intestine from the sacrificed fish were sampled and fixed in 10% neutral buffered formalin (NBF) for 24 h, after which the samples were dehydrated and embedded in melted paraffin wax. The tissues were sectioned (5 µm) and stained with haemotoxylin-eosin (H&E). The sections were observed and photographed using an Olympus FSX 100 phase contrast microscope equipped with a Canon EOS 500D digital camera (Fawole et al., 2017), and morphological measurements were carried out through ImageJ (version 1.51, National Institute of Health). The number of intraepithelial leucocytes (IELs) and goblet cells in the intestinal epithelium was estimated by averaging the numbers from all specimens over a standardized distance of 100 µm (10-fold per specimen).

Superoxide Dismutase, Catalase, and Malondialdehyde Assay

The superoxide dismutase (SOD) activity of liver tissue was estimated according to the procedure of Misra and Fridovich (1972) with little modification. A 10 mM epinephrine solution was prepared in 25 ml of 20 mM HCl. An aliquot of 30 µl enzyme was pipetted into a cuvette containing 1.5 ml 50 mM carbonate-bicarbonate buffer (pH 10.2) with 0.1 mM EDTA, and a

Table 3. Effects of graded levels of detoxified jatropha protein isolate on the serum biochemical parameters of rohu fingerlings.

	Dietary groups						Polynomial contrast		
Treatments	Control	JP 25	JP 50	JP 75	JP 100	SEM	Overall	Linear	Quadratic
Total protein (g dL^{-1})	2.24	2.56	2.34	2.03	2.66	0.138	0.024*	0.480	0.297
Glucose (mg dL^{-1})	136.15	150.58	137.88	145.09	154.26	7.509	0.383	0.207	0.741
Cholesterol (mg dL^{-1})	113.28	114.65	104.54	110.37	108.65	6.911	0.849	0.549	0.713
Triglyceride (mg dL^{-1})	147.40	149.03	137.41	148.62	136.80	5.735	0.382	0.261	0.853
Phosphorus (mg dL^{-1})	8.63	10.62	9.82	8.03	11.23	0.732	0.026*	0.268	0.604

Mean values with different superscripts in the same row differ significantly (*P<0.05*). SEM - Standard error of the mean (n=3).

Table 4. Effects of graded levels of detoxified jatropha protein isolate on the hepatic antioxidant enzyme, lipid peroxidation and histomorphometry of the intestine of rohu fingerlings.

	Dietary groups						Polynomial contrast		
Treatments	Control	JP 25	JP 50	JP 75	JP 100	SEM	Overall	Linear	Quadratic
SOD	26.52	27.41	32.45	25.84	32.97	1.801	0.050	0.075	0.906
Catalase	1.25	1.81	2.11	2.23	2.00	0.384	0.452	0.146	0.249
lipid peroxidation	1.65	1.63	1.59	1.15	1.77	0.216	0.355	0.736	0.298
Intestinal histology									
Goblet cells (per 100µm)	4.10	6.10	8.14	6.20	4.25	0.292	0.001*	0.858	<0.001*
IELs (per 100µm)	61.57	65.00	71.20	64.00	57.00	1.367	0.064	0.298	0.007*

Mean values with different superscripts in the same row differ significantly (*P<0.05*). SEM - Standard error of the mean. JP, detoxified Jatropha protein isolate; SOD, superoxide dismutase (µmol mg $protein^{-1}$ min^{-1} at 37°C); CAT, Catalase (mmol H_2O_2 decomposed min^{-1} mg $protein^{-1}$ at 37°C); MDA, lipid peroxidation (nanomoles MDA mg $protein^{-1}$); IELs, intraepithelial leucocytes.

known amount of epinephrine (0.5ml) was added to the mixture. The absorbance of the sample was taken at 480 nm (UV spectrophotometer) for 3 min at 30 sec intervals. The activity of catalase (CAT) enzyme for each sample was measured following the protocol of Takahara et al. (1960). Bradford (1976) method adapted to microplate, was used for the analysis of tissue soluble protein content. The hepatic malondialdehyde (MDA) content was quantified as thiobarbituric acid reactive substances (TBARs) as indicated by Sharma and Krishnamurthy (1968) and expressed in nanomoles of MDA mg protein^{-1}.

Data Analysis

Statistical analysis was carried out using IBM SPSS version 22 for windows. All data were checked for normality and homogeneity of variance using the Kolmogornov–Smirnov and Levene tests, respectively. When normal assumptions were met, the data was statistically analyzed by one-way analysis of variance (ANOVA) and polynomial contrast was used to determine the dietary impact of SP substitution with JP. The regression significance level (linear and quadratic) was set at $P < 0.05$, and the data were presented as mean ± pooled standard error of the mean (SEM).

Results

Weight gain and Haematological parameters

The weight gain performance among the dietary groups showed an overall significant (p = 0.031) effect and followed a linear (p = 0.03) trend, but no quadratic (p = 0.296) trend was observed. The fish fed the JP 100 diet had a significantly lower weight gain (WG) compared with the control and other JP-fed fish (Figure 1).

The haematological parameters of *L. rohita* fingerlings fed different diets are presented in Table 2. The haemoglobin (Hb) and red blood cell (RBC) values between the various dietary groups were not significantly different ($p > 0.05$). White blood cell (WBC) counts registered a significant overall effect and followed a quadratic trend ($p = 0.001$) while the haematocrit value showed overall significant effects ($p < 0.05$) but no linear or quadratic trends were observed ($p > 0.05$). The highest values of WBC and Hct were observed in JP 25 and JP 75 fed fish, respectively.

Innate immune response indicator

The total immunoglobulin value showed significantly higher overall effects ($p < 0.05$) in fish fed JP 100, however, the respiratory burst activity of the phagocyte did not differ significantly among the groups and did not show an observable trend ($p > 0.05$). Serum lysozyme activity increases linearly with increasing JP level and showed an overall significant effect ($p = 0.002$).

Serum protein and metabolites

The rohu fed the JP 100 diet registered the highest serum total protein value compared to other dietary groups and showed overall significant effects ($p < 0.05$). No significant effects or trends ($p > 0.05$) were observed for serum glucose, cholesterol, and triglyceride levels among the different dietary groups. However, phosphorus level showed an overall significant effect ($p < 0.05$) but no linear or quadratic trends (Table 3) were observed. The highest phosphorus level was observed in rohu fed JP 100.

Histomorphometry of mid-intestine and liver histology

The goblet cells (GCs) were found to be abundant in fish fed JP 50 and showed an overall significant and quadratic effect ($p \leq 0.001$), but no linear trend was observed. A similar observation was observed for intraepithelial leucocytes (IEL) with the highest recorded value in JP 50; however, the overall effect or linear trend was not significant, but it was found to have improved quadratically ($p = 0.007$) (Table 4). Histopathological examination of the liver showed a rarefied cytoplasm of hepatocytes and relatively small nuclear size in the control. The group fed JP 25 and JP 50 showed slight to moderate increase in the cytoplasmic content and nuclear size of the hepatocytes. However, the cytoplasmic content was found to be higher in the JP 100 group with centrally placed nuclei in almost all the hepatocytes. Nonetheless, no inflammatory or degenerative changes were observed in any of the experimental groups (Figure 2).

Antioxidant Enzymes and Malondialdehyde Level

The antioxidant enzymes such as SOD and CAT, and the oxidative stress status of *L. rohita* fingerlings as measured by MDA content are presented in Table 4. The hepatic activities of SOD and CAT enzymes, and MDA content among the various dietary groups did not differ significantly ($p > 0.05$).

Discussion

The results of the current studies showed that feeding JP up to 75% (130 g/kg) would not impair the growth performance of *L. rohita* fingerlings. The significant reduction in WG observed in JP 100 (174g/kg diet) could be due to a reduction in the lysine content in JP (Table 1), which is caused by the interaction of lysine with the soluble sugar during the alkaline extraction process, as reported by Fawole et al. (2018). However, this was not observed in the work of Musa et al. (2018) when *J. curcas* was subjected to hydrothermal processing. Our result is in line with the study of Jimoh et al. (2020), who recorded a lower weight gain in *Oreochromis niloticus* fed 40% (188.5g/kg diet) heat-treated *J. curcas* seed meal compared to those fed the

control diet. In another study, Okomoda et al. (2021) and Musa et al. (2018) observed a significant improvement in weight gain in *Clarias gariepinus* fed differently processed *J. curcas* kernel meal. The differences in fish response to *J. curcas* utilization could be related to the species of fish, the processing method used and the level of inclusion of *J. curcas*. Furthermore, isoleucine, one of the essential amino aci ds required for tissue repair and growth, was found to be lower in JP 100, and this could be partially responsib le for the lower growth observed in that group. Dietary isoleucine below the optimal requirement resulted in reduced growth and poor protein efficiency ratio in golden pompano, *Trachinotus ovatus* (Huang et al., 2015). Blood has been described as a pathophysiological reflector of the entire body and has been used in several studies to assess general health status in relation to ingredient substitution in fish diets (Gao et al., 2014; Fawole et al., 2017; Shamna et al., 2017). In this study, the Hb and RBC values showed no differences among the dietary groups, and this could be associated with the low level of tannin and phytic acid present in the diet. Tannin has been reported to have strong affinity for iron (Fe), hence inhibiting Fe absorption from the intestine, and resulting in low levels of Hb and RBC in rohu (Prusty et al., 2007), pig (Lee et al., 2010), and rat (Afsana et al., 2004). Shamna et al. (2017) discovered that feeding rohu with non-fermented jatropha protein concentrate (JPC) resulted in lower RBC and Hb, which they attributed to the high levels of tannin and phytic acid found in JPC. In addition, a significant decrease in RBC, Hb, and Hct was observed in Nile tilapia fed heat treated *Jatropha curcas* meal, and this effect was linked to the presence of antinutritional components (Jimoh et al., 2020). The higher Hct value recorded in JP 75 was consistent with the studies by Musa et al. (2018) and Okomoda et al. (2021) in *Clarias gariepinus* fed hydrothermally processed *J. curcas* kernel meal and

Table 1. Ingredient and proximate composition of the trial diets (dry matter basis).

	Diets				
Ingredients (g Kg^{-1})	Control	JP25	JP50	JP75	JP 100
Soy protein isolate (SP)[a]	180	135	90	45	0
Detoxified Jatropha protein isolate (JP)	0	43.5	86.9	130.3	173.9
Fish meal	60	60	60	60	60
Groundnut oil cake	150	150	150	150	150
Rice flour	236	236	236	236	236
Wheat flour	280	281.5	283.1	284.7	286.1
Oil	60	60	60	60	60
Vitamin/mineral mix	10	10	10	10	10
Dicalcium phosphate[b]	10	10	10	10	10
Carboxymethyl cellulose[b]	10	10	10	10	10
Choline chloride[b]	2	2	2	2	2
Butylated hydroxytoluene[b]	2	2	2	2	2
Total	1000	1000	1000	1000	1000
*Essential amino acid (g kg^{-1})**					
Arginine	26.4	27.0	27.6	28.1	28.7
Histidine	8.5	8.2	8.0	7.8	7.6
Isoleucine	15.0	14.2	13.4	12.6	11.8
Leucine	25.5	25.2	24.8	24.5	24.1
Lysine	18.0	16.1	14.3	12.5	10.7
Methionine+cysteine	10.2	10.4	10.5	10.7	10.8
Phenylalanine	16.8	16.2	15.6	15.1	14.5
Threonine	11.8	11.5	11.1	10.8	10.4
Valine	16.8	17.2	17.6	17.9	18.3
Proximate analysis (g Kg^{-1} dry matter)					
Crude protein	310	312	312	312	310
Crude lipid	70	65	65	70	70
Ash	60	60	55	60	60
***Antinutritional factors*					
Phorbol ester (mg Kg^{-1} diet)	0.00	0.52	1.04	1.56	2.09
Phytic acid (g Kg^{-1})	2.57	3.05	3.53	4.01	4.49
Tannin (g Kg^{-1} diet)	1.48	1.20	0.92	0.64	0.37

Composition of vitamin-mineral mix (PRE-EMIX PLUS) (quantity/kg): Vitamin A, 55, 00 000 IU; Vitamin B, 2 000 mg; Vitamin D3, 11, 00 000 IU; Vitamin E, 750 mg; Vitamin K, 1000 mg; Vitamin B6, 1000 mg; Vitamin B1, 2,6 mcg; Vitamin C, 100mg; 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.

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

[b]Himedia Laboratories, Mumbai, India; [c]Molychem Pvt, Mumbai, India

*Calculated based essential amino acid composition (g/kg) of FM, GNC, Wheat & Rice flour (NRC, 2011), and detoxified Jatropha protein isolate (arginine 82; histidine 19; isoleucine 26; leucine 66; lysine 15; phenylalanine 34; methionine 11; threonine 26; valine 54); and soybean protein isolate (arginine 65; histidine 23; isoleucine 43; leucine 72; lysine 55; phenylalanine 46; methionine 12; threonine 33; tryptophan 12; valine 44).

**Calculated based on phorbol esters, phytic acid, and tannin concentration in JP (0.012mg g^{-1}; 2.58%; 0.21g Kg^{-1}, respectively – Fawole et al. 2018) and SP (0.0mg g^{-1}; 1.43%; 0.82g Kg^{-1}, respectively)

soaked Jatropha kernel meal, respectively. Leucocyte is a useful index of fish health which play a part in innate immune defence response in fish (Whyte, 2007). In the current study, higher WBC was noticed in JP-based diet (except JP 100) and this implies that the innate immunity in rohu fingerlings fed JP diets were stimulated compared to the control. Furthermore, enhanced lysozyme activity has been reported as a sign of improved immunity in fish (Shamna et al., 2017), which is consistent with the current study, in which fish fed JP-based diets had higher lysozyme activity and increased with a corresponding increase in JP levels ($y = 0.0441x + 8.754$, $R^2 = 0.967$). Moreover, the significantly higher total immunoglobulin content observed in fish fed JP complements the evidence of an enhanced immune response following the feeding of rohu with a detoxified jatropha protein isolate-based diet. As a result, it could be deduced that replacing SPI with JP in the diets of *L. rohita* fingerlings will assist in improving the health status and wellbeing of the fish. A similar result was reported when detoxified *Jatropha curcas* kernel meal was fed to common carp (Kumar et al., 2010). Li et al. (2015) observed that feeding 30% partially detoxified kernel meal as a replacement for SBM had no effect on the haemato-immunological status of pigs.

Total protein is another important blood index used to assess health and nutritional status of fish (Kumar et al., 2010; Shamna et al., 2017). The present study showed that JP-fed fish, except JP 75, had higher serum total protein compared to the control, and this is an indication that the JP did not cause impaired protein metabolism, but rather enhanced its synthesis when SPI was replaced completely in rohu diet. The higher value recorded in JP 100 signifies that the level of ANFs and phorbol esters (PEs) in the diet would not affect protein synthesis, contrary to the report by Shamna et al. (2017), in which serum total protein was observed to decrease with increasing nontreated jatropha protein concentrate in the rohu diet. Phosphorus is an essential macronutrient required in energy metabolism and nucleic acids synthesis in fish (NRC, 2011; Tang et al., 2012), however, phosphorus in plant-based ingredients are often not available to fish because two-third of it are present in the form of phytate which cannot be digested by fish. Interestingly, in the present study, we found that the fish fed JP100-based diet recorded the highest serum phosphorus concentration compared to the control, and this could be associated with the detoxification processes (organic solvent + heat) which dramatically improved the release of phosphorus from the phytate-phosphorus bond thereby increases phosphorus bioavailability for the fish physiological need. The cholesterol-reducing effects of plant materials have been reported in several studies (Kaushik et al., 1995; Kumar et al., 2010; Fawole et al., 2016). However, in this study, no significant variation was observed in the cholesterol levels among the dietary groups, and this could be because the major protein of all the experimental diets, including the control, were of plant origin. A similar result was reported in great sturgeon *Huso huso,* in which the plasma cholesterol levels remain unchanged after being fed plant protein-based diets, including the control (Jahanbakhshi et al., 2013).

Goblet cells are important in mucus secretion, which creates a gel-like layer on the mucosal surfaces and acts as a barrier against microbial invasion to cause intestinal inflammation (Johansson et al., 2008; Knoop and Newberry, 2018). The depletion of goblet cells is one of the visible signs of intestinal abnormalities induced by nutritional imbalances or infections (Dharmani et al., 2009). In the present study, higher mucus-secreting GCs and IELs was found in rohu fed JP-based diet (except JP 100) compared with the control. Abundance of IELs and GCs have been reported to be associated with increased immune response in fish (Adeoye et al., 2020), thus the higher GCs and IELs found in JP-groups shows the beneficial impacts of feeding JP in place of SPI and its potential of increasing mucus production to protect the intestinal lining and improve gut health in rohu. Intestine of *C. gariepinus* fed raw *J. curcas* kernel showed loss of goblet cells and sloughing of the epithelial lining (Okomoda et al., 2021). Furthermore, the liver histology of the JP groups did not show significant changes compared to the control. A similar observation was also made in common carp fed detoxified *J. curcas* kernel meal (Kumar et al., 2010) and rats fed detoxified *J. curcas* seed cake protein isolate (Zhao et al., 2018). On the contrary, feeding raw *J. curcas* kernel caused severe necrosis in the liver of *C. gariepinus* (Okomoda et al., 2021).

Plant/plant products are rich in polyphenolic content that plays a vital role in attenuating oxidative stress at the cellular level (Isgor et al., 2018), and these effects have been attributed to their antioxidant capacity. Some findings show that feed ingredients or anti-nutrients present in the feed may alter the activity of antioxidant enzymes in fish, following ingestion (Lin et al., 2007; Tovar-Ramı'rez et al., 2010; Zheng et al., 2012). For example, Deng et al. (2015) stated that feeding a diet containing a high level of cyanide to hybrid tilapia (*Oreochromis niloticus x O aureus*) induced lipid peroxidation and triggered oxidative stress. Other studies have also reported similar observations in different fish species (Olsvik et al., 2011; Zheng et al., 2012; Shamna et al., 2017). Remarkably, in the present study, the activities of SOD, CAT, and the oxidative stress biomarker, MDA, in rohu hepatic tissue did not show any discernible changes. This observation could be attributed to the low level of PEs and other deterrent factors, suggesting that a higher inclusion (174 g Kg^{-1} diet) of detoxified JP in the diets for *L. rohita* would not trigger oxidative stress or alter the antioxidant status of the fish. Our results contrast with the findings of Shamna et al. (2017) who reported that feeding 20% JPC containing PEs resulted in higher hepatic SOD activity in rohu. Also, Zheng et al. (2012) reported that the high level of gossypol presents in cottonseed meal altered

the antioxidant enzyme systems in grass carp, *Ctenopharyngodon idellus,* when fed beyond 35% in replacement for soybean meal.

In conclusion, rohu fingerlings fed with detoxified jatropha protein isolate showed improved innate immune response, increased utilization of phosphorus, and comparable weight gain up to a 75% replacement level for soybean protein isolate. Furthermore, the use of JP in aquafeed for rohu would not have a detrimental impact on the histoarchitectural structure of the liver, as well as the status of hepatic antioxidant enzymes in the fish. Therefore, this study recommends the commercial production of jatropha seed cake protein isolate, a non-edible oil seed waste, to reduce the pressure on edible soybean meal, which is used in the production of soy protein isolate.

Ethical Statement

The study was approved by the institute ethics committee, and the sampling procedure was carried out according to ethical guidelines (No; 193-2012) prepared by ICAR-CIFE, Mumbai, which complies with all relevant local and/or international animal welfare laws, guidelines, and policies.

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Author Contribution

F.J.F: co-conceptualized, co-designed, conducted the fish feeding trial and lab work, interpretation of results and drafted the original manuscript. N.P.S: conceptualized, designed and supervised the research project. N.S and V.P: Helped in the analytical lab work and reviewed the manuscript. A.A.A and B.O.E: helped in histology micrograph, contributed to result interpretation, and reviewed the manuscript. All authors read the draft, corrected, and approved the final version of the manuscript for submission.

Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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