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Effects of detoxified *Jatropha curcas* protein isolate on growth performance, nutrient digestibility and physio-metabolic response of *Labeo rohita* fingerlings

F.J. Fawole^{1,2} | N.P. Sahu¹ | N. Shamna¹ | V. Phulia^{1,3} | B.O. Emikpe⁴ | A.A. Adeoye⁵ | A.Z. Aderolu⁶ | O.M. Popoola⁷

¹Fish Nutrition, Biochemistry and Physiology Division, ICAR-Central Institute of Fisheries Education, Mumbai, India

²Department of Fisheries and Wildlife Management, Osun State University, Osogbo, Nigeria

³Krishi Vigyan Kendra, SAS, Nagar, India

⁴Department of Veterinary Pathology, Faculty of Veterinary Medicine, University of Ibadan, Ibadan, Nigeria

⁵Department of Aquaculture and Fisheries Management, Federal University of Agriculture, Abeokuta, Nigeria

⁶Department of Marine Sciences, University of Lagos, Lagos, Nigeria

⁷Department Fisheries and Aquaculture Technology, Federal University of Technology, Akure, Nigeria

Correspondence

F.J. Fawole and N.P. Sahu, Fish Nutrition, Biochemistry and Physiology Division, ICAR-Central Institute of Fisheries Education, Mumbai, India.

Email: phem247@yahoo.com, npsahu@cife.edu.in

Abstract

Jatropha protein isolate was prepared following the principle of isoelectric precipitation. The isolated protein is low in fibre, lipid and ash, and high in protein and gross energy contents. The antinutritional factors such as phytic acid, tannin and trypsin inhibitor were found to decrease after protein extraction except phorbol esters (PEs), which remain bounded to the protein matrix. Thus, the protein isolate was detoxified and the PEs content reduced significantly. For the feeding trial, six isonitrogenous diets were formulated as DJP 0, DJP 25, DJP 50, DJP 75, DJP 100, or DJP 100 + lysine with detoxified *Jatropha* protein isolate (DJP) in replacement for soybean protein isolate (SPI) and fed to *L. rohita* fingerlings (3.25 ± 0.02 g) for 60 days. The weight gain percentage (WG %), specific growth rate and metabolic growth rate values among the groups showed no significant difference ($p > .05$), except DJP 100 fed group. The feed conversion ratio, protein efficiency ratio, protein productive value, and survival, digestive and hepatic aminotransferase enzymes activities were not significantly affected, whereas intestinal alkaline phosphatase and serum alanine transaminase differ significantly ($p < .05$). Thus, this study revealed that DJP supplemented with lysine could replace 100% SPI protein without causing any significant effects on fish performance and organ integrity.

KEYWORDS

aminotransferase enzymes, growth, *Jatropha curcas*, organ integrity, protein isolate, rohu

1 | INTRODUCTION

Aquaculture is one of the fastest growing animal sectors, but the shortage of ingredients and increasing cost of conventional feed resources is posing a great challenge to the sustainability of the industry. To reduce the resultant effects of ingredient shortage on fish productivity, several alternative ingredients both of animal and plant origins including single cell proteins have been tested in different commercial fish species, and the results were encouraging (Fawole et al., 2017; Kumar, Makkar, & Becker, 2012; Lin & Luo, 2011; Shamna et al., 2017). Among the most promising alternative protein sources, ingredients of plant origin became prominent due to their availability, supply and low cost. However, the growing demand for fish feed and the high

cost of conventional plant protein ingredient especially soybean meal have prompted researchers to focus on ingredients that do not conflict with human food security (Fawole et al., 2017; Teves & Ragaza, 2016). Therefore, in this perspective, non-edible oil seed resources became prominent, and in the last few decades, an appreciable number of protein-rich non-edible seeds have been tested in fish/shrimp feed preparation either to partially or totally replace fish meal or soybean meal (Fawole, Sahu, Jain, Gupta, Shamna, Phulia, et al., 2016; Harter et al., 2011; Kumar et al., 2012; Phulia et al., 2016).

Jatropha curcas is a non-edible, oil-yielding tree with good potential for bio-diesel fuel production, and provides a highly nutritious protein-rich seed cake for animal feed development (Kumar et al., 2012; Makkar & Becker, 2009; Wakandigara, Nhamo, & Kugara, 2013). Many countries



of the world have invested heavily on large-scale planting of *Jatropha* especially India, China, Indonesia, Madagascar, Myanmar, Cambodia and Vietnam, with the aim of using it as a source of clean energy (Ye, Li, Francis, & Makkar, 2009). Increasing *Jatropha* production as biofuel crops will ultimately increase the quantity of its by-product (i.e., seed cake). At present, oil is directly pressed from the seed without decortication and this produces seed cake of around 700–750 g/kg and oil 250–300 g/kg of the total biomass (Siang, 2009). This indicates that there is the possibility of producing adequate quantity of seed cake for aquafeed production. *Jatropha curcas* seed cake has good amino acid profile comparable to soybean meal and contains about 270–320 g/kg crude protein (Liberalino, Bambirra, Moraes-Santos, & Vieira, 1988; Shamna et al., 2015). Despite this enormous potential, *J. curcas* seeds cake (JSC) has a limited use in animal feed preparation owing to the presence of a toxic component, phorbol esters (PEs) and other deterrent factors (Makkar, Francis, & Becker, 2008). Aside from the toxic components and antinutritional factors (ANFs), the seed cake left after oil extraction contains a high level of indigestible material, which may limit their use in fish feed preparation. According to Devappa and Swamylingappa (2008), isolation of protein can be advantageous in the elimination of toxins and indigestible materials present in JSC to a safe level. Protein isolate has been described as a good substitute for soybean meal in monogastric animal diets including fish (Fawole, Sahu, Jain, Gupta, Shamna, Phulia, et al., 2016, 2017; Makkar, 2016). Being a pure protein fraction, the fibre, oil, non-starch polysaccharides and ANFs in protein isolates are very low compared to the original material, thus making them suitable for inclusion in the fish diet (Fawole, Sahu, Jain, Gupta, Shamna, Phulia, et al., 2016; Makkar, 2016).

Although many studies have been carried out on the utilization of *J. curcas* seed cake/kernel meal in different fish species, only a few studies (*Cyprinus carpio*- Kumar et al., 2012; Latif, Kumar, Stadtlander, Makkar, & Becker, 2015) have attempted to isolate protein from the raw JSC and examined its effect on the growth and nutrient utilization in fish. Furthermore, no study has attempted to examine the effects of *Jatropha* protein isolate on the hepatic aminotransferase enzyme activity in fish. Therefore, the aim of this study was (i) to isolate protein from JSC and determine its nutritional profile and antinutrient levels; (ii) to examine the effects of detoxified *Jatropha* protein isolate on the growth performance, nutrient utilization, apparent digestibility and physio-metabolic response of *Labeo rohita* (rohu) fingerlings, the most economically important freshwater fish in South-East Asia.

2 | MATERIALS AND METHODS

2.1 | Experiment I

2.1.1 | Collection of *Jatropha* seed cake and isolate preparation

Jatropha seed cake was obtained from A1 Oil India, Haridas Nagar, Borivali, Mumbai, India. The seed cake was finely powdered and defatted in hexane for 12 hr. The extraction was repeated and air-dried, and the resulting defatted *Jatropha* seed meal (DJSM) was stored at

–20°C until further use. The preparation of protein isolate was carried out following the method described in our earlier study (Fawole, Sahu, Jain, Gupta, & Shamna, 2016; Fawole, Sahu, Jain, Gupta, Shamna, Phulia, et al., 2016). A known quantity of DJSM was dispersed in a distilled water (1:10w/v) and stirred (SPINOT®, Tarson, India) for 2 hr with the pH of the dispersion adjusted to 12 using 1N NaOH. The solution was centrifuged at 5,300 g for 20 min, insoluble fraction discarded, and the resulting supernatant adjusted to pH 4.0 with 1N HCl to precipitate the protein. The precipitated protein was centrifuged (5,300 g, 15 min) to remove the soluble sugar and washed twice with distilled water to obtain the protein isolate. The wet protein isolate was freeze-dried (Scanvac™ cool safe 100-9 pro), and the dried *Jatropha* protein isolate (JPI) was analysed for proximate composition, antinutrient contents and amino acid profile.

2.1.2 | Proximate composition and amino acid analysis

Proximate analysis of the test ingredients was carried out as per the standard methods of the Association of Official Analytical Chemists (AOAC, 1995). The amino acid analysis was carried out by hydrolysing the samples with 6 M HCl for 24 hr under vacuum at 110°C, and precolumn derivatization was performed using phenylisothiocyanate prior to quantification by high-performance liquid chromatography (HPLC) (Bidlingmeyer, Cohen, & Tarvin, 1984).

2.1.3 | Detoxification of *Jatropha* protein isolate

Jatropha protein isolate was further detoxified using an organic solvent; 5 g of sample was extracted with 50 ml ethanol (90%) and kept on a magnetic stirrer (SPINOT®, Tarson, India) at 600 × g for 15 min at a temperature of 50°C. The slurry was filtered using Whatman No. 1 filter paper. The extraction was repeated 3 times and the filtrate discarded. The sample left over was used for the feed preparation and referred to as detoxified *Jatropha* protein isolate (DJP).

2.1.4 | Determination of antinutrient contents

The levels of phytic acid in DJSM and DJP were determined according to the colorimetric method (Gao et al., 2007). Tannin was estimated by Folin-Denis method (Schanderi, 1970) and the result expressed as tannic acid equivalents. Trypsin inhibitor activity (TIA) was determined following the method of Hamerstrand, Black, and Glover (1981) as modified by Jiang, Cai, and Xu (2013), and the results were expressed as mg trypsin inhibited per gram of dry sample (mg TI per g dry sample). For the phorbol esters (PEs) estimation, the method of Saetae and Santornsuk (2011) was followed with little modification; 5 g of sample was taken in 20 ml methanol (HPLC grade) and stirred in an orbital shaker at 200 rpm for 5 min followed by filtration using a Whatman No. 1 filter paper and the filtrate collected. The extraction was repeated four times, and the filtrate (i.e., extract fractions) was pooled together. The filtrate was concentrated using rotary evaporator (IKA® RV 10 basic) at a temperature of 60°C, rotor speed of

TABLE 1 Ingredient and nutrient composition of the experimental diets (DM basis) fed to *Labeo rohita* fingerlings

	Diets					
	DJP 0	DJP 25	DJP 50	DJP 75	DJP 100	DJP 100 + Lys
Ingredients (g/kg)						
Soy protein isolate ^a	180	135	90	45	0	0
Detoxified Jatropha protein isolate (DJP)	0	43.5	86.9	130.3	173.9	173.9
Fish meal ^b	60	60	60	60	60	60
Groundnut oil cake ^b	150	150	150	150	150	150
Rice flour ^b	236	236	236	236	236	236
Wheat flour ^b	280	281.5	283.1	284.7	286.1	286.1
Sunflower oil:cod liver oil (1:1) ^c	60	60	60	60	60	60
Vitamin/mineral mix	10	10	10	10	10	10
Dicalcium phosphate ^d	10	10	10	10	10	10
Carboxymethyl cellulose ^d	10	10	10	10	10	10
Choline Chloride ^d	2	2	2	2	2	2
Butylated hydroxytoluene ^d	2	2	2	2	2	2
Total	1,000	1,000	1,000	1,000	1,000	1,000
L-Lysine monohydrochloride ^e	—	—	—	—	—	5
Cr ₂ O ₃	5	5	5	5	5	5
Proximate analysis (g/kg DM)						
Dry matter	909	908	907	908	908	906
Crude protein	310	312	312	312	310	313
Crude lipid	70	65	65	70	70	70
Ash	60	60	55	60	60	65
Crude fibre	30	30	40	30	30	25
NFE ^f	530	534	529	539	531	527
Gross energy (MJ/kg) ^g	17.02	17.09	17.01	17.03	17.02	17.04
Phosphorus (mg P per g sample)	6.33	5.79	5.91	5.36	5.79	6.18

^aSoy Growth™ Medicamen organics limited, India; 84.5% crude protein, 3.5% crude lipid, 4% ash, 23 MJ/kg gross energy. Essential amino acid composition (g/kg): arginine 65; histidine 23; isoleucine 43; leucine 72; lysine 55; phenylalanine 46; methionine 12; threonine 33; tryptophan 12; valine 44.

^bPurchased from local animal feed ingredient dealer, Mumbai, India.

^cUniversal Medicare Pvt Ltd, Mumbai, India.

^dHimedia Laboratories, Mumbai, India.

^eMolychem Pvt, Mumbai, India.

^fNFE (Nitrogen free extract) = 1,000 – (Crude protein + Crude lipid + crude fibre + ash).

^gCalculated gross energy (MJ/kg) = [23.9 × CP (g/kg) + 39.8 × CL (g/kg) + 17.6 × NFE (g/kg)]/1,000.

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, 1,000 mg; Vitamin B6, 1,000 mg; Vitamin B1, 2,6 mcg; Vitamin C, 100 mg; Calcium Pantothenate, 2,500 mg; Nicotinamide, 10 g; Mn, 27,000 mg; I, 1,000 mg; Fe, 7,500 mg; Zn, 5,000 mg; Cu, 2,000 mg; Co, 450; L-lysine, 10 g; DL-Methionine, 10 g; Selenium, 125 mg.



50 rpm, and the resulting sample was quantified in high-performance liquid chromatography (HPLC—Waters, Massachusetts, USA) using UV detector at 280 nm.

2.2 | Experiment II—feeding experiment

2.2.1 | Experimental animal and design

Fingerlings of *Labeo rohita* (rohu) were obtained from Srushti Aquaculture, Sudhagad, Raigad District, Maharashtra, India, and transported to the Central Wet Laboratory, ICAR-Central Institute of Fisheries Education, Mumbai. At the wet laboratory, the fish stock was acclimatized under the aerated condition and fed with the control diet prior to the commencement of the experiment. A total of 216 fingerlings with the initial average weight of 3.25 ± 0.02 g were used in 60-day feeding trial. Fish were randomly assigned to six different diets with 18 replicate tubs containing 12 fish each. Feeding was carried out to satiation level, and feed intake was adjusted based on the observation of daily feed consumption. The daily ration was divided into two equal parts and fed at 10:00 hr and 18:00 hr. Fish in each tub were weighed at the start and 15-day interval throughout the experimental period. The water quality parameters were monitored and maintained within the range for freshwater fish viz; temperature $27.9^\circ\text{C} \pm 0.82$, pH 7.7 ± 0.42 , DO $6.9 \text{ mg/L} \pm 0.38$ and ammonia $0.08 \text{ ppm} \pm 0.03$.

2.2.2 | Feed preparation

Six isonitrogenous (311.3 g/kg) and isocaloric (17.12 MJ/kg) experimental diets were formulated to replace soybean protein isolate (SPI) with detoxified *Jatropha* protein isolate (DJP) at 0% (DJP 0), 25% (DJP 25), 50% (DJP 50), 75% (DJP 75), 100% (DJP 100) or 100% supplemented with L-Lysine (DJP 100 + LYS) on equivalent protein basis (Table 1). Apart from wheat flour, other ingredients such as groundnut oil cake, wheat bran, rice flour and fishmeal were included at the equal proportion in all the diets. All the ingredients were finely grinded and mixed thoroughly to form a homogenous blend, and water was added to form a dough. The dough was transferred to an aluminium container, which was then placed in a pressure cooker for cooking/steaming (15 min). The steamed dough was taken out and cooled, and then, oil and the vitamin–mineral mix were added. The dough was mixed properly and pelletized using a hand pelletizer (2 mm die), and the pellets were air-dried and stored at -20°C until further use. Cr_2O_3 (5 g/kg) was added to each diet as an indigestible marker for nutrient digestibility study.

2.2.3 | Growth response parameter and proximate composition analysis

Growth response and nutrient utilization indices such as percentage weight gain (WG %) = $[(\text{final weight} - \text{initial weight}) / \text{initial weight}] \times 100$; specific growth rate (SGR, %/day) = $100 \times (\ln \text{final body weight} - \ln \text{initial body weight}) / \text{experimental duration in days}$; metabolic growth rate (MGR, $\text{g kg}^{-0.8} \text{ day}^{-1}$) = $(\text{net weight gain in g}) / \{[(\text{initial body weight in g}/1,000)^{0.8} + (\text{final body weight in g}/1,000)^{0.8}]/2\}$

feeding days; Feed intake ($\text{g kg}^{-1} \text{ MBW day}^{-1}$) = total dry feed given per fish (g per fish)/MBW/experimental duration in days; mean metabolic body weight (MBW) = $\{[(\text{initial body weight in g}/1,000)^{0.75} + (\text{final body weight in g}/1,000)^{0.75}]/2\}$ (Deng, Kangsen, Liqiao, Haifeng, & Lu, 2015). Feed conversion ratio (FCR) = {feed consumption (g)/body weight gain (g)}; protein efficiency ratio (PER) = {net weight gain (g)/protein fed (g)}; Protein productive value (PPV) = $100 \times \{[(\text{final body weight} \times \text{final body protein content}) - (\text{initial body weight} \times \text{initial body protein content})] / (\text{protein content in diet} \times \text{total feed intake})\}$; and the survival (%) = $[(\text{Total number of fish harvested} / \text{total number of fish stocked}) \times 100]$ were determined at the end of the experiment. Feed sample was analysed for proximate composition following the standard methods of the Association of Official Analytical Chemists (AOAC, 1995). Before starting the experiment, a total of 12 fish were sacrificed and stored at -20°C for the analysis of initial whole body composition.

2.2.4 | Digestibility study

The procedures for faecal sample collection were similar to those described in our previous study (Fawole, Sahu, Jain, Gupta, Shamna, Phulia, et al., 2016). Analysis of crude protein and lipid contents was carried out according to AOAC (1995). Diet and faecal chromium contents were determined following the method of Furukawa and Tsukahara (1966). The apparent dry matter digestibility of diet and nutrients was calculated as given below:

$$\text{ADC of dry matter (\%)} = 100 \times \{1 - (\% \text{Cr}_2\text{O}_3 \text{ in diet}) / (\text{faecal } \% \text{Cr}_2\text{O}_3)\}$$

$$\text{ADC of nutrients (\%)} = 100 \times \{1 - \{(\% \text{Cr}_2\text{O}_3 \text{ in diet} / \text{faecal } \% \text{Cr}_2\text{O}_3) \times (\% \text{nutrients in faeces}) / (\% \text{nutrients in diet})\}\}.$$

2.2.5 | Fish sampling for serum collection and chemical analysis

At the end of the feeding trial, blood samples were collected from the caudal vein of a randomly selected fish (four per replicate) without anticoagulant into a dried Eppendorf tube and allowed to stand in a slanted position at room temperature. The sera were centrifuged (3,000 g, 10 min at 4°C) and transferred into another tube and kept in -20°C for immediate use. Serum ALT, AST, ALP and total bilirubin were estimated using a commercial kit (Erba® Diagnostic Mannheim, Transasia Bio-medicals Ltd, India). The liver and intestinal tissues were dissected out from the sacrificed fish and homogenized in cold 250 mM sucrose solution using a tissue homogenizer. The tubes were continuously kept in ice to avoid heating. The homogenate was centrifuged (5,000 g for 10 min at 4°C) and the supernatant stored at -80°C until analysis. Using the supernatant, the intestinal protease and amylase enzyme activities were measured following the methods of Drapeau (1974) and Rick and Stegbauer (1974), respectively. Metabolic enzymes such as ALT, AST (Wooten, 1964) and ALP (Garen & Levinthal, 1960) were also determined. The protein content of the tissue was measured using bovine serum albumin as the standard (Bradford, 1976).

2.2.6 | Histology and histomorphometric assessment of the intestine

A histological study was performed according to the method described in our previous studies (Fawole et al., 2017). The histomorphometric assessment was carried out as described by Moldal et al. (2014). Briefly, the height of the villi was measured from the villi fold apex to the bottom of the epithelium at the base of the folds. The width of the villi was assessed at two points in every fold. Six measurements of the villi height and twelve measurements of the villi width were recorded and statistically analysed using one-way ANOVA (SPSS version 20).

2.3 | Statistical analysis

All data were checked for normality using the Kolmogorov–Smirnov test, and homogeneity of variances among different treatments was tested using the Barlett's test. Then, data were statistically analysed by one-way ANOVA using IBM SPSS statistical package, version 20. Duncan multiple range test was used to determine the significant difference between the means. The significance level was defined at $p < .05$. Data are given as mean $\pm$ standard error of the mean.

3 | RESULTS

3.1 | Chemical composition and antinutritional factors

The chemical compositions of defatted Jatropha seed meal (DJSM) and detoxified Jatropha protein isolate (DJP) are presented in Table 2. The dry matter, protein and gross energy values of the isolate were higher than the corresponding meal (i.e., DJSM), while a reduction was noticed in the ash and carbohydrate contents of the isolate. The concentration of phytic acid (2.58), tannin (0.21) and trypsin inhibitors (12.30) in DJP was lower than those in DJSM (4.06, 0.74 and 26.64, respectively). The phorbol ester (PEs) content of DJSM was 0.274 mg/g but increased to 0.340 mg/g in the isolate (beneath Table 2). However, after further detoxification, the PEs concentration reduced to 0.012 mg/g.

3.2 | Amino acid (AA) composition

The AA results showed that the concentration of essential amino acids such as leucine, phenylalanine, methionine and valine increased after protein extraction (Table 3), while arginine, histidine, lysine and threonine showed a slight reduction. The sulphur-containing amino acid (DJP-2.7 g/kg; DJSM-2.0 g/kg) and aromatic amino acid (DJP-5.2 g/kg; DJSM-4.3 g/kg) contents of the protein isolate were higher than the meal.

3.3 | Growth performance, nutrient utilization and survival rate

The higher WG (%), SGR and MGR values recorded in the control (DJP 0) were statistically similar ($p > .05$) to those fed DJP 25, DJP

TABLE 2 Chemical composition and antinutritional factors of DJSM and DJP

Variables	DJSM	DJP
Dry matter (g/kg)	887.33 $\pm$ 4.91	938.25 $\pm$ 5.95
Crude protein (g/kg)	425.97 $\pm$ 1.73	875.23 $\pm$ 2.32
Crude lipid (g/kg)	10 $\pm$ 0.00	9.5 $\pm$ 0.50
Ash (g/kg)	76.67 $\pm$ 3.33	20 $\pm$ 0.00
NFE + fibre (g/kg)	487.36 $\pm$ 4.78	95.50 $\pm$ 2.40
Gross energy (MJ/kg)	19.16	22.97
Phorbol esters (mg/g)	0.274 $\pm$ 0.02	0.012 $\pm$ 0.00
Phytic acid (% phytic acid equivalent)	4.06 $\pm$ 0.01	2.58 $\pm$ 0.06
Tannin (g/kg tannic acid equivalent)	0.74 $\pm$ 0.03	0.21 $\pm$ 0.01
Trypsin inhibitor (mg TI per g)	26.64 $\pm$ 0.54	12.30 $\pm$ 0.17

Data expressed as Mean $\pm$ SE, $n = 3$.

DJSM, defatted Jatropha seed meal; DJP, detoxified Jatropha protein isolate. ANFs of non-detoxified Jatropha protein isolate: phorbol esters (0.340 mg/g), phytic acid (3.28%), tannin (0.46 g/kg).

50, DJP75 and DJP 100 + LYS (Table 4). As the inclusion level of DJP increased from 75% to 100%, there was a sharp decline in the growth parameters and differs significantly to the control ($p < .05$). However, lysine supplementation at 2.78% of DJP inclusion in the diets (DJPI 100 + LYS) had a positive impact on the growth performance indices compared to the corresponding DJP 100 fed group. No significant difference was observed in the feed intake among the experimental groups ($p > .05$). The nutrient utilization indices like FCR, PER, PPV and survival among the various groups did not differ significantly ($p > .05$). The weight gain (%) and protein efficiency ratio showed a positive linear correlation ($r = .90$, $p < .001$).

3.4 | Apparent digestibility coefficient (ADCs)

The apparent digestibility coefficients (ADCs) values for dry matter (DM), protein, lipid and phosphorus are shown in Table 5. The group fed DJP 50 and control showed a significantly higher ADC of DM compared to the lowest value recorded in DJP 75 ($p < .05$). The observed ADCs value for protein and phosphorus in groups fed DJP 50, DJP 100 + LYS and control was found to be similar and significantly higher than other fed groups ($p < .05$). ADC of lipid among the various groups showed no variation ($p > .05$).

3.5 | Intestinal digestive and brush border enzymes activities

The inclusion of DJP in replacement for SPI had no significant ($p > .05$) effects on the intestinal protease and amylase enzymes activities (Figures 1 and 2). The highest ALP activity in the intestine was observed in DJP 100 group, which was statistically similar to other fed groups except DJP 50 ($p < .05$) (Table 6).

**TABLE 3** Amino acid composition of DJSM and DJP

	DJSM	DJP
Essential amino acid (g/kg)		
Arginine	102	82
Histidine	23	19
Isoleucine	26	26
Leucine	50	66
Lysine	21	15
Phenylalanine	27	34
Methionine	8	11
Threonine	34	26
Tryptophan	ND	ND
Valine	39	54
Methionine + Cystine	20	27
Phenylalanine + Tyrosine	43	52
Non-essential amino acid (g/kg)		
Alanine	27	25
Glycine	28	28
Aspartate	71	75
Glutamic acid	116	106
Proline	30	27
Serine	45	38
Cystine	12	16
Tyrosine	16	18
TEAA	330	333
TNEEAA	345	333

TEAA, total essential amino acid; TNEAA, total non-essential amino acid; DJSM, defatted Jatropha seed meal; DJP, detoxified Jatropha protein isolate.

3.6 | Hepatic aminotransferase enzymes activities

In response to the dietary inclusion of DJP, hepatic ALT and AST enzyme activities among the various groups showed no significant variation ($p > .05$) (Table 6).

3.7 | Serum biochemical test

The serum biochemical test such as alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate transaminase (AST) and total bilirubin is given in Table 6. The serum ALP, AST and total bilirubin concentration among the various groups did not differ significantly ($p > .05$). However, a significantly higher serum ALT activity was observed in DJP 100 and control, which was similar to DJP 50, but differ significantly ($p < .05$) from other fed groups.

3.8 | Histological observation

The intestinal tissue revealed shorter villi in the intestinal epithelium of the control, whereas DJP 25, DJP 50 and DJP 75 fed groups showed increased villi length. DJP 100 fed group showed an increase in the thickness of intestinal villi. There were no significant differences among the various groups regarding the villi length (Table 7).

4 | DISCUSSION

Protein isolation methods have been used to improve the nutritional value of plant protein ingredients and reduce the level of deterrent factors as well as indigestible material, thus making them suitable for inclusion in animal diets (Fawole, Sahu, Jain, Gupta, Shamna, Phulia, et al., 2016; Makkar, 2016; Mwachireya, Beames, Higgs, & Dosanjh,

TABLE 4 Growth performance, nutrient utilization and survival rate of rohu fingerlings fed detoxified Jatropha protein isolate

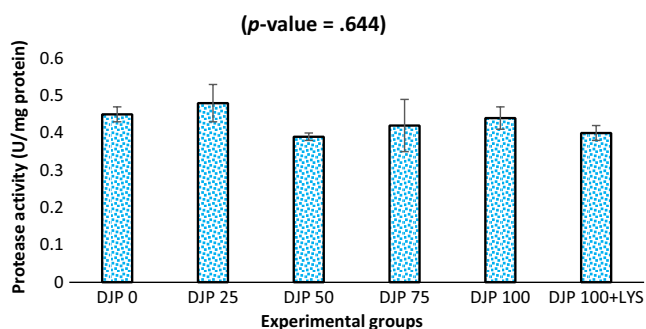
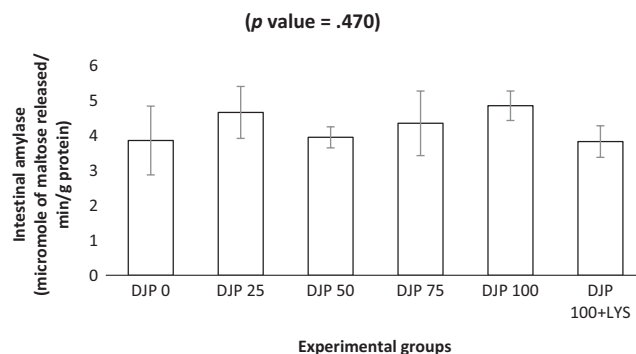
Variables	Treatments						<i>p</i> -Value
	DJP 0	DJP 25	DJP 50	DJP 75	DJP 100	DJP 100 + LYS	
Final weight (g)	8.52 ± 0.27	8.32 ± 0.20	8.34 ± 0.15	7.92 ± 0.12	7.55 ± 0.23	8.32 ± 0.32	.085
Weight gain (%)	161.80 ^a ± 8.00	154.71 ^a ± 5.87	157.47 ^a ± 3.38	142.78 ^{ab} ± 4.19	132.05 ^b ± 5.78	157.29 ^a ± 7.56	.035
Specific growth rate (%/day)	1.69 ^a ± 0.05	1.64 ^a ± 0.04	1.66 ^a ± 0.02	1.56 ^{ab} ± 0.03	1.48 ^b ± 0.04	1.66 ^a ± 0.05	.030
Metabolic growth rate (g kg ^{-0.8} day ⁻¹)	5.71 ^a ± 0.19	5.55 ^a ± 0.14	5.61 ^a ± 0.09	5.25 ^{ab} ± 0.10	4.97 ^b ± 0.16	5.60 ^a ± 0.17	.031
Feed intake (g kg ⁻¹ MBW day ⁻¹)	7.18 ± 0.29	7.03 ± 0.07	7.09 ± 0.02	7.30 ± 0.14	7.00 ± 0.17	7.28 ± 0.13	.687
Feed conversion ratio	1.63 ± 0.12	1.64 ± 0.06	1.63 ± 0.02	1.80 ± 0.02	1.83 ± 0.10	1.68 ± 0.09	.318
Protein efficiency ratio	1.99 ± 0.15	1.97 ± 0.07	1.97 ± 0.03	1.79 ± 0.02	1.77 ± 0.09	1.92 ± 0.09	.355
PPV (%)	32.10 ± 3.44	30.59 ± 2.50	31.79 ± 1.85	28.86 ± 0.76	28.40 ± 1.76	32.24 ± 1.90	.707
Survival (%)	97	97	97	100	100	100	.701

Mean value with different superscript in the same row differs significantly ($p < .05$). Data expressed as Mean ± SE, $n = 3$ (each replicate was stocked with 12 fish). PPV, protein productive value (%).

TABLE 5 Apparent digestibility coefficients of rohu fingerlings fed different diets

Treatments	Variables			
	Dry matter	Protein	Lipid	Phosphorus
DJP 0	86.92 ^a ± 0.04	89.00 ^{ab} ± 0.08	82.62 ± 2.66	79.66 ^a ± 0.34
DJP 25	75.37 ^c ± 0.54	82.55 ^c ± 0.85	78.17 ± 1.09	65.49 ^c ± 0.98
DJP 50	87.64 ^a ± 0.62	89.80 ^a ± 0.44	81.25 ± 1.57	78.73 ^a ± 0.66
DJP 75	68.36 ^d ± 0.33	76.72 ^e ± 0.06	80.59 ± 0.15	64.21 ^c ± 0.65
DJP 100	74.02 ^c ± 0.44	78.86 ^d ± 0.05	82.02 ± 2.91	71.50 ^b ± 0.56
DJP 100 + LYS	83.59 ^b ± 0.54	87.81 ^b ± 0.38	80.77 ± 0.04	79.71 ^a ± 0.04
p-Value	<.001	<.001	.620	<.001

Mean value with different superscript in the same column differs significantly ($p < .05$). Data expressed as Mean ± SE, $n = 3$.

**FIGURE 1** Protease enzyme activity of *Labeo rohita* fed different experimental diets**FIGURE 2** Amylase enzyme activity of *Labeo rohita* fed different experimental diets

1999). In the present study, the protein content of DJP was higher than DJSM, with a relative decrease in the fibre and ash contents. The increased protein content recorded in DJP compared with the corresponding meal was at the expense of the insoluble fibre, soluble sugar and other non-protein components present in the original material (i.e., DJSM), thereby allowing protein to aggregate at their isoelectric point (Fawole, Sahu, Jain, Gupta, Shamna, 2016; Garba, Kaur, & Phagwara, 2014). Furthermore, the ANFs were found to decrease after protein isolation and this could be due to the action of organic solvent and/or alkaline treatment, as reported by El Diwani, El Rafei, and Hawash (2011) that organic solvent could remove trypsin inhibitors, phytic acid and saponins present in plant-based ingredient. However, the phorbol esters (PEs) remain bound to the protein matrix, and its concentration in the isolate increased by 19.4% compared with the corresponding meal. The increase observed in the concentration of PEs may be due to its protein binding ability, thus solubilized and precipitated along with the proteins during the extraction process. Similar results were observed by Makkar et al. (2008). Nevertheless, to reduce the level of PEs in the protein isolate and make it suitable for animal feeding, organic solvent and heat treatments (50°C) were applied, and the level of PEs significantly reduced to 0.012 mg/g (96% reduction), which is below the permissible limit (0.09 mg/g; Gogoi, Niyogi, & Tyagi, 2014). The reduction observed in the lysine content of the isolated protein may be due to their interaction with the soluble sugar during the alkaline extraction process, similar to our earlier observation in rubber protein isolate (Fawole, Sahu, Jain, Gupta, Shamna, 2016).

The results of the feeding trial revealed that detoxified *Jatropha* protein isolate possesses good nutritional value and could replace up to 75% SPI protein in the diets of rohu without significantly affecting WG %, SGR and MGR. This is in line with the results of Latif et al. (2015) and Kumar et al. (2012) in common carp fed detoxified *Jatropha* protein isolate in replacement for fishmeal. The improvement in growth performance observed in DJP 100 supplemented with lysine, point to the fact that the depression in growth response noticed in DJP 100 group may be due to the amino acid imbalances. Thus, complete replacement of SPI protein with DJP in the diet of rohu is possible without causing any significant reduction in growth. The similar feed intake observed among the various groups indicates that the diets were well accepted by the fish and no palatability-mediated feed rejection was noticed during the feeding trial. Also, the insignificant differences observed in the FCR, PER and PPV values among the various groups signify optimum utilization of the dietary protein fed without any inhibitory effects of ANFs and are in line with other studies (Fawole, Sahu, Jain, Gupta, Shamna, Phulia, et al., 2016; Kumar et al., 2012). In contrast, Shamna et al. (2015) observed low performance in terms of weight gain (%), PER and higher FCR when non-detoxified *Jatropha* protein concentrate (JPC) was fed to *Labeo rohita* fingerlings, and the reduction was said to be due to the high concentration of dietary PEs (1.4 mg/g). Furthermore, severe toxicity symptoms and subsequent death have been reported in *Cyprinus carpio* (Becker & Makkar, 1998) and *Labeo rohita* (Shamna et al., 2015) fed diets containing PEs; however, in the current study, no phorbol ester-mediated



TABLE 6 Effects of different diets on ALP, ALT, AST and total bilirubin concentration of rohu fed graded levels of detoxified *Jatropha* protein isolate

Treatments	Variables						
	ALP ^a	ALT ^b	AST ^c	ALP ^d	ALT ^d	AST ^d	Total bilirubin ^e
	Intestine	Liver	Liver	Serum	Serum	Serum	Serum
DJP 0	176.66 ^{ab} ± 6.56	7.06 ± 1.25	32.28 ± 1.81	15.57 ± 2.17	1.36 ^a ± 0.10	15.98 ± 0.49	2.23 ± 0.28
DJP 25	186.68 ^{ab} ± 5.65	6.91 ± 0.79	35.31 ± 3.11	11.33 ± 1.25	1.14 ^b ± 0.08	13.95 ± 2.41	2.51 ± 0.29
DJP 50	130.98 ^c ± 4.61	8.22 ± 1.08	37.93 ± 4.17	15.75 ± 1.80	1.21 ^{ab} ± 0.03	11.25 ± 1.95	2.32 ± 0.15
DJP 75	163.70 ^b ± 9.06	8.70 ± 0.73	31.32 ± 4.13	14.74 ± 0.88	1.01 ^b ± 0.03	10.58 ± 1.27	2.08 ± 0.11
DJP 100	190.55 ^a ± 9.49	5.41 ± 1.30	31.50 ± 4.08	13.82 ± 1.88	1.37 ^a ± 0.06	17.22 ± 0.39	2.12 ± 0.75
DJP 100 + LYS	188.06 ^a ± 6.70	6.25 ± 0.95	34.39 ± 4.30	10.50 ± 1.44	1.10 ^b ± 0.04	15.75 ± 2.36	3.71 ± 0.05
p-Value	.001	.301	.781	.176	.007	.085	.056

Mean value with different superscript in the same column differs significantly ($p < .05$). Data expressed as Mean ± SE, $n = 3$.

^aAlkaline phosphatase (ALP): specific activities expressed as nanomoles *p*-nitrophenol released min⁻¹ mg protein at 37°C.

^bAlanine transaminase (ALT): specific activities expressed as nanomoles of sodium pyruvate formed mg protein⁻¹ min⁻¹ at 37°C.

^cAspartate transaminase (AST): specific activities expressed as nanomoles of oxaloacetate released min⁻¹ mg protein⁻¹ at 37°C.

^dALP, ALT, AST (U/100 ml).

^eTotal bilirubin (mg/dl).

TABLE 7 Changes in villi length of rohu fingerlings fed graded levels of detoxified *Jatropha* protein isolate

Treatment	Villi length (μm)	Villi width (μm)
DJP 0	244.80 ± 34.22	90.84 ± 1.47 ^a
DJP 25	251.90 ± 14.44	68.11 ± 6.75 ^b
DJP 50	289.49 ± 18.63	67.43 ± 4.14 ^b
DJP 75	272.43 ± 21.54	61.97 ± 3.03 ^b
DJP 100	242.74 ± 14.41	104.80 ± 3.31 ^a
DJP100 + LYS	260.07 ± 28.66	90.69 ± 9.56 ^a
p-Value	.699	.001

Mean value with different superscript in the same column differs significantly ($p < .05$).

Data expressed as Mean ± SE.

toxicity effect or death was noticed, and thus, higher survival was recorded among the various groups. This could be due to the low level of PEs present in DJP, which is comparatively lower to those found in non-toxic *Jatropha curcas* seed (0.11 mg/g, Makkar, Becker, Sporer, & Wink, 1997), or could be as a result of the high tolerance of rohu to PEs, which require further study.

Nutrient digestibility is an important factor in evaluating the quality of a feed or an ingredient, and their utilization potential (Kumar et al., 2012). In the current study, highest ADC of protein was recorded in DJP 50, which was similar to the control and lysine supplemented group. The insignificant lower ADC of protein recorded in fish fed DJP 75 and DJP 100 coincide with the result of protein productive value recorded for these group; hence, a positive linear relationship ($y = 0.2885x + 6.3963$, $R^2 = .91$) was observed between ADC of protein and PPV. A similar observation was made in other fish species fed plant protein-based diets (Bonaldo et al., 2011; Fawole, Sahu, Jain, Gupta, Shamna, Phulia, et al., 2016; Saha & Ghosh, 2013).

The higher ADC of phosphorus and low phytic acid level recorded in this study suggests the bioavailability of an adequate amount of phosphorus for the various metabolic uses of the fish. This may be due to the release of phosphorus from the phytate-bound phosphorus. Furthermore, the insignificant variation observed in the ADC of lipid among the various groups agrees well with the results of Kumar et al. (2012) and Fawole, Sahu, Jain, Gupta, Shamna, Phulia, et al. (2016) in *L. rohita* and common carp fed detoxified *Jatropha* and rubber protein isolates, respectively.

The presence of ANFs in plant-based protein could limit the activity of digestive enzymes, thereby hindering digestion processes and impaired intestinal absorption of nutrients for muscle growth. Trypsin inhibitor, tannin and phytic acid present in raw de-oiled *Jatropha* seed meal and *Jatropha* protein concentrate have been reported to elicit an adverse effect on the protease, amylase and lipase enzymes activities in *L. rohita* fingerlings (Saha & Ghosh, 2013; Shamna et al., 2015). However, in the present study, no variation was observed in the activities of protease and amylase enzymes, and this could be ascribed to the low level of the ANFs present in the treatment diets which if above the tolerance limit could inhibit the activity of protein-digesting enzyme and hinder nutrient/mineral utilization. Alkaline phosphatase (ALP) is one of the most abundant intestinal brush border membrane-bound enzymes and is involved in the transport of nutrient in fish (Debnath et al., 2007; Molina et al., 2005). In the current study, the higher intestinal ALP activity recorded in DJP 100 and DJP 100 + lysine fed groups signifies better phosphorus metabolism and nutrient absorption, hence resulting in an improved phosphorus utilization and growth. Furthermore, any disturbances in the membrane due to toxins or toxicants can lead to an alteration in the activities of ALP and consequently drainage of the enzyme from the tissues into the bloodstream, thus lowering the tissue level activity (Safahieh, Hedayati, Savari, & Movahedinia, 2010). Based on the results obtained in this study, the non-significant ALP activity in

the serum indicates no leakage of the enzyme into the bloodstream and the level of PEs and other ANFs present in the DJP-based diet were not sufficient to cause damaging effects on the intestinal membrane stability nor decrease the nutrient absorptive capacity of the enterocytes.

In general, ALT and AST enzymes catabolize amino acids and transfer amino group to alpha-keto acids, thus forming new amino acids with the amino group from the pre-existing ones. Through this process, the non-essential amino acids required for growth and other metabolic activities are produced. In the present study, the hepatic ALT and AST enzyme activities in the treatment groups were found to be similar to the control group, suggesting that the dietary protein fed was sufficient to meet the fish requirement and most of the amino acids synthesized were utilized for muscle growth. This is in congruent with the result of Fawole, Sahu, Jain, Gupta, Shamna, Phulia, et al. (2016) in *L. rohita* fed rubber protein isolate. Conversely, Shamna et al. (2015) observed a significant decrease in the hepatic ALT and AST enzyme activities of *L. rohita* fingerlings fed *Jatropha* protein concentrate. The reason was attributed to the toxic effects of PEs (1.4 mg/g) on the liver cells, thus resulting in the leakage of the enzyme into the bloodstream. However, such effects were not observed in the present study and the serum level activity of AST enzyme showed no significant variation. Although DJP 100 group recorded higher serum ALT activities, the ALP, AST and total bilirubin values among the various groups showed no differences compared to the control; hence, it could be said that feeding DJP to rohu would not cause any alteration or impairment in the liver functions.

Increased area of absorption of the intestine and hence the absorption of nutrients could influence feed utilization efficiency and growth in fish (Bello, Emikpe, & Olaifa, 2012). According to Moldal et al. (2014), shortening of the intestinal villi length may reduce the total surface area of the intestine, thereby influencing the growth of the fish. Although in the current study, the groups fed DJP 25, DJP 50 and DJP 75 showed shorter villi width compared to other groups, but no significant variation was observed in the villi length, and this may be responsible for the similar growth and feed conversion efficiency noticed among the various groups. Additionally, the histo-architecture of the intestine showed no discernible changes in DJP-fed groups compared with the control. A similar observation was reported in *L. rohita* fingerlings fed fermented *Jatropha curcas* kernel meal as a substitute for soybean meal (Phulia et al., 2016).

5 | CONCLUSION

The results of this study revealed that protein extraction strategy could enhance the protein content of *Jatropha curcas* seed meal with a concomitant reduction in the ANFs level and indigestible material, but further detoxification is required to eliminate or reduce the PEs to a safe level in order to enhance the dietary nutritional value of the isolated protein. Furthermore, the results of the feeding trial revealed that DJP-fed fish exhibited comparable growth performance and nutrient utilization to SPI-fed control without any negative effects on the digestive, brush border and aminotransferase enzymes activities, and organ integrity of the fish. Thus, this study suggests the utilization

of detoxified *Jatropha curcas* protein isolate as an alternative protein source for animal feeding to replace heavily-dependent soybean meal in the diet of *L. rohita* fingerlings.

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ORCID

F.J. Fawole  <http://orcid.org/0000-0003-4645-6962>

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