EVALUATION OF CASTOR SEED PROTEIN ISOLATE IN THE LEAF MEAL BASED DIET OF *LABEO ROHITA* (HAMILTON, 1822)

Thesis submitted in partial fulfillment
of the requirements
for the degree of

Ph.D. (Fish Nutrition and Feed Technology)

by

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DEDICATED TO MY PARENTS…

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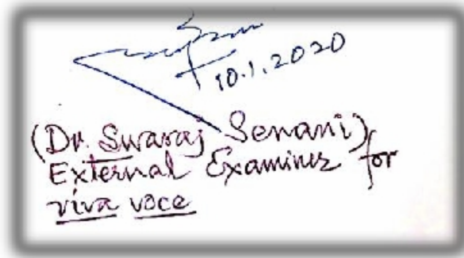
CERTIFICATE

Certified that the thesis entitled **"EVALUATION OF CASTOR SEED PROTEIN ISOLATE IN THE LEAF MEAL BASED DIET OF *LABEO ROHITA* (HAMILTON, 1822)"** is a record of independent bonafide research work carried out by **Mr. Manish Jayant** during the period of study from September 2013 to August, 2019 under our supervision and guidance for the degree of **Doctor of Philosophy (Fish Nutrition and Feed Technology)** and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title.



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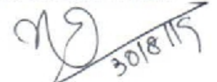

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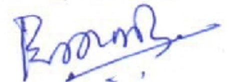

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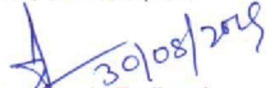

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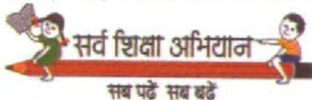

DECLARATION

I hereby declare that the dissertation entitled "**EVALUATION OF CASTOR SEED PROTEIN ISOLATE IN THE LEAF MEAL BASED DIET OF *LABEO ROHITA* (HAMILTON, 1822)**" is an authentic record of the work done by me and that no part thereof has been presented for the award of any degree, diploma, associateship, fellowship or any other similar title.

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Mumbai ***(Manish Jayant)***

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सारांश

लेबियो रोहिता (रोहू) कि अंगुलिकाऔ पर पत्ती आधारित आहार में अरंडी बीज से आइसोलेटेड प्रोटीन (सीपीआई) के पोषण का मूल्यांकन करने के लिए चार प्रयोग किए गए। पहले प्रयोग में, प्रोटीन आइसोलेट की विधी को मानकीकृत किया गया, प्राप्त परिणामों से पता चला कि प्रोटीन आइसोलेट में प्रोटीन की मात्रा के साथ प्रोटीन की प्राप्ति क्रमशः 12 और 5 के पीएच संयोजन से सर्वाधिक हुआ। तैयार आइसोलेट का पोषण मूल्य वसा रहित अरंडी बीज के खल्ली से बेहतर है और मानक प्रोटीन (अंडे का सफेद भाग) और सोयाबीन प्रोटीन आइसोलेट (एसपीआई) के तुलनीय है। सीपीआई में लाइसिन अधिक कमी वाला एमिनो एसिड था। दूसरे प्रयोग में, पांच आइसो–नाइट्रोजनस (34.31±0.04 % क्रूड प्रोटीन) और आइसो–कैलोरिक (421.76± 0.24 Kcal 100 g^{-1}) आहार रोहू कि अंगुलिकाऔ को प्रतिदिन दो बार 60 दिनों के लिए खिलाया गया। एसपीआई नियंत्रण आहार में प्रोटीन मुख्य स्रोत था, जिसे सीपीआई द्वारा प्रोटीन के आधार पर क्रमशः 25%, 50%, 75% और 100% द्वारा क्रमिक रूप से प्रतिस्थापित किया गया। परिणामों से पता चला कि 75% समूह में सर्वश्रेष्ठ वृध्दि, आहार रूपांतरण और पोषक तत्वों का उपयोग देखा गया। एमिनोट्रांस्फरेज (एलटीएच को छोड़कर), एलडीएच (मांसपेशियों को छोड़कर) की गतिविधियां काफी प्रभावित हुईं, जबकि एमडीएच और तनाव एंजाइम सीपीआई के आहार समावेश स्तर के साथ भिन्न नहीं थे। सीपीआई के बढ़ते स्तर के साथ आंत के म्यूकोसल फोल्ड़ और सेलुलर अतःसरण के संलयन में वृद्धि हुई। तीसरे प्रयोग में, अमीनो एसिड को कम मात्रा (एल–लाइसिन और डीएल–मेथियोनीन) को देखते हुए रोहू की पोषण संबंधी आवश्यकता को पूरा करने के लिए आहार में पूरक किया गया। प्राप्त परिणाम सें पता चला है कि 75% समूहों में सर्वश्रेष्ठ वृध्दि और पोषक तत्वों का उपयोग हुआ, लेकिन 100% में वृद्धि नियंत्रण समूह के समान पायी गयी। इसी तरह, पाचन एंजाइम, चयापचय एंजाइम और तनाव एंजाइम 100% और नियंत्रण समूह के बीच भिन्न नहीं थे। हिस्टोलॉजिकल जॉच से पता चला कि एल–लाइसिन और डीएल–मेथियोनीन के पुरक आहार वर्ग में कोशिकीय श्लेष्मा फोल्ड के कम संलयन और प्रयोग दो, की तुलना में कम इंट्रासेल्युलर अतःसरण हुआ है। चौथे प्रयोग में, *चेटोमियम गोलोबोसुम* के साथ शकरकंद की पत्ती (एसपीएलएम) के ठोस अवस्था किण्वन कर के क्रूड फाइबर एवॅ अकारक पोषक तत्वों को कम किया गया। परिणामों से पता चला कि आहार सीपीआई और किण्वित एसपीएलएम (एफएसपीएलएम) मछलियों में वृध्दि, पोषक तत्वों के उपयोग, अस्तित्व, पाचन एंजाइम और चयापचय एंजाइम गतिविधियों, हेमाटो–इम्यूनोलॉजिकल और हेमेटोलॉजिकल मापदंडों को प्रभावित नहीं करता है। चौथे प्रयोग के परिणामों के आधार पर, यह अनुमान लगाया गया की है कि CPI और FSPLM रोहू की अंगलिकाऔ के आहार में वैकल्पिक प्रोटीन स्रोत के रूप में काम मे लाया जा सकता है। इस प्रयोग से प्राप्त परिणाम कृषि अवशेष या गैर–खाद्य तेल बीजों पाप्त खल्ली या इसके उत्पादों सें प्राप्त प्रोटीन का उपयोग मछलियों के भोजन के रूप में करने की उपयोगिता भी दर्शाता है।

ABSTRACT

Four experiments were conducted in sequence to evaluate the nutritional value of castor seed protein isolate (CPI) in leaf meal based diet of *Labeo rohita* fingerlings. In first experiment, the process for the preparation of protein isolate was standardized and results revealed that best dry matter and protein recovery along with protein content in the protein isolate was achieved at pH combination of 12 and 5 for solubilization and precipitation of protein, respectively from defatted castor kernel meal. The nutritional value (in terms of nutritional indices and anti-nutritional factors) of CPI is better than the defatted kernel meal and comparable to standard protein (egg white) and soybean protein isolates (SPI). Lysine was the most deficient amino acid in the CPI. In second experiment, five iso-nitrogenous (34.31±0.04 % crude protein) and iso-caloric (421.76± 0.24 Kcal GE 100 g^{-1}) diets were fed to *L. rohita* fingerlings for 60 days twice daily. SPI was the main protein source in the control diet which was successively substituted by 25%, 50%, 75% and 100% by CPI on protein equivalent basis and designated as CPI_{25}, CPI_{50}, CPI_{75} and CPI_{100}, respectively. Results revealed that best growth performance, feed conversion and nutrient utilization were observed in CPI_{75} group. Activities of aminotransferase (except ALT in muscle), LDH (except in muscle) were significantly affected. Histological study of liver did not exhibit any significant variation (P>0.05) while fusion of mucosal fold and cellular infiltration of lamina propria in the intestine were increased with the increasing level of dietary CPI. In third experiment, deficient amino acids (L-lysine and DL-methionine) were supplemented in the diets to fulfill the nutritional requirement of rohu and five iso-nitrogenous (35.45±0.14 % crude protein) and iso-caloric (421.82±0.16 Kcal GE 100g^{-1}) experimental diets were prepared and fed for 60 days twice daily. SPI was the main protein source in the control diet which was successively replaced with 25%, 50%, 75% and 100% CPI on protein equivalent basis and designated as $ACPI_{25}$, $ACPI_{50}$, $ACPI_{75}$ and $ACPI_{100}$, respectively. Best growth performance and nutrient utilization were reported in $ACPI_{50}$ and $ACPI_{75}$ groups but growth in $ACPI_{100}$ was similar to the control group. Similarly, digestive enzyme, metabolic enzymes and stress enzymes did not vary between $ACPI_{100}$ and control. Histological examination revealed no significant variation in liver tissue and dietary supplementation of L-lysine and DL-methionine improved the cellular integrity along with less fusion of mucosal folds and less intracellular infiltration of lamina propria in intestine compared to experiment II. In experiment IV, solid state fermentation of sweet potato leafmeal (SPLM) with *Chaetomium golobosum* improved the nutritional values in terms of increase in crude protein with reduction of crude fibre content concomitant with reduced anti-nutritional factor contents. Four iso-nitrogenous (35.13±0.09 % crude protein) and iso-caloric (420.95±0.46 Kcal GE 100g^{-1}) experimental diets were prepared by supplementing with deficient L-lysine and DL-methionine and fed to rohu fingerlings for 60 days twice daily. The results revealed that dietary CPI and fermented SPLM (FSPLM) did not affect the growth performance, nutrient utilization, survival, digestive enzyme & metabolic enzyme activities, hemato-immunological and hematological parameters. Based on the results of experiment IV, it can be inferred that CPI may serve as an alternate protein source along with FSPLM in the diet of *Labeo rohita* fingerlings. Further, it is suggested that protein isolates produced from by-product of agro-industrial wastes and/or nonedible oil seed cakes or meals may be studied as an alternate protein source in animal/fish feed.

CONTENTS

SL.NO.	PARTICULARS	PAGE NO.
1	**INTRODUCTION**	1-5
2	**REVIEW OF LITERATURE**	6-26
2.1	Aquaculture Growth	6
2.2	Production of *Labeo rohita* (rohu)	6
2.3	Utilization of non-conventional feed ingredients in fish feed	7
2.4	Utilization of castor seed cake or meal as protein source in fish/animal feed	9
2.4.1	Castor bean or castor seed	9
2.4.2	Castor seed cake	10
2.4.3	Utilization of castor seed cake or meal in the animal/fish feed	13
2.4.4	Antinutritional factors	15
2.4.4.1	Ricin	15
2.4.4.2	Ricinine	17
2.4.4.3	Allergen or Castor bean allergen-1	18
2.4.5	Effect of processing on ricin reduction in castor seed meal	18
2.5	Protein isolates/concentrate of oilseeds as protein source in fish or animal feed	19
2.6	Leaf Meal as an Aquafeed Ingredient	22
2.7	Sweet Potato Leaf Meal (SPLM)	24
2.8	Strategies to Improve the Utilization of Leaf Meal in Fish Diet	25

3	**MATERIALS AND METHODS**	27-66
3.1	Site of the Experiment	27
EXPERIMENT I		
3.2	Standardization of the Process for Preparation of Protein Isolate and its Quality Evaluation	28
3.2.1	Preparation of defatted castor kernel meal	28
3.2.2	Preparation of protein isolate from defatted castor kernel meal	28
3.2.3	Amino acid analysis	34
3.2.4	Amino acid score or chemical score	34
3.2.5	Essential amino acid index (EAAI) and biological value (BV)	34
3.2.6	Nutritional index (NI)	34
3.2.7	Predicted protein efficiency ratio (P-PER)	34
3.2.8	*In-vitro* protein digestibility	35
3.3	Estimation of Anti-nutritional Factors	35
3.3.1	Total tannin	35
3.3.2	Phytic acid	35
3.3.3	Trypsin inhibitor	36
3.3.4	Oxalates	36
3.3.5	Alkaloids	36
3.3.6	Hydrogen cyanide (HCN)	37
3.4	Statistical Analysis	37
3.5	Feeding Experiment	38
3.5.1	Experimental animals	38
3.5.2	Experimental units	38
3.6	Chemicals and glassware	40
3.7	Physico- chemical Parameters of Water	40

3.7.1	Temperature	40
3.7.2	pH	40
3.7.3	Dissolved oxygen	40
3.7.4	Free carbon dioxide	40
3.7.5	Carbonate hardness	41
3.7.6	Ammonia	41
3.7.7	Nitrite-N	41
3.7.8	Nitrate-N	41

Experiment- II Feeding Trial to Evaluate the Nutritional Value of Castor Seed Protein Isolate in the Diet of *Labeo rohita* Fingerlings

3.8	Feed Preparation	42
3.9	Experimental Design and Feeding	44
3.1	Digestibility Studies	44
3.10.1	Faecal matter collection	44
3.10.2	Determination of chromium oxide	45
3.10.3	Apparent digestibility coefficient (ADC)	46

Experiment- III Nutritional Evaluation of Castor Seed Protein Isolate Supplemented with Amino Acids (L-Lysine and DL-Methionine) in the Diet of *L. rohita* Fingerlings

3.11	Feed Preparation	47
3.12	Experimental Design and Feeding	47

Experiment- IV Feeding Trial to Evaluate the Nutritional Value of Protein Isolate Prepared from Defatted Castor Kernel Meal in the Leafmeal Based Diet of *L. rohita* Fingerlings

3.13	Preparation of Sweet Potato Leaf Meal	50
3.14	Solid State Fermentation of Sweet Potato Leaf Meal	50
3.15	Feed Preparation	51

3.16 Experimental Design and Feeding 54
3.17 Growth and Nutrient Utilization 54
3.17.1 Weight gain (%) 54
3.17.2 Specific growth rate (SGR) 54
3.17.3 Feed conversion ratio 54
3.17.4 Feed efficiency ratio 55
3.17.5 Protein efficiency ratio 55
3.17.6 Apparent net protein utilization 55
3.17.7 Hepato-somatic index 55
3.17.8 Viscero-somatic Index 55
3.17.9 Survival 55
3.18 Proximate Analysis 55
3.18.1 Moisture 55
3.18.2 Crude Protein (CP) 56
3.18.3 Ether Extract (EE) 56
3.18.4 Total ash 56
3.18.5 Crude fibre (CF) 57
3.18.6 Nitrogen free extract 57
3.18.7 Gross energy (GE) 57
3.19 Sampling and Tissue Homogenate Preparation 57
3.2 Tissue Protein Estimation 58
Enzyme Assays
3.21 Digestive Enzymes 58
3.21.1 Protease 58
3.21.2 Amylase 59
3.21.3 Lipase 59
3.22 Metabolic Enzymes 60

3.22.1	Aspartate aminotransferase (AST)	60
3.22.2	Alanine aminotransferase (ALT)	61
3.22.3	Lactate dehydrogenase (LDH)	61
3.22.3	Malate dehydrogenase (MDH)	61
3.23	Enzymes of Oxidative Stress	62
3.23.1	Superoxide dismutase (SOD)	62
3.23.2	Catalase (CAT)	62
3.24	Biochemical and Haemato-Immunological Parameters	62
3.24.1	Collection of blood and serum	62
3.24.2	Biochemical parameters	63
3.24.2.1	Serum protein	63
3.24.2.2	Albumin	63
3.24.2.3	Globulin	64
3.24.2.4	Albumin globulin ratio (A:G ratio)	64
3.24.3	Biochemical Test	64
3.24.4	Immuno-hematological parameters	65
3.24.4.1	Serum glucose	65
3.24.4.2	Nitro blue tetrazolium (NBT) assay	65
3.24.5	Haematological parameters	65
3.25	Histopathology	66
3.26	Statistical Analysis	66
4	**RESULTS**	67-142
Experiment- I		
4.1	Protein Isolate, Dry Matter and Protein Recoveries	67
4.2	Chemical Composition (Dry Weight Basis) and anti-nutritional factors of Defatted Castor Kernel Meal (DCKM) and Castor Seed Protein Isolate	69

	(CPI)	
4.3	Amino Acid Composition of Defatted Castor Kernel Meal and Castor Seed Protein Isolates (CPI)	70
4.4	Nutritional indices of Defatted Castor Kernel Meal and Protein Isolates	72
EXPERIMENT- II		
4.5	Physico-Chemical Parameters of Water	74
4.6	Proximate Composition of the Experimental Diets	74
4.7	Growth Performance and Nutrient Utilization	77
4.8	Body Indices	82
4.9	Survival (%)	82
4.1	Whole Body Composition	83
4.11	Apparent Digestibility Coefficients (ADCs)	85
4.12	Digestive Enzymes Activities	85
4.13	Metabolic Enzymes	86
4.13.1	Aspartate aminotransferase (AST)activity	86
4.13.2	Alanine aminotransferase (ALT) Activity	87
4.13.3	Lactate dehydrogenase (LDH)	87
4.13.4	Malate dehydrogenase (MDH)	87
4.14	Antioxidant Enzymes Activities	89
4.14.1	Superoxide dismutase (SOD) and Catalase (CAT)	89
4.15	Biochemical and Haemato-Immunological Parameters	90
4.15.1	Serum protein biochemistry	90
4.15.2	Hemato-immunological parameters	90
4.15.3	Nitrobluetetrazolium (NBT) assay	90
4.16	Haematological Parameters	90
4.17	Histopathology	93

4.17.1	Liver	93
4.17.2	Intestine	93
Experiment- III		
4.18	Physico-Chemical Parameters of Water	99
4.19	Proximate Composition of the Experimental Diets	101
4.2	Growth Performance and Nutrient Utilization:	102
4.20.1	Body weight (g)	102
4.20.2	Weight gain (%)	102
4.20.3	Specific growth rate (SGR)	102
4.20.4	Feed conversion ratio (FCR)	102
4.20.5	Feed efficiency ratio (FER	103
4.20.6	Protein efficiency ratio (PER)	103
4.20.7	Apparent net protein utilization (ANPU)	103
4.21	Body Indices and Survival	107
4.21.1	Hepatosomatic Index (HSI)	107
4.21.2	Viscero-somatic Index (VSI)	107
4.21.3	Survival	107
4.22	Whole Body Composition	108
4.23	Digestive Enzymes Activities	110
4.24	Metabolic Enzymes Activities	110
4.24.1	Aspartate aminotransferase (AST) activity	110
4.24.2	Alanine aminotransferase (ALT) Activity	111
4.24.3	Lactate dehydrogenase (LDH)	111
4.24.4	Malate dehydrogenase (MDH)	111
4.25	Antioxidant enzymes Activities	113
4.25.1	Superoxide dismutase (SOD) and Catalase (CAT)	113
4.26	Biochemical and Haemato-Immunological Parameters	113

4.26.1	Serum protein biochemistry	113
4.26.2	Hemato-immunological parameters	114
4.26.3	Nitrobluetetrazolium (NBT) assay	114
4.27	Haematological Parameters	114
4.28	Histopathology	117
4.28.1	Liver	117
4.28.2	Intestine	117
Experiment- IV		
4.29	Proximate Composition of Sweet Potato Leafmeal (SPLM) and Fermented Sweet Potato Leafmeal (FSPLM)	123
4.3	Anti-nutritional factors of Sweet Potato Leafmeal (SPLM) and Fermented Sweet Potato Leafmeal (FSPLM)	123
4.31	Physico-Chemical Parameters of Water	126
4.32	Proximate Composition of the Experimental Diets	126
4.33	Growth Performance and Nutrient Utilization:	129
4.33.1	Body weight	129
4.33.2	Weight gain (%) and specific growth rate (SGR)	129
4.33.3	Feed conversion ratio (FCR) and feed efficiency ratio (FER)	129
4.33.4	Protein efficiency ratio (PER)and apparent net protein utilization (ANPU)	129
4.34	Body Indices and Survival	130
4.34.1	Hepatosomatic Index (HSI) and Viscero-somatic Index (VSI)	130
4.34.2	Survival	130
4.35	Whole Body Composition	134

4.36	Digestive Enzymes Activities	136
4.37	Metabolic Enzymes Activities	136
4.37.1	Aspartate aminotransferase (AST)activity	136
4.37.2	Alanine aminotransferase (ALT) Activity	136
4.37.3	Lactate dehydrogenase (LDH)	137
4.37.4	Malate dehydrogenase (MDH)	137
4.38	Antioxidant Enzymes Activities	137
4.38.1	Superoxide dismutase (SOD) and catalase (CAT)	137
4.39	Biochemical and Haemato-Immunological Parameters	139
4.39.1	Serum protein biochemistry	139
4.39.2	Hemato-immunological parameters	139
4.39.3	Nitrobluetetrazolium (NBT) assay	141
4.40	Haematological Parameters	141
5	**DISCUSSION**	143-178
6	**SUMMARY**	179-185
7	**REFERENCES**	186-215
	APPENDICES	

LIST OF TABLES

Table No.	Name of Table	Page No.
1	Proximate composition of castor seed cake (% dry weight basis)	11
2	Amino acid profile of un-decorticated and decorticated castor seed cake compared with soybeans as standard plant protein.	12
3	Amino acid profile of castor protein isolate, boiled castor protein isolate and lime treated castor protein isolate (g kg^{-1} protein)	22
4	Composition of the experimental diets (g $100g^{-1}$ dry matter basis) fed to *Labeo rohita* fingerlings during the experimental period	43
5	Amino acid content of experimental diets (g amino acids 100 g^{-1} feed)	48
6	Composition of the experimental diets (g $100g^{-1}$ dry matter basis) fed to *L. rohita* fingerlings during the experimental period	49
7	Amino acid content of experimental diets (g 100 g^{-1} feed)	52
8	Composition of the experimental diets (g $100g^{-1}$ dry matter basis) fed to *L. rohita* fingerlings during the experimental period	53
9	Protein contents, dry matter (DM) and protein recoveries (%) of protein isolates prepared from defatted castor seed meal under different conditions	68
10	Chemical composition (%) of defatted castor kernel meal (DCKM) and castor seed protein isolate (CPI) on dry matter basis	69
11	Anti-nutritional factors in defatted castor kernel meal (DCKM) and castor seed protein isolate (CPI)	70
12	Amino acid content (g/100 g) of defatted castor kernel meal (DCKM) and castor seed protein isolate (CPI)	71
13	Nutritional indices of defatted castor kernel meals and	72

	protein isolates	
14	Physico-chemical parameters of water during the experimental period of 60 days for different experimental groups.	75
15	Proximate composition of the experimental diets (% DM basis)	76
16	Body weight (g) of *L. rohita* fingerlings fed with different experimental diets (15 days interval)	79
17	Growth performance and nutrient utilization parameters of *L. rohita* fingerlings fed with different experimental diets	81
18	Body indices of *L. rohita* fingerlings fed with different experimental diets	83
19	Whole body composition of *L. rohita* fingerlings fed with different experimental diets (% wet weight basis)	84
20	Apparent digestibility coefficients of *L. rohita* fingerlings fed with different experimental diets	85
21	Digestive enzymes activities of *L. rohita* fingerlings fed with different experimental diets	86
22	Metabolic enzymes, aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH) and Malate dehydrogenase (MDH) enzyme activities of *L. rohita* fingerlings fed with different experimental diets	88
23	Superoxide dismutase (SOD) and catalase (CAT) enzyme activities of *L. rohita* fingerlings fed with different experimental diets	89
24	Effects of different experimental diets on the serum protein biochemistry of *L. rohita* fingerlings	91
25	Effect of different diets on serum glucose, alanine transaminase, aspartate transaminase, and NBT in *L. rohita* fingerlings	91
26	Hematological parameters of *L. rohita* fingerlings fed with	92

	different experimental diets	
27	Physico-chemical parameters of water during the experimental period of 60 days for different experimental groups	100
28	Proximate composition of the experimental diets (% dry weight basis)	101
29	Body weight (g) of *L. rohita* fingerlings fed with different experimental diets (15 days interval)	104
30	Growth performance and nutrient utilization parameters and survival rate of *L. rohita* fingerlings fed with different experimental diets	106
31	Body indices and survival rate of *L. rohita* fingerlings fed with different experimental diets	107
32	Whole body composition of *L. rohita* fingerlings fed with different experimental diets	109
33	Digestive enzymes activities of *L. rohita* fingerlings fed with different experimental diets	110
34	Metabolic enzymes, aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH) and Malate dehydrogenase (MDH) enzyme activities of *L. rohita* fingerlings fed with different experimental diets	112
35	Superoxide dismutase (SOD) and catalase (CAT) enzyme activities of *L. rohita* fingerlings fed with different experimental diets	113
36	Effects of different experimental diets on the serum protein biochemistry of *L. rohita* fingerlings	115
37	Effect of different diets on serum glucose, alanine transaminase, aspartate transaminase, and nitrobluetetrazolium (NBT) assay in *L. rohita* fingerlings	115
38	Haematological parameters of *L. rohita* fingerlings fed with different experimental diets	116
39	Proximate composition (Dry weight basis) of SPLM and FSPLM	124
40	Anti-nutritional factors profile of SPLM and FSPLM	124

41	Physico-chemical parameters of water during the experimental period of 60 days for different experimental groups.	127
42	Proximate composition of the experimental diets (% DM basis)	128
43	Body weight (g) of *L. rohita* fingerlings fed with different experimental diets (15 days interval)	131
44	Growth performance and nutrient utilization parameters and survival rate of *L. rohita* fingerlings fed with different experimental diets	133
45	Body indices and survival rate of *L. rohita* fingerlings fed with different experimental diets	134
46	Whole body composition of *L. rohita* fingerlings fed with different experimental diets	135
47	Digestive enzymes activities of *L. rohita* fingerlings fed with different experimental diets	136
48	Metabolic enzymes, aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH) and Malate dehydrogenase (MDH) enzyme activities of *L. rohita* fingerlings fed with different experimental diets	138
49	Superoxide dismutase (SOD) and catalase (CAT) enzyme activities of *L. rohita* fingerlings fed with different experimental diets	139
50	Effects of different experimental diets on the serum protein biochemistry of *L. rohita* fingerlings	140
51	Serum glucose, alanine transaminase, aspartate transaminase, and nitrobluetetrazolium (NBT) assay in *L. rohita* fingerlings fed with different experimental diets	140
52	Hematological parameters of *L. rohita* fingerlings fed with different experimental diets	142

LIST OF FIGURES

Figure No.	Title	Page No.
1	Flow-chart of preparation of protein isolate from defatted castor kernel meal	31
2	BSA standard curve to determine the tissue protein concentration showing a strong linear correlation between the concentration of protein and optical density ($Y = 0.0167x + 0.0766$, $R^2 = 0.9627$)	60
3	Amino acid score of defatted castor kernel meal and protein and castor seed protein isolate	73
4	Average body weight (g) of *L. rohita* fingerlings fed different experimental diets	80
5	Body weight gain (g) of *L. rohita* fingerlings fed different experimental diets	105
6	Anti-nutritional factors profile of SPLM and FSPLM	125
7	Body weight gain (g) of *L. rohita* fingerlings fed different experimental diets	132

LIST OF PLATES

Plate No.	Title	Page No.
1	Intracellular uptake of ricin and ribosome inactivation	16
2	Toxicity mechanism of ricin in target cell	17
3	Castor seed	31
4	Castor seed kerel	31
5	Defatted castor kernel meal (DCKM)	32
6	Castor seed protein isolates (CPI)	32
7	Preparation of protein isolate from defatted castor kernel meal	33
8	Experimental units	39
9	Solid State Fermentation of Sweet Potato Leaf Meal	51
10	Histological section of liver in the control group. Scale bar = 32 μm	94
11	Histological section of liver of CPI_{25} group.No discernable change was noticed. Scale bar = 32 μm	94
12	Histological section of liver of CPI_{50} group. No discernable change was noticed.Scale bar = 32μm	95
13	Histological section of liver of CPI_{75} group. No discernable change was noticed. Scale bar = 32μm	95
14	Histological section of liver in CPI_{100} fed fish showing no marked significant change. Scale bar = 32 μm	96

15	Histological section of intestine of normal fish (Control). Scale bar = 32 µm	96
16	Histological section of intestine of CPI_{25} group revealed normal histo-architecture, Scale bar = 32 µm	97
17	CPI_{50} showed normal histo-architecture. Scale bar = 32 µm	97
18	CPI_{75} showed fused mucosal folds and intracellular filtration in lamina propria in intestine. Scale bar = 32 µm	98
19	CPI_{100} showed fusion of mucosal folds and intracellular filtration in lamina propria. Scale bar = 32 µm	98
20	Histological section of liver in the control group. Scale bar = 32 µm	118
21	Histological section of liver of $ACPI_{25}$ group. No discernable change was noticed. Scale bar = 32 µm	118
22	Histological section of liver of $ACPI_{50}$ group. No discernable change was noticed. Scale bar = 32µm	119
23	Plate 13: Histological section of liver of $ACPI_{75}$ group. No discernable change was noticed. Scale bar = 32µm	119
24	Histological section of liver in $ACPI_{100}$ fed fish showing no marked significant change. Scale bar = 32 µm	120
25	Histological section of intestine of normal fish (Control). Scale bar = 32 µm	120
26	Histological section of intestine of $ACPI_{25}$ group revealed normal histo-architecture, Scale bar = 32 µm	121
27	Histological section of intestine of $ACPI_{50}$ showed normal histo-architecture. Scale bar = 32 µm	121
28	Histological section of intestine of $ACPI_{75}$ showed fused mucosal folds Scale bar = 32 µm	122
29	Histological section of intestine of $ACPI_{100}$ group revealed normal histo-architecture. Scale bar = 32 µm	122

1. INTRODUCTION

Feed represents the single largest input in aquaculture operation, accounting 40-60% of total cost of fish production. Presently, the global production of commercial aquafeed stands at about 1.07 billion tons of which 40 million tons contributed by global aquafeed during 2017-2018 (4% of total global feed production). China is the leading country and produces 15.7 million tons aquafeed, followed by Vietnam (3.9 million tons), India (2.1 million tons), Norway and Indonesia (both 1.8 million tons) (Alltech, 2019). Unlike terrestrial animal, the protein requirement of fish is higher. Hence, there is more demand of protein rich ingredients in aqua-feed. However, the price of conventional ingredients is increasing due to increasing demand from animal production sector. The cost of finished feed is determined by the cost of conventional feed ingredients. The basic feed ingredients such as soybean meal, groundnut oil cake, mustard oil cake, cotton seed oilcake, de-oiled rice bran, wheat bran, and corn flour etc. are being used in fish feed. Competition with human/ animal food producing sectors for commonly available ingredients, led to reduce the availability of feed ingredients which has accentuated the search for an alternative protein sources that is nutritionally compatible for cultured species, and cost efficient for sustainable aqua-feed development.

The rapid expansion of aquaculture and increasing cost of fishmeal has fuelled the demand for the high-quality plant protein source. Soybean meal is the most studied and commonly used alternative protein source for many aquaculture species, in the diets of freshwater herbivorous/omnivorous fish species (Yue and Zhou, 2008). This is due to its high protein and energy contents, high digestibility and relatively well balanced amino acid profile (Hertrampf and Piedad-Pascual, 2000). It’s feeding value is unsurpassed by any other plant protein source and it is the standard to which other protein sources are compared (Cromwell, 1999). De-oiled rice bran (DORB) is one of the most commonly used ingredients incorporated in aquaculture feed formulation and acts as a dietary carbohydrate/ energy source in the diet (Tsvetanov and Duneva, 1990). Rice bran is layer and germ of rice grains with hulls

or broken rice at levels that are unavoidable in milling rice grain. Due to its good nutritional profile, rice bran is one of the most common agricultural by-product used in fish feed. Rapid expansion along with intensification of aquaculture has put more pressure on demand of conventional plant ingredients, resulted in increased cost of conventional plant ingredients, therefore, making these uneconomic for aqua-feed production. Hence, there is need for its replacement with affordable non-conventional plant protein sources, which are available in large quantity, and would be viable in reducing the aqua-feed cost. Therefore, in the present scenario, non-edible oil seed cakes or kernel and leaf meals would be most preferred choice provided they are made free of any toxic and anti-nutritional factors (Agbede and Aletor, 2004; Marrufo-Estrada *et al*., 2013; Vhanalakar and Muley, 2014).

Castor seed cake, obtained from castor seed (*Ricinus communis*) after removal of oil, is a potential non-conventional feed-stuff after removal of ricin. India ranks first in castor seed production and contribute 80% of total global castor seed production. It's cultivation occupies about 8.23 lakh ha area and production is estimated about 14.33 lakh tons in India during 2017-18. In India, it is cultivated in Gujarat, Rajasthan, Andhra Pradesh and Telangana of which, Gujarat is the leading producer with 85% production of total production of India (SEA, 2018). Castor seed cake has a relatively high protein 35-48% and can be a promising alternative plant protein source ingredient for aquafeed production. However, despite the high protein content, incorporation of castor seed cake in animal feed is not encouraging due to its adverse effect on growth owing to the presence of toxic factors mainly ricin, ricinine, castor bean allergens, cyanogenic glycosides, whose metabolic product is hydrogen cyanide that imparts negatively on the physio-metabolic responses of fish. However, these anti-nutritional factors can be reduced to a tolerant level for the animal with better palatability by boiling, moist heat treatment, use of lime, sodium hydroxide, sodium chloride and soaking in water for different duration (Anandan *et al.,* 2005).

Besides this, the preparation of protein isolates from non-edible seed is an ideal approach for utilization of this seed. Production of plant protein isolate from defatted seed cake has been described as a way of reducing the contents of toxic

components (Marrufo-Estrada *et al*., 2013) with high levels of protein, which often have digestibility similar or higher than that of fish meal protein (Makkar *et al*., 2008; Nepal *et al*., 2010). Protein isolates are rich in total proteins with low amounts of lipids, soluble carbohydrate, phenols or fibers. These are good alternative protein source for fish owing to their high nutritional values, high protein and good amino acids makeup, better digestibility, low anti-nutritional components and consistent quality (Dersjant-Li, 2002). Also protein isolation produces more than three-fold increase in protein content than the raw ingredients, and are often used to fortify and formulate food products, thereby making them an important protein ingredients in human and animal feeds as well. However, since non-conventional ingredients like castor seed cakes contain less protein, large amount of indigestible materials and anti-nutrients, there is need to convert them into a better utilizable products by isolating their protein for optimum utilization in fish. According to Kumar *et al.* (2011b), fish fed with detoxified Jatropha protein isolate exhibited better growth, protein and energy digestibility, and nutrient utilization than fish fed soybean meal based diets. Consequently using plant protein isolate/concentrate as protein source will assure availability of quality protein for better growth performance of fish compared to using raw seed cake directly as protein source in fish feed (Kaushik *et al*., 1995). Technique for production of protein isolate from plants or legumes is relatively well known and well documented. Among these, most popular processes are alkaline extraction and isoelectric pH precipitation, solubilized extraction, alcohol extraction, etc. Protein isolates/concentrates from soybean, pea and canola meal are commercially available; but to the best of our knowledge little or no information is available on the protein isolate from castor seed cake.

At present, leaf meal can serve as cheapest sources of protein and energy and can substitute DORB in fish feed (Adewolu, 2008). Several attempts have been made for incorporation of leaf meal in fish feed (Osman, 2007; Vhanalakar and Muley, 2014). Among leaf meals, sweet potato (*Ipomoea batatas*) leaf can serves as an efficient protein and energy source for the preparation of fish diets. Sweet potato leaf meal comprises 23-33% crude protein with the high amino acid score (Antia *et al*., 2006; Adewolu, 2008; Abonyi *et al*., 2012) but presence of high crude fibre and

anti-nutritional factors (ANFs) limits its use in animal feed (Franklin, 1993; Antia *et al*., 2006). Antinutrients present in sweet potato leaf meal (SPLM) are phytate, trypsin inhibitor, alkaloid, oxalate, tannin and cyanide (Antia *et al.,* 2006). Several attempts have been made to study the nutritive value of sweet potato leaf meal (Adewolu, 2008; Ishida *et al*., 2000) in fish and animal feed.

Several methods such as moist heat treatments, water soaking, and fermentation etc. have been used and documented to neutralize the ANFs from leaf meal (Campbell and Bedford, 1992; Almazan, 1995; Kim *et al*., 1999; Mwachireya *et al.,* 1999). Fermentation with micro-organism could be an innovative approach to minimize the ANFs concentration as well as digestion of crude fibre in SPLM (Kim *et al*., 1999; Mahesh and Mohini, 2013; Keishing and Banu, 2013; Keishing *et al*., 2015). The use of fermented leaf meal in feed of monogastric animals has been practiced in chicken (Hirabayashi *et al*., 1998), piglets (Kiers *et al.,* 2003) and also in fish feed (Yamamoto *et al*., 2010; Yuan *et al*., 2013). Solid-state fermentation (SSF) is a process of biological detoxification of agro-industrial by-product and plant ingredients with different microorganisms. The basic aim of SSF is to increase the nutritional values of agro-industrial by-product and plant ingredients and to neutralize or minimize the ANFs concentration. SSF of agro-industrial by-product and plant ingredients also reduces the crude fibre content of these products and also increases the bio-availability of the nutrients (Onyimba *et al*., 2015; Meshram *et al*., 2018). Keeping this in view, the present study was aimed to evaluate the protein isolate prepared from defatted castor kernel meal in the leafmeal based diet of *Labeo rohita* fingerlings with the following objectives

Objectives

1. To standardize the method for castor seed protein isolate preparation and its quality evaluation
2. To evaluate the effects of feeding castor seed protein isolate on nutrient utilization and growth of *Labeo rohita*

3. To study the hemato-biochemical, histological changes and immunological responses of *L. rohita* fed with castor seed protein isolate
4. To evaluate the leaf meal based feed enriched with castor seed protein isolate in *Labeo rohita*

2. REVIEW OF LITERATURE

2.1 Aquaculture Growth

The increasing world population is projected to reach 9.7 billion by 2050 (UNDESA, 2015). Hence, the global demand for protein rich di*et al*so increases to ascertain the nutritional security. Aquaculture production continues to grow at a relatively high rate compared to other animal production sector. The average annual increase in global food-fish consumption (3.2%), outpaced the population growth (1.6 percent) and exceeded the meat consumption from all terrestrial animals (2.8 percent) during 1961 and 2016 (FAO, 2018). Global fish production has reached 170.9 million tons of which share of aquaculture and capture fisheries is 47% (80.0 million tons) and 53% (90.9 million tons), respectively. Per capita fish consumption has grown from 9.0 kg in 1961 to 20.5 kg in 2017, at an average rate of about 1.5 percent per year. As capture fishery production is relatively constant since 1980s, therefore, aquaculture has to play an important role to increase sustainable production which may be able to fulfill the increasing demand of fish for human consumption (FAO, 2018). The total fish production in India was estimated at 11.41 million metric tons, where aquaculture production contributed 5.7 million tons with a 7.1% growth rate during 2016-17 (DAHD&F, 2017; FAO, 2018).

2.2. Production of *Labeo rohita* (rohu)

The original habitat of the Indian major carps is the rivers and the backwaters of northern India. The three fourth of the total freshwater fish production is contributed by Indian major carps, which are *Catla catla* (catla), *Labeo rohita* (rohu) and *Cirrhinus mrigala* (mrigal), out of which rohu contributes around 35-40% (FAO, 2018). Among the cyprinidae, rohu (*Labeo rohita*) is of prime importance as a cultivable fish along with other major carps, catla (*Catla catla*) and mrigal (*Cirrhinus mrigala*). The global production of rohu, *L. rohita* has increased from 1.13 to 1.84

million tons during 2011 to 2016, respectively. Global production of rohu ranked at 10th position among the finfish production in 2016 and contributed 3% of global fish production (FAO, 2018). According to FAO (2012), India was the biggest contributor with a share of 44.1% of global rohu production. The growth rate of rohu, *L. rohita* is faster than mrigal but slower than catla in pond conditions and it can attain a size of 500-1000g in one year under good farming conditions. Rohu is the natural resident of the riverine system of northern and central India, and the rivers of Bangladesh, Pakistan and Myanmar. Rohu, is being a column feeder and prefer to grow well in deeper ponds (2-3 m water depth), rather than in shallower ponds.

Now-a-days, the culture of rohu, *L. rohita* has gained popularity among the Indian farmers. Several attributes like higher growth rate, taste and consumer preference have made it a species of choice for Indian aquaculture. Apart from India the species has also been established in many other South-East Asian countries, including Sri Lanka, the former USSR (Union of Soviet Socialist Republics), Japan, China, Philippines, Malaysia, Nepal and some countries of Africa (Jena, 2006). As it plays an important role in Indian aquaculture, several efforts has been made to improve its growth rate and a new strain i.e. genetically improved rohu (popularly known as “Jayanti rohu”) through selective breeding has been developed by ICAR-Central Institute of Freshwater Aquaculture, Bhubaneswar, India.

2.3 Utilization of Non-conventional Feed Ingredients in Fish Feed

Intensification of aquaculture system from traditional or extensive to semi-intensive or intensive system has put a pressure on aquafeed industry for production of cost effective feed. However, non-availability of the conventional ingredients along with high price is the major challenge for the aquafeed industry. Among the ingredients the protein rich ingredients are the most expensive. Hence, use of fishmeal has become a limiting factor in expansion of aquaculture because of huge declination in supply, which has resulted increased feed cost (Wu *et al.,* 1999). The basic feed ingredients used in fish feed are fish meal, fish oil, soybean oilcake or

meal, groundnut oilcake, mustard oilcake, deoiled ricebran, wheat bran, corn flour, and wheat flour, etc. But there is a huge competition for feed ingredients among the animal feed production sectors This has resulted in surging the price of fish feed and thereby affecting the sustainability of this enterprise (Tacon and Metian, 2008; Shamna *et al.,* 2015; Coffey *et al*., 2016; Fawole *et al*., 2016b).

Presently, soybean meal is one of the most commonly used protein source for many aquaculture species. It is owing to high protein and energy contents, high digestibility and relatively well-balanced amino acid profile (Hertrampf and Piedad-Pascual, 2000; Yue and Zhou, 2008). Among the energy rich ingredients, ricebran is most commonly used ingredient in fish feed (Tsvetanov and Duneva, 1990). Due to its good nutritional profile, rice bran is one of the most common agricultural by-products used in fish feed. It contains appreciable amount of nutrients like protein, fat and dietary fibre (Ranjan *et al.,* 2018). Hence, there is a need for its replacement with potential non-conventional plant protein sources for cost efficient and sustainable aquafeed development.

The quest for alternate protein sources has become a necessity for the sustainable aquaculture production including inland open water fisheries. Hence, the need arises to explore the good quality, cheaper and readily available alternative resources i.e. agro-industrial wastes and by-products to replace the costly ingredients in the fish feed. The global costs of producing equivalent amounts of animal protein from intensive farming is more than the production of plant protein in terms of area and water demand (2.4-33 times), and greenhouse gases emissions (2.4-240 times) (Di Paola *et al*., 2017). The main reason for such difference is due to very low conversion efficiency of animal protein production i.e. only 15% of plant protein could be converted into animal protein source (Albanese *et al*., 2018). Therefore, there is a need to use of agro-industrial wastes and by-products to feed livestock to minimize the global cost of production.

Agro-industrial wastes and by-products i.e. non-conventional plant ingredients may fill the gap of scarcity of conventional feed ingredients for feed based aquaculture. There are a number of non-conventional feed resources, which are rich

in protein, most of which are of plant origin. The utilization of plant protein in aquafeed is generally limited due to their low levels of digestible protein and the presence of anti-nutritional factors (ANFs), which interfere with nutrient bio-availability and utilization (Abowei and Ekubo, 2011; Kumar *et al.* 2011a). These anti-nutritional factors must be eliminated or inactivated prior to their use in the feed because the long-term exposure of these toxic factors at relatively low levels may have harmful effects on metabolism. Therefore, in the present scenario, non-edible oil seed cake or kernel and leaf meal would serve as one of the most favorable choices being nutritionally compatible to cultured species after removal of anti-nutritional factors (Marrufo-Estrada *et al*., 2013; Suprayudi *et al*., 2015). The different methods used for detoxification of anti-nutritional factors are moist heat treatments, water soaking, and fermentation etc. (Kim *et al*., 1999; Mwachireya *et al.,* 1999; Kumar *et al*., 2012; Shamna *et al*., 2015; Fawole *et al*., 2016a; Meshram *et al*., 2018).

2.4 Utilization of Castor Seed Cake or Meal as Protein Source in Fish/Animal Feed

2.4.1 Castor bean or castor seed

Castor (*Ricinus communis*) is a well-established oilseed crop with many industrial applications. It is mainly cultivated in tropical countries like India, Brazil and China, which are the major producers. As a crop, the main advantage of castor is its tolerance to drought stress and adaptation to several growing conditions (Lima *et al*., 2007; Babita *et al*., 2010; Lima *et al*., 2016). India ranks first in castor seed production and contribute 80% of total global castor seed production. It is cultivated in about 8.23 lakh ha and production is estimated to about 14.33 lakh tons in India during in 2017-18. It is cultivated in Gujarat, Rajasthan, Andhra Pradesh and Telangana of which, Gujarat is the leading producer with 85% share of total production of India (SEA, 2018). Castor seed contains about 45-55% oil (dry weight basis), containing more than 85% ricinoleic acid (12-hydroxy 9-octadecenoic acid) (Ogunniyi, 2006; Miller *et*

al., 2009; Wang *et al*., 2011; Naik *et al.,* 2018). Castor oil and its derivatives are used in the manufacturing of soaps, lubricants, hydraulic and brake fluids, paints, dyes, coatings, inks, cold resistant plastics, waxes and polishes, nylon, pharmaceuticals and perfumes (Mutlu and Meier, 2010). Castor oil as the major ingredient of castor seed, is soluble in both methanol and fatty methyl esters, therefore, castor bean is a promising candidate for biodiesel production (Maleki *et al.,* 2013).

Taxonomic classification:

Kingdom: Plantae

Phylum: Spermatophyta

Class: Dicotyledonae

Order: Euphorbiales

Family: Euphorbiaceae

Genus: Ricinus

Species: *Ricinus communis*

2.4.2 Castor seed cake

Castor seed cake or meal is a by-product of castor oil extraction, which contains 35 to 48% crude protein (Carrera *et al.,* 2012; Medeiros *et al.,* 2015) and has great potential for use as a protein source in animal feed. But, the presence of anti-nutritional factors such as ricin, ricinine, castor bean allergen, phytic acid, tannin, and hydrogen cyanide limits its use in animal feed (Nagalakshmi and Dhanalakshmi, 2015; Akande *et al.,* 2016).

Table 1: Proximate composition of castor seed cake (% dry weight basis)

Nutrients	Undecorticated		Decorticated	
	Full-fat castor seed meal	**Castor seed cake**	**Full-fat castor seed meal**	**Castor seed cake**
Dry matter	92.97±0.90	98.15±1.03	98.13±0.62	98.00±0.99
Crude protein	20.78±0.97	31.06±0.90	21.87±0.48	35.43±0.44
Crude fat	51.20±0.47	19.40±0.73	55.50±2.66	25.10±0.21
Crude fibre	4.98±0.20	11.90±0.57	2.50±0.05	4.43±0.05
Total ash	7.75±0.13	11.10±0.03	9.40±0.40	7.14±0.07
Nitrogen free extract	7.96	24.69	8.86	24.88
Gross energy (Kcal g^{-1})	6.5	5.14	6.53	5.6

(Source: Annongu and Joseph, 2008)

The castor cake is a good source of protein and energy but its chemical composition depends on the method of processing and oil extraction (Ogunniyi, 2006; Nagalakshmi and Dhanalakshmi, 2015). The essential amino acids such as lysine, methionine, tryptophan, valine, alanine, isoleucine and cysteine are deficient in castor meal (Harnold, 2002; Olayeni *et al.,* 2006; Santos *et al.,* 2015) while castor meal is richer in arginine (Santos *et al.,* 2015) and Leucine (Annongu and Joseph, 2008).

Though castor seed meal or cake contains a moderate nutritional profile but major issue in the utilization of castor seed meal in animal feed is the presence of anti-nutritional factors. Ricin is the major toxin present in the cake (Darby *et al.*, 2001; Olsnes, 2004; Kozlov *et al.,* 2006; El-Nikhely *et al*., 2007). Anandan *et al.* (2005) reported that ricin is the most lethal of the toxins present in castor bean residue or cake up to 1.5% (w/w, in defatted cake). In addition, the other anti-nutritional factors present in seed cake are agglutinins (Bigalke and Rummel, 2005), ricinine and allergen (Darby *et al.* 2001; Olsnes, 2004), tannins, lectin, oxalate and phytate (Akande *et al.,* 2011). The quality of castor seed cake or meal can be further

enhanced by processing technologies with an appreciable reduction in the anti-nutritional factors.

Table 2: Amino acid profile of un-decorticated and decorticated castor seed cake compared with soybeans as standard plant protein.

Amino acids (g/16gN)	Undecorticated castor seed cake	Decorticated castor seed cake	Soybeans cake
Essential amino acids			
Arginine	8	9.36	8.1
Histidine	1.38	1.88	2.7
Isoleucine	3.09	1.61	7.9
Leucine	5.9	8.56	5.1
Lysine	4.11	4.48	6.3
Methionine	2.06	2.86	1.6
Threonine	3	3.88	3.9
Tryptophan	ND	ND	1.3
Valine	5.46	4.93	5.1
Non-essential amino acids			
Alanine	3.96	5.02	4.3
Glycine	0.56	1.11	4.4
Aspartic acid	6.42	7.66	11.8
Glutamic acid	13.19	15.64	17.9
Proline	2.76	3.08	5.9
Serine	2.13	3.02	5.2
Cystine	0.53	0.86	1.6

(Source: Annongu and Joseph, 2008); ND- Not determined

2.4.3 Utilization of castor seed cake or meal in the animal/fish feed

Castor seed cake or meal has been suggested as a potential feed ingredient for livestock and aquaculture feed production by different researchers. Balogun *et al*. (2005) reported that castor seed (*Ricinus communis*) meal (CBM) can be used as feed ingredient for *Oreochrornis niloticus* (39% inclusion level) when it was boiled for 50 to 65 min at 100°C. Growth rate (weight gain percentage and SGR) and feed conversion was found to be highest in diet boiled for 65 min. Similarly, Cai *et al*. (2005) determined the effect of diet processing and replacement of fishmeal by detoxified castor bean meal (DCBM) at levels of 0, 40 and 100% on growth performance, body composition and phosphorus availability of juvenile grass carp (*Ctenopharyngodon idellus*) and observed no significant difference in grass carp juveniles fed with 50 g kg^{-1} DCBM and control diet.

Feeding of raw castor seed meal to broiler chickens is toxic and 100% mortality was observed at 5-20% inclusion level within 7 to 14 days. Different methods for detoxification of anti-nutritional factors in castor seed meal such as boiling for 30 min, boiling for 30 min with fermentation for 3 days, water soaking for 72 hr, and water soaking for 72 hr followed by boiling for 30 min and revealed that boiling cum fermentation method was the best method for detoxification of anti-nutritional factors and 10% inclusion of detoxified castor seed meal did not affect the growth and health performance of broiler chicks (Mustapha *et al.,* 2015). Santos *et al*. (2015) studied characterization and digestibility of detoxified castor oil meal using different methods of extraction and processing of castor seed meal. In first method, castor meal extracted in alcohol at 80°C for 20 min, followed by drying at 80°C. In second method, castor seed meal extracted in alcohol at 80°C for 6 min, neutralization with 5% NaOH followed by sun drying. In third method, extraction of castor seed meal in alcohol at 80°C for 6 min, and neutralization with 5% NaOH followed by pelletization and fourth method included the extraction in alcohol at 110°C for 15 min followed by drying at 110°C and thus obtained castor seed meal were designated as CMA, CMB, CMC, and CMD, respectively. The author revealed that CMD perform best based on digestibility values and lower concentration of ricin (based on cytotoxicity test) than

other methods. CMC exhibited better amino acid profile than other meals. Best growth performance and feed conversion in Japanese quails was reported in CMA but did not show any significant difference with CMC and CMD. Moreover, Akande *et al*. (2013) applied different methods for detoxification of anti-nutritional factors (boiling, calcium oxide treatment, and natural fermentation) and revealed that dietary inclusion of treated castor seed kernel meal significantly lowers the growth performance and feed conversion as compared to control in broiler chickens. It was reported that processed castor bean meal (CBM) can be included in the diet of broiler chicks at 10% level without DL-methionine supplementation and at 15% level with DL-methionine supplementation without any adverse effects on growth, nutrient utilization, carcass composition. (Note: castor seed meal cooking at 100°C for 50 min and then drying at 100°C for overnight in an oven (Ani and Okorie, 2013). Nsa *et al*. (2013) revealed that complete substitution of dietary soybean meal with boiled castor seed meal is possible in laying hens without compromising performance in egg production and egg qualities. Akande and Odunsi (2012) concluded that detoxification of castor kernel cake is possible with heating (60 min) followed by alkaline pH treatment with calcium oxide (pH 9.5) and inclusion up to 15% in the diet of broiler chick is possible without any deleterious effect in growth and feed conversion. It was reported that 5% inclusion of natural fermented castor oil seed meal in diets for cockerel chicks is possible without any deleterious effect on growth, nutrient digestibility, haematological parameters and carcass yield (Oso *et al*., 2011). Addition of β-xylanase improved the apparent nitrogen and fibre absorption as well as feed transit time. Up to 150 g kg^{-1} boiled castor seed meal could be incorporated into pullet chick diets without any adverse effect on growth performance (Babalola *et al*., 2006).

Borja *et al.* (2017) observed that the use of 10 g kg^{-1} CaO along with 30 min of autoclaving led to the complete detoxification of the castor seed meal and 20% inclusion of detoxified castor seed meal in lamb diets increased the growth performance and feed conversion efficiency without any toxicity. However, in another study, no significant effect on growth performance, nutrient utilization, hematology and biochemical indices was observed in lambs fed with 10% raw castor seed meal for 150 days. But histological examination of different tissues (liver, kidney, lungs and

intestines) revealed that the feeding raw castor seed meal has adverse effects on their vital organs (histopathological lesions) and these lesions are due to presence of anti-nutritional factors (ricin) (Nagalakshmi and Dhanalakshmi, 2015).

2.4.4 Antinutritional factors

Anti-nutritional factors present in castor seed meal or cake are ricin, ricinine, and castor bean allergens of which ricin is the most toxic (Deus-de-Oliveira *et al*., 2011; Severino *et al*., 2012; Bozza *et al*., 2015). The concentration of ricin in castor seed also depends on the variety of castor seed and size of the seed (Durowaiye, 2015).

2.4.4.1 Ricin

Ricin component of the castor seeds remain a serious impediment limiting the application of castor seed meal in animal feed (EFSA, 2008). Ricin is the most notorious deadly poison found in abundance in the seed and in smaller amounts in the remaining plant parts. Ricin is a highly toxic naturally occurring protein found in castor plant although the lethal dose in adults is considered to be four to eight seeds as reports of actual poisoning are relatively rare (Horton and Maurice, 1989). The concentration of ricin in castor seed cake varies between 1-1.5% on dry weight basis (Anandan *et al.,* 2005).

Ricin is a toxic glycoprotein (Toxalbumin– a carbohydrate binding protein) and type 2 ribosome-inactivating proteins (RIP) found in castor seed. It comprises two polypeptide chains A and B, linked by a disulfide bond. The A-chain of ricin is the toxic portion of the protein while ricin gains cellular entry through the lectin binding properties associated with the 34 kDa ricin B-chain (Rutenber and Robertus, 1991; FAO, 2012). Ricin disrupts the protein synthesis mechanism of the cell. The B-chain of ricin makes possible the entry of the A-chain into the target cell (Plate 1). The B-chain consists of a galactose-binding region capable of binding a target cell's membrane and initiating cell entry. Without the B-chain's functionality, the A-chain cannot enter the cell. However, once ricin A chain (32 kDa) enters in cell, it attacks

ribosomes and inactivate up to 1777 ribosomes per minute (Endo and Tsurugi, 1988; Sandvig *et al.*, 2010), which blocks protein synthesis and activates cell death pathways (Stirpe and Battelli, 2006; Walsh *et al.*, 2013). The cytotoxic action of the A chain is so high that a simple molecule causes the destruction of the cell ribosomes at a rate higher than it can produce new ones (Olsnes and Kozlov, 2001), leading to cell death (Plate 2). This unique action makes ricin highly toxic and LD_{50} dose of ricin for human is 5–10 µg kg^{-1} body weight (inhaled or injected) and 1–20 mg kg^{-1} body weight (ingested) (Kreuzer *et al.*, 2013); while in animals such as in pigs, 13-65 mg kg^{-1} body weight; in rabbits, 9–45 mg kg^{-1} body weight; in horses, from 1 to 5 mg kg^{-1} body weight; and in chickens, 140–170 mg kg^{-1} body weight (Garland and Bailey, 2006).

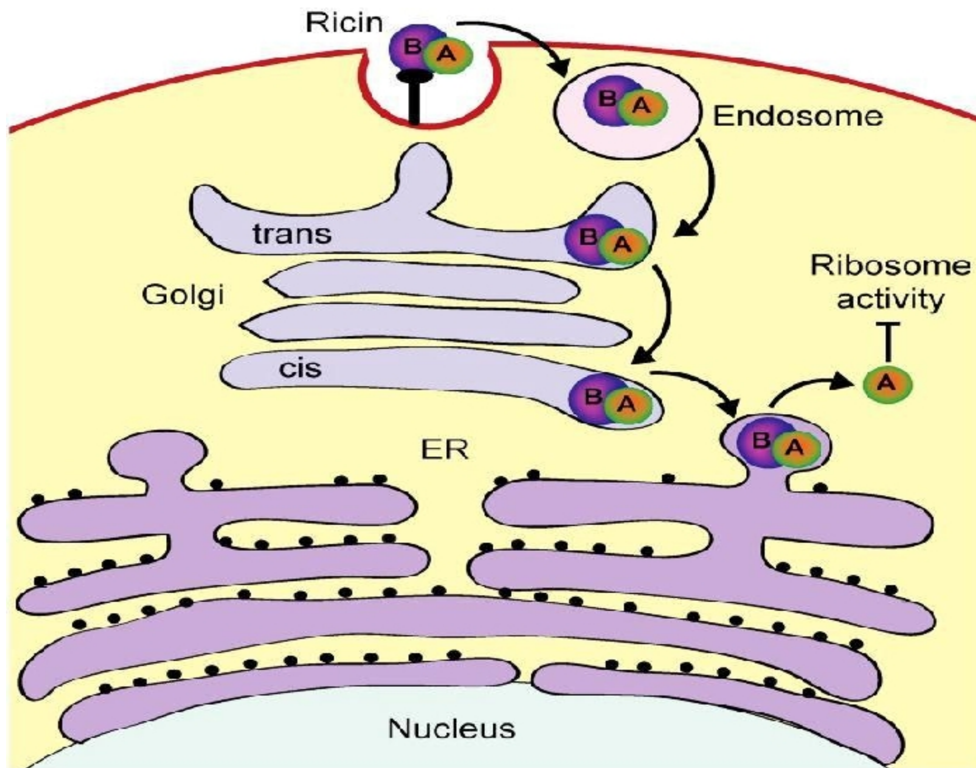

Plate 1: Intracellular uptake of ricin and ribosome inactivation (Bozza *et al.*, 2015)

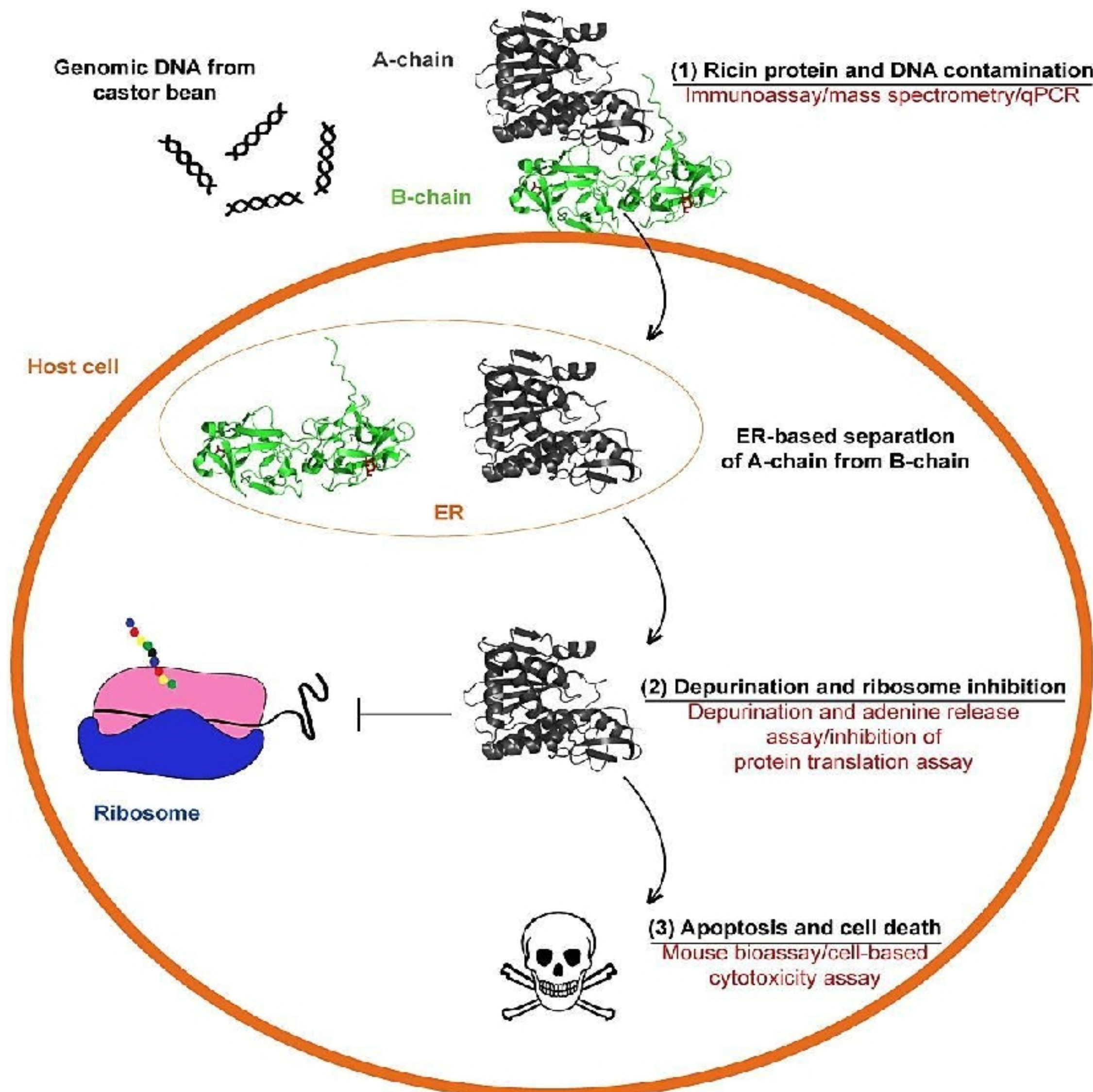

Plate 2: Toxicity mechanism of ricin in target cell (Bozza *et al.*, 2015)

2.4.4.2 Ricinine

Ricinine or ricinus communis agglutinin (RCA) is a soluble alkaloid, and one of the toxic components found in seed, leaves and stems. It is mostly concentrated in seed coat and bitter in taste (EFSA, 2008). It is only mild toxic to human being. Viola and Anekwe (2001) revealed that RCA does not penetrate the intestinal wall and not affect red blood cell if ingested but it causes hemolysis and agglutination of red blood cells if injected intravenously.

2.4.4.3 Allergen or castor bean allergen-1 (CB1A)

Coattrenec *et al*. (2017) reported that castor bean allergen-1 (CB1A) is the principal allergen of the castor bean. It is virtually non-toxic, does not cause death though may cause allergic reactions. CB1A is among one of the most heat-stable proteins found in normal heating conditions.

2.4.5 Effect of processing on ricin reduction in castor seed meal

The readily available agro-industrial by-products with high protein content and minimal competition of castor seed meal (CSM) by other industries warrants the need to research the processing techniques to improve its utilization in animal feed as a protein source. Different detoxification methods including physical, chemical (Anandan *et al*., 2005; Diniz *et al.,* 2010; Santos *et al.,* 2015) and microbial fermentation (Madeira *et al*., 2011; Akande *et al*., 2011; Akande *et al*., 2016) have been reported to reduce the ricin content of CSM at different degrees.

Autoclaving at 15 psi for 30 min and 60 min can remove 85% and 100% ricin content in CSM (Anandan *et al*., 2005) and Oliveira *et al.* (2011) observed complete removal of ricin in CSM when autoclaved at 15 psi for 90 min. Autoclaving for longer duration might affect the protein quality through possible maillard reactions but supplementation of deficient amino acids could help it’s utilization as in animal feed. Application of dry heat did not have more effect in detoxification but moist heat treatment and boiling in water were reported to be much more effective (Anandan *et al*., 2005; Alexander *et al*., 2008). Water soaking of CSM for 3hr and 6 hr can reduce the ricin concentration by 65% and 85%, respectively (Anandan *et al*., 2005). Vilhjalmsdottir and Fisher (1971) reported that water soaking of CSM is a better method than heating. These findings suggest that ricin is both water soluble, and heat labile but it is lost more through solubility.

It has been observed that efficacy of ricin reduction increases with the concentration of lime or sodium hydroxide. An amount of 60 or 90 g sodium hydroxide in 1.5 L or higher than 1.5 L water per kg of castor cake was effective for

neutralizing ricin content (de Andrade *et al*., 2019). Treatment of CSM with calcium hydroxide at (40 g kg^{-1} cake or meal) could completely denature the ricin (Anandan *et al*., 2005). Oliveira *et al*. (2011) also reported that treatment with 60 g calcium hydroxide or calcium oxide per kg of cake (cake: water, 1:10) was an effective in removing the ricin content of CSM. Solid-state fermentation (SSF) of CSM with several microorganisms has also found to be an efficient method of detoxification. SSF with *Penicillium simplicissimum* completely detoxified castor residue (Godoy *et al*., 2009). Solid-state fermentation (SSF) of the castor cake with *Aspergilus niger* for 24 hr eliminates the ricin concentration completely (Fernandes *et al*., 2012).

2.5 Protein Isolates/ Concentrate of Oilseeds as Protein Source in Fish or Animal Feed

The preparation of protein isolates from non-edible seed is an ideal approach for utilization of these seeds. Production of plant protein isolate from defatted seed cake has been described as a way of reducing the contents of anti-nutrient and toxic components (Marrufo-Estrada *et al*., 2013) with high levels of protein, which often have digestibility similar or higher than that of fishmeal protein (Makkar *et al*., 2008; Nepal *et al*., 2010). Many studies have been carried out on the nutritional value of protein isolate/concentrates in different fish species with an improved feed intake, digestibility, nutrient utilization and growth performance without any adverse effects (Puttaraj *et al.*, 1994; Devappa and Swamylingappa, 2008; Makkar *et al*., 2008, 2012; Kumar *et al*., 2011b; Nagel *et al*., 2012; Shamna *et al*., 2015, 2017; Fawole *et al*., 2016a, 2016b, 2017, 2018).

Fawole *et al*. (2018) studied the effects of detoxified *Jatropha curcas* protein isolate supplemented with lysine on growth performance, nutrient digestibility and physio-metabolic response in *L. rohita* fingerlings and reported that the growth performance, feed conversion and nutrient utilization did not show significant difference among different dietary treatment except at 100% inclusion level but it can be resolved with lysine supplementation. Fawole *et al*. (2016a) evaluated the

nutritional value of protein isolates prepared from rubber seed meal and observed that crude protein, dry matter and protein recoveries of defatted rubber meal protein isolates (RPI) were 90.8% to 98.7%, 14.3% to 17.5%, and 31.2% to 36.8%, respectively. Essential amino acids such as histidine, arginine, and phenylalanine plus tyrosine concentration were increased while lysine concentration was decreased in RPI. The predicted biological value, nutritional index, essential amino acid index and predicted protein efficiency ratio were found to be high in the isolate and comparable with other plant-based protein isolates. Based on these indices RPI could be used as a source of protein for animal feed development. Fawole *et al.* (2016b) evaluated the rubber seed protein isolate (RSPI) in the diet of *Labeo rohita* and concluded that RSPI could serve as a potential replacer for soybean protein isolate and did not had any adverse effects on growth performance, feed conversion, nutrient utilization, and physio-metabolic responses. Feeding rubber protein isolate did not cause any detrimental effect on growth, haemato-biochemical indices, innate immunity, antioxidant status and liver histology (Fawole *et al.*, 2017). The nutritional importance of fermented Jatropha protein concentrate compared to soy protein concentrate in *Labeo rohita* fingerlings. The authors concluded that jatropha protein concentrate (FJPC) detoxified by solid state fermentation with *Aspergillus niger* is a promising protein source for aquafeed and can be included up to 20% (complete replacement of soy protein concentrate) in the diets of *Labeo rohita* without compromising growth rate and feed conversion (Shamna *et al.*, 2015) and 20% inclusion level had no negative effects on heamato-immunological and physiological response (Shamna *et al.*, 2017). Similarly, Nagel *et al.* (2012) evaluated the nutritional potential of rapeseed protein isolate (RPI) as fish meal substitute in juvenile turbot (*Psetta maxima* L.) and observed that growth performance and feed conversion was maximum at 33% replacement level of RPI and it was similar to control group. Growth performance, feed intake, feed conversion were decreased at 66% and 100% replacement level. Dietary RPI inclusion did not affect the blood parameters except total protein in juvenile turbot. Author concluded that 66% of RPI could substitute fish meal in diet without affecting physiological parameters of turbot. Common carp exhibited better growth performance and nutrient utilization when fed with detoxified

Jatropha protein isolate (DJPI) as compared to soy protein isolates and fishmeal based diets. The study concluded that DJPI is a good quality protein source for carp and can replace up to 75% fish meal protein without sacrificing fish yield (Kumar *et al.*, 2011a). Makkar *et al.* (2012) reported protein contents of the protein isolates were 76.0 and 87.0% for Jatropha concentrate (JC) and Defatted Jatropha Concentrate (DJC), respectively. The dry matter yield obtained for protein concentrate was 17.2%, and the protein digestibility was approximately 90%. Makkar *et al.* (2008) revealed that the recovery of protein concentrate was highest when the proteins from the seed cakes were solubilized at pH 11 for 1 hr at 60°C and precipitated at pH 4. The crude protein in the protein concentrates obtained from the defatted oilseed cake was 76%, and 82%, respectively.

Devappa and Swamylingappa (2008) evaluated the Jatropha protein isolate prepared by steam injection heating method and reported that the yield of protein isolates was 70–77%, with a protein content of 95.5–97.0%. Anti-nutritional factors such as trypsin inhibitor, phytate, tannin and saponin were reduced by 90–97%, 90%, 85%, and 98%, respectively in protein isolates while phorbol esters and cyanogenic glucosides decreased to undetectable levels. The chemical scores for the meals and isolates were similar and methionine and cystine were the limiting amino acids in meals and isolates, respectively. The *in-vitro* digestibility and calculated nutritional indices (essential amino acid index, predicted biological value, nutritional index and computed protein efficiency ratio) of the protein isolates were higher than the respected meals. Puttaraj *et al.* (1994) evaluated the effect of dietary castor seed protein isolate (CPI) on its nutritional quality in rats. The crude proteins in castor protein isolate (CPI), boiled castor protein isolate (BCPI), and lime treated castor protein isolate (LCPI) was 91.7%, 85.7% and 81.3%, respectively. The amino acid scores suggested that lysine was the first limiting amino acid in castor flour and CPI while threonine was the first limiting amino acid in BCPI and LCPI. The chemical scores of both treated isolates were similar but nutritional indices (essential amino acid index) and PER were higher for boiled isolate than that was for lime cum heat treated isolates of castor seed. It was concluded that detoxification by boiling was better than lime cum heat treatment for CPI.

Table 3: Amino acid profile of castor protein isolate, boiled castor protein isolate and lime treated castor protein isolate (g kg^{-1} protein)

Amino acids	Castor protein isolate	Boiled castor protein isolate	Lime treated castor protein isolate
Leucine	86	83	91
Isoleucine	139	148	139
Valine	123	115	121
Tryptophan	77	84	78
Lysine	64	71	71
Threonine	75	65	68
Phenylalanine + Tyrosine	121	125	120
Methionine + Cystine	76	78	75

(Source: Puttaraj *et al*., 1994)

2.6 Leafmeal as an Aquafeed Ingredient

Presently there is a huge competition between various food and feed industries for ingredients like soybean meal and DORB (de-oiled rice bran). As a practice, DORB is most commonly used feed ingredient for carp culture. In carp culture, DORB is used either as mash feed or a combination of mash and pelleted feed (Singh *et al.,* 2004). DORB is cheapest agricultural by-product available throughout the year which is predominantly used along with oil cakes in the feed formulation and is well accepted by the carps (Veerina *et al*., 1993). The production of rice bran is insufficient to meet the requirement of animal feed industry. Thus, the quest for alternate sources has become a necessity for the sustainable aquaculture production including inland open water fisheries. There is a need to explore the good quality, cheaper and readily available alternative resources i.e. agro-industrial wastes and by-products to replace the costly ingredients in the fish feed.

Among the plant ingredients, leaf meal constitutes one of the most unexplored ingredients and is one of the cheapest sources of protein and energy that may act as a potential alternative in future to reduce the cost of fish feed. As an additional source of protein, leaf protein should be given serious attention because leaves are abundantly available in the tropics. The utilization of leaf meals may aid in enhancing farmers income with the prevailing practice of feeding. Leaf meal could be made from both terrestrial and aquatic plants. The utilization of different leaf meals in fish feed has been attempted to replace conventional feed ingredients. Maiti (2018) observed significantly higher growth performance and feed conversion in *L. rohita* fingerlings fed with 10% *Hygrophila spinosa* leaf meal as compared to other dietary treatments but the growth performance and feed conversion at 30% dietary inclusion did not vary with control group. Up to 30% dietary inclusion of *Vigna mungo* is possible in the diet of *L. rohita* fingerlings without affecting the growth performance and feed conversion (Sahoo, 2018). In a different study Ali *et al*. (2018) evaluated fermented Ipomea leaf meal has potential to replace fish meal up to 25% in *L. rohita* diets without any adverse effect on growth performance, daily protein retention and nutrient deposition. Singh *et al*. (2016) used jute (*Corchorus olitorius*) leaf powder as a dietary protein source for *L. rohita* fingerlings and concluded that jute leaf powder can be incorporated in the feed of rohu fingerlings up to 20% along with rice bran, soybean meal, mustard oil cake, vegetable oil and vitamin-mineral mixture. Diet with 12.5% replacement of groundnut oil cake (GNOC) by *Moringa oleifera* leaf meal resulted in the better growth and feed conversion in *Clarias gariepinus* fingerling (Olaniyi *et al*., 2013). Rajeswari *et al*. (2012) observed a significantly higher growth rate in *Fenneropenaeus indicus* by incorporation of *Hygrophila spinosa* leaf extract and its active immune-stimulating compounds helped to decrease the coagulation time and improved the total haemocyte count (THC), phagocytosis, phenol oxidase (PO) activity, haemagglutinin activity and bacterial clearance in white shrimp. Feeding eichhornia leaf meal fermented with fish gut bacteria exhibited better extracellular enzyme activity and it can be incorporated up to 40% level in the diet of *L. rohita* fingerlings without any adverse effect on growth of the fish, and thus aided to produce cost effective formulated fish feed (Saha *et al*., 2011). Konyeme *et al*. (2006) reported

the use of water hyacinth to replace 10 and 20% of fishmeal in the diet of *Clarias gariepinus* fingerlings. Ritcher *et al.* (2003) tested the use of Moringa leaf meal to replace 10% of fishmeal in the diet of Tilapia (*O. niloticus*) and found that growth was comparable to fishes fed control diet.

2.7 Sweet Potato Leafmeal (SPLM)

Taxonomic classification

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Eudicotyledones

Order: Solanales

Family: Convolvulaceae

Genus: Ipomea

Species: Batatas

Though many researchers (Osman, 2007; Rajeswari *et al.,* 2012; Olaniyi *et al.*, 2013; Vhanalakar and Muley, 2014; Singh *et al.*, 2016; Meshram *et al.*, 2018; Ahmad *et al.,* 2019) have worked on utilization of leaf meal in fish feed for commercial fishes and leaf meals are yet to be adequately studied and utilized in fish feed. Among leaf meals, sweet potato (*Ipomoea batatas*) leaf can serves as an efficient protein and energy source for the preparation of fish feed (Antia *et al.*, 2006; Adewolu, 2008; Meshram *et al.*, 2018; Ahmad *et al.*, 2019). Sweet potato is a traditional crop in tropical countries and is extensively grown in many countries, especially in China and Southeast Asia.

Besides being used for human consumption, the SPLM serves as fodder for cattle, sheep, goats, pigs and other domestic animals (Antia *et al.,* 2006). There are very few reports on the utilization of these leaves for fish feed. The crude

protein and crude fibre content of the foliage of sweet potato vary largely with the variety and the plant (leaves or stem). The leaf contain some unique phytochemicals as polyphenol (anthocyanins and phenolic acid) as reported by Islam *et al*. (2002a, 2002b) and Islam (2006) and these have established health benefit. Crude protein contents ranges from 23.0% to 33.0% and 10.4 to 14.1% in leaves and stems, respectively on dry matter basis (Antia *et al*., 2006; Preston, 2006; Adewolu, 2008; Abonyi *et al*., 2012; Meshram *et al*., 2018; Ahmad *et al*., 2019). But the presence of high crude fibre and anti-nutritional factors (ANFs) limits its use in animal feed (Franklin, 1993; Antia *et al*., 2006). Antinutrients present in sweet potato leaf meal (SPLM) are phytate, trypsin inhibitor, alkaloid, oxalate, tannin and cyanide (Antia *et al.,* 2006; Meshram *et al*., 2018; Ahmad *et al.,* 2019). Several attempts have been conducted to study the nutritive value of sweet potato leaf meal (Ishida *et al*., 2000; Adewolu, 2008; Meshram *et al*., 2018; Ahmad *et al*., 2019) in fish and animal feed.

2.8 Strategies to Improve the Utilization of Leaf Meal in Fish Diet

Leafy shrubs and aquatic weeds growing abundantly in nature can serve as a potential feed ingredient for the fishes. However, most of the leaves are known to contain certain amount of anti-nutritional factors like phytate, trypsin inhibitor, alkaloid, oxalate, tannin and cyanide as a defence mechanism of the plants. However, these ANFs can be eliminated or reduced to the tolerable limit of fish by following different strategies. Several methods such as moist heat treatments, water soaking, and fermentation etc. have been used and documented to neutralize the ANFs from leaf meal (Campbell and Bedford, 1992; Almazan, 1995; Kim *et al*., 1999; Mwachireya *et al.,* 1999; Meshram *et al*., 2018; Ahmad *et al*., 2019). Water soaking of plant based ingredients is known to help in reduction of anti-nutritional factors. Solid-state fermentation with micro-organism could be an innovative approach to minimize the ANFs concentration as well as digestion of crude fibre in SPLM (Kim *et al*., 1999; Mahesh and Mohini 2013; Keishing *et al*., 2015) and it has been suggested that SSF of leaf meal with several micro-organisms improve the nutritional value of sweet

potato leaf meal in fish and animal feed (Ramachandran *et al*., 2005; Yuan *et al*., 2013; Meshram *et al.,* 2018). Solid-state fermentation (SSF) is a process of biological detoxification of agro-industrial by-products and plant ingredients with different microorganisms. The basic aim of SSF is to increase the nutritional values of agro-industrial by-products and plant ingredients and to neutralize or minimize the ANFs concentration. SSF of agro-industrial by-products and plant ingredients also reduces the crude fibre content of these products and also increases the bio-availability of the nutrients (Onyimba *et al*., 2015; Meshram *et al*., 2018). The use of fermented leaf meal in feed of monogastric animals has been practiced in chicken (Hirabayashi *et al*., 1998), piglets (Kiers *et al.,* 2003) and also in fish feed (Yamamoto *et al*., 2010; Yuan *et al*., 2013; Meshram *et al*., 2018; Ahmad *et al*., 2019).

3. MATERIALS AND METHODS

Four experiments were conducted to evaluate the nutritional potential of castor seed protein isolate in the leaf meal based diet of *Labeo rohita* fingerlings. The experiments were as follow:

Experiment I: Standardization of the process for preparation of protein isolate and its quality evaluation.

Experiment II: Feeding trial to evaluate the nutritional value of castor seed protein isolate in the diet of *Labeo rohita* fingerlings.

Experiment III: Nutritional evaluation of castor seed protein isolate supplemented with amino acids (L-lysine and DL-methionine) in the diet of *L. rohita* fingerlings.

Experiment IV: Feeding trial to evaluate the nutritional value of protein isolate prepared from defatted castor kernel meal in the leafmeal based diet of *L. rohita* fingerlings.

3.1. Site of the Experiment

The experiment I and different bio-chemical analysis were carried out at Fish Nutrition, Biochemistry and Physiology division laboratory of the ICAR-Central Institute of Fisheries Education, Mumbai, India-400 061. The feeding trials of experiment II, III, and IV were conducted at Central Wet Laboratory of the ICAR-Central Institute of Fisheries Education, Mumbai, India 400061.

EXPERIMENT I

3.2 Standardization of the Process for Preparation of Protein Isolate and its Quality Evaluation

3.2.1 Preparation of defatted castor kernel meal

Castor seeds were procured from Sri Ganganagar District of Rajasthan and transported to ICAR-CIFE, Mumbai in airtight polythene bags. The seeds were dried at 60°C and decorticated using a hammer and powdered in a warring blender. The castor seeds were defatted with hexane for 12 hrs in soxhlet's apparatus, air dried and sieved through a 60-mesh sieve (250 µ). The extraction process was repeated twice to get defatted castor kernel meal (DCKM) and stored at room temperature for preparation of protein isolate.

3.2.2 Preparation of protein isolate from defatted castor kernel meal

Protein isolate from defatted castor kernel meal was prepared by alkali extraction and acid precipitation at its iso-electric point following the method of Fawole *et al.* (2016a) with little modification (Figure 1). 100 g defatted castor kernel meal was distributed in 2 L distilled water (1:20 w/v) and the pH of the mixture was adjusted to 10.0 using 1 N NaOH. The pH of the mixture was checked and adjusted to 10.0 by using 1 N NaOH for every 15 min and stirred for 2 hrs at room temperature. The slurry was centrifuged at 7000 rpm for 20 min at 25°C and supernatant was collected. The pH of the supernatant was adjusted to 4.0 using 1 N HCl, stirred for 10 min and kept in water bath at 60°C for 15-20 min to precipitate the proteins. The precipitated proteins were centrifuged at 7000 rpm for 20 min at 25°C and supernatant was discarded. The wet pellet was washed twice with distilled water to obtain protein isolate. The protein isolate was freeze-dried (ScanvacTM cool safe 100-9 pro) and the weight of the protein isolate was recorded and analysed for the protein content (Cunniff, 1995). The percentage dry matter and protein recovery were determined by using the formulae described by Makkar *et al.* (2008). The resulted protein isolate was designated as 10.0/4.0. The same process was repeated for preparation of protein isolates from defatted castor seed meal at pH 10.0/4.5, 10.0/5.0, 10.0/5.5, 10.5/4.0, 10.5/4.5, 10.5/5.0, 10.5/5.5,

11.0/4.0, 11.0/4.5, 11.0/5.0, 11.0/5.5, 11.5/4.0, 11.5/4.5, 11.5/5.0, 11.5/5.5, 12.0/4.0, 12.0/4.5, 12.0/5.0, 12.0/5.5, 12.5/4.0, 12.5/4.5, 12.5/5.0 and 12.5/5.5. The process is illustrated in Figure 1 and all the extractions were carried out in triplicate and the mean values calculated.

Dry matter recovery (%)

$$\text{Dry matter recovery(\%)} = \frac{\text{Weight of protein isolate obtained (g)}}{\text{Initial dry weight of kernel meal (g)}} \times 100$$

Protein recovery (%)

$$\text{Protein recovery(\%)} = \frac{\text{Protein in protein isolate obtained (g)}}{\text{Protein in kernel meal (g)}} \times 100$$

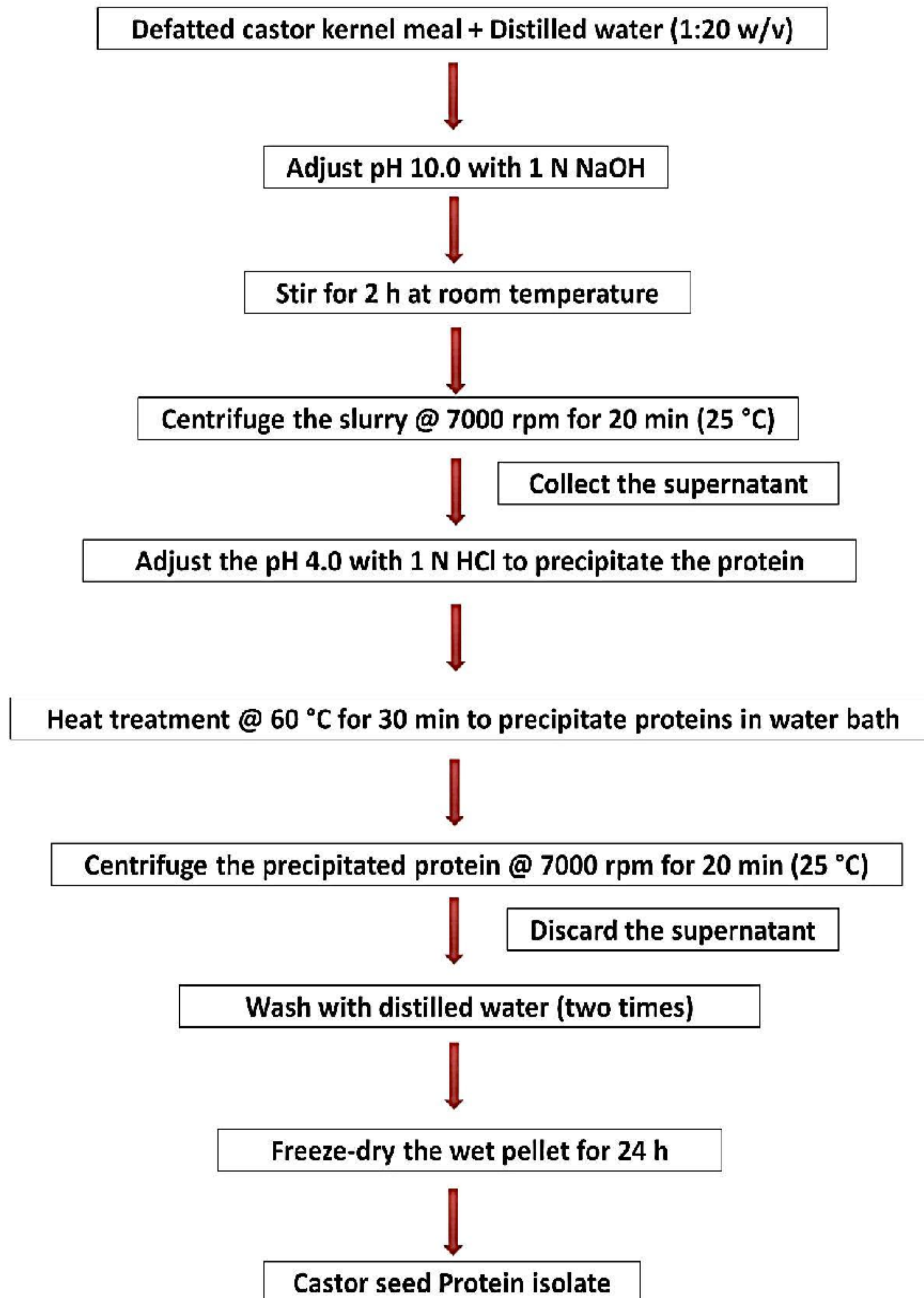

Figure 1: Flow-chart of preparation of protein isolate from defatted castor kernel meal

Plate 3: Castor seed

Plate 4: Castor seed kernel

Plate 5: Defatted castor kernel meal (DCKM)

Plate 6: Castor seed protein isolates

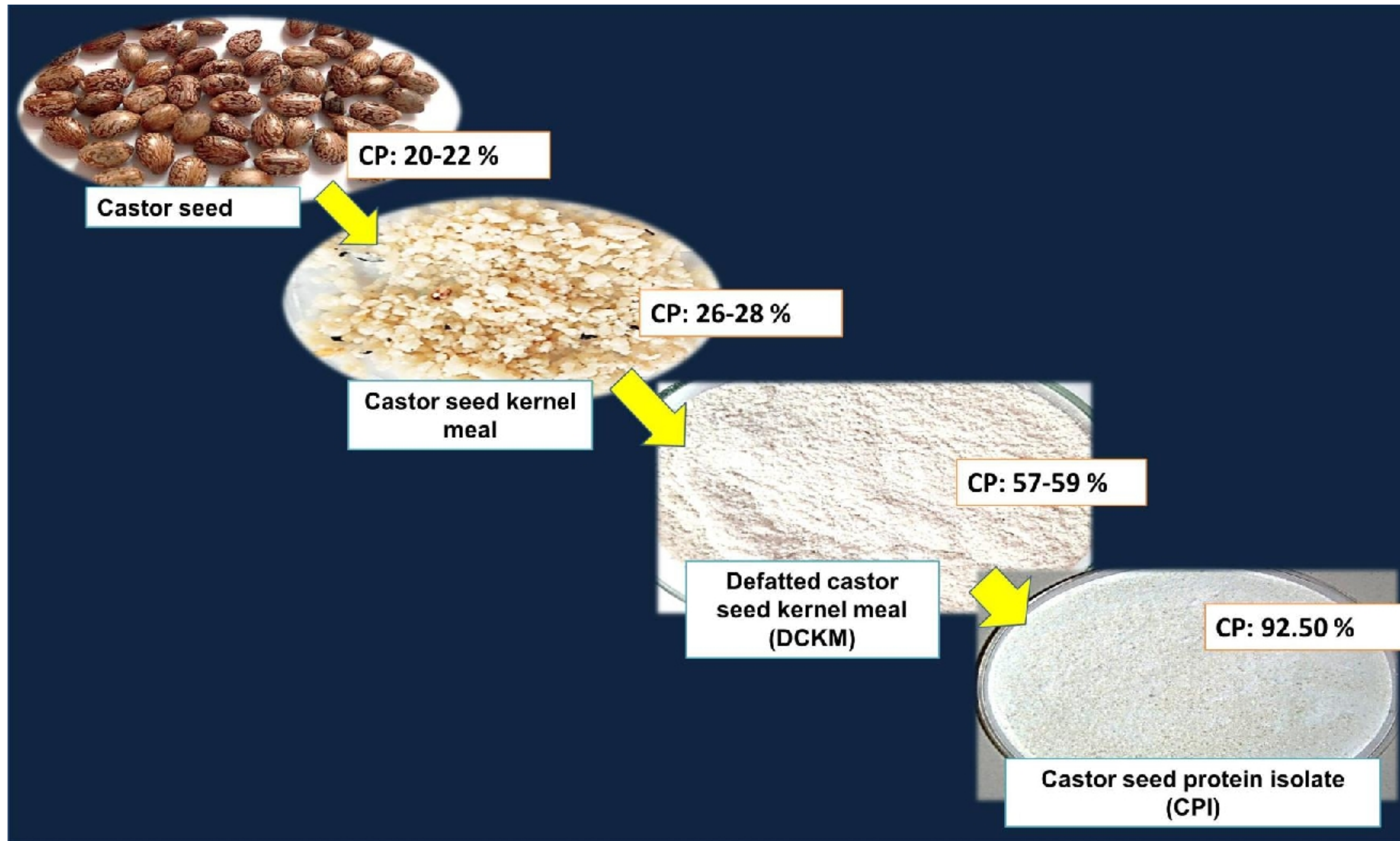

Plate 7: Preparation of protein isolate from defatted castor kernel meal

3.2.3 Amino acid analysis

Sample containing 5 mg protein were hydrolysed with 6 M HCl for 24 hr under vacuum at 110°C and pre-column derivatisation was done using phenyl isothiocyanate prior to quantification by high performance liquid chromatography (HPLC). The total and free amino acids were analysed using a GC2010 Plus instrument (Shimadzu, Tokyo, Japan) equipped with a flame ionization detector (FID), and an automatic liquid sampler (Devappa and Swamylingappa, 2008).

3.2.4 Amino acid score or chemical score

The chemical score was calculated by using the ratio of a gram of the most limiting amino acid in the test ingredient to the same amount of the corresponding amino acid in the reference protein (egg white) multiplied by 100 (Caire-Juvera *et al.,* 2013).

3.2.5 Essential amino acid index (EAAI) and biological value (BV)

EAAI was calculated according to the method of Oser (1951) and BV was calculated using the formula of Oser (1959).

$$BV = 1.09 \times (\text{EAA Index}) - 11.7$$

3.2.6 Nutritional index (NI)

Nutritional index was calculated using formula of Crisan and Sands (1978).

$$NI = (\text{EAA index} \times \%\ \text{protein}) / 100$$

3.2.7 Predicted protein efficiency ratio (P-PER)

The predicted protein efficiency ratio (P–PER) was estimated by using the equation of Alsmeyer *et al.* (1974).

$$P\text{–}PER = -0.468 + 0.454\ (\text{Leucine}) - 0.105\ (\text{Tyrosine})$$

3.2.8 *In-vitro* protein digestibility

In vitro protein digestibility of different feed ingredients was assessed using the pH drop method described by Ali *et al.* (2009). Fresh tissue of alimentary canal of the fish was homogenised under cold conditions and diluted with distilled water (1:10 w/v). Enzyme was extracted by centrifugation at 12000 rpm for 15 min at 4°C. An equivalent amount of each ingredient that provided 160 mg of crude protein was weighed and dispersed in 20 ml of distilled water and kept at 40°C overnight. 2 ml of enzyme extract was added to produce suspension of 8 mg crude protein per ml and pH was adjusted to 8.0 with the addition of dilute sodium hydroxide (NaOH) or hydrochloric acid (HCl). The pH drop was recorded at every min interval for 10 min by pH meter (SI Analytics TitroLine® 5000 titrator; Xylem Analytics, Germany) and casein was used as the reference protein.

The Relative protein digestibility (RPD) was calculated by the following formula:

$$RPD\% = \frac{\Delta pH\ of\ ingredient}{\Delta pH\ of\ casein} X\ 100$$

3.3 Estimation of Anti-nutritional Factors

3.3.1 Total tannin

Total tannin content was determined by following the spectrophotometric method (Makkar et al., 2007a). The total tannin content was calculated using the calibration curve of tannic acid standard and expressed as the tannic acid equivalents.

3.3.2 Phytic acid

The phytic acid content was estimated by using the method given by Gao *et al.* (2007). The assay comprises of addition of 10 ml, 3.5% HCl to 0.5 g sample followed by shaking for 1 hr at 200 rpm (ORBITEK shaker; Scigenics, India). Then this sample extract was centrifuged (Heraeus Megafuge 8R, Thermo Fisher Scientific, USA) at 1677 g for 10 min, and the supernatant was collected

and mixed with 1 ml, Wade reagent (0.03% $FeCl_3$ $6H_2O$ + 0.3% sulphosalicylic acid) and centrifuged at 1,677 g for 10 min. The supernatant was collected and absorbance was recorded at 500 nm using spectrophotometer (Thermoscientific, USA). A standard calibration curve was prepared using phytic acid standard.

3.3.3 Trypsin inhibitor

Trypsin inhibitor concentration was determined by using a modified method of Makkar *et al.* (2007b). The assay comprises of the addition of 50 ml, 0.01M NaOH to 1 g of finely ground sample and stirring for 3 hr (ORBITEK shaker; Scigenics, India) at room temperature and pH range between 8.4 to 10.0. The volume of aspirated aliquots of 0, 0.6, 1.4 and 1.8 ml were made up to 2 ml with distilled water. To this added 2 ml trypsin (Sigma, St. Louis, USA) and incubated at 37°C for 10 min. The reaction was stopped by addition of 1 ml, 30% acetic acid and centrifuged it and the supernatant was collected. The absorbance was measured at 410 nm in UV-visible spectrophotometer (Shimadzu, UV1800, Kyoto, Japan) against the reagent blank. The activity was expressed as trypsin inhibitor units (TIU).

3.3.4 Oxalates

Oxalates content was determined following the method given by Nwosu (2011). The assay comprises of addition of 20 ml, 0.03 N HCl to 1 g sample, followed by heating using magnetic hot plate cum stirrer. The mixture was allowed to cool and diluted to 100 ml using distilled water. An aliquot of 5 ml was mixed with 1ml of 5 N NH_4OH followed by addition of glacial acetic acid to make its pH acidic. Then, added 1 ml, 5% $CaCl_2$ and 3 drops of phenolphthalein indicator and allowed to precipitate for 3 hrs. Centrifuged at 604 g for 15 min and discarded the supernatant. The precipitate was dissolved in 2 ml, 3 N H_2SO_4 and titrated against 0.01 N $KMnO_4$ for end point of faint pink colour solution persisted for at least for 30 sec. The oxalates content of sample was calculated considering 1 ml, 0.01 N $KMnO_4$ as equivalent to 2.2 mg oxalate.

3.3.5 Alkaloids

For quantitative estimation of alkaloids, the gravimetric method suggested by Harborne (1973) was used. The assay comprises of addition of 50

ml, 10% acetic acid in ethanol solution to 5 g sample and followed by shaking for 4 hrs at 200 rpm (ORBITEK shaker; Scigenics, India). The filtrate was evaporated to its one quarter volume and precipitated with concentrated ammonium sulphate. A pre-weighed filter paper was used to filter off the precipitate and it was followed by washing with 1% NH_4OH solution. The filter paper with precipitate was oven-dried at 60°C for 30 min and allowed to cool in desiccator. The weight of the alkaloid was determined by the weight difference of the filter paper.

3.3.6 Hydrogen cyanide (HCN)

The hydrogen cyanide was determined by using the alkaline titration method of AOAC (1984). The assay of the method comprises the addition of 100 ml distilled water to 5 g sample and stirring for 2 hrs at room temperature. The solution was filtered and steam-distilled with sodium hydroxide solution. The distillate was collected and treated with diluted potassium iodide solution. Then, this solution was titrated against 0.02M $AgNO_3$ solution till end point i.e. the change from clear to faint and finally a permanent turbid solution. The Hydrogen cyanide concentration was determined by taking 1 ml, 0.02 M $AgNO_3$ equivalent to 1.08 mg HCN.

3.4 Statistical Analysis

All the data were statistically analyzed using one way analysis of variance by using IBM-SPSS Statistics 22.0 and the significant differences between mean were determined by using Duncan’s multiple range test (DMRT) (Duncan, 1955). The significance level was made at $p<0.05$. All data were expressed as mean± standard error of the mean.

3.5 Feeding Experiment

3.5.1 Experimental animals

Experimental animals i.e. *Labeo rohita* fingerlings were procured from Matasayabeej Kendra, Thakarpada, Dapchari, Palghar District, Maharashtra, India and were transported in well oxygenated polythene bags to the Central Wet Laboratory of the ICAR- Central Institute of Fisheries Education, Mumbai. The fish were transferred to three circular fibre tanks (1000 L) and were left undisturbed the whole night. In order to ameliorate the handling stress the fishes were given a mild salt treatment and vitamin C treatment the next day. The stock was acclimatized under aerated conditions for a period of 15 days and fed with a control diet containing 30% crude protein. After 15 days of acclimatization, fish were transferred to the experimental rearing tanks for the commencement of the experiment II and the rest of the fish were continuously fed with control diet till the commencement of experiment III and experiment IV.

3.5.2 Experimental units

Fifteen plastic rectangular tanks (180 L) were used as experimental units for the experiment II, III and IV. The tanks were initially washed and filled with 4 ppm potassium permanganate ($KMnO_4$) solution and left overnight to disinfect the tanks. The $KMnO_4$ solution was drained out next morning and again the tanks thoroughly washed with water and allowed to sun dry for 12 hr. The experimental tanks were filled with 150 L chlorine free bore-well water and continuous aeration was maintained throughout the experimental trial. The aeration pipe in each tub was provided with an air stone and a plastic regulator to control the air pressure uniformly in all the tubs. All the experimental tanks were covered with perforated lids to prevent fish from jumping out.

Plate 8: Experimental units

3.6 Chemicals and Glasswares

The glasswares used throughout the experiment were neutral glass of Borosil and Qualichem. Chemicals (analytical grade) of various companies *viz.* SIGMA, SISCO research laboratory (SRL), Hi-media, Qualigens, Erba®, Merck etc. were used for analytical purposes.

3.7 Physico- chemical Parameters of Water

Water quality parameters viz. temperature, pH, dissolved oxygen, free carbon dioxide, total hardness, ammonia, nitrite and nitrate were recorded during the experimental period of all the experiments II, III and IV.

3.7.1 Temperature

The water temperature of all the experimental tubs were recorded using dissolved oxygen meter (MERCK, Germany), which also showed the temperature.

3.7.2 pH

The pH was measured by a digital pH meter for all the experimental tubs.

3.7.3 Dissolved oxygen

The dissolved oxygen was measured by membrane electrode method using dissolved oxygen meter (MERCK, Germany) for all the experimental tubs.

3.7.4 Free carbon dioxide

The dissolved free carbon dioxide was measured by titrimetric method (APHA, 1998) and calculated using the following formula.

$$\text{Free carbon dioxide (mg/L)} = \frac{\text{A} \times \text{N} \times 44 \times 1000}{\text{Volume of sample (ml)}}$$

Where, A = Volume of titrant (NaOH)

N = Normality of titrant (N/44)

3.7.5. Carbonate hardness

Carbonate hardness was estimated by carbonate hardness test kit (Carbonate hardness test, MERCK, Germany).

3.7.6. Ammonia

Un-ionized ammonia concentration was estimated spectrophotometrically at 635 nm wavelength by phenate method (APHA, 1998) and compared with standard graph. The concentration was expressed as mg L^{-1}.

3.7.7. Nitrite-N

Nitrite concentration was estimated spectrophotometrically at 543 nm wavelength (APHA, 1998) and compared with standard graph. The concentration was expressed as mg L^{-1}.

3.7.8. Nitrate-N

Nitrate concentration was estimated spectrophotometrically at 543 nm wavelength (APHA, 1998) and compared with standard graph. The concentration was expressed as mg L^{-1}.

Experiment II: Feeding Trial to Evaluate the Nutritional Value of Castor Seed Protein Isolate in the Diet of *Labeo Rohita* Fingerlings

3.8 Feed Preparation

Five iso-nitrogenous (34.31±0.04 % crude protein) and iso-caloric (421.76±0.24 kcal $100g^{-1}$) experimental diets were formulated by using the different feed ingredients as given in Table 4. Fish meal, soybean protein isolate, castor seed protein isolate and groundnut oilcake were used as protein sources. De-oiled rice bran (DORB), wheat flour and corn flour were used as carbohydrate sources. Sunflower oil and fish oil were used as dietary lipid source. Carboxymethyl cellulose, choline chloride, and BHT (butylated hydroxyl toluene) were used as binder, attractants, and antioxidant, respectively.

A control diet was formulated using soybean protein isolate (SPI) as the major protein source without castor seed protein isolate (CPI). Four experimental diets were prepared with graded levels of castor seed protein isolate substituting 25% SPI first experimental diet (CPI_{25}) and thereafter 50%, 75%, and 100% SPI in experimental diets CPI_{50}, CPI_{75}, and $CPI_{100,}$ respectively (Table 4). The CPI thereby contribute 0%, 11.73%, 22.67%, 33.20% and 43.73% of the total dietary protein. The required quantities of various dietary ingredients were weighed as per feed formulation (except fish oil and sunflower oil, choline chloride, BHT and vitamin-mineral mix) and kept in a plastic tray. The weighed ingredients were mixed to form a homogenous blend and steam cooked in a pressure cooker at 15 psi for 20 min to ensure proper gelatinization of starch and allowed to cool. Other ingredients, such as fish oil and sunflower oil, choline chloride, BHT and vitamin-mineral mix were added and mixed uniformly. Then the mixture was pressed through a pelletizer to get uniform sized pellets (1.0 mm diameter and 3.4 mm in length), which were spread on a tray and dried in oven at 60°C. Then the dried pellets were packed in an airtight polythene bag, labelled and stored at room temperature.

Table 4: Composition of the experimental diets (g $100g^{-1}$ dry matter basis) fed to *Labeo rohita* fingerlings during the experimental period

Ingredients	Control	CPI_{25}	CPI_{50}	CPI_{75}	CPI_{100}
Fish meal[c]	6.00	6.00	6.00	6.00	6.00
Soybean protein isolate[b]	17.80	13.20	8.50	4.15	0.00
Castor seed protein isolate[a]	0.00	4.40	8.50	12.45	16.40
Ground nut oilcake[c]	24.00	24.00	23.80	23.60	23.00
De-oiled rice bran[c]	30.00	30.00	30.00	30.00	30.00
Corn flour[c]	8.16	8.36	9.16	9.76	10.56
Wheat flour[c]	7.00	7.00	7.00	7.00	7.00
Fish oil[c]	2.00	2.00	2.00	2.00	2.00
Sunflower oil[c]	2.00	2.00	2.00	2.00	2.00
***Vitamin- min premix**	2.00	2.00	2.00	2.00	2.00
Choline chloride[d]	0.02	0.02	0.02	0.02	0.02
Butylated hydroxytoluene[d]	0.02	0.02	0.02	0.02	0.02
Carboxymethyl cellulose[d]	1.00	1.00	1.00	1.00	1.00
Total	100.00	100.00	100.00	100.00	100.00
Chromium oxide	0.50	0.50	0.50	0.50	0.50

[a]Prepared in fish nutrition, Biochemistry and Physiology Laboratory, ICAR-Central Institute of Fisheries Education, Mumbai, India

[b]Soybean protein isolate– Nusowin protein powder, Win-Medicare Pvt Ltd, New Delhi, India

[c]Purchased from local animal feed ingredient dealer, Mumbai, India

[d]Himedia Pvt, Mumbai, India

*Composition of vitamin mineral mix (Agrimin) (quantity/kg): Vitamin A- 55,00,000 IU; vitamin D3- 11,00,000 IU; vitamin B2- 2,000 mg; vitamin E- 750 mg; vitamin K- 1,000 mg; vitamin B6- 1,000 mg; vitamin B12- 6 mcg; calcium pantothenate- 2,500 mg; nicotinamide- 10 g; choline chloride- 150 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.

3.9 Experimental Design and Feeding

One hundred fifty fingerlings of *L. rohita* (initial average weight, 3.33±0.04 g) were randomly distributed into five distinct experimental groups in triplicates following a completely randomized design. Ten fish were stocked in each tank (15 tanks) and all groups were fed twice a day (09:00 am and 05:00 pm) to satiation level during the entire feeding trial of 60 days. Feed intake was adjusted based on the observation of daily feed consumption. The experimental tubs were cleaned manually and siphoning was done every day in order to remove uneaten feed pellets and the faecal matter. An equal volume of clean bore well water was maintained to replace the siphoned water. This was carried out throughout the experimental period. The body weight was measured at an interval of 15 days to assess the growth. The fishes were starved overnight before taking the bodyweight.

3.10 Digestibility Studies

The digestibility study was carried out by using indirect method. Chromium oxide (Cr_2O_3) was used an inert marker (Cho and Slinger, 1979; Alexander *et al*., 2011; Fawole *et al*., 2016b). The diets were prepared by incorporating 0.5% Cr_2O_3 and the marker containing feed was fed to *L. rohita* fingerlings for 15 days after completion of feeding trial. Before starting the digestibility trial, the fish were acclimated for a period of 10 days to the respective diet.

3.10.1 Faecal matter collection

Faecal matter samples were collected daily by siphoning for 15 days. *L. rohita* fingerlings were fed 1% of their body weight once daily at 9:00 am and after 2 hr. There was no feed left out in the tub. Faecal samples were collected in a plastic petridish after 6 hr of feeding and centrifuged at 5000 rpm for 15 min to separate water. The wet faecal sample was stored at -20 ^{0}C and lypholized (Shiau and Liang, 1994; Usmani *et al.,* 2003). Crude protein and lipid content of faecal sample were determined according to Cunniff (1995).

3.10.2 Determination of chromium oxide

Chromium oxide content of the feed and faecal matters was determined according to the method of Furukawa and Tsukahara (1966). 100mg sample was weighed and kept in a 100ml Kjeldahl flask, to which 5 ml HNO_3 was added and digested with gentle boiling for 30 min or until yellowish vapors become colourless. If the quantity of liquid reduces significantly and nitrous vapor is still giving off, 5ml more nitric acid was added and continued with the digestion. A clear greenish solution with no nitrous vapours indicated completion of the digestion. After cooling, 3 ml of perchloric acid was added and sample digested again until the solution turned from green to lemon yellow and contents were allowed to cool. A reddish ring around the solution should appear; if not, or if the liquid turns green again, digestion process was repeated until the change is permanent. The solution was transferred to a 25 ml volumetric flask and volume made up with distilled water. A blank was run without sample at the same time using only acid and distilled water. The absorbance was measured at 350 nm using spectrophotometer. The calculation was made as per the formula given by Furukawa and Tsukahara (1966).

Calculation

a. Calculate the amount of chromic oxide (mg) present in the sample:

$$x = \frac{1}{4} \cdot \frac{y - 0.0032}{0.2089}$$

Where: y = absorption at 350 nm, and

0.0032 and 0.2089= are constants.

b. Calculate the % of chromic oxide in the sample

$$O.C.\% = 100\frac{X}{Y}$$

Where: X = weight of chromic oxide; Y = weight of sample.

3.10.3 Apparent digestibility coefficient (ADC)

Apparent digestibility coefficients (ADCs) of diets and nutrients were determined by the following equations (Law, 1986)

ADC of dry matter of diet (%) = 100× {1-(% Cr_2O_3 in feed)/ (% Cr_2O_3 in faeces)}

ADC of nutrients of the diets (%) = 100× [1 – {(D/F) × (FCr/DCr)}]

NOTE: Where, F is the percent of nutrients in the faeces, D is the percent of nutrients in the diet, DCr is the percent of chromic oxide in the diet, and FCr is the percent of chromic oxide in faeces.

Experiment III: Nutritional Evaluation of Castor Seed Protein Isolate Supplemented with Amino Acids (L-Lysine and DL-Methionine) in the Diet of *L. rohita* Fingerlings

3.11 Feed Preparation

Five iso-nitrogenous (35.45±0.14% crude protein) and iso-caloric (421.82±0.16 kcal $100g^{-1}$) experimental diets were formulated by using the different feed ingredients as given in Table 6. Amino acid analysis exhibited the deficiency of L-lysine and DL-methionine in all experimental diets (Table 5). Therefore, in the present experiment, the limiting amino acids (L-lysine and DL-methionine) were supplemented to fulfil the nutritional requirement of *L. rohita* fingerlings (NRC, 2011). A control diet was prepared using soybean protein isolate (SPI). The CPI protein successively replaced 25%, 50%, 75%, and 100% of SPI protein and designated as $ACPI_{25}$, $ACPI_{50}$, $ACPI_{75}$, and $ACPI_{100}$, respectively (Table 6). The required quantities of various dietary ingredients were weighed as per feed formulation (except fish oil and sunflower oil, choline chloride, BHT and vitamin-mineral mix) and kept in a plastic tray. The weighed ingredients were mixed to form a homogenous blend and steam cooked in a pressure cooker at 15 psi for 20 min to ensure proper gelatinization of starch and allowed to cool. Other ingredients, such as fish oil and sunflower oil, choline chloride, BHT, L-lysine, DL-methionine and vitamin-mineral mix were added and mixed uniformly. Then the mixture was pressed through a pelletizer to get uniform sized pellets (1.0 mm diameter and 3.4 mm in length), which were spread on a tray and dried in oven at 60°C. The dried pellets were packed in an airtight polythene bag, labelled and stored at room temperature.

3.12 Experimental Design and Feeding

One hundred fifty fingerlings of *L. rohita* (initial average weight, 7.17±0.02 g) were randomly distributed into five distinct experimental groups in triplicates following a completely randomized design. Ten fish were stocked in each tank (15 tanks) and all groups were fed twice a day (09:00 am and 05:00 pm)

to satiation level, during the entire length of the feeding trial of 60 days. Feed intake was adjusted based on the observation of daily feed consumption. The experimental tubs were cleaned manually and siphoning was done every day in order to remove uneaten feeds and the remaining faecal matters. An equal volume of clean bore well water was maintained and used to replace the siphoned water. This was carried out throughout the experimental period. The body weight was measured at intervals of 15 days to assess the growth. The fishes were starved overnight before taking the bodyweight.

Table 5: Amino acid content of experimental diets (g amino acids 100 g^{-1} feed)

Amino acids	Control	CPI_{25}	CPI_{50}	CPI_{75}	CPI_{100}	Requirement (NRC, 2011)
Arginine	3.03	2.86	2.67	2.49	2.32	1.70
Histidine	0.84	0.83	0.81	0.80	0.79	0.90
Isoleucine	1.46	1.54	1.61	1.67	1.74	1.00
Leucine	2.58	2.45	2.30	2.16	2.03	1.40
Lysine	1.77	1.61	1.43	1.28	1.12	2.20
Methionine	0.53	0.50	0.47	0.44	0.41	0.70
Phenylalanine	1.71	1.56	1.39	1.23	1.07	1.30
Tryptophan	0.36	0.35	0.33	0.32	0.30	0.30
Threonine	1.31	1.53	1.71	1.90	2.08	1.50
Valine	1.64	1.58	1.50	1.43	1.36	1.40

Table 6: Composition of the experimental diets (g $100g^{-1}$ dry matter basis) fed to *Labeo rohita* fingerlings during the experimental period

Ingredients	Control	$ACPI_{25}$	$ACPI_{50}$	$ACPI_{75}$	$ACPI_{100}$
Fish meal[c]	6.00	6.00	6.00	6.00	6.00
Soybean protein isolate[b]	17.80	13.20	8.50	4.15	0.00
Castor seed protein isolate[a]	0.00	4.40	8.50	12.45	16.40
Ground nut oilcake[c]	24.00	24.00	23.80	23.60	23.00
De-oiled rice bran[c]	30.00	30.00	30.00	30.00	30.00
Corn flour[c]	7.51	7.56	8.11	8.60	9.16
Wheat flour[c]	7.00	7.00	7.00	7.00	7.00
Fish oil[c]	2.00	2.00	2.00	2.00	2.00
Sunflower oil[c]	2.00	2.00	2.00	2.00	2.00
*Vitamin- min premix	2.00	2.00	2.00	2.00	2.00
Choline chloride[d]	0.02	0.02	0.02	0.02	0.02
Butylated hydroxytoluene[d]	0.02	0.02	0.02	0.02	0.02
Carboxymethyl cellulose[d]	1.00	1.00	1.00	1.00	1.00
L-Lysine[d]	0.45	0.60	0.80	0.90	1.10
DL-Methionine[d]	0.20	0.20	0.25	0.26	0.30
Total	100.00	100.00	100.00	100.00	100.00

[a]Prepared in fish nutrition, Biochemistry and Physiology Laboratory, ICAR-Central Institute of Fisheries Education, Mumbai, India

[b]Soybean protein isolate– Nusowin protein powder, Win-Medicare Pvt Ltd, New Dehli, India

[c]Purchased from local animal feed ingredient dealer, Mumbai, India

[d]Himedia Pvt, Mumbai, India

*Composition of vitamin mineral mix (Agrimin) (quantity/kg): Vitamin A- 55,00,000 IU; vitamin D3- 11,00,000 IU; vitamin B2- 2,000 mg; vitamin E- 750 mg; vitamin K- 1,000 mg; vitamin B6- 1,000 mg; vitamin B12- 6 mcg; calcium pantothenate- 2,500 mg; nicotinamide- 10 g; choline chloride- 150 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.

Experiment IV: Feeding Trial to Evaluate the Nutritional Value of Protein Isolate Prepared from Defatted Castor Kernel Meal in the Leafmeal Based Diet of *L. rohita* Fingerlings

3.13 Preparation of Sweet Potato Leaf Meal

The fresh sweet potato leaves were collected locally, and washed, dried in shade and brought to the Fish Nutrition and Feed Technology Laboratory, ICAR- Central Institute of Fisheries Education, Mumbai. The leaves were oven-dried at 40-45°C for 24 hrs. The dried leaves were ground and sieved through 250 mm mesh and stored in airtight container.

3.14 Solid State Fermentation of Sweet Potato Leaf Meal

Solid-state fermentation (SSF) of sweet potato leaf meal was performed by using *Chaetomium globosum* (MTCC-4179) (Meshram *et al*., 2018). The process of solid state fermentation of leaf meal comprises of the addition of water to increase the moisture content of SPLM in a conical flask up to 50%, followed by sterilization of leaf meal in autoclave for 15-20 min at 121°C and 15 psi pressure. The sterilized leaf meal was allowed to cool and followed by inoculation of *C. globosum* (3×10^5 cells/g) to SPLM and mixed properly for uniform distribution of inoculum. Then it was incubated at 28-30°C for 120 hrs for solid state fermentation. After incubation, the fermented sweet potato leaf meal was dried in an oven at 60°C to inactivate the *C. globosum* spores for 12 hrs as; active *C. globosum* spores would utilize nutrients from leaf meal and result in decreasing crude protein.

Plate 9: Solid State Fermentation of Sweet Potato Leaf Meal

3.15 Feed Preparation

Amino acid analysis exhibited that the experimental diets were deficient in L-lysine and DL-methionine (Table 7). Therefore, the limiting amino acids (lysine and methionine) were supplemented to fulfil the nutritional requirement of *L. rohita* fingerlings. Four iso-nitrogenous (35.13±0.09% crude protein) and iso-caloric (420.95±0.46 kcal 100g^{-1}) experimental diets were formulated by using the different feed ingredients as given in Table 8.

In experiment III, dietary castor seed protein isolate (16.40% inclusion) could completely substitute the soybean protein isolate (17.80% inclusion). Based on the results obtained in experiment III, first diet (control diet) was prepared with 17.80% CPI and 30% de-oiled ricebran (DORB) and designated as SPI+DORB. In second diet, comprised of 17.80% SPI inclusion and 30% fermented sweet potato leafmeal (FSPLM) and designated as SPI+SPLM.

Similarly, third and fourth diet contained 16.40% CPI and 30% DORB & 30% FSPLM, respectively and designated as CPI+DORB and CPI+SPLM, respectively (Table 8). The required quantities of various dietary ingredients were weighed as per feed formulation (except fish oil and sunflower oil, choline chloride, BHT and vitamin-mineral mix) and kept in a plastic tray. The weighed ingredients were mixed to form a homogenous blend and steam cooked in a pressure cooker at 15 psi for 20 min to ensure proper starch gelatinization and allowed to cool. Other ingredients, such as fish oil and sunflower oil, choline chloride, BHT, L-lysine, DL-methionine and vitamin-mineral mix were added and mixed uniformly. Then the mixture was pressed through a pelletizer to get uniform sized pellets (1.0 mm diameter and 3.4 mm in length), which were spread on a tray and dried in oven at 60°C temperature. Then the dried pellets were packed in an airtight polythene bag, labelled and stored at room temperature.

Table 7: Amino acid content of experimental diets (g 100 g^{-1} feed)

Essential amino acids	SPI+ DORB	SPI+ SPLM	CPI+ DORB	CPI+ SPLM	Requirement (NRC, 2011)
Arginine	3.3	2.47	2.59	1.76	1.7
Histidine	0.9	0.79	0.85	0.75	0.9
Isoleucine	1.55	1.45	1.83	1.74	1
Leucine	2.75	2.7	2.2	2.14	1.4
Lysine	1.87	1.82	1.22	1.17	2.2
Methionine	0.56	0.5	0.44	0.38	0.7
Phenylalanine	1.85	1.7	1.21	1.06	1.3
Tryptophan	0.39	0.29	0.33	0.24	0.3
Threonine	1.38	1.26	2.15	2.03	1.5
Valine	1.75	1.59	1.47	1.31	1.4

Table 8: Composition of the experimental diets (g $100g^{-1}$ dry matter basis) fed to *Labeo rohita* fingerlings during the experimental period:

Ingredients	SPI+DORB	SPI+SPLM	CPI+DORB	CPI+SPLM
Fish meal[c]	6.00	6.00	6.00	6.00
Soybean protein isolate[b]	17.80	17.80	0.00	0.00
Castor seed protein isolate[a]	0.00	0.00	16.40	16.40
Ground nut oilcake[c]	30.00	10.40	29.00	9.50
De-oiled rice bran[c]	30.00	0.00	30.00	0.00
#FSPLM[a]	0.00	30.00	0.00	30.00
Corn flour[c]	4.56	14.16	5.26	14.76
Wheat flour[c]	4.00	14.00	5.00	15.00
Fish oil[c]	2.00	2.00	2.00	2.00
Sunflower oil[c]	2.00	2.00	2.00	2.00
***Vitamin- min premix**	2.00	2.00	2.00	2.00
Choline chloride[d]	0.02	0.02	0.02	0.02
Butylated hydroxytoluene[d]	0.02	0.02	0.02	0.02
Carboxymethyl cellulose[d]	1.00	1.00	1.00	1.00
L-Lysine[d]	0.40	0.40	1.00	1.00
DL-Methionine[d]	0.20	0.20	0.30	0.30
Total	100.00	100.00	100.00	100.00

[a]Prepared in fish nutrition, Biochemistry and Physiology Laboratory, ICAR-Central Institute of Fisheries Education, Mumbai, India

[b]Soybean protein isolate– Nusowin protein powder, Win-Medicare Pvt Ltd, New Delhi, India

#FSPLM- Fermented sweet potato leaf meal

[c]Purchased from local animal feed ingredient dealer, Mumbai, India

[d]Himedia Pvt, Mumbai, India

*Composition of vitamin mineral mix (Agrimin) (quantity/kg): Vitamin A- 55,00,000 IU; vitamin D3- 11,00,000 IU; vitamin B2- 2,000 mg; vitamin E- 750 mg; vitamin K- 1,000 mg; vitamin B6- 1,000 mg; vitamin B12- 6 mcg; calcium pantothenate- 2,500 mg; nicotinamide- 10 g; choline chloride- 150 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.

3.16 Experimental Design and Feeding

One hundred twenty fingerlings of *L. rohita* (initial average weight, 9.25±0.02 g) were randomly distributed into four distinct experimental groups in triplicates following a completely randomized design. Ten fish were stocked in each tank (12 tanks) and all groups were fed twice a day (09:00 am and 05:00 pm) to satiation, during the entire length of the feeding trial of 60 days. Feed intake was adjusted based on the observation of daily feed consumption. The experimental tubs were cleaned manually and siphoning was done every day in order to remove excess feed pellets and the remaining faecal matter. An equal volume of clean bore well water was maintained at the same temperature was used to replace the siphoned water. This was carried out throughout the experimental period of the experiment. The body weight was measured at intervals of 15 days to assess the growth. The fishes were starved overnight before taking the bodyweight.

General Procedures

3.17 Growth and Nutrient Utilization

Growth performance and nutrient utilization indices were assessed by taking fish weight at every 15 days interval. The growth performance and nutrient utilization indices were calculated using the following formula:

3.17.1 Weight gain (%)

$$\mathrm{WG(\%)} = \frac{\text{Final weight} - \text{Initial Weight}}{\text{Initial weight}} \times 100$$

3.17.2 Specific growth rate (SGR)

$$\mathrm{SGR} = \frac{\mathrm{Log_e}\ \text{Final weight} - \mathrm{Log_e}\ \text{Initial weight}}{\text{Number of days}} \times 100$$

3.17.3 Feed conversion ratio

$$\mathrm{FCR} = \frac{\text{Feed intake (Dry Weight)}}{\text{Body Weight Gain (Wet Weight)}}$$

3.17.4 Feed efficiency ratio

$$\text{FER} = \frac{\text{Body Weight Gain (Wet Weight)}}{\text{Feed intake (Dry Weight)}}$$

3.17.5 Protein efficiency ratio

$$\text{PER} = \frac{\text{Net Weight Gain (Wet Weight)}}{\text{Protein Fed}}$$

3.17.6 Apparent net protein utilization

$$\text{ANPU (\%)} = \frac{\text{Final carcass protein} - \text{Initial carcass protein}}{\text{Protein intake}} \times 100$$

3.17.7 Hepato-somatic index

$$\text{HSI (\%)} = \frac{\text{Liver Weight (g)}}{\text{Weight of fish (g)}} \text{X } 100$$

3.17.8 Viscero-somatic Index

$$\text{VSI (\%)} = \frac{\text{Viscera weight (g)}}{\text{Weight of fish (g)}} \text{X } 100$$

3.17.9 Survival

$$\text{Survival (\%)} = \frac{\text{Total number of fishes harvested}}{\text{Total number of fishes stocked}} \text{X } 100$$

3.18 Proximate Analysis

Proximate analysis of ingredients, experimental diets, and fish body composition were carried out as per the standard methods of the Association of Official Analytical Chemists (Cunniff, 1995).

3.18.1 Moisture

The moisture content of the ingredients, experimental diets and carcass tissue was determined by taking a known weight of the sample in the petri

dish and drying it in a hot air oven at 100- 105^0 C till a constant weight was achieved. The difference in weight of the sample gave the moisture content, which was calculated by using the following formula

$$\text{Moisture (\%)} = \frac{\text{Wet weight of sample} - \text{Dried weight of sample}}{\text{Wet weight of sample}} \times 100$$

3.18.2 Crude protein (CP)

The crude protein content (N x 6.25) was determined by the Kjeldahl method and the assay comprised of acid digestion (concentrated sulphuric acid), alkali distillation (40% NaOH) using an auto Kjeldahl System (Kelplus auto digester and distillation unit; Pelican, Chennai, India) followed by titration (SI Analytics TitroLine® 5000 titrator; Xylem Analytics, Germany). The crude protein percentage was obtained by multiplying the nitrogen percentage by a factor of 6.25.

Crude protein (%) = Nitrogen (%) X 6.25.

3.18.3 Ether extract (EE)

The ether extract was estimated by the solvent extraction method (Socsplus, SCS-08-As, Pelican equipment, Chennai, India) and the assay comprises extraction of lipid with an organic solvent (diethyl ether) at 40-60º C temperature in soxhlet apparatus. The calculation was made as follows.

$$\text{EE (\%)} = \frac{\text{Initial weight of sample} - \text{Weight of sample after extraction}}{\text{Initial weight of sample}} \times 100$$

3.18.4 Total ash

Total ash of the experimental diets and carcass tissue content was determined by incinerating the moisture-free sample at 550ºC temperature for 6 hr in a muffle furnace. The calculation was done as follows:

$$\text{Ash (\%)} = \frac{\text{Weight of ash}}{\text{Weight of sample}} \times 100$$

3.18.5 Crude fibre (CF)

The assay of crude fiber of experimental diets comprises of acid digestion (1.25% sulphuric acid) of a fat-free sample, washing with distilled water and alkali digestion (1.25% sodium hydroxide) followed by incineration in the muffle furnace. The difference in weight after calculation indicates the quantity of fibre present in the sample.

$$\text{Crude fibre (\%)} = \frac{\text{A} - \text{B}}{\text{C}} \times 100$$

Where,

A=Weight of crucible with dry residue

B=Weight of crucible with ash

C=Weight of sample

3.18.6 Nitrogen free extract

Nitrogen free extract was determined by subtracting the other nutrients from 100.

NFE (%) = 100- {CP (%)+ EE (%)+ total ash (%)+ CF (%)}

3.18.7 Gross energy (GE)

Gross energy (GE) content of feed was measured using a Parr bomb calorimeter (Model # 1241, Moline, IL). Energy of the dried material was determined by placing 1 g weighed sample in the ignition cup of the bomb. Oxygen was added at ~35 psi and the bomb immersed in 2 L of water. The sample was ignited in the presence of oxygen and the heat released due to ignition of sample is absorbed by water in an adiabatic chamber. Gross energy was calculated using a boric acid calibration standard and expressed as kcal $100g^{-1}$.

3.19 Sampling and Tissue Homogenate Preparation

At the end of the experiment, 3 fishes were randomly collected from each tank and anaesthetized with clove oil ($50\mu L.L^{-1}$). Fishes were then dissected and

the tissues *viz.*, liver, gills, intestine, and muscle, were carefully removed and weighed. The tissue were homogenised with chilled 250 mM sucrose solution in a plastic tube using teflon coated mechanical homogeniser (MICCRA D-9, ART Prozess and Labotechnik, Germany). The whole procedure was carried out in ice cold condition. Homogenised samples were centrifuged at 5000 rpm for 10 min at -4^0C. The collected supernatant was stored in deep freezer (-20^0C) till further analysis.

3.20 Tissue Protein Estimation

Quantification of protein of the different tissues was carried out by using Bradford method (Bradford, 1976). The Bradford assay relies on the binding of the dye Coomassie brilliant blue G250 to protein. Tissue homogenate (20µl) was taken along with 180µl distilled water and 250µl 1N NaOH added. After that 5ml Bradford reagent (100 mg Coomassie brilliant blue G250 in 50 ml, 95% ethanol mixed with 85% phosphoric acid and volume made up to 1 L) added, vortexed and kept for 5min at room temperature. A blank was run using 20 µL distilled water without tissue sample. Following the same procedure, a protein standard curve was prepared using bovine serum albumin (BSA). The absorbance of samples was measured at 595nm against the blank. The absorbance readings of BSA standards at different concentrations were fitted into the standard curve to determine their protein concentrations.

Enzyme Assays

3.21 Digestive Enzymes

3.21.1 Protease

Protease activity was determined by the casein digestion method (Drapeau, 1976). The enzyme reaction mixtures consisted of 1% casein in 0.05 M Tris PO_4 buffer (pH 7.8) and incubated for 5 min at 37^0C. Then, the tissue

homogenate add to the enzyme mixture after ten minutes reaction was stopped by adding 10% TCA and the whole content were filtered. The reagent blank was made by adding tissue homogenate just before stopping the reaction and without incubation. One unit of enzyme activity was defined as the amount of enzyme needed to release acid soluble fragments equivalent to $\Delta 0.001A_{280}$ per minute at 37^0C and pH 7.8.

3.21.2 Amylase

The reducing sugars produced due to the action of glucoamylase and amylase on carbohydrate was estimated using Dinitro-salicylic acid (DNS) method (Rick and Stegbauer, 1974). The reaction mixtures consisted of 1% (w/v) starch solution, phosphate buffer (pH 6.9) and the tissue homogenate. The reaction mixtures were incubated at 37^0C for 30 min. DNS was added after incubation and kept in boiling water bath for 5 min. After cooling, the reaction mixture was diluted with distilled water and absorbance was measured at 540 nm. Maltose was used as the standard. Amylase activity was expressed as mole of maltose released from starch per min at 37^0C temperature.

3.21.3 Lipase

The lipase activity was determined by the titrimetric method of Cherry and Crandell (1932), which is based on the measurement of fatty acids released by the enzymatic hydrolysis of triglycerides present in a stabilized emulsion of olive oil. The amount of a standard sodium hydroxide solution used to titrate the fatty acids released was taken as an index of lipase activity of the crude enzyme extract. The assay system consisted of 1.5 ml of stabilized lipase substrate and 1.5 ml of 0.1 M Tris-HCl buffer at pH 8.0, to which 1.0ml of the crude enzyme extract was added. The assay mixture was incubated for 24 hr at 4^oC, after which the reaction was stopped by addition of 3 ml, 95% etanol. The mixture was then titrated with 0.01 N NaOH and 0.9% (w/v) phenolphthalein was used as an indicator.

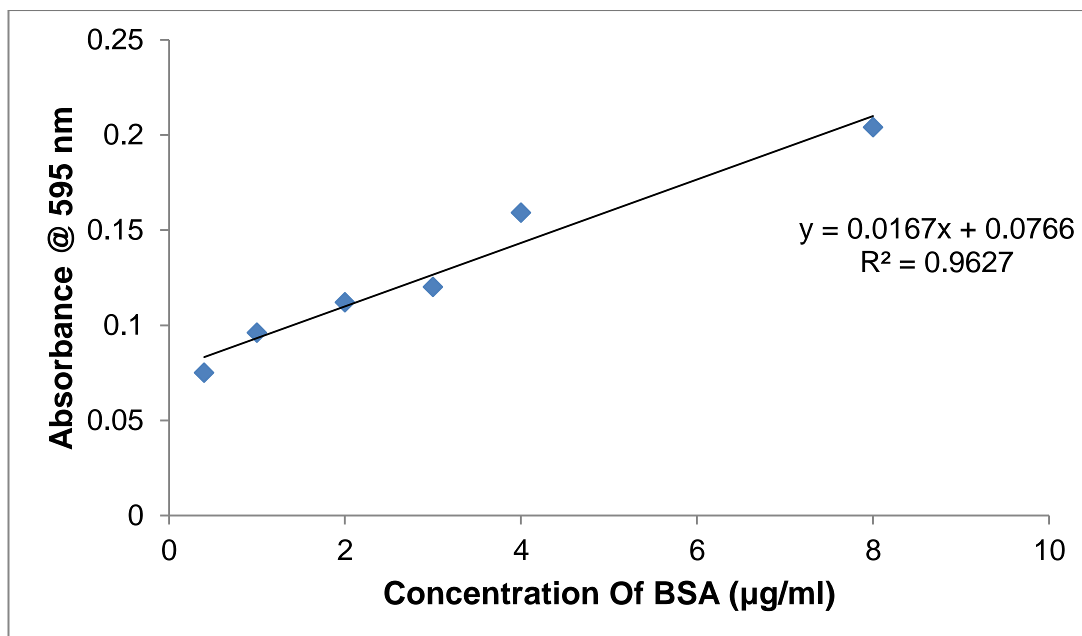

Figure 2: BSA standard curve to determine the tissue protein concentration showing a strong linear correlation between the concentration of protein and optical density (Y = 0.0167x + 0.0766, R^2 = 0.9627)

3.22 Metabolic Enzymes

3.22.1 Aspartate aminotransferase (AST)

(L- aspartate: 2oxaloglutarate aminotransferase, E.C.2.6.1.1)

AST activity was assayed in different tissue homogenates as described by Wooton (1964). The substrate comprised of 0.2M DL- aspartic acid and 2mM α-ketoglutarate in 0.05M phosphate buffer (pH 7.4). In the treatment and control tubes, 0.5ml of substrate was added. The reaction was started by adding 0.1ml of tissue homogenate in the treatment tube. The assay mixture was incubated at 37^{0}C for 60 min. The reaction was terminated by adding 0.5ml of 1mM 2, 4-dinitrophenyl hydrazine (DNPH). In the control tubes the enzyme source was added after DNPH solution. The tubes were held at room temperature for 20 minutes with occasional shaking. Then 5ml of 0.4ml NaOH solution was added and the contents were thoroughly mixed. After 10 minutes, the OD was recorded

at 540nm against blank. The AST activity was expressed as nanomoles oxaloacetate released mg protein^{-1} min^{-1} at 37°C.

3.22.2 Alanine aminotransferase (ALT)

(L- alanine: 2 oxaloglutarate aminotransferase; E.C.2.6.1.2)

The procedure adopted for the estimation of ALT activity was same as that for AST activity estimation except that the substrate comprised of 0.2 M D, L-alanine instead of aspartic acid. The ALT activity was expressed as nanomoles pyruvate released mg protein^{-1} min^{-1} at 37°C.

3.22.3 Lactate dehydrogenase (LDH)

(L- Lactate NAD+ oxidoreductase; E.C.1.1.1.27)

The LDH activity was assayed in different tissue homogenate according to the method of Wroblewski and Ladue (1955). 3 ml of the total reaction mixture comprised of 2.7ml of 0.1M phosphate buffer (pH 7.5), 0.1ml of NADH solution (2 mg NADH dissolved in 1ml of phosphate buffer solution), 0.1ml of tissue homogenate and 0.1ml of sodium pyruvate. The reaction started after addition of substrate sodium pyruvate. OD was recorded at 340 nm at 15 seconds interval for 3 minutes. LDH activity was expressed as micro moles of NAD released mg protein^{-1} min^{-1} at 37°C.

3.22.3 Malate dehydrogenase (MDH)

The malate dehydrogenase (MDH) activity was assayed in different tissues by the method of Ochoa (1955) and the specific enzymatic activity was expressed as unit mg protein^{-1} min^{-1} at 25°C where 1 unit was equal to Δ0.01 OD min^{-1}.

3.23 Enzymes of Oxidative Stress

3.23.1 Superoxide dismutase (SOD)

Superoxide dismutase (E.C.1.15.1.1) activity was estimated according to the method described by Mishra and Fridovich (1972), based on the oxidation of epinephrine–adrenochrome transition by the enzyme. 50µl of the sample was taken in the cuvette and 1.5 ml 0.1M carbonate – bicarbonate buffer containing 57 mg dl^{-1} EDTA (pH 10.2) and 0.5 ml epinephrine (3mM) was added and mixed well. Change in optical density at 480 nm was read immediately for 3 min in a Shimadzu® – UV spectrophotometer. One unit of SOD activity was the amount of protein required to give 50% inhibition of epinephrine auto oxidation. SOD (superoxide dismutase) activity is expressed as 50% inhibition of epinephrine auto- oxidation/mg protein/min

3.23.2 Catalase (CAT)

Catalase was assayed according to the method described by Takahara *et al.* (1960). To a reaction mixture of 2.45 ml phosphate buffer (50 mM, pH 7.0), enzyme source was added and the reaction was started by the addition of 1.0 ml of H_2O_2 solution. The decrease in absorbance was measured at 240 nm at 15 sec intervals for 3 min. The enzyme blank was run simultaneously with 1.0 ml distilled water instead of H_2O_2 solution. Enzyme activity was expressed as nano moles H_2O_2 decomposed min^{-1} mg^{-1} protein

3.24 Biochemical and Haemato-Immunological Parameters

3.24.1 Collection of blood and serum

Each fish was anesthetized in clove oil (50 µl of clove oil per liter of water) before taking blood. Blood was withdrawn from caudal vein (Vena caudalis) using a medical syringe, which was previously, rinsed with 2.7% EDTA solution. Collected blood was then transferred immediately to test tube containing thin layer of EDTA powder (as an anticoagulant) and shake well in order to prevent coagulation of blood. The blood was used for the determination of haemoglobin

(Hb), total erythrocyte (RBC) and leucocyte counts (WBC), and nitroblue tetrazolium (NBT) assay.

Another fresh blood sample was collected from the caudal vein (Vena caudalis) without using 2.7% EDTA solution by a medical syringe. Blood collected was then transferred immediately to a dried eppendorf tube. The tubes were allowed to stand in tilted position at room temperature for an hour to allow the blood to clot. After clotting the blood, the sera was centrifuged at 5000 rpm for 10 min in a cooling centrifuge (REMI CPR-24, India) and the yellow straw color serum was carefully collected and transferred to another eppendorf tube and kept at -20^{0}C for further analysis. The serum was used for the estimation of biochemical and immunological parameters.

3.24.2 Biochemical parameters

3.24.2.1 Serum protein

Serum protein was estimated by biuret method (Reinhold, 1953) using kit. Proteins present in the plasma binds with copper ions in an alkaline medium of the biuret reagent and produce a purple colored complex, whose observance is proportional to the protein concentration. Three test tubes labelled as Blank (B), Standard (S) and Test (T) were taken. Into all the tubes, 1ml of biuret reagent and 2ml of distilled water were added. 0.05 ml of protein standard was taken in the test tube labelled as standard and 0.05ml of plasma was added into the test tube labelled as a test. It was then mixed well and incubated at 37°C for 10 min. The absorbance of standard (S) and test (T) were measured against the blank (B) in a spectrophotometer at 630 nm. The calculation was done as follows:

$$\text{Total proteins (g/dl)} = \frac{\text{Absorbance of test (T)}}{\text{Absorbance of standard (S)}} \times \text{C}$$

Where, C= concentration of standards (6 g dl^{-1})

3.24.2.2 Albumin

Albumin was estimated by Bromocresol green binding method (Doumas *et al.*, 1971). Albumin in a buffered medium binds with Bromocresol

Green (BCG) and produces a green color whose observance is proportional to the albumin concentration. Three test tubes labelled as Blank (B), Standard (S) and Test (T) were taken. Into all the tubes, 1ml of the buffered dye reagent and 2ml of distilled water were added. 0.01ml of albumin standard was taken in the test tube labelled as standard and 0.01ml of plasma was added into the test tube labelled as a test. It was then mixed well and incubated at 37°C for 10 min. The absorbance of standard (S) and test (T) were measured immediately against blank (B) in a spectrophotometer at 630nm. The calculation was done as follows:

$$\text{Albumin (g/dl)} = \frac{\text{Absorbance of test (T)}}{\text{Absorbance of standard (S)}} \times \text{C}$$

Where, C= concentration of standards (3.6 g dl^{-1})

3.24.2.3 Globulin

Globulin was calculated by subtracting albumin values from total plasma protein.

$$\text{Globulins (g dl}^{-1}\text{) = Total protein (g dl}^{-1}\text{) - Albumin (g dl}^{-1}\text{)}$$

3.24.2.4 Albumin globulin ratio (A:G ratio)

A/G ratio was calculated by dividing albumin values by globulin values

$$\text{A: G ratio} = \frac{\text{Albumin (g/dl)}}{\text{Globulin (g/dl)}}$$

3.24.3 Biochemical test

Serum sample was used for the determination of alanine aminotransferase (ALT/SGPT) and aspartate aminotransferase (AST/SGOT) using a diagnostic kits (Erba® Diagnostic Mannheim, Transasia Biomedicals Ltd, Solan, HP, India).

3.24.4 Immuno-hematological parameters

3.24.4.1 Serum glucose

Serum was estimated by the method of Nelson (1944). Five hundred microliter of blood samples was deproteinised by mixing with 4.75ml of zinc sulfate followed by the addition of 4.75ml of barium hydroxide. The solution was mixed vigorously and filtered using a filter paper and the filtrate was collected in a dry test tube and 1ml of alkaline copper sulfate was added to it. The test tubes were placed in a boiling water bath for 20 min. The test tubes were then cooled to room temperature and 1ml arsenomolybdate reagent was added. The absorbance was recorded at 540nm against blank.

3.24.4.2 Nitro blue tetrazolium (NBT) assay

Nitro blue tetrazolium assay was done by the modified method of Stasiack and Bauman (1996). Blood was collected from the fish by severing the caudal vein in a test tube containing 2.7% EDTA as an anticoagulant. Fifty microlitres of blood was placed in the wells of 'U' bottom microtitre plates and incubated at 37°C for 1 hr to facilitate adhesion of cells. Then the supernatant was removed and the loaded wells were washed three times in PBS. After washing, 50 microlitres of 0.2% NBT was added and was incubated for further 1 hr. The cells were then fixed with 100% methanol for 2-3 min and again washed thrice with 30% methanol. The plates were then air dried. Sixty microlitres 2N KOH and 70 µl dimethyl sulphoxide were added into each well to dissolve the formazon blue precipitate formed. The OD of the turquoise blue coloured solution was then read in ELISA reader (Quant; Bio-Tek Instrument, Winooski, VT, USA).

3.24.5 Haematological parameters

Haematological parameters such as haemoglobin (Hb), red blood cells (RBC), white blood cells (WBC), haematocrit (Hct)/packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were analysed in the laboratory of Suburban Diagnostics, Seven Bunglows, Mumbai, using an

automated blood analyser (Pentra Xl 80, Penra 60c+, Biorad D-10HPLC, Automated Coaglometer).

3.25 Histopathology

The liver and intestine tissue sample of *L. rohita* fingerlings were taken from the treatment groups as well as control group and fixed in neutral buffered formalin fixative for 24 hr and were dehydrated in 90% alcohol for an hour and three times in absolute alcohol for 45 min each, separately. The samples were then cleared two times in xylene for 30 min each and embedded in paraffin for 45 min. The samples were then blocked, allowed to cool, cut on a rotatory microtome at 5 µm and mounted sections were de-waxed in xylene and dehydrated serially in alcohol and then the slides were washed in tap water for 1 min, stained in haematoxylene for 12 min, washed with tap water, dipped in 2% acid alcohol and again washed in tap water. The section were dehydrated through 50%, 70% and 90% alcohol for 2 min each and stained in eosin for 4 min and dipped in absolute alcohol for 1 min. Finally the stained sections were cleared in xylene for 5 min and mounted with DPX. Histological slides were observed under an Olympus FSX-100 phase contrast microscope equipped with a canon EOS 500D digital camera and photographs were taken.

3.26 Statistical Analysis

All the data were statistically analysed using one way analysis of variance by using IBM-SPSS Statistics 22 and the significant differences between mean were determined by using Duncan's multiple range test (DMRT) (Duncan, 1955). The significance level was made at $p<0.05$. All data were expressed as mean± standard error of the mean.

4. RESULTS

Experiment I

4.1 Protein Isolate, Dry Matter and Protein Recoveries

The protein content, recoveries of dry matter (DM) and protein in the protein isolates prepared from defatted castor kernel meal (DCKM) under different pH conditions are given in Table 9. The protein content of the castor seed protein isolate CPI) under different pH conditions ranged between 81.52% and 92.55%. The maximum value of protein was observed in pH 12.0/5.0 followed by 12.0/4.0 and 12.5/5.0, while pH 10.0/4.0 registered the lowest protein content. However, the dry matter and protein recoveries were highest when protein from DCKM were solubilized at pH 12.0 and precipitated at pH 5.0. From the results obtained, the recoveries (DM and protein) were found maximum when precipitated at pH 5.0 in all combinations. The dry matter and protein recoveries values ranged from 31.12% to 49.83% and 43.31% to 78.68%, respectively.

Based on the dry matter and protein recoveries, and protein contents of CPI, the pH combination of 12.0 and 5.0 used for extraction and precipitation of proteins, respectively, exhibited significantly ($P<0.05$) better results than other pH combinations, which may be used for the large scale production of castor seed protein isolates for feed preparation.

Table 9: Protein contents, dry matter (DM) and protein recoveries (%) of protein isolates prepared from defatted castor seed meal under different conditions

Conditions (pH combinations)	Variables		
	Dry matter recovery (%)	Protein recovery (%)	Protein contents of castor protein isolate (%)
10.0/4.0	$33.03^{c}\pm0.14$	$45.93^{b}\pm0.14$	$81.52^{a}\pm0.52$
10.0/4.5	$33.52^{c}\pm0.22$	$46.64^{b}\pm0.22$	$81.56^{a}\pm0.72$
10.0/5.0	$31.86^{b}\pm0.16$	$45.06^{ab}\pm0.16$	$82.91^{ab}\pm2.14$
10.0/5.5	$31.12^{a}\pm0.16$	$43.31^{a}\pm0.16$	$81.58^{a}\pm0.67$
10.5/4.0	$42.75^{de}\pm0.27$	$60.34^{c}\pm0.27$	$82.75^{ab}\pm1.52$
10.5/4.5	$44.45^{ijk}\pm0.34$	$63.49^{efg}\pm0.34$	$83.69^{ab}\pm0.82$
10.5/5.0	$43.68^{fgh}\pm0.23$	$62.99^{defg}\pm0.23$	$84.53^{bcd}\pm1.20$
10.5/5.5	$42.58^{de}\pm0.23$	$60.81^{cd}\pm0.23$	$83.71^{ab}\pm0.66$
11.0/4.0	$43.47^{fg}\pm0.24$	$62.45^{cde}\pm0.24$	$84.22^{abc}\pm0.58$
11.0/4.5	$43.03^{ef}\pm0.15$	$62.70^{def}\pm0.15$	$85.39^{bcde}\pm0.82$
11.0/5.0	$42.73^{de}\pm0.24$	$63.59^{efg}\pm0.24$	$87.22^{defg}\pm0.43$
11.0/5.5	$42.60^{de}\pm0.18$	$61.99^{cde}\pm0.18$	$85.27^{bcde}\pm0.38$
11.5/4.0	$46.08^{l}\pm0.25$	$68.10^{ij}\pm0.25$	$86.61^{cdef}\pm1.13$
11.5/4.5	$44.58^{jk}\pm0.08$	$66.60^{hi}\pm0.08$	$87.56^{efgh}\pm0.36$
11.5/5.0	$45.09^{k}\pm0.16$	$68.96^{j}\pm0.16$	$89.63^{gh}\pm0.74$
11.5/5.5	$43.77^{ghi}\pm0.20$	$65.14^{gh}\pm0.20$	$87.22^{defg}\pm0.64$
12.0/4.0	$48.69^{n}\pm0.18$	$74.04^{lm}\pm0.18$	$89.11^{fgh}\pm0.80$
12.0/4.5	$48.94^{n}\pm0.22$	$75.49^{m}\pm0.22$	$90.41^{hi}\pm0.54$
12.0/5.0	$49.83^{o}\pm0.32$	$78.68^{n}\pm0.32$	$92.55^{i}\pm0.31$
12.0/5.5	$47.70^{m}\pm0.10$	$73.31^{l}\pm0.10$	$90.08^{ghi}\pm0.36$
12.5/4.0	$47.49^{m}\pm0.29$	$71.09^{k}\pm0.29$	$87.74^{efgh}\pm0.61$
12.5/4.5	$44.28^{hij}\pm0.06$	$67.38^{ij}\pm0.06$	$89.18^{fgh}\pm0.89$
12.5/5.0	$42.15^{d}\pm0.49$	$64.85^{fgh}\pm0.49$	$90.15^{hi}\pm0.83$
12.5/5.5	$43.76^{ghi}\pm0.13$	$66.69^{hi}\pm0.13$	$89.32^{fgh}\pm0.82$
***P*-value**	0.000	0.000	0.000

Data expressed as Mean ± SE n=3; Mean values in the same column with different superscript differ significantly ($P<0.05$)

4.2 Chemical Composition (Dry Weight Basis) and anti-nutritional factors of Defatted Castor Kernel Meal (DCKM) and Castor Seed Protein Isolate (CPI)

The proximate composition of defatted castor kernel meal (DCKM) and protein isolate prepared from defatted castor kernel meal (CPI) are given in Table 10. The CKM contained 4.85% moisture, 26.02% crude protein, 56.35% ether extract, 3.15% crude fibre, 11.52% nitrogen free extract and 2.96% total ash. The DCKM contained 8.51% moisture, 58.67% crude protein, 3.00% ether extract, 6.28% crude fibre, 25.98% nitrogen free extract and 6.13% total ash. While, CPI contained 1.47% moisture, 92.55% crude protein, 1.96% ether extract, 1.05% crude fibre, 3.45% nitrogen free extract and 1.00% total ash. The nutritional value of CPI has increased in terms of all nutrients compared to DCKM while gross energy value was decreased from 517.4 to 442.3 kcal $100g^{-1}$.

The results of anti-nutritional factors of DCKM and CPI were shown in Table 11. The tannin, phytic acid, hydrogen cyanide and trypsin inhibitor concentration of DCKM were 46.05 mg $100g^{-1}$, 7.75 mg $100g^{-1}$, 145.8 mg kg^{-1}, and 20.29 TI mg^{-1} protein respectively. While, the tannin, phytic acid contents, hydrogen cyanide and trypsin inhibitor concentration of protein isolates were 14.59 mg $100g^{-1}$, 2.12 mg $100g^{-1}$, 24.30 mg kg^{-1}, and 2.32 TI mg^{-1} protein, respectively.

Table 10: Chemical composition (%) of defatted castor kernel meal (DCKM) and castor seed protein isolate (CPI) on dry matter basis

Variables	CKM	DCKM	CPI
Moisture	4.85±0.03	8.51±0.24	1.67±0.07
Crude protein	26.02±0.10	58.61±0.46	92.35±0.31
Ether extract	56.35±0.40	3.00±0.14	1.96±0.08
Crude fibre	3.15±0.0.05	6.28±0.24	1.05±0.04
Nitrogen free extract	11.52±0.43	25.98±0.76	3.45±0.32
Total ash	2.96±0.08	6.13±0.16	1.00±0.06
GE (kcal $100g^{-1}$)	631.42	517.40	442.30

Data expressed as Mean ± SE n=3

Table 11: Anti-nutritional factors in defatted castor kernel meal (DCKM) and castor seed protein isolate (CPI)

Variables	DCKM	CPI	Reduction (%)
Tannin (mg 100g^{-1})	46.05±1.15	14.59±0.58	68.34
Phytic acid (mg 100g^{-1})	7.75±0.01	2.12±0.02	72.65
Hydrogen cyanide (mg HCN kg^{-1})	145.8±3.12	24.30±2.70	83.56
Trypsin inhibitor (TI mg^{-1} protein)	20.29±1.13	2.32±0.10	88.58

Data expressed as Mean ± SE n=3

4.3 Amino Acid Composition of Defatted Castor Kernel Meal and Castor Seed Protein Isolates (CPI)

Results pertaining to amino acid profile of defatted castor kernel meal and castor seed protein isolates (CPI) are given in Table 12. The concentration of essential amino acids of protein isolates prepared from defatted castor kernel meal exhibited an increase (except arginine, phenyl alanine, and methionine), compared to DCKM. Arginine, phenyl alanine and tryptophan exhibited a slight decrease in their concentration of 25.90%, 23.45%, and 10.11%, respectively while methionine exhibited a significant decrease of 71.39%. Among the essential amino acids, arginine was most predominantly present in DCKM, while threonine in CPI. Similarly, highest glycine content was found among the non-essential amino acids in both DCKM and glutamic acid in CPI. The sulphur containing amino acids were found to decrease in protein isolates compared to DCKM.

Table 12: Amino acid content (g/100 g) of defatted castor kernel meal (DCKM) and castor seed protein isolate (CPI)

Amino acids (g $100g^{-1}$)	DCKM	CPI	Change (%)	FAO/WHO reference protein[a]	Soybean Protein isolate[b]
Essential amino acids					
Arginine	5.70	4.22	-25.90	-	6.79
Histidine	3.02	3.03	0.34	1.9	2.44
Isoleucine	3.66	8.84	141.80	2.8	3.65
Leucine	2.69	6.05	124.61	6.6	6.81
Lysine	2.30	2.67	16.29	5.8	5.21
Methionine	2.42	0.69	-71.39	-	1.21
Phenylalanine	1.85	1.42	-23.45	3.4	4.32
Tryptophan	1.40	1.26	-10.11	-	1.04
Threonine	3.19	11.65	264.90	1.1	3.11
Valine	3.56	4.16	16.72	3.5	3.74
Methionine + Cysteine	4.66	3.37	-27.78	2.5	2.19
Phenylalanine + Tyrosine	5.37	6.87	28.11	6.3	7.42
Non-essential amino acids					
Alanine	1.91	2.48	29.93	-	4.09
Glycine	6.60	10.29	55.84	-	4.4
Proline	4.14	8.16	97.27	-	5.02
Serine	1.08	1.21	11.95	-	4.60
Glutamic acid	4.16	13.06	213.54	-	17.49
Glutamine	1.02	1.16	13.68	-	-
Asparagine	1.46	1.26	-13.82	-	-
Aspartic Acid	2.69	2.80	4.27	-	12.28
Cysteine	2.24	2.67	19.30	-	0.98
Tyrosine	3.52	5.46	55.23	-	3.10

[a]FAO/WHO protein quality evaluation (1985); [b]Kumar *et al.* (2011)

4.4 Nutritional Indices of Defatted Castor Kernel Meal and Protein Isolates

Nutritional quality parameters of defatted castor kernel meal and protein isolates were assessed in terms of amino acid score, essential amino acid index (EAAI), predicted biological value (P-BV), nutritional index (NI) and predicted protein efficiency ratio (P-PER) (Table 13). Based on the essential amino acid composition, the protein isolates exhibited higher amino acid score than the kernel meal, thus it revealed that lysine was first limiting amino acid in protein isolates as well as in kernel meal of defatted castor seed. All nutritional indices such as EAAI, P-BV, NI and P-PER were found to be higher in protein isolate of defatted castor seed than defatted castor kernel meal. *In-vitro* digestibility of protein isolates prepared from defatted castor kernel meal was 94.47% and it was higher than defatted castor kernel meal (25.94%).

Table 13: Nutritional indices of defatted castor kernel meals and protein isolates

Variables	DCKM	CPI
In-vitro digestibility	25.94	94.47
First limiting amino acid	Lysine	Lysine
Amino acid score	39.64	46.10
Essential amino acid index	60.44	82.50
Predicted biological value	54.18	78.22
Nutritional index	35.43	72.39
Predicted protein efficiency ratio	0.39	1.70

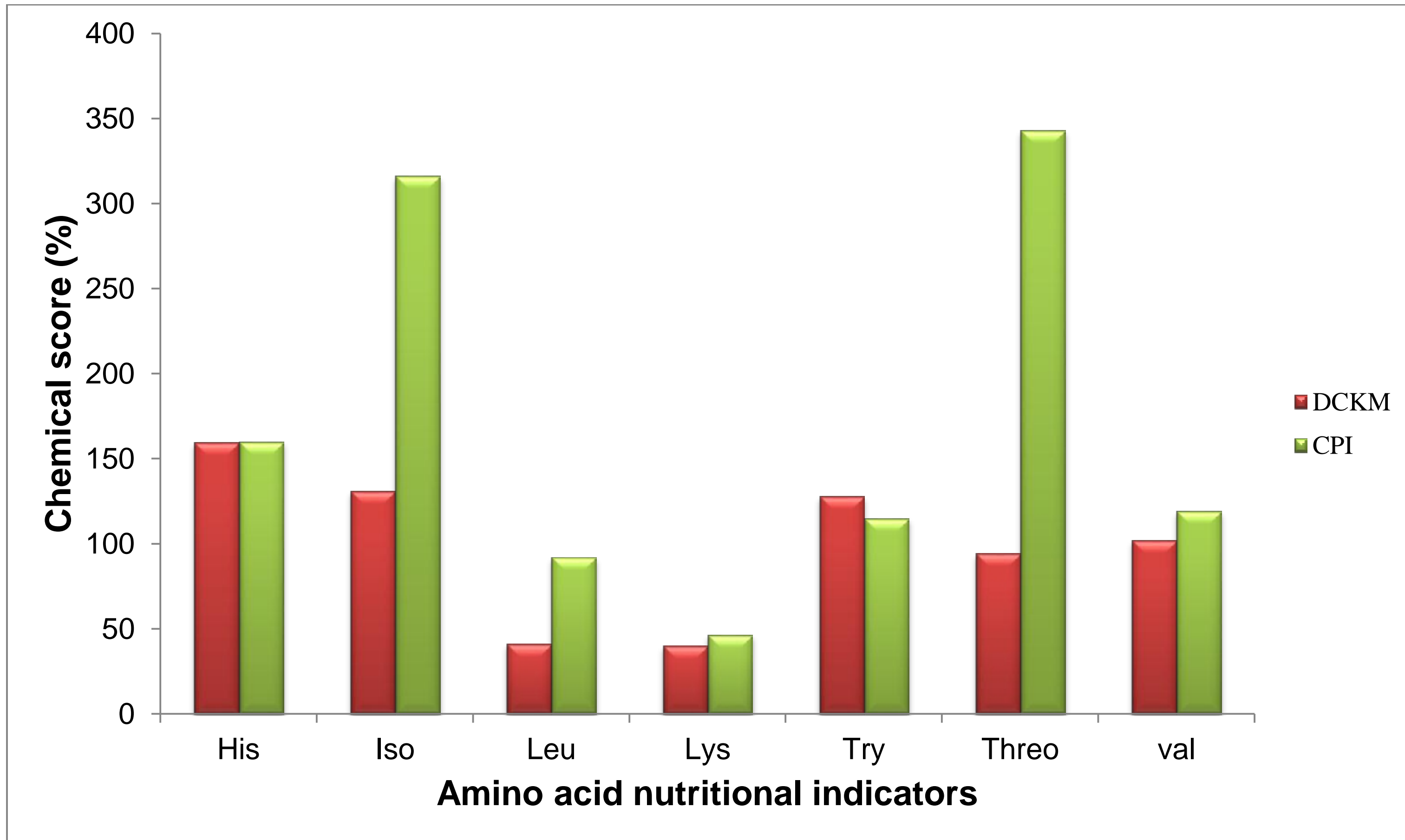

Figure 3: Amino acid score of defatted castor kernel meal and protein and castor seed protein isolate

EXPERIMENT II

The present experiment was carried out to evaluate the nutritional potential of protein isolates prepared from defatted castor kernel meal (CPI) in *L. rohita* fingerlings. Five iso-nitrogenous (34.31±0.04% crude protein) and iso-caloric (421.76± 0.24 kcal 100 g^{-1}) with substitution of soybean protein isolate (SPI) at 0%, 25%, 50%, 75% and 100% levels with CPI were formulated, prepared and fed to *L. rohita* fingerlings twice daily till satiation level for 60 days. Growth performance, survival, nutrient utilization, feed conversion, body indices, digestive enzymes and physio-metabolic changes were measured at the end of experiment and used as response parameters.

4.5 Physico-chemical Parameters of Water

The physico-chemical parameters of water such as temperature, pH, dissolved oxygen, total hardness, ammonia, Nitrite-N, and Nitrate–N were measured and found in the range from 27.2^{0}C to 28.0^{0}C, 7.5 to 8.2, 6.3 to 7.2 mg L^{-1}, 228 - 242 mg L^{-1}, 0.14 to 0.25 mg L^{-1}, 0.001 to 0.005 mg L^{-1}, and 0.02 to 0.06 mg L^{-1}, respectively during the experimental period of 60 days. The free carbon dioxide in water was found to be negligible during the experimental period of 60 days Table 14.

4.6 Proximate Composition of the Experimental Diets

The proximate composition of the experimental diets is given in the Table 15. The moisture content (%) of the experimental diet was recorded in the range of 5.22±0.03 and 5.67±0.07. The crude protein (%) in the diet varied from 34.22±0.11 to 34.42±0.06. The ether extract (%) varied from 6.24±0.05 to 6.33±0.02. The crude fibre (%) in the diets varied from 6.63±0.02 to 6.74±0.04, whereas nitrogen free extract (%) in the experimental diets was found in the range of 44.56±0.06 and 44.90±0.31. The total ash (%) of the diet varied from 7.91±0.05 to 8.13±0.02.

Table 14: Physico-chemical parameters of water during the experimental period of 60 days

Parameters	Control	CPI_{25}	CPI_{50}	CPI_{75}	CPI_{100}
Temperature (^{0} C)	27.2-27.7	27.3-27.5	27.5-27.8	27.4-27.9	27.6-28.0
pH	7.7-8.0	7.5-8.2	7.6-7.9	7.8-8.1	7.5-7.9
DO (mgL^{-1})	6.5-7.2	6.3-7.5	6.5-7.0	6.4-7.2	6.3-6.9
Free CO_2 (mgL^{-1})	ND	ND	ND	ND	ND
Hardness (mgL^{-1})	228-238	233-240	230-241	234-240	231-242
Ammonia-N (mgL^{-1})	0.17-0.21	0.19-0.25	0.14-0.21	0.18-0.23	0.17-0.21
Nitrite-N (mgL^{-1})	0.001-0.002	0.001-0.002	0.002-0.004	0.002-0.003	0.002-0.005
Nitrate-N (mgL^{-1})	0.03-0.04	0.02-0.04	0.02-0.06	0.02-0.05	0.03-0.06

ND-Not detected

Table 15: Proximate composition of the experimental diets (% DM basis)

Variables	Control	CPI_{25}	CPI_{50}	CPI_{75}	CPI_{100}
Moisture (%)	5.46±0.07	5.67±0.01	5.65±0.13	5.22±0.03	5.49±0.04
Crude protein (%)	34.23±0.16	34.22±0.11	34.33±0.01	34.42±0.06	34.36±0.03
Ether extract (%)	6.29±0.02	6.32±0.03	6.33±0.02	6.24±0.05	6.28±0.03
Crude fibre (%)	6.67±0.23	6.74±0.04	6.63±0.02	6.70±0.12	6.66±0.10
Nitrogen free extract (%)	44.90±0.31	44.80±0.06	44.62±0.10	44.61±0.16	44.56±0.06
Total ash (%)	7.91±0.05	7.92±0.10	8.09±0.05	8.03±0.05	8.13±0.02
Gross energy (kcal $100g^{-1}$)	421.7	421.9	421.2	422.6	421.4
Antinutritional factors in the diet					
Hydrogen cyanide (mg HCN kg^{-1})	0.00±0.00	1.07±0.12	2.07±0.23	3.03±0.34	3.95±0.44
Phytic acid (g kg^{-1})	3.20±0.05	3.22±0.06	3.19±0.12	3.28±0.15	3.22±0.13
Tannin (g kg^{-1})	0.90±0.08	0.88±0.08	0.87±0.08	0.89±0.09	0.84±0.04

Data expressed as Mean ± SE n=3.

4.7 Growth Performance and Nutrient Utilization

4.7.1 Body weight

The body weight of the experimental groups was recorded at 15 days interval as shown in the Table 16 and Figure 4. The initial and final average body weight among the treatment groups varied from 3.21±0.03 g to 3.42±0.01 g and 6.22±0.22 g to 6.73±0.15 g, respectively.

4.7.2 Weight gain (%)

The body weight gain was expressed in percentage so as to avoid the initial variation in body weight and is given in Table 17. There was significant difference (P<0.05) in the body weight gain among the different treatment groups at the end of the experimental period. The higher values of weight gain were observed in the control, CPI_{50} and CPI_{75} group and the lowest was in the CPI_{100} group. The weight gain percentage was found to be significantly different (P<0.05) among the various treatment groups. Highest weight gain (%) was recorded in control (108.02±4.05) and CPI_{75} group (106.62±3.55), followed by $CPI_{25,}$ CPI_{50} group and the lowest growth were recorded in CPI_{100} group (86.27±2.70), which was significantly different from the other groups.

4.7.3 Specific growth rate (SGR)

The SGR of the different experimental groups was shown in the Table 17. The mean of SGR of the control (1.22±0.03%) and CPI_{75} group (1.21±0.03%) were significantly higher (P<0.05) than the other groups. The lowest SGR value was found in CPI_{100} group (1.01±0.01), which was significantly different from other groups.

4.7.4 Feed conversion ratio (FCR)

The FCR values of the different experimental groups were shown in the Table 17. The FCR of different experimental groups varied significantly (P<0.05). The feed conversion ratio was recorded significantly lower in control (2.29±0.03) and

CPI_{75} group (2.32±0.03), whereas higher FCR value was found in CPI_{100} fed group (2.88±0.03).

4.7.5 Feed efficiency ratio (FER)

The FER of different treatment groups are given in Table 17. The mean FER values of different treatments varied significantly ($P<0.05$) with the highest value in the control (0.44±0.007) and CPI_{75} group (0.43±0.006), which was significantly different from other groups. The lowest FER value was found in CPI_{100} group (0.34±0.003).

4.7.6 Protein efficiency ratio (PER)

The PER of different treatment groups is given in Table 17. The mean PER value was significantly different ($P<0.05$) among the different treatment groups. The highest PER value was recorded in control (1.28±0.02) and CPI_{75} group (1.25±0.02) followed by $CPI_{25,}$ CPI_{50} groups. The lowest value was found in CPI_{100} group (1.01±0.01), which was significantly different from other groups.

4.7.7 Apparent net protein utilization (ANPU)

The ANPU of different treatment groups is given in Table 17. The mean ANPU value was significantly different ($P<0.05$) among the different treatment groups. The highest ANPU value was recorded in control (35.52±1.39) and CPI_{75} fed group (35.29±0.67) followed by CPI_{50} group. The lowest value was found in CPI_{25} (31.30±1.24) and CPI_{100} (30.27±0.76), which was significantly different from other groups.

Table 16: Body weight (g) of *L. rohita* fingerlings fed with different experimental diets (15 days interval)

Treatments	Initial	15th day	30th day	45th day	60th day
Control	3.21±0.03	3.91±0.04	4.94±0.21	5.56±0.09	6.67±0.10
CPI_{25}	3.40±0.10	4.19±0.19	5.12±0.22	5.81±0.41	6.64±0.24
CPI_{50}	3.42±0.01	4.35±0.12	4.97±0.17	5.71±0.26	6.73±0.15
CPI_{75}	3.23±0.06	4.01±0.10	4.82±0.07	5.63±0.27	6.67±0.13
CPI_{100}	3.40±0.13	4.09±0.14	5.08±0.24	5.49±0.21	6.22±0.22
P-value	0.228	0.215	0.822	0.927	0.251

Data expressed as Mean ± SE n=3.

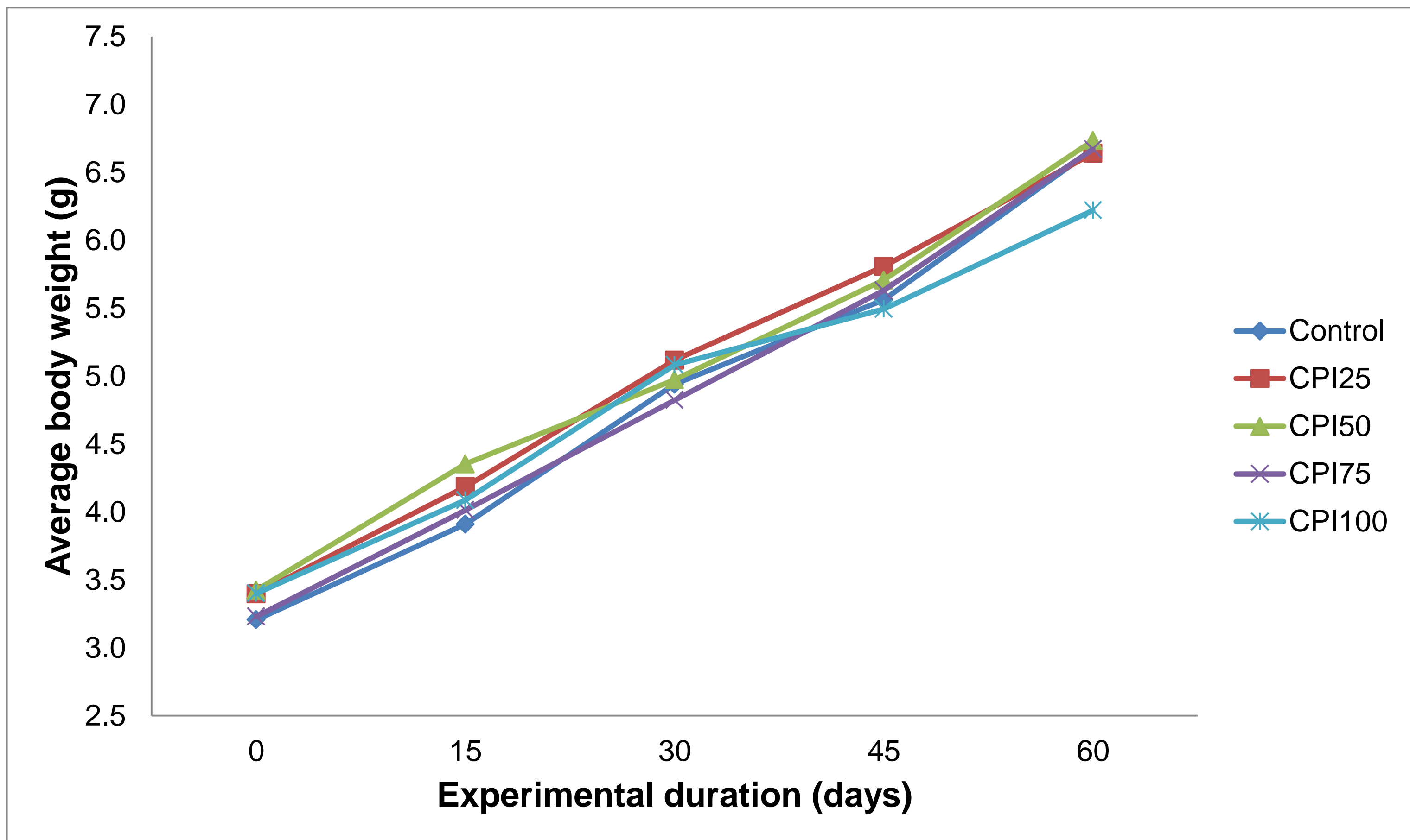

Figure 4: Average body weight (g) of *L. rohita* fingerlings fed with different experimental diets

Table 17: Growth performance, feed conversion and nutrient utilization parameters of *L. rohita* fingerlings fed with different experimental diets

Treatments	Variables						
	Weight gain (g)	WG (%)	SGR (% day^{-1})	FCR	FER	PER	ANPU (%)
Control	3.46^{b}±0.11	108.02^{c}±4.05	1.22^{c}±0.03	2.29^{a}±0.03	0.44^{c}±0.007	1.28^{c}±0.02	35.52^{b}±1.39
CPI_{25}	3.25^{bc}±0.19	95.26^{b}±3.05	1.11^{b}±0.03	2.57^{b}±0.04	0.39^{b}±0.006	1.14^{b}±0.02	31.30^{a}±1.24
CPI_{50}	3.28^{b}±0.16	98.33^{bc}±3.22	1.14^{bc}±0.03	2.47^{b}±0.05	0.41^{b}±0.009	1.18^{b}±0.03	33.03^{ab}±1.31
CPI_{75}	3.44^{b}±0.11	106.62^{c}±3.55	1.21^{c}±0.03	2.32^{a}±0.03	0.43^{c}±0.006	1.25^{c}±0.02	35.29^{b}±0.67
CPI_{100}	2.82^{a}±0.10	83.05^{a}±1.53	1.01^{a}±0.01	2.88^{c}±0.03	0.34^{a}±0.003	1.01^{a}±0.01	30.27^{a}±0.76
P-value	0.049	0.002	0.001	0.000	0.000	0.000	0.026

Data expressed as Mean ± SE n=3; Mean values in the same column with different superscript differ significantly ($P<0.05$)

WG- Percentage weight gain; SGR- Specific growth rate; FCR-Feed conversion ratio; FER- Feed efficiency ratio; PER- Protein efficiency ratio; ANPU- Apparent net protein utilization

4.8 Body Indices

4.8.1 Hepatosomatic Index (HSI) and Viscero-somatic Index (VSI)

The HSI (%) of different treatments are given in given in Table 18. The HSI and VSI value of different treatment did not vary significantly (P>0.05) among the different treatments.

4.9 Survival (%)

The survival rate of the fishes in the different experimental groups is given in the Table 18. The survival rate among the experimental groups did not vary significantly (P>0.05). Maximum and minimum survival rates were found in CPI_{50} (97.22±2.78) and CPI_{100} group (86.11±2.78), respectively among the different treatments.

Table 18: Body indices and survival of *L. rohita* fingerlings fed with different experimental diets

Treatments	Variables		
	HSI (%)	VSI (%)	Survival (%)
Control	1.39±0.07	4.09±0.31	91.67±0.00
CPI_{25}	1.55±0.06	3.74±0.33	94.44±5.56
CPI_{50}	1.61±0.08	4.03±0.29	97.22±2.78
CPI_{75}	1.41±0.05	3.45±0.22	94.44±2.78
CPI_{100}	1.44±0.10	3.38±0.21	86.11±2.78
P- value	0.174	0.258	0.239

Data expressed as Mean ± SE n=6.

4.10 Whole Body Composition

Data pertaining to the biochemical composition of *L. rohita* fingerlings in all the experimental treatments in terms of moisture, protein, lipid and total ash is given in Table 19. Whole body composition of *L. rohita* fingerlings *viz*. moisture content, crude protein, ether extract, total carbohydrate and total ash content were found to be independent of the dietary treatments.

The moisture content of the experimental fishes varied from 74.08±0.03 to 75.27±0.03. The observed crude protein (% wet matter basis) of different experimental groups varied from 15.23±0.05 to 15.35±0.03. The ether extract of different groups varied from 3.90±0.09 to 4.05±0.10. The total ash content varied from 4.07±0.07to 4.16±0.13. Total carbohydrate content of the experimental fishes varied from 1.41±0.10 to 2.59±0.20.

Table 19: Whole body composition of *L. rohita* fingerlings fed with different experimental diets (% wet weight basis)

Treatments	Variables				
	Moisture	Crude protein	Ether extract	Total carbohydrate	Total ash
Control	74.44±0.27	15.34±0.03	3.96±0.10	2.16±0.23	4.10±0.04
CPI_{25}	74.40±0.33	15.27±0.02	4.05±0.10	2.19±0.32	4.10±0.09
CPI_{50}	74.94±0.45	15.32±0.06	3.92±0.04	1.66±0.43	4.16±0.13
CPI_{75}	75.27±0.03	15.35±0.03	3.90±0.09	1.41±0.10	4.07±0.07
CPI_{100}	74.08±0.03	15.23±0.05	3.97±0.16	2.59±0.20	4.14±0.06
P-value	0.076	0.266	0.871	0.087	0.952

Data expressed as Mean ± SE n=3.

4.11 Apparent Digestibility Coefficients (ADCs)

The apparent digestibility coefficient values of dry matter and nutrients (protein and lipid) showed no significant variation (P>0.05) among the dietary treatment groups (Table 20). ADC of DM, protein, and lipid values ranges from 68.73% to 70.71%, 79.44% to 84.02%, and 86.16% to 87.54%, respectively.

Table 20: Apparent digestibility coefficients of *L. rohita* fingerlings fed with different experimental diets

Treatments	Apparent digestibility coefficients (ADC)		
	DM	Protein	Ether extract (lipid)
Control	69.99±0.80	84.02±1.37	86.16±1.47
CPI_{25}	70.71±1.46	82.58±1.67	87.54±1.80
CPI_{50}	69.87±1.44	82.14±1.51	85.72±1.58
CPI_{75}	69.63±1.43	83.80±0.52	86.39±1.79
CPI_{100}	68.73±0.92	79.44±1.04	86.16±1.38
P-value	0.853	0.168	0.944

Data expressed as Mean ± SE n=3.

ADC – Apparent digestibility coefficients; DM–Dry matter.

4.12 Digestive Enzyme Activities

The specific activities of protease, amylase, and lipase in the intestine of the fish of different experimental groups are shown in the Table 21. Digestive enzyme activities in intestine of *L. rohita* fingerlings did not affect significantly by dietary CPI (P>0.05).

Table 21: Digestive enzyme activities of *L. rohita* fingerlings fed with different experimental diets

Treatments	Digestive enzymes		
	Protease[1]	Amylase[2]	Lipase[3]
Control	0.14±0.003	3.56±0.13	0.18±0.003
CPI_{25}	0.13±0.009	3.33±0.26	0.18±0.007
CPI_{50}	0.13±0.007	2.64±0.20	0.19±0.008
CPI_{75}	0.14±0.002	3.21±0.07	0.21±0.008
CPI_{100}	0.13±0.003	2.95±0.29	0.21±0.023
P-value	0.728	0.078	0.225

Data expressed as Mean ± SE n=6.

[1]Protease activity expressed as micromole of tyrosine released/ min/mg protein

[2]Amylase as micromol of maltose released/min/mg protein

[3]Lipase as units/mg protein

4.13 Metabolic Enzymes

4.13.1 Aspartate aminotransferase (AST) activity

The AST activity of *L. rohita* fingerlings fed with different experimental diet is shown in Table 22**.** The activity of enzyme in liver and muscle varied significantly ($P<0.05$). In liver, higher activity of the enzyme was recorded in control and CPI_{75} group than the other dietary treatments, whereas the lowest activity was observed in CPI_{25} group. In the muscle, higher activity ($P<0.05$) was recorded in control and CPI_{75} group than the other dietary treatments, whereas the lowest activity was observed in CPI_{25} and CPI_{100} groups.

4.13.2 Alanine aminotransferase (ALT) Activity

The ALT activity of *L. rohita* fingerlings fed with different experimental diet is shown in Table 22. The activity of enzyme in liver and muscle varied significantly (P<0.05) among the different treatment groups. In the liver, significantly higher value was recorded in CPI_{25} and CPI_{100} group and the lowest value in control and CPI_{75} groups. In the muscle, the highest activity was found in control and CPI_{75} groups followed by CPI_{50} and lowest activity in CPI_{100} group, which was significantly (P<0.05) different from the other groups.

4.13.3 Lactate dehydrogenase (LDH)

The lactate dehydrogenase activity in the liver and muscle of *L. rohita* fingerlings of the different experimental groups are presented in the Table 22. Significant difference (P<0.05) of LDH activity in liver was recorded among the various treatment groups. In the liver, highest activity was found in the CPI_{100} and lowest activity in control and CPI_{75} groups, which was significantly different among the different groups, whereas LDH activity in muscle did not vary (P>0.05) among the various treatment groups.

4.13.4 Malate dehydrogenase (MDH)

The malate dehydrogenase activity in the liver and muscle of *L. rohita* fingerlings of the different experimental groups are presented in the Table 22. MDH activity in liver and muscle did not vary significantly (P>0.05) among the various treatment groups.

Table 22: Metabolic enzymes, aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH) and Malate dehydrogenase (MDH) activities of *L. rohita* fingerlings fed with different experimental diets

Treatments	Variables							
	[1]AST		[2]ALT		[3]LDH		[4]MDH	
	Liver	Muscle	Liver	Muscle	Liver	Muscle	Liver	Muscle
Control	$16.37^{c}\pm0.64$	$9.65^{c}\pm0.21$	$2.60^{a}\pm0.12$	3.62±0.11	$2.60^{a}\pm0.12$	3.62±0.11	4.89±0.04	3.84±0.16
CPI_{25}	$13.78^{a}\pm0.30$	$7.08^{a}\pm0.30$	$3.81^{c}\pm0.13$	3.87±0.11	$3.81^{c}\pm0.13$	3.87±0.11	4.77±0.14	4.09±0.17
CPI_{50}	$15.95^{bc}\pm0.32$	$8.81^{b}\pm0.27$	$3.20^{b}\pm0.11$	3.75±0.18	$3.20^{b}\pm0.11$	3.75±0.18	4.80±0.07	3.85±0.10
CPI_{75}	$16.50^{c}\pm0.42$	$9.69^{c}\pm0.18$	$2.70^{a}\pm0.18$	3.78±0.19	$2.70^{a}\pm0.18$	3.78±0.19	4.86±0.10	4.08±0.22
CPI_{100}	$14.90^{ab}\pm0.46$	$7.68^{a}\pm0.20$	$4.34^{d}\pm0.12$	3.82±0.09	$4.34^{d}\pm0.12$	3.82±0.09	4.71±0.09	4.25±0.18
P-value	0.007	0.000	0.000	0.787	0.000	0.787	0.675	0.432

Data expressed as Mean ± SE n=6; Mean values in the same column with different superscript differ significantly (P<0.05)

[1]ALT: specific activities expressed as Nano moles of sodium pyruvate formed/mg protein/minute at 37^{0}C.

[2]AST specific activities expressed as Nano moles of oxaloacetate released/min/mg protein at 37^{0}C.

[3]LDH: specific activity expressed as Units/ min/ mg protein at 37^{0}C.

[4]MDH: specific activity expressed as Units/ min/ mg protein at 37^{0}C.

4.14 Antioxidant Enzymes Activities

4.14.1 Superoxide dismutase (SOD) and catalase (CAT)

The superoxide dismutase and catalase activities in the liver and gills of *L. rohita* fingerlings of the different experimental groups are presented in the Table 23. The SOD enzyme activity in liver was affected by dietary treatments (P<0.05). Significantly lower SOD activity in CPI_{25}, CPI_{50} and CPI_{75} were observed than CPI_{100} group but not with the control group. While, SOD activity in gills did not vary significantly (P>0.05) among the different dietary treatment groups. Dietary CPI did not affect the catalase activity in liver and gills of *L. rohita* fingerlings (P>0.05) among the different treatment groups.

Table 23: Superoxide dismutase (SOD) and catalase (CAT) activities of *L. rohita* fingerlings fed with different experimental diets

Treatments	Anti-oxidant enzymes			
	[1]SOD		[2]Catalase	
	Liver	Gill	Liver	Gill
Control	3.20^{a}±0.08	4.45±0.31	2.86±0.12	3.01±0.19
CPI_{25}	3.28^{a}±0.07	4.47±0.21	2.47±0.22	2.51±0.06
CPI_{50}	3.34^{a}±0.10	5.08±0.27	2.49±0.21	2.59±0.13
CPI_{75}	3.36^{a}±0.13	4.53±0.32	2.25±0.15	2.28±0.16
CPI_{100}	3.67^{b}±0.05	5.01±0.22	2.14±0.16	2.58±0.21
P-value	0.040	0.320	0.112	0.083

Data expressed as Mean ± SE n=6; Mean values in the same column with different superscript differ significantly (P<0.05)

[1]SOD (superoxide dismutase) activity is expressed as 50% inhibition of epinephrine auto-oxidation/mg protein/min.

[2]CAT (catalase) activity is expressed as nanomoles of H_2O_2 decomposed/min/mg protein.

4.15 Biochemical and Haemato-Immunological Parameters

4.15.1 Serum protein biochemistry

The serum total protein, albumin, globulin and albumin globulin ratio of *L. rohita* fingerlings fed different experimental diets are given in Table 24. The CPI_{25}, and CPI_{75} fed groups exhibited higher concentration of total protein than the other groups and total protein concentration was affected by dietary treatment groups ($P<0.05$). While, other indices such as albumin, globulin and albumin globulin ratio were not affected ($P>0.05$) by dietary treatment groups.

4.15.2 Hemato-immunological parameters

The results of effect of different diets on serum glucose, alanine transaminase, aspartate transaminase, and NBT in *L. rohita* fingerlings are presented in Table 25. Serum glucose concentration and aspartate aminotransferase activity in rohu fingerlings did not vary with the different dietary treatments ($P>0.05$). Dietary CPI affects the serum alanine aminotransferase activity in *L. rohita* fingerlings ($P<0.05$) and maximum activity was observed in CPI_{100} group.

4.15.3 Nitroblue tetrazolium (NBT) assay

Respiratory burst activity of the phagocytes (NBT assay) in *L. rohita* fingerlings is given in Table 25. The activity was significantly ($P<0.05$) different among the groups and shown to increase with the dietary CPI level of inclusion in the feed and maximum activity was observed in CPI_{100} group.

4.16 Haematological Parameters

The haematological parameters of *L. rohita* fingerlings fed with different experimental diets are given in Table 26. There was a significant difference ($P<0.05$) in total erythrocyte counts (RBC) and haemoglobin concentration in *L. rohita* fingerlings fed with different experimental diets. Highest RBC count was observed in CPI_{75} group, while CPI_{25}, CPI_{50}, and CPI_{100} groups did not show any variation with the

control group (P>0.05). Maximum and minimum haemoglobin concentration was observed in CPI_{75} and CPI_{25} groups, respectively. Total leucocyte count (WBC), haematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) values did not differ significantly (P>0.05) among dietary treatment groups.

Table 24: Effects of different experimental diets on the serum protein biochemistry of *L. rohita* fingerlings

Treatments	Serum parameters (g dl^{-1})			
	Total protein	Albumin	Globulin	A:G Ratio
Control	2.34^{a}±0.03	1.14±0.02	1.21±0.04	0.95±0.052
CPI_{25}	2.48^{b}±0.03	1.15±0.03	1.33±0.05	0.87±0.049
CPI_{50}	2.43^{a}±0.03	1.13±0.01	1.30±0.02	0.87±0.006
CPI_{75}	2.32^{a}±0.02	1.15±0.02	1.17±0.03	0.99±0.038
CPI_{100}	2.52^{b}±0.05	1.19±0.02	1.33±0.05	0.89±0.044
P-value	0.008	0.315	0.057	0.256

Data expressed as Mean ± SE n=6; Mean values in the same column with different superscript differ significantly (P<0.05)

Table 25: Effect of different diets on serum glucose, alanine transaminase, aspartate transaminase, and NBT in *L. rohita* fingerlings

Treatments	Serum parameters			
	Glucose (mg dl^{-1})	SGOT (U L^{-1})	SGPT (U L^{-1})	NBT
Control	82.48±1.42	9.29±0.47	17.62^{b}±0.31	0.15^{ab}±0.006
CPI_{25}	85.37±1.71	9.64±0.62	14.05^{a}±0.63	0.13^{a}±0.006
CPI_{50}	83.59±1.63	9.46±0.47	15.24^{a}±0.63	0.14^{ab}±0.009
CPI_{75}	81.89±0.45	9.29±0.48	17.98^{b}±0.31	0.16^{c}±0.008
CPI_{100}	85.37±1.42	8.93±0.47	23.93^{c}±0.82	0.18^{d}±0.008
P-value	0.327	0.856	0.000	0.001

Data expressed as Mean ± SE n=6; Mean values in the same column with different superscript differ significantly (P<0.05).

Table 26: Hematological parameters of *L. rohita* fingerlings fed with different experimental diets

Treatments	Hematological parameters						
	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	$6.77^{bc}\pm0.13$	$1.86^{a}\pm0.08$	204.00±6.35	23.73±1.53	127.38±6.44	36.43±1.62	28.69±1.38
CPI_{25}	$6.37^{ab}\pm0.09$	$1.89^{ab}\pm0.05$	188.67±9.29	21.57±1.28	113.72±4.10	33.65±0.67	29.70±1.49
CPI_{50}	$6.47^{bc}\pm0.28$	$1.76^{a}\pm0.06$	191.73±12.94	22.93±1.79	130.19±6.33	36.80±0.68	28.36±1.05
CPI_{75}	$6.93^{c}\pm0.09$	$2.09^{b}\pm0.09$	205.60±5.77	24.60±0.70	117.91±2.68	33.27±1.17	28.21±0.44
CPI_{100}	$5.95^{a}\pm0.09$	$1.80^{a}\pm0.03$	173.60±7.39	20.80±1.15	116.11±8.12	33.18±0.96	28.74±1.18
P-value	0.010	0.035	0.141	0.323	0.263	0.088	0.905

Data expressed as Mean ± SE n=3; Mean values in the same column with different superscript differ significantly ($P<0.05$)

Hb- Hemoglobin; RBC- Red blood cells; WBC-white blood cells; Hct- Hematocrit values; MCV; Mean cell volume; MCH-mean cell hemoglobin; MCHC- Mean cell hemoglobin concentration

4.17 Histopathology

4.17.1 Liver

The histological examination of the liver revealed polygonal hepatocytes and prominent nuclei and nucleoli, and granular cytoplasm in the control group (Plate 10). CPI25, CPI50, CPI75 and CPI100 did not show any discernible changes compared to the control and no significant changes were observed in dietary treatments and control groups (Plate 11-14).

4.17.2 Intestine

The histological examination of the intestine exhibited cellular changes in all dietary treatment and control groups (Plate 15-19). Histo-intestinal architecture of *L. rohita* fingerlings revealed the gradual increase in the cellular changes with the increased dietary levels of CPI across the treatments. The noticeable changes were increased fusion of mucosal folds, reduced number of supranuclear absorptive vacuoles in enterocytes and increased width and intracellular infiltration in the lamina propria of intestine.

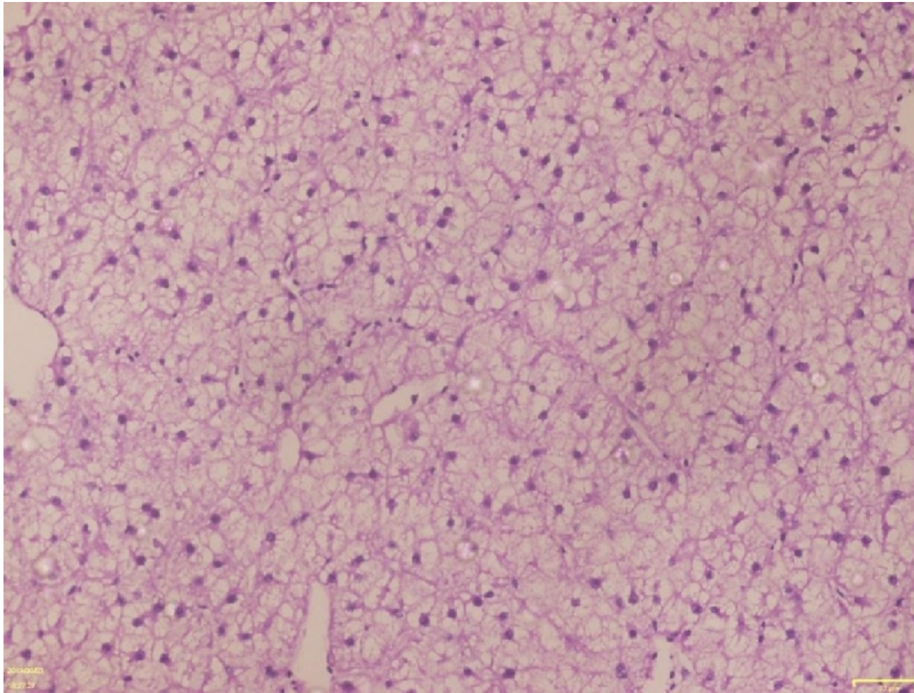

Plate 10: Histological section of liver in the control group. (Scale bar = 32 µm, 20X)

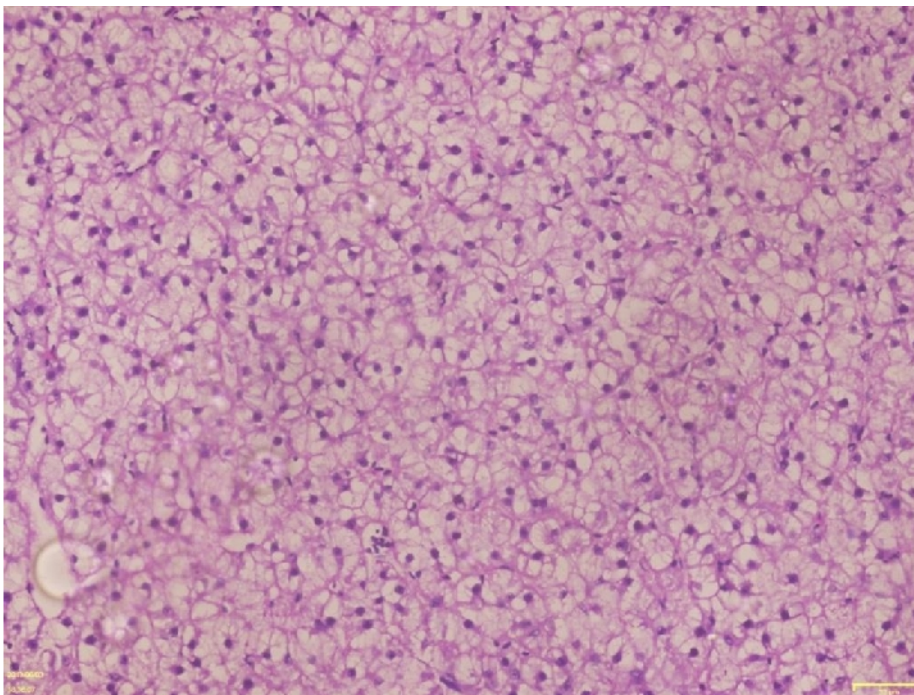

Plate 11: Histological section of liver of CPI_{25} group. No discernable change was noticed. (Scale bar = 32 µm, 20X)

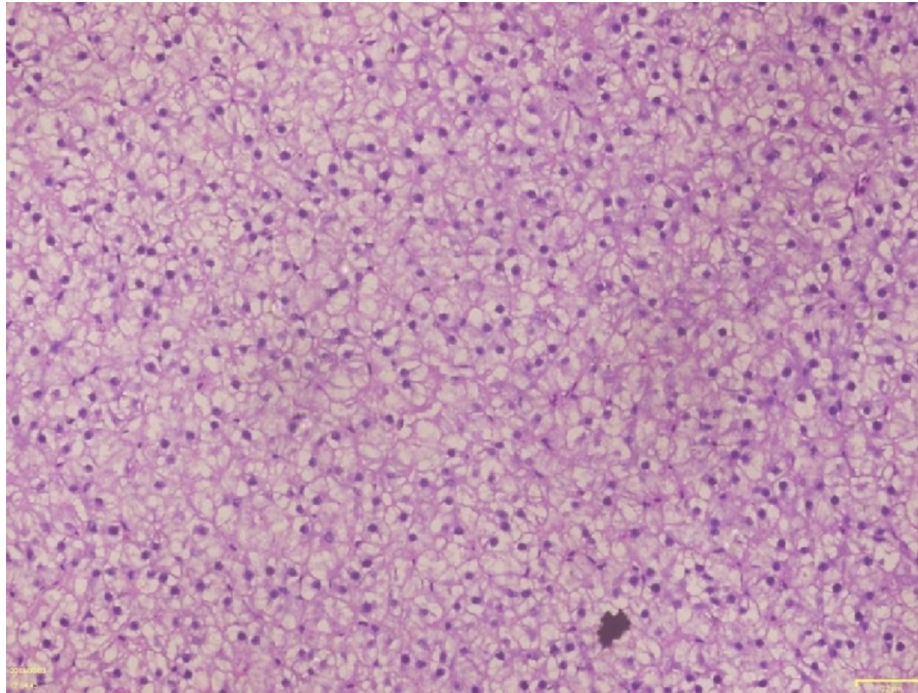

Plate 12: Histological section of liver of CPI_{50} group. No discernable change was noticed. (Scale bar = 32 µm, 20X)

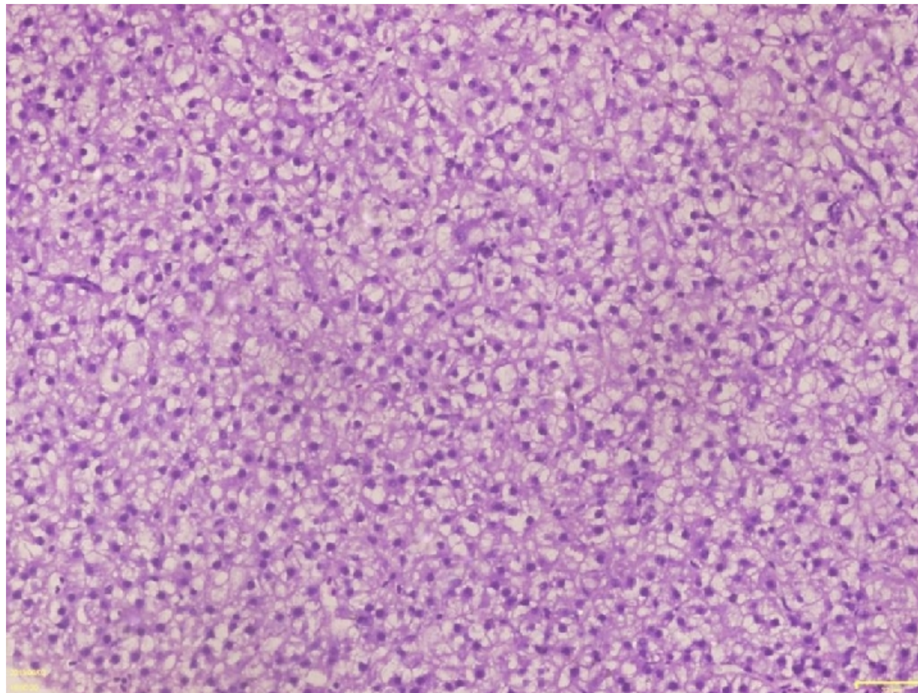

Plate 13: Histological section of liver of CPI_{75} group. No discernable change was noticed. (Scale bar = 32 µm, 20X)

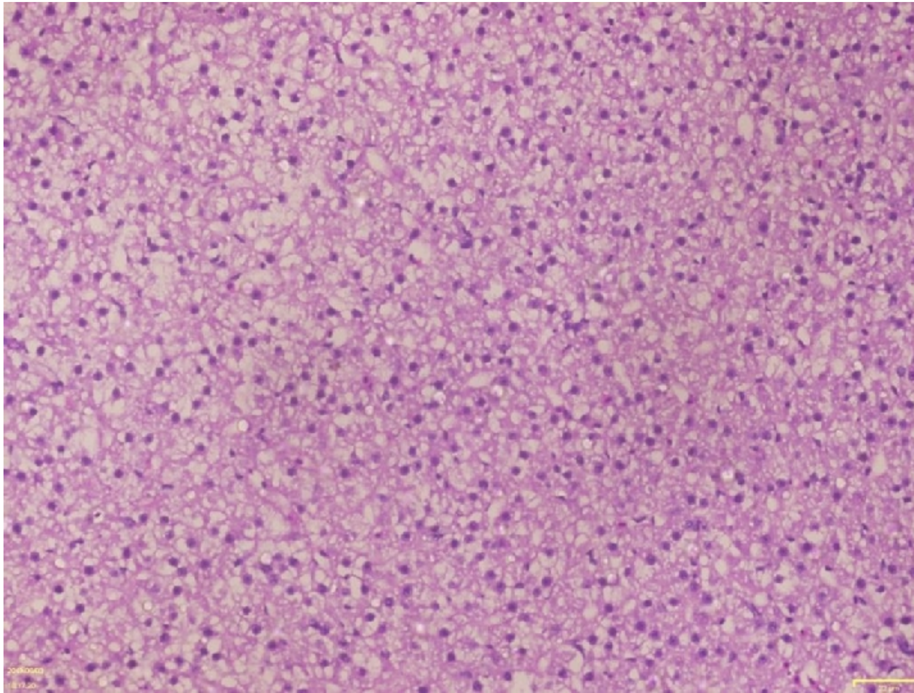

Plate 14: Histological section of liver in CPI_{100} fed fish showing no marked significant change. (Scale bar = 32 µm, 20X)

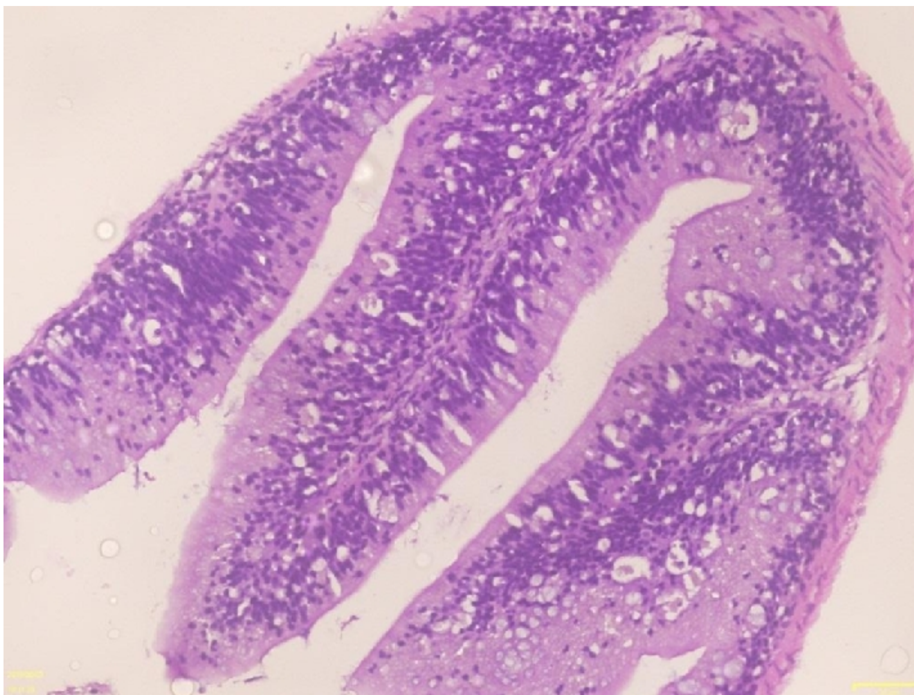

Plate 15: Histological section of intestine of normal fish (Control). (Scale bar = 32 µm, 20X)

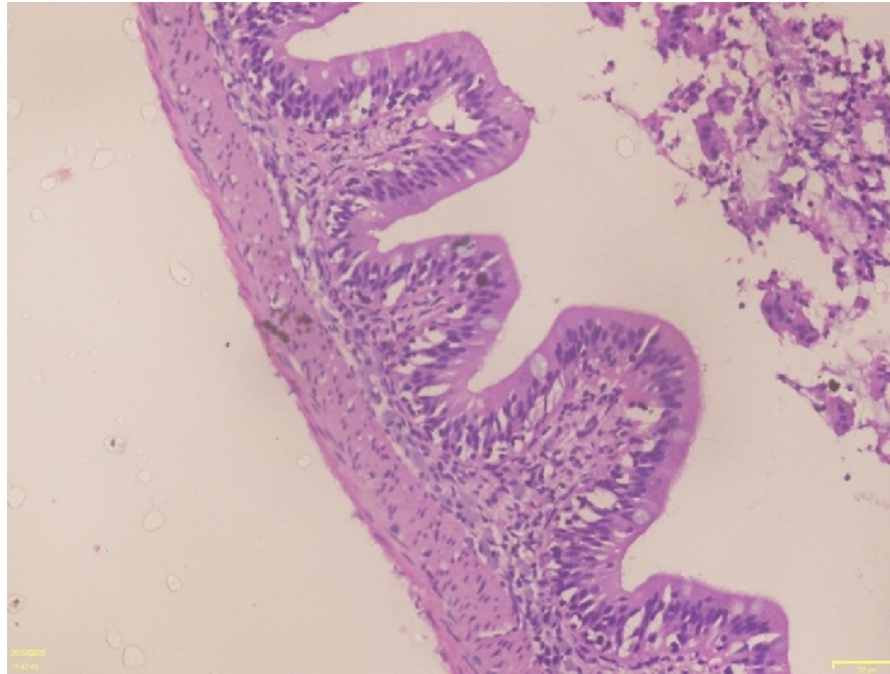

Plate 16: Histological section of intestine of CPI_{25} group revealed normal histo-architecture. (Scale bar = 32 µm, 20X)

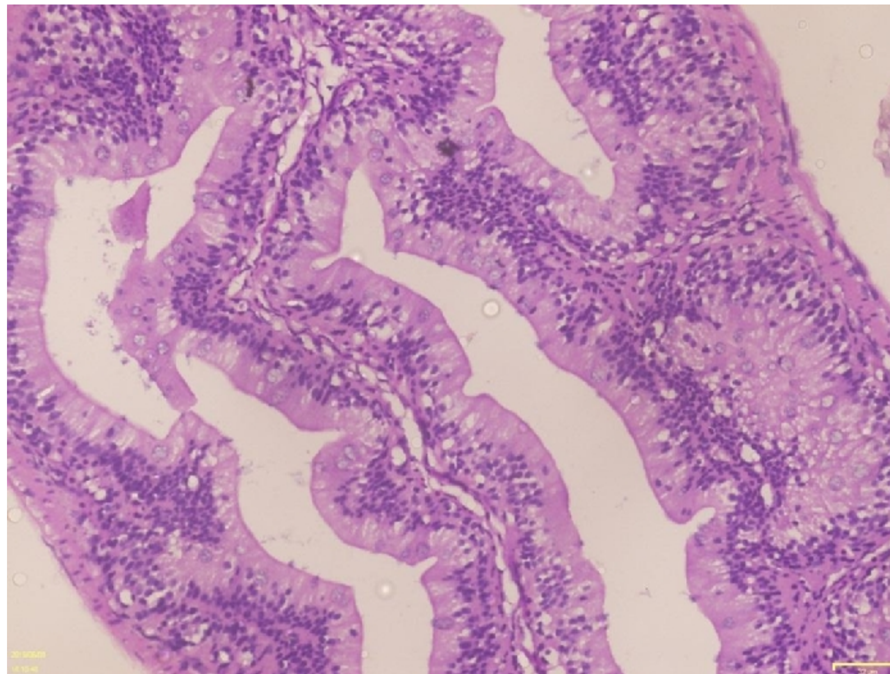

Plate 17: CPI_{50} showed normal histo-architecture. (Scale bar = 32 µm, 20X)

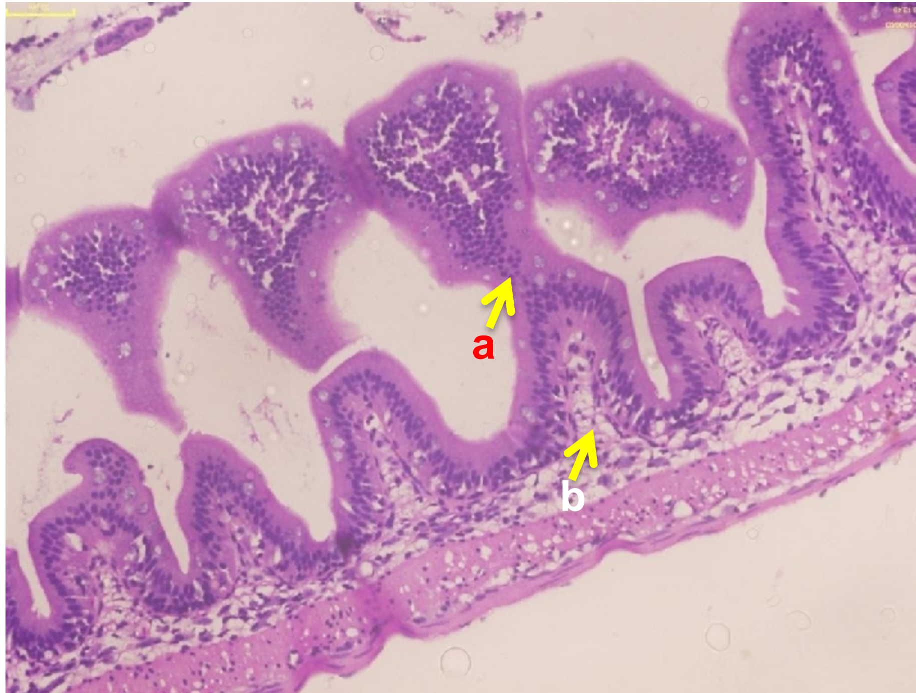

Plate 18: CPI_{75} showed fused mucosal folds (a) and intracellular filtration in lamina propria (b) in intestine. (Scale bar = 32 µm, 20X)

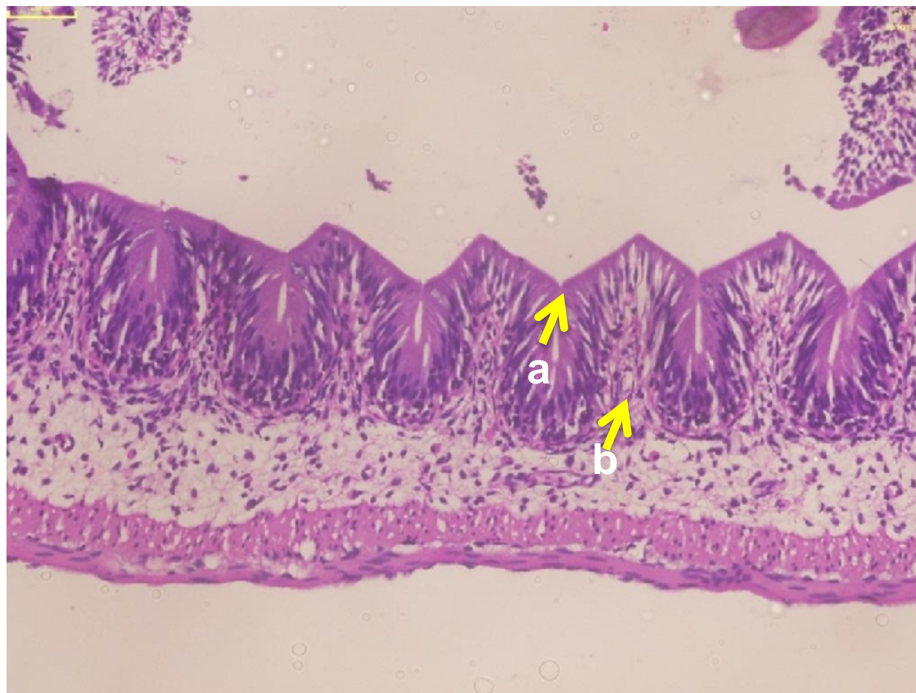

Plate 19: CPI_{100} showed fusion of mucosal folds (a) and intracellular filtration in lamina propria (b). (Scale bar = 32 µm, 20X)

Experiment III

The present experiment was carried out to evaluate the nutritional potential of protein isolates prepared from defatted castor kernel meal (CPI) supplemented with deficient L-lysine and DL-methionine in *L. rohita* fingerlings. In experiment II, amino acid profile indicated that L-lysine and DL-methionine was deficient in all experimental diets (Table 5). The amino acid requirement of *L. rohita* fingerlings was fulfilled by supplementing the deficient amino acids as suggested by NRC (2011). Five iso-nitrogenous (35.45±0.14% crude protein) and isocaloric (421.82±0.16 kcal $100g^{-1}$) experimental diets with substitution of soybean protein isolate (SPI) at 0%, 25%, 50%, 75% and 100% levels with CPI were formulated, prepared and fed to *L. rohita* fingerlings twice daily till satiation level for 60 days. Growth performance, survival, nutrient utilization, feed conversion, body indices, digestive enzymes and physio-metabolic changes were measured at the end of experiment and used as response parameters.

4.18 Physico-Chemical Parameters of Water

The physico-chemical parameters of water such as temperature (^{0} C), pH, dissolved oxygen, total hardness, ammonia, Nitrite - N, and Nitrate - N were recorded during the experimental period of 60 days and these parameter varied in the range of 28.4 ^{0} C to 29.3 ^{0} C, 7.4 to 8.1, 6.2 to 7.2 mg L^{-1}, 231 - 244 mg L^{-1}, 0.15 to 0.23 mg L^{-1}, 0.001 to 0.005 mg L^{-1}, and 0.02 to 0.07 mg L^{-1}, respectively. The free carbon dioxide in water was found to be negligible during the whole experimental period of 60 days (Table 27).

Table 27: Physico-chemical parameters of water during the experimental period of 60 days for different experimental groups

Parameters	Control	$ACPI_{25}$	$ACPI_{50}$	$ACPI_{75}$	$ACPI_{100}$
Temperature (^0C)	28.5-29.1	28.4-29.0	28.5-29.1	28.3-29.0	28.6-29.1
pH	7.5-8.1	7.4-8.0	7.4-7.9	7.6-8.1	7.4-7.9
DO (mgL^{-1})	6.3-7.1	6.4-7.2	6.2-7.0	6.2-7.1	6.3-7.0
Free CO_2 (mgL^{-1})	ND	ND	ND	ND	ND
Hardness (mgL^{-1})	231-242	234-244	236-240	233-240	232-239
Ammonia-N (mgL^{-1})	0.15-0.20	0.16-0.23	0.15-0.21	0.17-0.22	0.15-0.22
Nitrite-N (mgL^{-1})	0.001-0.003	0.002-0.003	0.001-0.004	0.001-0.005	0.002-0.005
Nitrate-N (mgL^{-1})	0.03-0.07	0.02-0.06	0.03-0.07	0.03-0.05	0.02-0.06

ND-Not detected

4.19 Proximate Composition of the Experimental Diets

The proximate composition of the experimental diets is given in the Table 28. The moisture content (%) of the experimental diet was recorded in the range of 6.92±0.10 and 7.54±0.14. The crude protein (%) in the diet varied from 35.17±0.52 to 35.79±0.30. The ether extract (%) varied from 6.36±0.05 to 6.49±0.03. The crude fibre (%) in the diet varied from 6.54±0.01 to 6.64±0.10, nitrogen free extract was found in the range from 42.92±0.45 to 43.66±0.40. The total ash (%) of the diet varied from 8.06±0.02 to 8.23±0.05.

Table 28: Proximate composition of the experimental diets (% dry weight basis)

Variables	Control	$ACPI_{25}$	$ACPI_{50}$	$ACPI_{75}$	$ACPI_{100}$
Moisture	6.92±0.10	7.17±0.16	7.54±0.14	7.44±0.01	7.53±0.08
Crude protein	35.17±0.52	35.26±0.35	35.46±0.31	35.60±0.24	35.79±0.30
Ether extract	6.36±0.05	6.43±0.03	6.44±0.08	6.38±0.13	6.49±0.03
Crude fibre	6.64±0.10	6.63±0.09	6.54±0.01	6.59±0.03	6.58±0.34
Nitrogen free extract	43.66±0.40	43.48±0.46	43.49±0.22	43.22±0.29	42.92±0.45
Total ash	8.17±0.01	8.18±0.01	8.06±0.02	8.21±0.03	8.23±0.05
Gross energy ($kcal.100g^{-1}$)	421.70	422.10	421.30	422.20	421.80
Antinutritional factors in the diet					
Hydrogen cyanide (mg HCN kg^{-1})	0.00±0.00	1.17±0.13	1.98±0.22	3.04±0.16	4.03±0.19
Phytic acid (g kg^{-1})	3.18±0.09	3.20±0.15	3.19±0.15	3.21±0.18	3.17±0.10
Tannin (g kg^{-1})	1.11±0.03	1.11±0.07	1.06±0.10	1.09±0.09	1.08±0.05

Data expressed as Mean ± SE n=3.

4.20 Growth Performance and Nutrient Utilization

4.20.1 Body weight (g)

The body weight of *L. rohita fingerlings* fed with different experimental diets was recorded at 15 days interval as shown in the Table 29 and Figure 5**.** The initial average body weight among the treatment group varied from 7.13±0.05 to 7.23±0.04 g and the final body weight varied from 13.48±0.07 to 14.56±0.09 g.

4.20.2 Weight gain (%)

The body weight gain expressed in percentage so as to avoid the initial variation in body weight and is given in Table 30. There was significant difference ($P<0.05$) in the body weight gain among different treatment groups at the end of the experimental period. The higher weight gain was observed in the $ACPI_{50}$ and $ACPI_{75}$ groups than others. The weight gain percentage was found to be significantly different ($P<0.05$) among various treatment groups. Higher values were recorded in $ACPI_{50}$ and $ACPI_{75}$ groups than other groups but weight gain percentage in *L. rohita* fingerlings fed with $ACPI_{25}$ and $ACPI_{100}$ was found to be similar ($P>0.05$) with control group.

4.20.3 Specific growth rate (SGR)

The SGR of the different experimental groups was shown in the Table 30. The mean of SGR of the $ACPI_{50}$ (1.17±0.03) and $ACPI_{75}$ groups (1.18±0.01) were significantly higher ($P<0.05$) than the control and other groups. But, SGR values in ACPI25 and ACPI100 were found similar to the control group.

4.20.4 Feed conversion ratio (FCR)

The FCR values of the different experimental groups were shown in the Table 30. The FCR of different experimental groups varied significantly ($P<0.05$). The better feed conversion was observed in *L. rohita* fingerlings of $ACPI_{50}$ and $ACPI_{75}$ groups.

4.20.5 Feed efficiency ratio (FER)

The FER of different treatment groups are given in Table 30. The mean FER values of different treatments varied significantly ($P<0.05$) with the highest in the $ACPI_{50}$ and $ACPI_{75}$ groups which was significantly different from the other groups. The lower FER value was found in control, $ACPI_{25}$ and $ACPI_{100}$ groups.

4.20.6 Protein efficiency ratio (PER)

The PER of different treatment groups are given in Table 30. The mean PER value was significantly different ($P<0.05$) among the different treatment groups. Significantly higher PER value was recorded in *L. rohita* fingerlings fed with $ACPI_{50}$ and $ACPI_{75}$ groups than the other groups.

4.20.7 Apparent net protein utilization (ANPU)

The ANPU of different treatment groups are given in Table 30. The mean ANPU value was significantly different ($P<0.05$) among the different treatment groups. The better protein retention was recorded in $ACPI_{75}$ (29.50±1.05) followed by $ACPI_{25}$ (26.79±1.20) and $ACPI_{50}$ (27.53±0.89) group. The lowest value was found in control (25.04±1.02) and $ACPI_{100}$ (24.70±0.51), which was significantly different from other groups.

Table 29: Body weight (g) of *L. rohita* fingerlings fed with different experimental diets (15 days interval)

Treatments	Initial	0-15 days	15-30 days	30-45 days	45-60 days
Control	7.13±0.05	8.40±0.02	9.89±0.05	11.92±0.06	13.48^{a}±0.07
$ACPI_{25}$	7.15±0.15	8.36±0.07	9.80±0.09	11.93±0.14	13.73^{a}±0.07
$ACPI_{50}$	7.17±0.05	8.34±0.05	9.80±0.08	12.67±0.13	14.51^{b}±0.34
$ACPI_{75}$	7.16±0.05	8.26±0.04	9.78±0.16	12.12±0.26	14.56^{b}±0.09
$ACPI_{100}$	7.23±0.04	8.40±0.03	9.57±0.03	11.91±0.23	13.51^{a}±0.22
P-value	0.671	0.415	0.212	0.057	0.004

Data expressed as Mean ± SE n=3; Mean values in the same column with different superscript differ significantly ($P<0.05$)

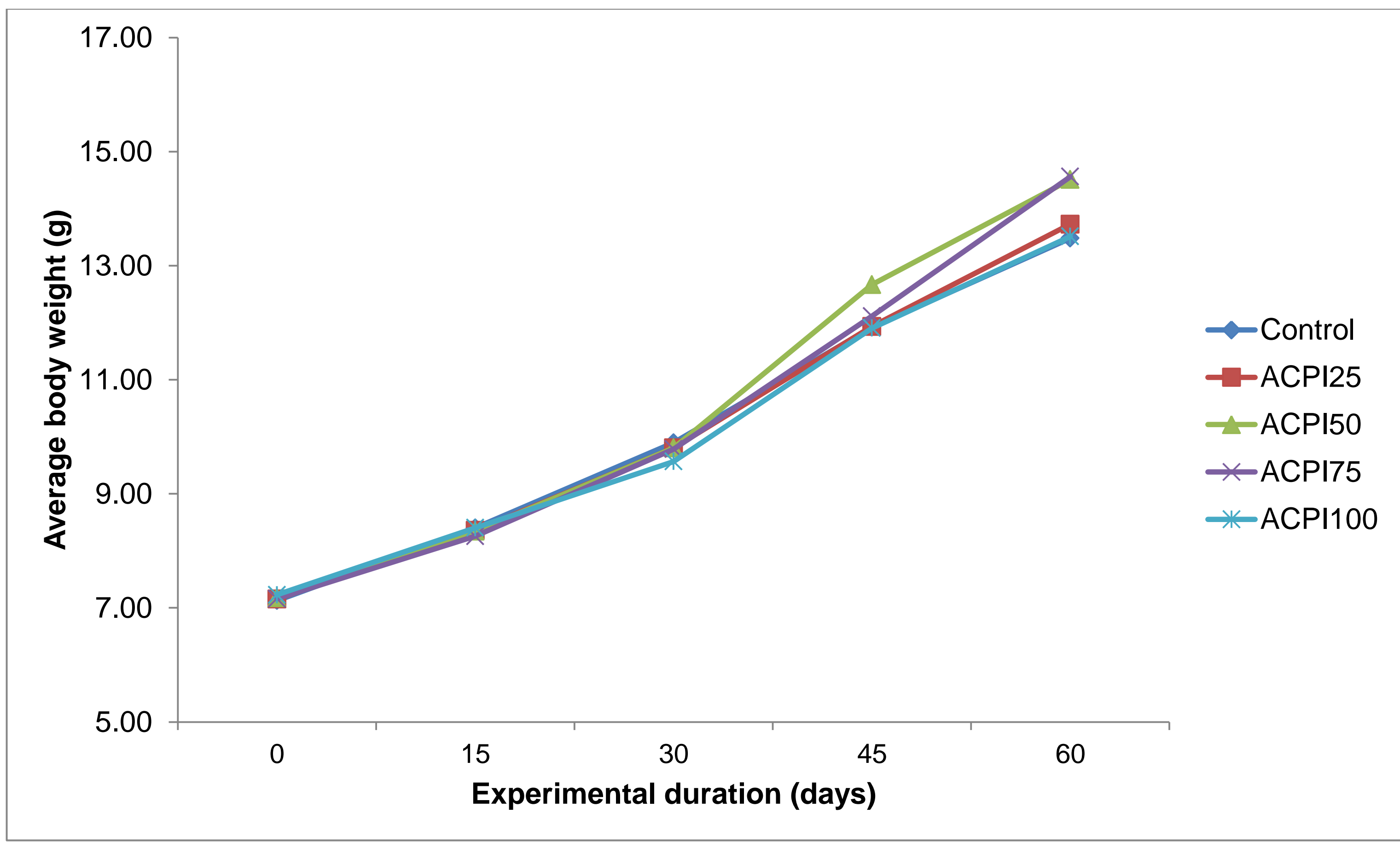

Figure 5: Body weight gain (g) of *L. rohita* fingerlings fed with different experimental diets

Table 30: Growth performance, feed conversion and nutrient utilization parameters of *L. rohita* fingerlings fed with different experimental diets

Treatments	Variables						
	Weight gain (g)	WG (%)	SGR (% day^{-1})	FCR	FER	PER	ANPU (%)
Control	6.35^{a}±0.06	89.16^{a}±1.25	1.06^{a}±0.01	2.65^{b}±0.03	0.38^{a}±0.003	1.08^{a}±0.01	25.04^{a}±1.02
$ACPI_{25}$	6.57^{a}±0.11	91.90^{a}±2.18	1.08^{a}±0.02	2.55^{b}±0.04	0.39^{a}±0.009	1.11^{a}±0.02	26.79^{ab}±1.20
$ACPI_{50}$	7.34^{b}±0.29	102.23^{b}±3.44	1.17^{b}±0.03	2.34^{a}±0.09	0.43^{b}±0.017	1.21^{b}±0.04	27.53^{ab}±0.89
$ACPI_{75}$	7.40^{b}±0.08	103.38^{b}±1.21	1.18^{b}±0.01	2.27^{a}±0.05	0.44^{b}±0.009	1.24^{b}±0.02	29.50^{b}±1.05
$ACPI_{100}$	6.29^{a}±0.18	89.96^{a}±2.09	1.04^{a}±0.02	2.66^{b}±0.05	0.38^{a}±0.007	1.05^{a}±0.02	24.70^{a}±0.51
P-value	0.001	0.001	0.001	0.002	0.002	0.002	0.031

Data expressed as Mean ± SE n=3; Mean values in the same column with different superscript differ significantly (P<0.05)

WG%- Weight gain (%); SGR- Specific growth rate; FCR-feed conversion ratio; FER- Feed efficiency ratio; PER- Protein efficiency ratio; ANPU- apparent net protein utilization

4.21 Body Indices and Survival Rate

4.21.1 Hepatosomatic Index (HSI)

The HSI (%) of different treatments are given in given in Table 31**.** The HSI value of different treatment was found to be similar (P>0.05) among the different treatments.

4.21.2 Viscero-somatic Index (VSI)

The VSI (%) of different treatments are given in given in Table 31**.** The higher value was found in ACP_{25} (2.72±0.10) group, whereas lower values were observed in control (2.50±0.02) and ACP_{75} (2.50±0.04). The VSI among the experimental groups did not vary significantly (P>0.05).

4.21.3 Survival

The survival rates of the fishes in the different experimental groups are given in the Table 31. The survival rate among the experimental groups did not vary significantly (P>0.05).

Table 31: Body indices and survival of *L. rohita* fingerlings fed with different experimental diets

Treatments	**Variables**		
	HSI (%)	**VSI (%)**	**Survival (%)**
Control	1.03±0.02	3.50±0.02	96.67±3.33
$ACPI_{25}$	1.05±0.03	3.72±0.10	96.67±3.33
$ACPI_{50}$	1.05±0.02	3.61±0.07	93.33±3.33
$ACPI_{75}$	1.04±0.03	3.50±0.04	96.67±3.33
$ACPI_{100}$	1.07±0.04	3.66±0.01	93.33±3.33
P-value	0.890	0.077	0.871

Data expressed as Mean ± SE n=6.

4.22 Whole Body Composition

Data pertaining to the biochemical composition (% wet weight basis) of *L. rohita* fingerlings in all the experimental treatments in terms of moisture, protein, lipid and total ash are given in Table 32. Whole body composition parameter of *L. rohita* fingerlings *viz*. moisture content, ether extract, total carbohydrate and total ash content were found to be independent of the dietary treatments (P>0.05) while crude protein content of whole tissue of *L. rohita* fingerlings was found to be significantly different among the treatments.

The moisture content of the experimental fishes varied from 74.04±0.13 to 74.94±0.45%. The observed crude protein of different experimental groups varied from 16.42±0.06 to 16.71±0.08%. The ether extract of different groups that were recorded varied from 3.60±0.07 to 3.80±0.05%. Total carbohydrate content of the experimental fishes varied from 1.18±0.32 to 2.12±0.31%. The total ash content varied from 3.43±0.09 to 3.59±0.14 %.

Table 32: Whole body composition of *L. rohita* fingerlings fed with different experimental diets

Treatments	Variables				
	Moisture (%)	Crude protein (%)	Ether extract (%)	Total carbohydrate (%)	Total ash (%)
Control	74.80±0.57	16.42^{a}±0.06	3.78±0.03	1.57±0.45	3.43±0.09
$ACPI_{25}$	74.04±0.13	16.53^{ab}±0.07	3.80±0.05	2.12±0.31	3.51±0.14
$ACPI_{50}$	74.50±0.21	16.61^{ab}±0.05	3.78±0.06	1.59±0.31	3.53±0.17
$ACPI_{75}$	74.94±0.45	16.71^{b}±0.08	3.60±0.07	1.18±0.32	3.56±0.10
$ACPI_{100}$	74.62±0.31	16.42^{a}±0.03	3.61±0.08	1.76±0.15	3.59±0.14
P-value	0.506	0.028	0.084	0.405	0.916

Data expressed as Mean ± SE n=3; Mean values in the same column with different superscript differ significantly (P<0.05)

4.23 Digestive Enzyme Activities

The specific activities of protease, amylase and lipase in the intestine of *L. rohita* fingerlings of different experimental groups are shown in the Table 33. The digestive enzymes activities of intestine such as protease, amylase and lipase did not vary (P>0.05) among different dietary treatments.

Table 33: Digestive enzyme activities of *L. rohita* fingerlings fed with different experimental diets

Treatments	Digestive enzymes		
	[1]Protease	[2]Amylase	[3]Lipase
Control	0.15±0.004	2.33±0.16	0.12±0.003
$ACPI_{25}$	0.12±0.002	2.54±0.18	0.12±0.008
$ACPI_{50}$	0.13±0.015	2.49±0.13	0.13±0.003
$ACPI_{75}$	0.15±0.002	2.32±0.14	0.13±0.009
$ACPI_{100}$	0.14±0.005	2.23±0.06	0.14±0.006
P-value	0.095	0.541	0.512

Data expressed as Mean ± SE n=6.

[1]Protease as micromol of tyrosine released/ min/mg protein.

[2]Amylase as micromol of maltose released/min/mg protein.

[3]Lipase as units/mg protein.

4.24 Metabolic Enzymes Activities

4.24.1 Aspartate aminotransferase (AST) activity

The AST activity of *L. rohita* fingerlings fed with different experimental diet is shown in Table 34. The activity of enzyme in liver and muscle differ significantly (P<0.05). In liver, higher activity of the enzyme was recorded in $ACPI_{25}$ followed by $ACPI_{50}$, whereas the lowest activity was observed in control, $ACPI_{75}$ and ACP_{100} group. In the muscle, higher activity of the enzyme was recorded in $ACPI_{25}$

followed by $ACPI_{50}$, $ACP_{100,}$ whereas lower activity was observed in control and $ACPI_{75}$ fed group.

4.24.2 Alanine aminotransferase (ALT) Activity

The ALT activity of *L. rohita* fingerlings fed with different experimental diet is shown in Table 34. The activity of enzyme in muscle differ significantly ($P<0.05$) whereas, ALT activity did not vary in liver ($P>0.05$) among different treatment groups. In the muscle, the highest activity was found in control and $ACPI_{100}$ followed by $ACPI_{25}$ and $ACPI_{50}$ groups and lowest activity was observed in $ACPI_{75}$ group which was significantly different from the other groups.

4.24.3 Lactate dehydrogenase (LDH)

The lactate dehydrogenase activity in the liver and muscle of *L. rohita* fingerlings of the different experimental groups is presented in the Table 34. Significant difference ($P<0.05$) in LDH activity in liver and muscle were recorded among the various treatment groups. In the liver, highest activity was found in the $ACPI_{50}$ and lower activity was recorded in control, $ACPI_{75}$ and $ACPI_{100}$ groups, which varied significantly among the different groups. In the muscle, significantly higher LDH activity was observed in $ACPI_{25}$ group, followed by $ACPI_{50}$ and minimum activity was recorded in control, $ACPI_{75}$ and $ACPI_{100}$ groups.

4.24.4 Malate dehydrogenase (MDH)

The malate dehydrogenase activity in the liver and muscle of *L. rohita* fingerlings of the different experimental groups is presented in the Table 34. MDH activity in liver did not vary significantly ($P>0.05$) among the various treatment groups. Whereas, significantly higher MDH activity in muscle was observed in $ACPI_{50}$ group, while minimum activity was observed in control, $ACPI_{75}$ and $ACPI_{100}$ groups.

Table 34: Metabolic enzymes, aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH) and Malate dehydrogenase (MDH) enzyme activities of *L. rohita* fingerlings fed with different experimental diets

Treatments	Metabolic enzymes							
	[1]AST		[2]ALT		[3]LDH		[4]MDH	
	Liver	Muscle	Liver	Muscle	Liver	Muscle	Liver	Muscle
Control	8.05^{a}±0.37	17.61^{a}±0.06	4.90±0.10	31.87^{c}±0.10	1.80^{a}±0.09	8.15^{a}±0.40	2.54±0.11	8.28^{a}±0.30
$ACPI_{25}$	13.98^{c}±0.35	39.48^{d}±0.77	4.76±0.13	28.14^{b}±0.13	2.97^{b}±0.10	12.65^{c}±0.70	2.64±0.13	10.36^{b}±0.20
$ACPI_{50}$	12.51^{b}±0.29	34.38^{c}±1.87	4.87±0.24	26.87^{b}±0.24	3.29^{c}±0.15	11.25^{b}±0.25	2.76±0.03	11.76^{c}±0.25
$ACPI_{75}$	8.03^{a}±0.19	18.64^{a}±0.41	5.01±0.19	22.17^{a}±0.19	1.83^{a}±0.10	8.04^{a}±0.18	2.69±0.10	8.23^{a}±0.24
$ACPI_{100}$	8.01^{a}±0.15	26.45^{b}±0.54	4.96±0.07	32.22^{c}±0.07	1.77^{a}±0.06	8.14^{a}±0.12	2.59±0.12	8.33^{a}±0.19
P-value	0.000	0.000	0.817	0.000	0.000	0.000	0.487	0.000

Data expressed as Mean ± SE n=6; Mean values in the same column with different superscript differ significantly (P<0.05)

[1]ALT: specific activities expressed as Nano moles of sodium pyruvate formed/mg protein/minute at 37^{0}C.

[2]AST specific activities expressed as Nano moles of oxaloacetate released/min/mg protein at 37^{0}C.

[3]LDH: specific activity expressed as Units/ min/ mg protein at 37^{0}C.

[4]MDH: specific activity expressed as Units/ min/ mg protein at 37^{0}C.

4.25 Antioxidant enzymes Activities

4.25.1 Superoxide dismutase (SOD) and catalase (CAT)

The Superoxide dismutase and catalase activities in the liver and gills of *L. rohita* fingerlings of the different experimental groups are presented in the Table 35. The SOD and CAT enzyme activities in liver and gills were not affected by dietary treatments (P>0.05).

Table 35: Superoxide dismutase (SOD) and catalase (CAT) enzyme activities of *L. rohita* fingerlings fed with different experimental diets

Treatments	Antioxidant enzymes			
	[1]SOD		[2]Catalase	
	Liver	Gill	Liver	Gill
Control	2.20±0.06	2.96±0.08	1.70±0.14	2.22±0.15
$ACPI_{25}$	2.22±0.05	3.25±0.14	1.76±0.11	1.94±0.10
$ACPI_{50}$	2.27±0.10	3.33±0.09	1.57±0.10	1.99±0.25
$ACPI_{75}$	2.34±0.09	3.05±0.09	1.63±0.10	1.81±0.06
$ACPI_{100}$	2.29±0.06	3.08±0.11	1.39±0.10	1.84±0.09
P-value	0.674	0.138	0.249	0.359

Data expressed as Mean ± SE n=6; Mean values in the same column with different superscript differ significantly (P<0.05)

[1]SOD (superoxide dismutase) activity is expressed as 50% inhibition of epinephrine auto-oxidation/mg protein/min.

[2]CAT (catalase) activity expressed as nanomoles H_2O_2 decomposed/min/mg protein.

4.26 Biochemical and Haemato-Immunological Parameters

4.26.1 Serum protein biochemistry

The serum total protein, albumin, globulin and albumin globulin ratio of *L. rohita* fingerlings fed different experimental diets are given in Table 36. Dietary

inclusion of CPI supplemented with lysine and methionine did not affect the total protein, albumin, globulin concentration and albumin globulin ratio in *L. rohita* fingerlings (P>0.05).

4.26.2 Hemato-immunological parameters

The results of the effect of different dietary treatments on serum glucose, alanine transaminase, aspartate transaminase, and NBT in *L. rohita* fingerlings are presented in Table 37. Serum glucose concentration, aspartate aminotransferase alanine aminotransferase activity in rohu fingerlings did not vary with different dietary treatments (P>0.05).

4.26.3 Nitroblue tetrazolium (NBT) assay

Respiratory burst activity of the phagocytes (NBT assay) in *L. rohita* fingerlings is given in Table 37. The respiratory burst activity was found to be maximum (P<0.05) in $ACPI_{25}$ and $ACPI_{100}$ group followed by $ACPI_{50}$ and minimum in control and $ACPI_{75}$ groups.

4.27 Haematological Parameters

The haematological parameters of *L. rohita* fingerlings fed with different experimental diets are given in Table 38. The haemoglobin concentration in *L. rohita* fingerlings was significantly affected by the different dietary treatments (P<0.05). Maximum haemoglobin concentration was observed in control and $ACPI_{75}$, while minimum concentration in $ACPI_{25}$ groups. Total erythrocyte count, total leucocyte count (WBC), haematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) values did not vary significantly among the treatment groups (P>0.05).

Table 36: Effects of different experimental diets on the serum protein biochemistry of *L. rohita* fingerlings

Treatments	Serum protein			
	Total protein (g dl^{-1})	Albumin (g dl^{-1})	Globulin (g dl^{-1})	A:G Ratio
Control	2.62±0.07	1.09±0.03	1.53±0.10	0.72±0.060
$ACPI_{25}$	2.60±0.04	1.13±0.05	1.47±0.04	0.77±0.048
$ACPI_{50}$	2.66±0.05	1.14±0.01	1.52±0.04	0.75±0.015
$ACPI_{75}$	2.68±0.07	1.13±0.04	1.55±0.10	0.74±0.073
$ACPI_{100}$	2.47±0.02	1.08±0.02	1.38±0.02	0.78±0.020
P-value	0.141	0.679	0.471	0.891

Data expressed as Mean ± SE n=6; Mean values in the same column with different superscript differ significantly (P<0.05)

Table 37: Effect of different diets on serum glucose, alanine transaminase, aspartate transaminase, and nitroblue tetrazolium (NBT) assay in *L. rohita* fingerlings

Treatments	Serum parameters			
	Glucose (mg dl^{-1})	SGOT (U L^{-1})	SGPT (U L^{-1})	NBT
Control	90.13±4.98	43.93±1.64	52.86±1.29	0.29^{a}±0.031
$ACPI_{25}$	95.10±2.46	45.36±3.05	53.58±1.86	0.42^{b}±0.037
$ACPI_{50}$	96.44±2.86	44.65±0.36	52.15±0.36	0.36^{ab}±0.034
$ACPI_{75}$	93.84±0.73	42.86±2.23	51.79±0.94	0.31^{a}±0.025
$ACPI_{100}$	100.89±3.55	45.72±0.94	52.86±1.89	0.40^{b}±0.019
P-value	0.282	0.828	0.903	0.019

Data expressed as Mean ± SE n=6; Mean values in the same column with different superscript differ significantly (P<0.05)

Table 38: Haematological parameters of *L. rohita* fingerlings fed different experimental diets

Treatments	Haematological parameters						
	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	7.03^b±0.22	1.94±0.04	179.10±8.15	23.13±1.34	119.34±5.23	36.30±0.40	30.53±1.31
$ACPI_{25}$	5.63^a±0.18	1.79±0.09	178.67±9.50	21.47±0.94	120.78±9.80	31.53±0.74	26.36±1.54
$ACPI_{50}$	6.57^{ab}±0.45	1.93±0.06	191.73±7.18	23.13±1.48	119.68±5.09	33.96±1.49	28.37±0.22
$ACPI_{75}$	7.27^b±0.29	2.06±0.06	191.87±12.21	24.93±0.73	121.02±0.29	35.28±1.08	29.15±0.85
$ACPI_{100}$	6.27^{ab}±0.42	1.85±0.08	183.60±4.61	20.90±0.75	113.25±1.29	33.88±0.85	29.93±1.10
P-value	0.038	0.124	0.694	0.153	0.847	0.055	0.145

Data expressed as Mean ± SE n=3; Mean values in the same column with different superscript differ significantly (P<0.05)

Hb- Hemoglobin; RBC- Red blood cells; WBC-white blood cells; Hct- Hematocrit values; MCV; Mean cell volume; MCH-mean cell hemoglobin; MCHC- Mean cell hemoglobin concentration

4.28 Histopathology

4.28.1 Liver

The histological examination of the liver revealed polygonal hepatocytes and prominent nuclei and nucleoli, and granular cytoplasm in the control group (Plate 20). $ACPI_{25}$, $ACPI_{50}$, $ACPI_{75}$ and $ACPI_{100}$ did not show any noticeable changes as compared to the control and no significant changes were observed in dietary treatments and control groups (Plate 21-24).

4.28.2 Intestine

The histological examination of the intestine exhibited cellular changes in all dietary treatment and control groups (Plate 25-29). Ultrastructure of the intestine exhibited improved structural integrity in the intestine of *L. rohita* fingerlings when fed with dietary CPI supplemented with deficient amino acids (L-lysine and DL-methionine). The noticeable changes were comparable; less fusion of mucosal folds, increased number of vacuoles in enterocytes and less intracellular infiltration in the lamina propria of intestine.

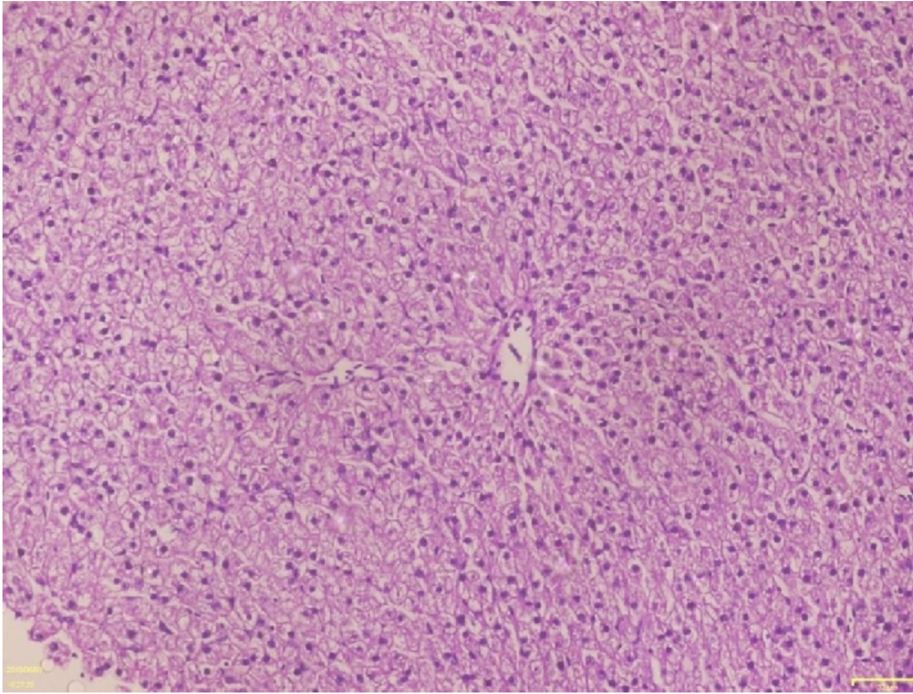

Plate 20: Histological section of liver in the control group. (Scale bar = 32 µm, 20X)

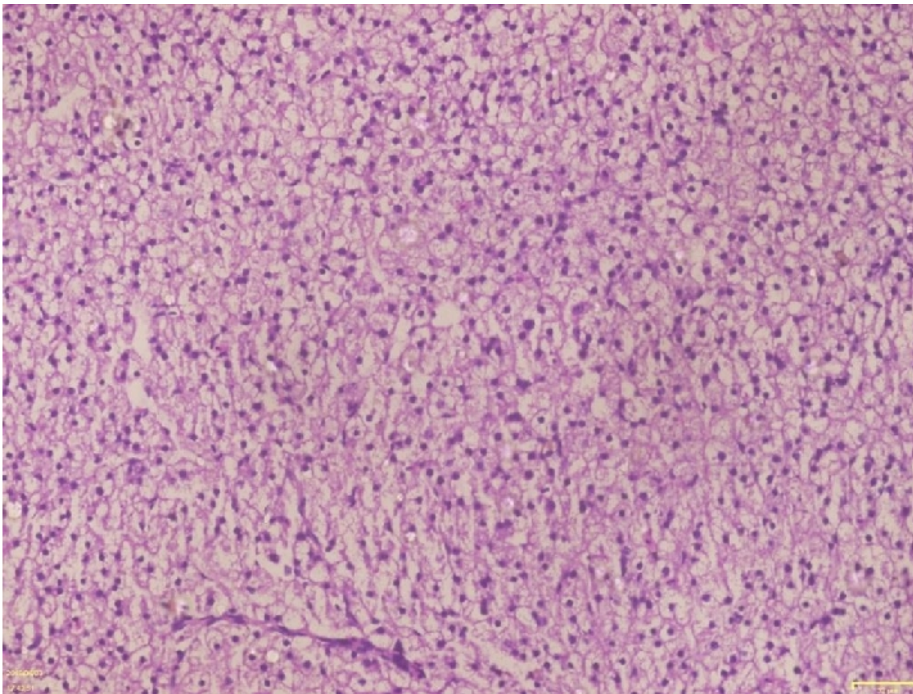

Plate 21: Histological section of liver of $ACPI_{25}$ group. No discernable change was noticed. (Scale bar = 32 µm, 20X)

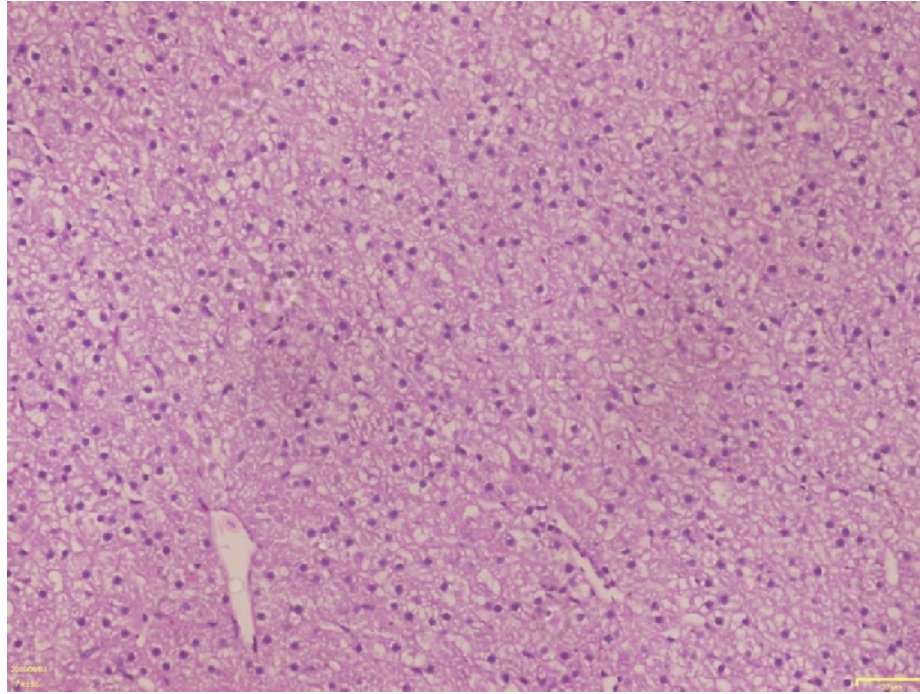

Plate 22: Histological section of liver of $ACPI_{50}$ group. No discernable change was noticed. (Scale bar = 32 µm, 20X)

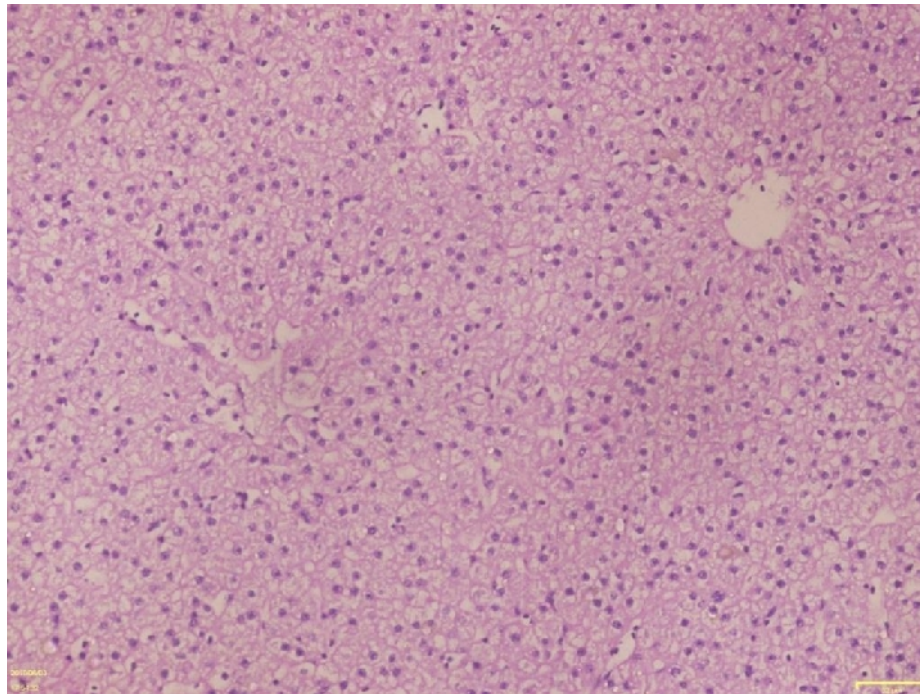

Plate 23: Histological section of liver of $ACPI_{75}$ group. No discernable change was noticed. (Scale bar = 32 µm, 20X)

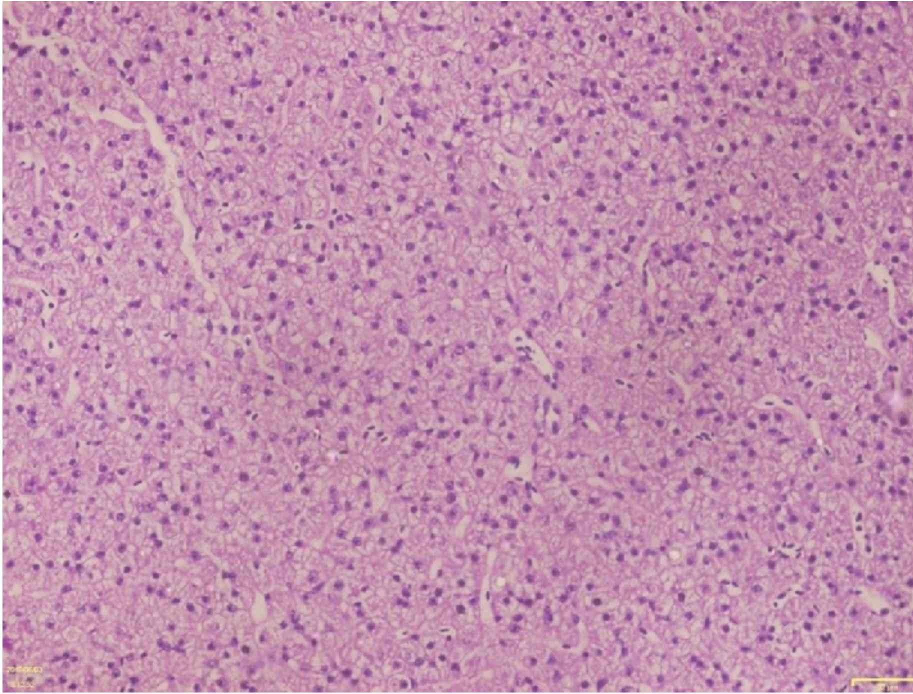

Plate 24: Histological section of liver in $ACPI_{100}$ fed fish showing no marked significant change. (Scale bar = 32 µm, 20X)

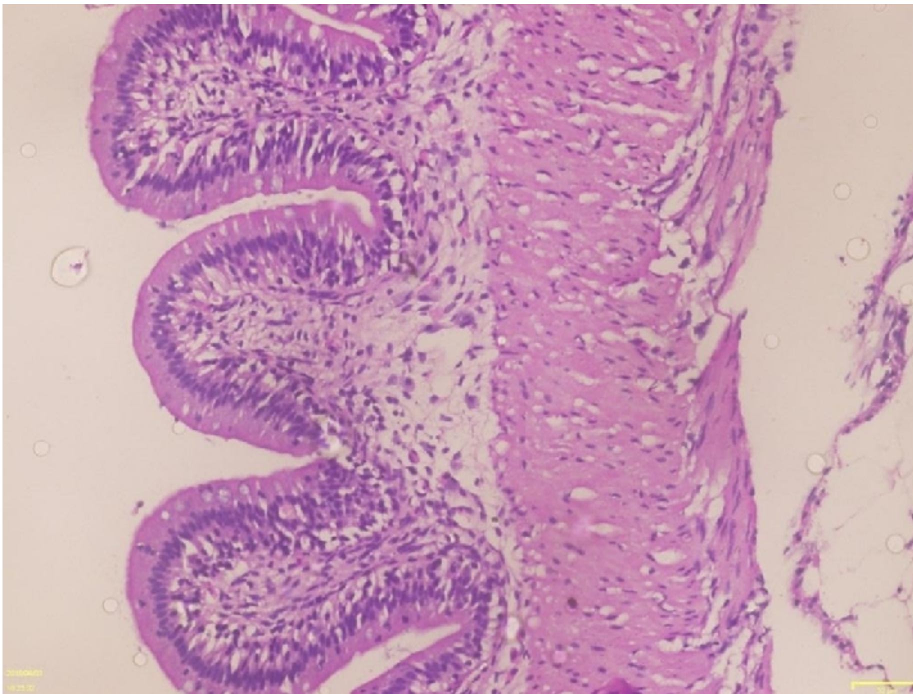

Plate 25: Histological section of intestine of normal fish (Control). (Scale bar = 32 µm, 20X)

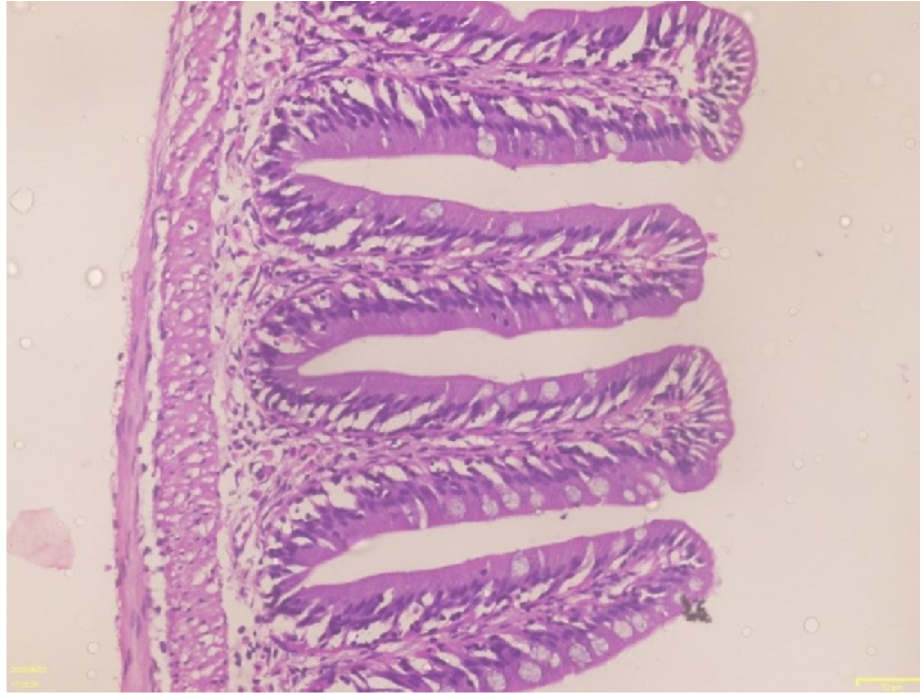

Plate 26: Histological section of intestine of $ACPI_{25}$ group revealed normal histo-architecture. (Scale bar = 32 µm, 20X)

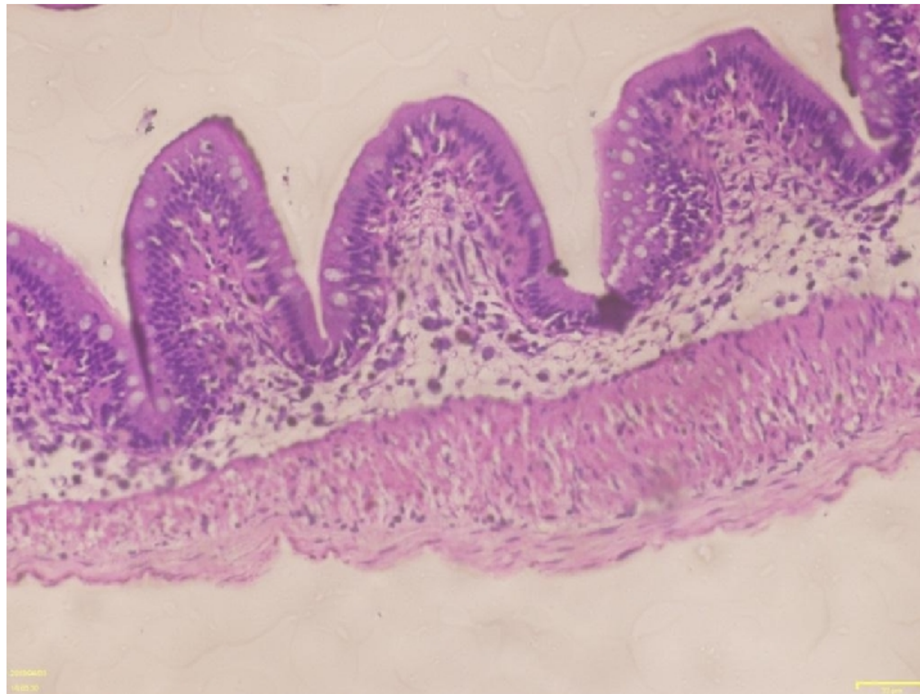

Plate 27: Histological section of intestine of $ACPI_{50}$ showed normal histo-architecture. (Scale bar = 32 µm, 20X)

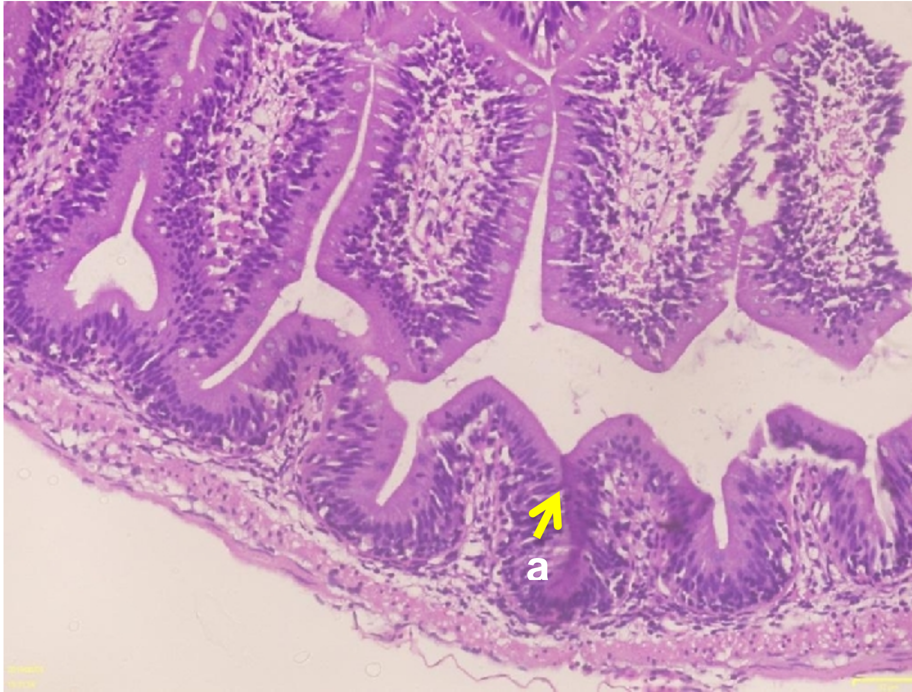

Plate 28: Histological section of intestine of $ACPI_{75}$ showed fused mucosal folds (a). (Scale bar = 32 µm, 20X)

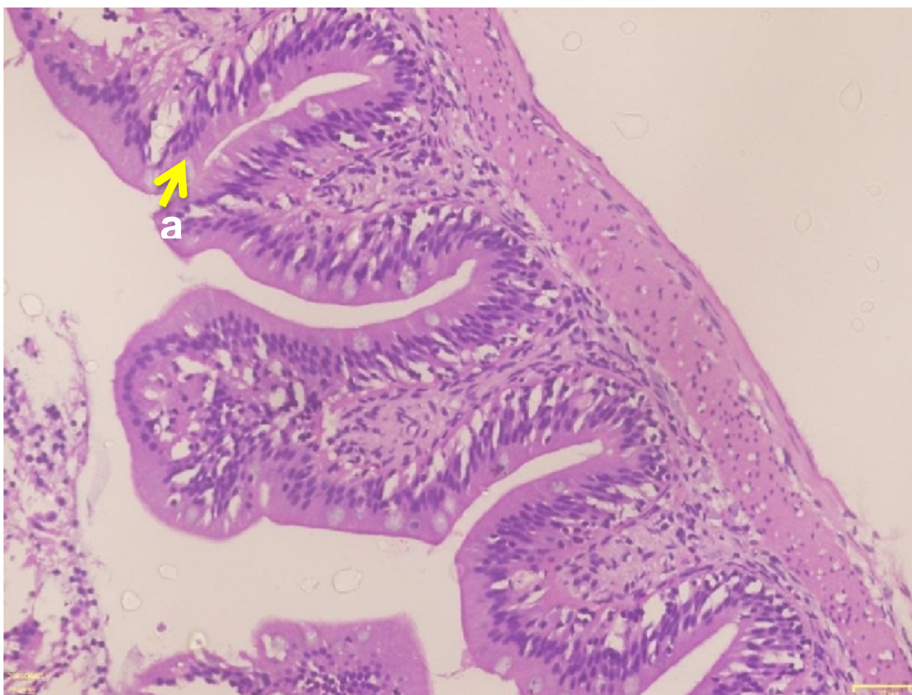

Plate 29: Histological section of intestine of $ACPI_{100}$ group revealed fused mucosal folds (a). (Scale bar = 32 µm, 20X)

Experiment IV

The present experiment was conducted to evaluate the CPI in the fermented sweet leaf meal based diet of *L. rohita* fingerlings. Sweet potato leaf meal was fermented to improve the nutritional value and to detoxify the anti-nutritional factors. The prepared experimental diets were deficient in L-lysine and DL-methionine in *L. rohita* fingerlings. Based on amino acid profiles of experimental diets (Table 7), amino acid requirement of the L-lysine and DL-methionine of *L. rohita* fingerlings was fulfilled by supplementing the deficient amino acids as suggested by NRC (2011). Four iso-nitrogenous (35.45±0.14 % crude protein) and isocaloric (420.95±0.46 kcal $100g^{-1}$) experimental diets were formulated, prepared (Table 8) and fed to *L. rohita* fingerlings twice daily till satiation level for 60 days. Growth performance, survival, nutrient utilization, feed conversion, body indices, digestive enzymes and physio-metabolic changes were measured at the end of experiment and were used as response parameters.

4.29 Proximate Composition of Sweet Potato Leafmeal (SPLM) and Fermented Sweet Potato Leafmeal (FSPLM)

Proximate composition of SPLM and FSPLM are shown in Table 39. The crude protein content of fermented sweet potato leafmeal was increased from 21.47% to 31.20%, whereas the crude fibre content decreased from 19.43% to 7.22%.

4.30 Anti-nutritional factors of Sweet Potato Leafmeal (SPLM) and Fermented Sweet Potato Leafmeal (FSPLM)

Anti-nutritional factors concentrations of SPLM and FSPLM were shown in Table 40. The concentration of anti-nutritional factors such as tannin, phytates, trypsin inhibitor, oxalates, alkaloid and hydrogen cyanide (HCN) were observed to decrease from 25.05 to 8.78 (mg $100g^{-1}$),16.90 to 7.18 (mg $100g^{-1}$),

3.07 to 2.60 (mg g^{-1}),1.42 to 0.89 (%), 1.40 to 0.70 (mg g^{-1}), and 0.47 to 0.18 (mg $100g^{-1}$), respectively during solid-state fermentation.

Table 39: Proximate composition (Dry weight basis) of SPLM and FSPLM

Variables	SPLM	FSPLM
Moisture (%)	8.51±0.24	7.07±0.30
Crude protein (%)	21.47±0.32	31.20±0.17
Ether extract (%)	5.10±0.12	7.17±0.15
Crude fibre (%)	19.43±0.46	7.22±0.14
Nitrogen free extract (%)	43.60±0.71	44.11±0.41
Total ash (%)	10.40±0.09	10.30±0.09
Gross energy (kcal $100g^{-1}$)	353	359.5

Data expressed as Mean ± SE n=3;

SPLM-Sweet potato leaf meal; FSPLM- Fermented sweet potato leaf meal

Table 40: Anti-nutritional factors profile of SPLM and FSPLM

Variables	SPLM	FSPLM	Reduction (%)
Tannin (mg $100g^{-1}$)	25.05±0.46	8.78±0.24	64.95
Phytates (mg $100g^{-1}$)	16.90±0.47	7.18±0.06	57.51
Trypsin inhibitor (mg g^{-1})	3.07±0.22	2.60±0.06	15.31
Oxalate (%)	1.42±0.01	0.89±0.02	37.32
Alkaloid (mg g^{-1})	1.40±0.02	0.70±0.03	50.00
Hydrogen cyanide (mg $100g^{-1}$)	0.47±0.036	0.18±0.036	61.70

Data expressed as Mean ± SE n=3;

SPLM-Sweet potato leaf meal; FSPLM- Fermented sweet potato leaf meal

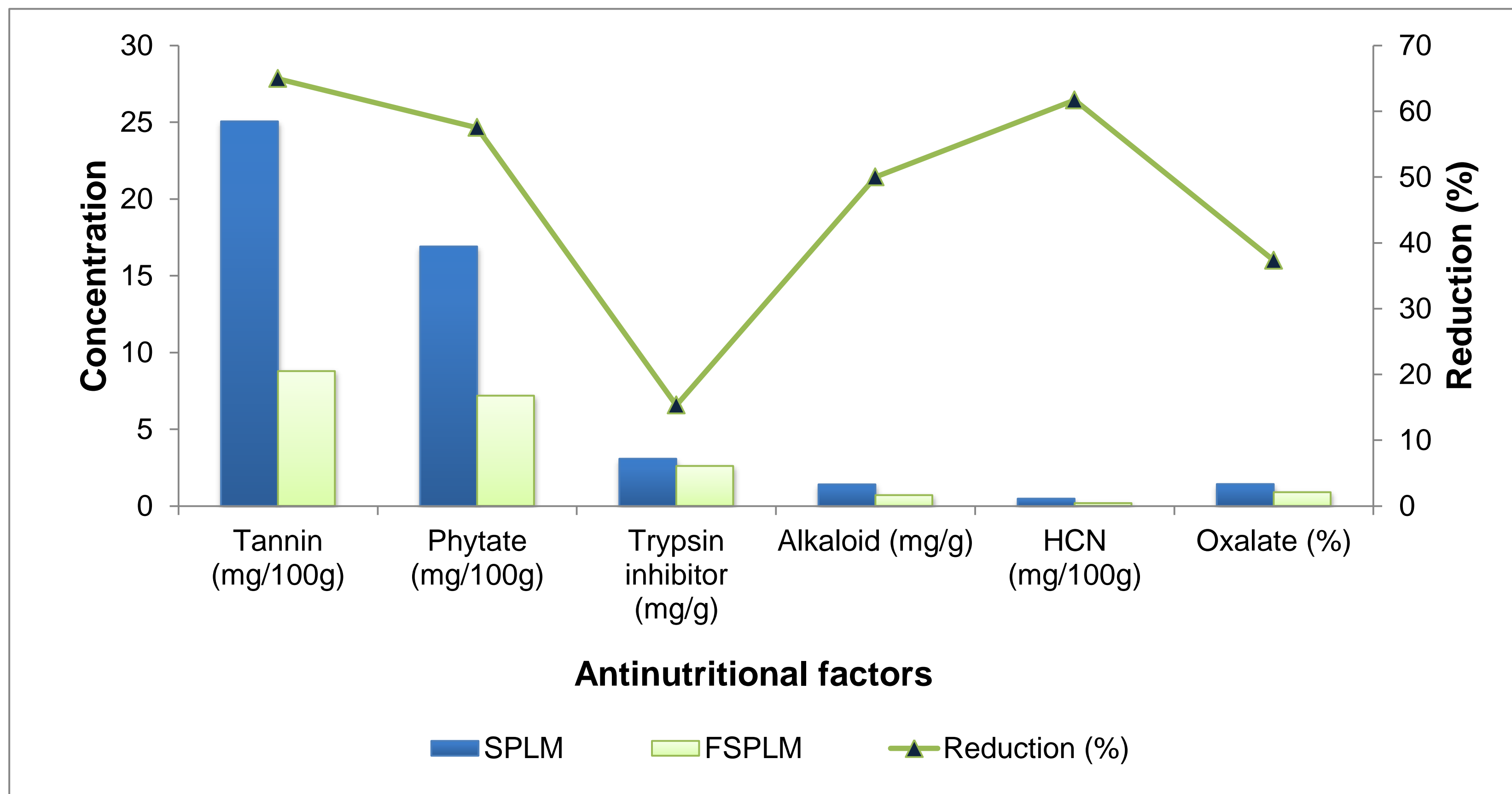

Figure 6: Anti-nutritional factors profile of SPLM and FSPLM

4.31 Physico-Chemical Parameters of Water

The physico-chemical parameters of water such as temperature (^{0} C), pH, dissolved oxygen, total hardness, ammonia, Nitrite - N, and Nitrate - N were recorded during the experimental period of 60 days and these varied in the range of 27.2 to 28.2 ^{0}C, 7.2 to 8.2, 6.4 to 7.7 mg L^{-1}, 228 - 240 mg L^{-1}, 0.11 to 0.22 mg L^{-1}, 0.001 to 0.006 mg L^{-1}, and 0.01 to 0.07 mg L^{-1}, respectively. The free carbon dioxide in water was found to be negligible during the whole experimental period of 60 days (Table 41).

4.32 Proximate Composition of the Experimental Diets

The proximate composition of the experimental diets is given in the Table 42. The moisture content (%) of the experimental diet was recorded in the range of 7.25±0.40 and 7.67±0.27. The crude protein (%) in the diet varied from 35.10±0.36 to 35.18±0.09. The ether extract% varied from 6.51±0.08 to 6.61±0.04. The crude fibre (%) in the diet varied from 7.01±0.03 to 7.12±0.06 whereas nitrogen free extract (%) in the experimental diets was found in the range of 42.62±0.06 and 42.93±0.11. The total ash (%) of the diet varied from 8.33±0.16 to 8.50±0.06.

Table 41: Physico-chemical parameters of water during the experimental period of 60 days for different experimental groups.

Parameters	SPI+DORB	SPI+SPLM	CPI+DORB	CPI+SPLM
Temperature (^{0}C)	27.5-28.3	27.2-28.0	27.5-28.1	27.3-28.2
pH	7.2-8.0	7.4-8.2	7.1-7.9	7.2-8.2
DO (mgL^{-1})	6.5-7.6	6.4-7.5	6.6-7.6	6.4-7.3
Free CO_2 (mgL^{-1})	ND	ND	ND	ND
Hardness (mgL^{-1})	228-235	230-239	232-240	229-237
Ammonia-N (mgL^{-1})	0.11-0.20	0.13-0.21	0.12-0.22	0.14-0.22
Nitrite-N (mgL^{-1})	0.001-0.005	0.002-0.006	0.003-0.004	0.002-0.005
Nitrate-N (mgL^{-1})	0.01-0.07	0.03-0.06	0.02-0.07	0.01-0.04

ND-Not detected

Table 42: Proximate composition of the experimental diets (% DM basis)

Variables	SPI+DORB	SPI+SPLM	CPI+DORB	CPI+SPLM
Moisture	7.25±0.40	7.67±0.27	7.56±0.40	7.63±0.51
Crude protein	35.10±0.36	35.18±0.09	35.11±0.13	35.15±0.06
Ether extract	6.51±0.08	6.61±0.04	6.59±0.17	6.53±0.04
Crude fibre	7.06±0.12	7.09±0.06	7.01±0.03	7.12±0.06
Nitrogen free extract	42.93±0.11	42.62±0.06	42.96±0.42	42.83±0.03
Total ash	8.40±0.06	8.50±0.06	8.33±0.16	8.37±0.05
Gross energy (kcal $100g^{-1}$)	421.7	420.2	421.8	420.1
Antinutritional factors in diet				
Tannin (g kg^{-1})	1.14±0.03	1.28±0.01	1.15±0.02	1.26±0.01
Phytic acid (g kg^{-1})	3.26±0.10	3.66±0.07	3.35±0.15	3.69±0.13
HCN (mg HCN kg^{-1})	4.03±0.05	4.57±0.15	4.06±0.08	4.60±0.18

Data expressed as Mean ± SE n=3.

4.33 Growth Performance and Nutrient Utilization

4.33.1 Body weight

The body weight of *L. rohita fingerlings* fed with different experimental diets was recorded at 15 days interval as shown in the Table 43 and Figure 7**.** The initial and final average body weight among the treatment group varied from 9.23±0.02 to 9.28±0.01 g and from 20.12±0.31 to 20.73±0.55 g, respectively.

4.33.2 Weight gain (%) and specific growth rate (SGR)

The body weight gain expressed in percentage so as to avoid the initial variation in body weight, is given in Table 44. There was no significant difference (P>0.05) in the body weight gain among different treatment groups at the end of the experimental period.

The weight gain percentage and specific growth rate (SGR) of the different experimental groups is presented in Table 44 and it did not vary significantly among the different dietary treatments (P>0.05).

4.33.3 Feed conversion ratio (FCR) and feed efficiency ratio (FER)

The FCR and FER values of the different experimental groups were shown in the Table 44. The FCR of different experimental groups did not vary significantly (P>0.05).

The mean FER values of different treatments did not vary significantly (P>0.05) among different dietary treatment groups (P>0.05).

4.33.4 Protein efficiency ratio (PER) and apparent net protein utilization (ANPU)

The PER and ANPU values of different treatment groups are given in Table 44. The mean PER value was not found significantly different (P>0.05) among the different treatment groups.

The mean ANPU value did not differ significantly (P>0.05) among the different treatment groups.

4.34 Body Indices and Survival

4.34.1 Hepatosomatic Index (HSI) and Viscero-somatic Index (VSI)

The HSI and VSI (%) of different treatments are given in Table 45. The difference of HSI value under various treatment was found non-significant (P>0.05). The VSI among the experimental groups did not vary significantly (P>0.05).

4.34.2 Survival

The survival rate in the different experimental groups is given in the Table 45. The survival rate among the experimental groups did not vary significantly (P>0.05).

Table 43: Body weight (g) of *L. rohita* fingerlings fed different experimental diets (15 days interval)

Treatments	0th day	15th days	30th days	45th days	60th days
SPI+DORB	9.28±0.01	13.37^{b}±0.22	15.24±0.18	17.27±0.46	20.73±0.55
SPI+SPLM	9.26±0.01	13.05^{b}±0.18	14.47±0.23	16.93±0.52	20.32±0.63
CPI+DORB	9.25±0.08	12.83^{ab}±0.08	14.79±0.72	16.86±0.96	20.19±0.65
CPI+SPLM	9.23±0.02	12.37^{a}±0.23	14.38±0.32	16.77±0.25	20.12±0.31
P-value	0.817	0.028	0.498	0.938	0.865

Data expressed as Mean ± SE n=3.

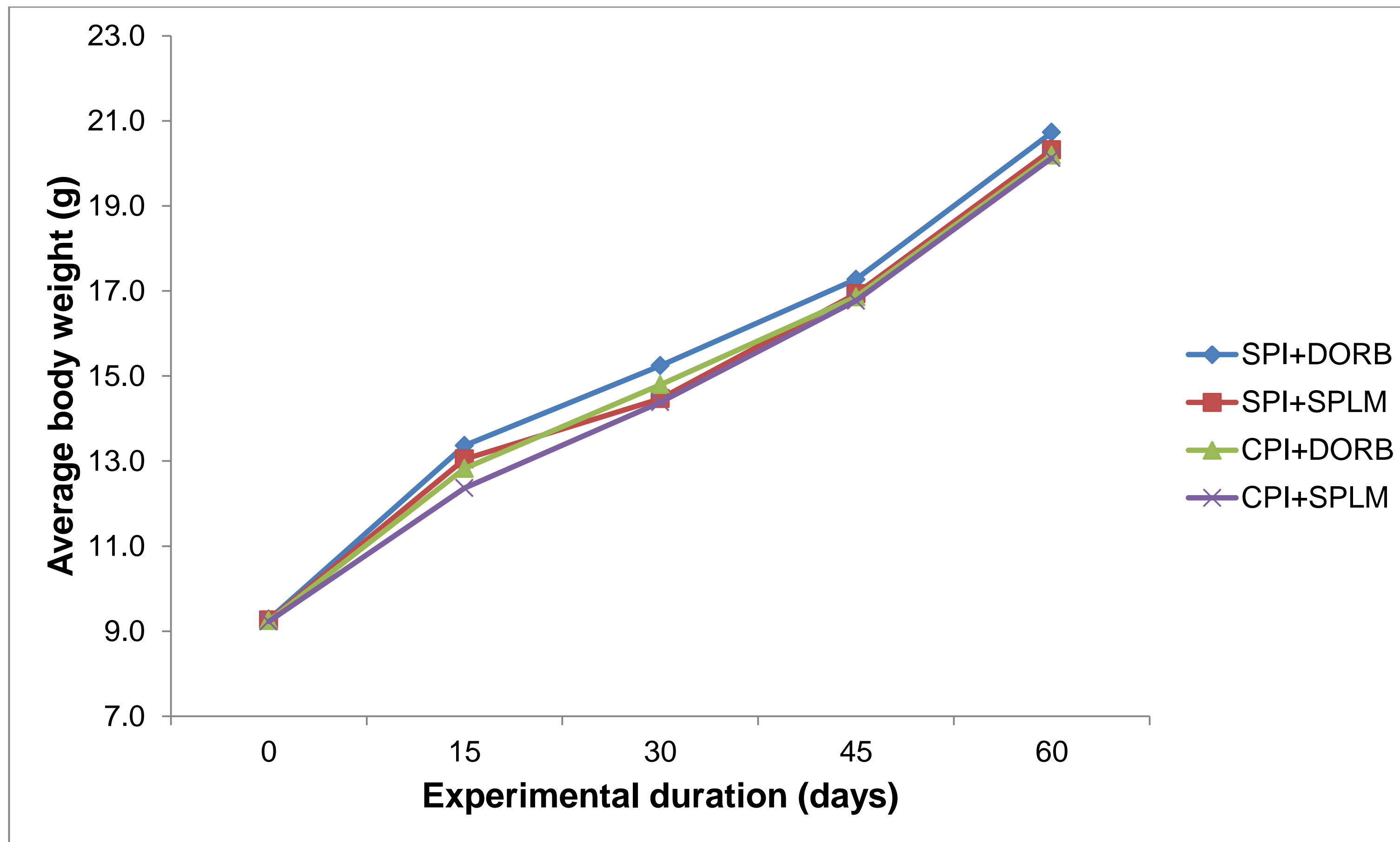

Figure 7: Body weight gain (g) of *L. rohita* fingerlings fed different experimental diets

Table 44: Growth performance, feed conversion and nutrient utilization parameters of *L. rohita* fingerlings fed different experimental diets

Treatments	Variables						
	Weight gain	WG (%)	SGR (% day^{-1})	FCR	FER	PER	ANPU (%)
SPI+DORB	11.45±0.55	123.37±6.06	1.34±0.05	2.18±0.09	0.46±0.02	1.31±0.06	24.71±0.92
SPI+SPLM	11.06±0.64	119.45±7.03	1.31±0.05	2.20±0.11	0.46±0.02	1.30±0.07	24.57±1.08
CPI+DORB	10.95±0.58	118.31±5.28	1.30±0.04	2.21±0.06	0.45±0.01	1.29±0.03	24.16±0.60
CPI+SPLM	10.90±0.28	118.07±2.76	1.30±0.02	2.18±0.07	0.46±0.01	1.31±0.04	24.43±0.71
P-value	0.879	0.895	0.897	0.989	0.987	0.986	0.971

Data expressed as Mean ± SE n=3.

WG- Weight gain (%); SGR- Specific growth rate; FCR-feed conversion ratio; FER- Feed efficiency ratio; PER- Protein efficiency ratio; ANPU- Apparent net protein utilization

Table 45: Body indices and survival rate of *L. rohita* fingerlings fed different experimental diets

Treatments	Variables		
	HSI (%)	VSI (%)	Survival rate (%)
SPI+DORB	1.26±0.06	3.75±0.24	100.00±0.00
SPI+SPLM	1.16±0.03	3.69±0.11	100.00±0.00
CPI+DORB	1.15±0.04	3.66±0.09	96.67±3.33
CPI+SPLM	1.15±0.03	3.82±0.12	93.33±3.33
P-value	0.194	0.872	0.219

Data expressed as Mean ± SE n=6.

4.35 Whole Body Composition

Data pertaining to the biochemical composition (% wet weight basis) of *L. rohita* fingerlings in all the experimental treatments in terms of moisture, protein, lipid and total ash is given in Table 46. Whole body composition parameter of *L. rohita* fingerlings *viz*. moisture content, crude protein, ether extract, total carbohydrate and total ash content were found to be independent with the dietary treatments (P>0.05). The moisture content (%) of the experimental fishes varied from 74.22±0.26 to 74.68±0.32%. The observed crude protein (%) of different experimental groups varied from 16.61±0.27 to 16.70±0.23 %. The ether extract (%) of different groups that were recorded varied from 3.89±0.01 to 3.99±0.07%. Total carbohydrate content (%) of the experimental fishes varied from 1.09±0.42 to 1.42±0.09%. The total ash content (%) varied from 3.67±0.15 to 3.76±0.06%.

.**Table 46: Whole body composition of *L. rohita* fingerlings fed different experimental diets**

Treatments	Whole body composition (% wet weight basis)				
	Moisture	**Crude protein**	**Ether extract**	**Total carbohydrate**	**Total ash**
SPI+DORB	74.68±0.32	16.68±0.06	3.89±0.01	1.09±0.42	3.67±0.15
SPI+SPLM	74.32±0.24	16.70±0.23	3.93±0.06	1.30±0.12	3.76±0.21
CPI+DORB	74.28±0.27	16.64±0.18	3.95±0.10	1.39±0.37	3.73±0.16
CPI+SPLM	74.22±0.26	16.61±0.27	3.99±0.07	1.42±0.09	3.76±0.06
P-value	0.654	0.991	0.742	0.844	0.969

Data expressed as Mean ± SE n=3

4.36 Digestive Enzyme Activities

The specific activities of protease, amylase and lipase in the intestine of *L. rohita* fingerlings of different experimental groups are shown in the Table 47. The digestive enzymes activities of intestine such as protease, amylase and lipase did not vary (P>0.05) among different dietary treatments.

Table 47: Digestive enzyme activities of *L. rohita* fingerlings fed different experimental diets

Treatments	Digestive enzymes		
	[1]Protease	[2]Amylase	[3]Lipase
SPI+DORB	0.17±0.011	5.83±0.09	0.14±0.012
SPI+SPLM	0.16±0.010	6.41±0.34	0.15±0.004
CPI+DORB	0.16±0.006	5.81±0.31	0.15±0.013
CPI+SPLM	0.14±0.004	5.74±0.18	0.14±0.011
P-value	0.276	0.287	0.661

Data expressed as Mean ± SE n=6.

[1]Protease as micromol of tyrosine released/ min/mg protein.

[2]Amylase as micromol of maltose released/min/mg protein.

[3]Lipase as units/mg protein.

4.37 Metabolic Enzymes Activities

4.37.1 Aspartate aminotransferase (AST) activity

The AST activity of *L. rohita* fingerlings fed with different experimental diet is shown in Table 48. The activity of enzyme in liver and muscle did not differ significantly (P>0.05) among the different dietary treatments.

4.37.2 Alanine aminotransferase (ALT) activity

The ALT activity of *L. rohita* fingerlings fed with different experimental diet is shown in Table 48. ALT activity did not vary in liver and muscle (P>0.05) among different treatment groups.

4.37.3 Lactate dehydrogenase (LDH)

Lactate dehydrogenase activity in the liver and muscle of *L. rohita* fingerlings of the different experimental groups is presented in the Table 48. Significant difference (P<0.05) in LDH activity in liver were recorded among the various treatment groups. In the liver, higher activity was found in the SPI+SPLM and CPI+SPLM and lower activity was recorded in SPI+DORB and CPI+DORB groups, which was significantly different among the different groups. In the muscle, LDH activity did not vary significantly among dietary treatment groups (P>0.05).

4.37.4 Malate dehydrogenase (MDH)

Malate dehydrogenase activity in the liver and muscle of *L. rohita* fingerlings of the different experimental groups is presented in the Table 48. MDH activity in liver and did not vary significantly (P>0.05) among the various treatment groups.

4.38 Antioxidant Enzymes Activities

4.38.1 Superoxide dismutase (SOD) and catalase (CAT)

The SOD and CAT activities in the liver and gills of *L. rohita* fingerlings of the different experimental groups are presented in the Table 49. The enzyme activities in liver and gills were not affected by the dietary treatments (P>0.05).

Catalase activity in liver and gills did not vary (P>0.05) among the various treatment groups.

Table 48: Metabolic enzymes, aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH) and Malate dehydrogenase (MDH) enzyme activities of *L. rohita* fingerlings fed different experimental diets

Treatments	Metabolic enzymes							
	[1]AST		[2]ALT		[3]LDH		[4]MDH	
	Liver	Muscle	Liver	Muscle	Liver	Muscle	Liver	Muscle
SPI+DORB	3.61±0.15	7.57±0.43	1.34±0.04	14.36±0.70	0.20^{a}±0.006	0.34±0.031	0.33±0.010	0.90±0.024
SPI+SPLM	3.51±0.18	8.19±0.20	1.40±0.08	15.20±0.69	0.26^{b}±0.018	0.33±0.025	0.30±0.012	0.95±0.035
CPI+DORB	3.60±0.18	7.65±0.16	1.42±0.08	14.33±0.54	0.20^{a}±0.012	0.35±0.026	0.29±0.019	0.81±0.056
CPI+SPLM	3.48±0.15	6.80±0.28	1.43±0.05	13.64±0.14	0.27^{b}±0.017	0.34±0.031	0.32±0.012	0.81±0.046
P-value	0.915	0.052	0.746	0.347	0.009	0.949	0.258	0.100

Data expressed as Mean ± SE n=6; Mean values in the same column with different superscript differ significantly ($P<0.05$)

[1]ALT: specific activities expressed as Nano moles of sodium pyruvate formed/mg protein/minute at 37^{0}C.

[2]AST specific activities expressed as Nano moles of oxaloacetate released/min/mg protein at 37^{0}C.

[3]LDH: specific activity expressed as Units/ min/ mg protein at 37^{0}C.

[4]MDH: specific activity expressed as Units/ min/ mg protein at 37^{0}C.

Table 49: Superoxide dismutase (SOD) and catalase (CAT) enzyme activities of *L. rohita* fingerlings fed different experimental diets

Treatments	Antioxidant enzymes			
	[1]SOD		[2]Catalase	
	Liver	Gill	Liver	Gill
SPI+DORB	1.98±0.08	3.21±0.28	0.94±0.04	6.21±0.43
SPI+SPLM	2.06±0.13	2.92±0.19	0.83±0.04	7.40±0.36
CPI+DORB	1.93±0.11	3.17±0.18	0.85±0.05	7.57±0.32
CPI+SPLM	2.28±0.15	2.88±0.16	0.94±0.04	7.72±0.31
P-value	0.259	0.585	0.201	0.062

Data expressed as Mean ± SE n=6.

[1]SOD (superoxide dismutase) activity is expressed as 50% inhibition of epinephrine auto-oxidation/mg protein/min.

[2]CAT (catalase) activity expressed as nanomoles H_2O_2 decomposed/min/mg protein.

4.39 Biochemical and Haemato-Immunological Parameters

4.39.1 Serum protein biochemistry

The serum total protein, albumin, globulin and albumin globulin ratio of *L. rohita* fingerlings fed different experimental diets are given in Table 50. Dietary treatments did not affect the total protein, albumin, globulin concentration and albumin globulin ratio in *L. rohita* fingerlings (P>0.05).

4.39.2 Hemato-immunological parameters

The results of effect of different dietary treatments on serum glucose, alanine transaminase, aspartate transaminase, and NBT in *L. rohita* fingerlings are presented in Table 51. Serum glucose concentration, aspartate aminotransferase and alanine aminotransferase activity in rohu fingerlings did not vary significantly with the different dietary treatments (P>0.05).

Table 50: Effects of different experimental diets on the serum protein biochemistry of *L. rohita* fingerlings

Treatments	Serum parameters			
	Total protein (g dl^{-1})	Albumin (g dl^{-1})	Globulin (g dl^{-1})	A:G Ratio
SPI+DORB	3.80±0.09	1.32±0.05	2.49±0.14	0.54±0.048
SPI+SPLM	3.81±0.06	1.29±0.02	2.52±0.05	0.51±0.007
CPI+DORB	3.83±0.07	1.27±0.04	2.57±0.06	0.49±0.020
CPI+SPLM	3.77±0.03	1.29±0.04	2.48±0.07	0.52±0.029
P-value	0.924	0.826	0.891	0.804

Data expressed as Mean ± SE n=6.

Table 51: Serum glucose, alanine transaminase, aspartate transaminase, and nitroblue tetrazolium (NBT) assay in *L. rohita* fingerlings fed with different experimental diets

Treatments	Serum parameters			
	Glucose (mg dl^{-1})	SGOT (U L^{-1})	SGPT (U L^{-1})	NBT
SPI+DORB	86.26±2.98	58.10±4.52	49.05±1.72	0.18±0.005
SPI+SPLM	91.16±2.00	56.19±1.49	50.48±2.12	0.15±0.015
CPI+DORB	88.16±3.37	46.43±4.46	49.05±3.13	0.16±0.016
CPI+SPLM	89.18±5.68	54.77±2.75	43.81±2.27	0.17±0.013
P-value	0.828	0.347	0.295	0.346

Data expressed as Mean ± SE n=6.

4.39.3 Nitroblue tetrazolium (NBT) assay

Respiratory burst activity of the phagocytes (NBT assay) in *L. rohita* fingerlings is given in Table 51. The respiratory burst activity did not vary significantly (P>0.05) in *L. rohita* fingerlings fed with different experimental diets.

4.40 Haematological Parameters

The haematological parameters of *L. rohita* fingerlings fed with different experimental diets are given in Table 52. Haemoglobin concentration, total erythrocyte count, total leucocyte count (WBC), haematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) values did not differ significantly among dietary treatment groups ($P>0.05$).

Table 52: Hematological parameters of *L. rohita* fingerlings fed different experimental diets

Treatments	Hematological parameters						
	HB ($g\ dl^{-1}$)	RBC (10^6 cells mm^{-3})	WBC (10^5 cells mm^{-3})	HCT (%)	MCV (fL)	MCH (pg)	MCHC ($g\ dl^{-1}$)
SPI+DORB	6.03±0.13	1.83±0.08	1.88±0.15	23.40±1.08	127.68±2.55	33.13±2.24	25.93±1.63
SPI+SPLM	7.70±0.72	1.94±0.08	2.01±0.06	26.83±4.42	137.18±17.45	39.59±2.12	29.46±2.42
CPI+DORB	6.83±0.38	1.80±0.11	1.87±0.12	23.23±3.44	127.86±11.74	38.17±1.98	30.60±4.30
CPI+SPLM	6.57±0.67	1.77±0.12	1.84±0.09	23.57±3.81	132.28±17.08	37.02±2.38	28.43±1.75
P-value	0.245	0.671	0.702	0.858	0.952	0.263	0.675

Data expressed as Mean ± SE n=3.

Hb- Hemoglobin; RBC- Red blood cells; WBC-white blood cells; Hct- Hematocrit values; MCV; Mean cell volume; MCH-mean cell hemoglobin; MCHC- Mean cell hemoglobin concentration

5. DISCUSSION

Experiment I

Extraction of proteins from defatted seed cake or meal has been defined as a method of reducing the anti-nutrients and improving their nutritional values (Marrufo-Estrada *et al.,* 2013). Preparation of protein isolates comprises of two steps, of which first step includes aqueous solubilization of proteins in alkaline medium and another step includes precipitation of protein using different methods such as membrane processing, dialysis, ultrafiltration, diafiltration, ion-exchange, protein micellar mass (PMM) and iso-electric precipitation (Diosady *et al*., 1987; Tzeng *et al*., 1988a, 1988b, 1990; Devappa and Swamylingappa, 2008; Saetae and Suntornsuk, 2011; Shamna *et al*., 2015; Fawole *et al*., 2016a). Devappa and Swamylingappa (2008) reported that the isolated proteins from oilseeds are the best and most pure forms with higher nutritional values and generally regarded as SAFE by the Food and Drugs Administration (FDA). It can be used to fortify, formulate and impart food products (Saetae and Suntornsuk, 2011). The protein content in isolated proteins are more than or equal to 90% and characterized by high protein and amino acid concentration, high digestibility, low dietary fibres & lipids and lower concentration of anti-nutritional factors (Dersjant-Li, 2002; Lin *et al.,* 2006; USSEC, 2008). For plant oil seeds, aqueous extraction followed by isoelectric precipitation at different pH combination is the most preferred method, which is used in the present study. Hence, the present study was aimed to standardize the method for preparation of the protein isolates from defatted castor kernel meal, and to evaluate its nutritional quality.

5.1 Protein Isolate Prepared from Defatted Castor Kernel Meal

The seeds were manually decorticated to separate hulls and kernels and the kernel/hull ratio was 72:28 (w/w). The protein content was increased from 20-22% to 26-28% due to decortication. Akande *et al*. (2012) reported that kernel/hull ratio in castor seed varies from 25:75 to 31:69. Castor kernels were defatted with hexane for 24 hr and the crude protein was increased up to 57-59%, which was used

for the preparation of protein isolates. In the present experiment, different combination of pH were used to isolate the protein from defatted castor kernel meal and it was observed that all pH combinations exhibited higher protein content varied from 81.52% and 92.55%. The protein content in castor seed protein isolate (CPI) is 1.4 to 1.6 times more than DCKM. The high protein content of CPI might be due to removal of soluble and insoluble carbohydrates and other non-proteinaceous components present in the DCKM during alkali extraction followed by centrifugation. Fawloe *et al.* (2016a, 2018) observed the solubilization of proteins at alkaline pH and aggregation and precipitation at iso-electric pH, where net charge on proteins become zero and protein tends to precipitate due to absence of electrostatic repulsion, thereby resulting in higher proportion of protein (Garba and Kaur, 2014). The higher protein content in protein isolates (81.52% and 92.55) are in agreement with other reports on different oil seeds such as canola (90.8%, Mwachireya *et al.*, 1999), lupin and soybean (80.5% and 89.3% respectively, Glencross *et al.,* 2005), jatropha seed (95.5% -97.0%, Devappa and Swamylingappa, 2008; 87.7%-97.75%, Fawole *et al.*, 2018), and castor seed (91.7%, Puttaraj *et al.*, 1994). The dry matter recovery and protein recovery of protein isolate ranged from 31.12% to 49.83% and 43.31% and 78.68%, respectively in this study and these values are higher than the earlier reported values by other researchers (Villanueva *et al.,* 1999; Marnoch and Diosady, 2006; Devappa and Swamylingappa, 2008; Makkar *et al.*, 2008; Kumar *et al.*, 2011a; Fawole *et al.*, 2016a and Fawole *et al.*, 2018). Of the protein present in DCKM, 44-79% proteins were recovered in CPI in this study. The values of protein recovery in the present study are similar to 70–77% in Jatropha protein isolate reported by Devappa and Swamylingappa (2008). The higher recoveries in the present study might be due to the centrifugation of protein extract before protein precipitation and heat treatment after precipitation. The highest recoveries of dry matter and protein were obtained at pH 5.0 while, other researchers reported highest recoveries at pH 4.0 (Makkar *et al.*, 2008; Kumar *et al.,* 2011a; Fawole *et al.*, 2016a; Fawole *et al.*, 2018), pH 4.5 (Schulz *et al.,* 2007; Marrufo-Estrada *et al.*, 2013), pH 5.0 (Marnoch and Diosady, 2006) and pH 5.5 (Devappa and Swamylingappa, 2008). Based on the protein content, recoveries of dry matter and protein, pH combination of 12.0/5.0 at

room temperature was the best and could be used for preparation of protein isolate at mass scale.

5.2 Proximate Composition and Anti-nutritional Factors

Castor seed and cake (screw press produced) contains 20-22% and 31-36% crude protein, respectively (Annongu and Joseph, 2008). While other researchers has reported that crude protein content of castor seed meal or cake is 35 to 48% and it varies as per the processing method used for extraction of castor oil (Ogunniyi, 2006; Nagalakshmi and Dhanalakshmi, 2015). In the present study, castor kernel meal (CKM) and defatted castor kernel meal (DCKM) contained 26.02% and 58.61% crude protein, respectively. The more crude protein contents in DCKM might be due to the solvent extraction method being used to remove oil from CKM. Further, the carbohydrate and total ash contents were increased in DCKM after solvent extraction of CKM. It is in agreement with the findings of other researchers (Devappa and Swamylingappa, 2008; Shamna *et al*., 2015; Fawole *et al*., 2016a). The CPI protein content was observed as 92.35% and it was similar with the results obtained during the standardization process. Dersjant-Li (2002) reported that protein isolates were characterised by high protein with better amino acid profile and reliable nutritional quality over fish meal and other protein concentrates. Other constituents such as ether extract, crude fibre, and total ash contents were found to decrease in CPI. Similar results were also reported in rapeseed protein isolate (Nagel *et al*., 2012); Jatropha protein isolate (Makkar *et al.,* 2008, Kumar *et al.,* 2011a; Shamna *et al*., 2015; Fawole *et al.,* 2018) and rubber protein isolate (Fawole *et al*., 2016a).

Plant based agro-industrial wastes and by-products have good nutritional profile and potential to replace conventional feed ingredients in animal or fish feed but, the presence of anti-nutritional factors limits their use. In the present study, the tannin content was decreased from 46.05 mg $100g^{-1}$ in the DCKM to 14.59 mg $100g^{-1}$ in CPI. The values of tannin contents in DCKM are lower to the values reported by Akande *et al*. (2011) in castor seed cake. The low concentration of tannin content in DCKM is due to the removal of hulls (outer coat). Tannin content was

significantly reduced in CPI (68.34% reduction). Devappa and Swamylingappa (2008) also observed the reduction in tannin content from 1.03 to 0.15 g $100g^{-1}$ after alkali extraction and steam injection process during preparation of jatropha protein isolate. The phytic acid content was reported as 7.75 and 2.12 mg $100g^{-1}$ in DCKM and CPI, respectively and the reduction was to the time of 72.65%. Phytic acid contents were shown to decrease during preparation of protein isolate (Saetae and Suntornsuk, 2011; Shamna *et al*., 2015; Fawole *et al*., 2016a) and the reason of reduction in phytic acid content might be the binding of phytate ions to sodium ions during extraction and solubilization of protein in alkali medium. The hydrogen cyanide (HCN) content was reduced by 83.56% in CPI (from 145.8 to 24.30 mg kg^{-1}). Cyanide concentration were reported to decrease in protein isolates prepared from plant protein sources compared to respective oilseed meal by many researchers (Shamna *et al*., 2015; Fawole *et al*., 2016a; Fawole *et al*., 2018). The trypsin inhibitor (TI) concentration in DCKM was 20.29 TIU mg^{-1} protein, which was similar to the findings of Ramos *et al*. (2013) in castor seed meal while concentration in CPI was observed as 2.32 TIU mg^{-1} protein (88.58% reduction). It may be attributed to the heat treatment applied during precipitation of proteins. The concentration of trypsin inhibitor in the present study was lower than the tolerance limit (5 mg g^{-1}) for fish (Francis *et al.,* 2001).

5.3 Amino Acid Composition

The amino acid composition of defatted castor kernel meal exhibited similar values as reported by other researchers (Puttaraj *et al.,* 1994; Onwuliri and Anekwe, 2001; Annongu and Joseph, 2008). The essential amino acids like arginine, methionine, lysine, histidine, cysteine, threonine, isoleucine and valine were found to be higher in DCKM while leucine was similar to the value reported by Annongu and Joseph (2008). The reason for slight variation in amino acids concentration might be attributed to differences in geographical parameters like weather condition, origin and soil type (Ratnaningsih *et al*., 2014; Ogunniyi, 2006; Nagalakshmi and Dhanalakshmi, 2015). The essential amino acids such as lysine, methionine, tryptophan, valine, alanine, isoleucine and cysteine (Harnold, 2002; Olayeni *et al.,* 2006; Santos *et al.,*

2015) are deficient in castor meal while castor seed cake is richer in arginine (Santos *et al.,* 2015) and Leucine (Annongu and Joseph, 2008).

Indispensable amino acid profile of protein isolates obtained from defatted castor kernel meal revealed the similarity with the egg white (FAO/WHO, 1985) and soybean protein isolate(SPI) (Kumar *et al.,* 2012). The amino acids profile exhibited that concentrations of arginine, leucine, lysine, methionine, phenyl alanine, and valine were lower in CPI than SPI while concentrations of histidine, isoleucine, tryptophan, and threonine were higher than SPI. Amino acid concentrations of lysine, methionine, and phenyl alanine were found to be lower than FAO/WHO (1985) reference protein. The lysine content in CPI was higher than DCKM but it was deficient in both DCKM and CPI. Conversely, reduced lysine concentration was reported in protein isolates prepared from oilseeds and it may be due to the interaction of soluble sugars with amino acids under alkaline pH. (Villanueva *et al*., 1999; FAO, 2011; Jannat Alipour *et al*., 2010).

5.4 Nutritional Indices of Defatted Castor Kernel Meal and Protein Isolates

Nutritional indices are the indicators for protein quality of an ingredient. The nutritional indices of DCKM and CPI were determined based on the amino acid profile of egg white (reference protein). Lysine was the first limiting amino acid in both DCKM and CPI as lysine comprises of minimal amino acid score 39.64% and 46.10% for DCKM and CPI, respectively. Our findings are in agreement with the results of Puttaraj *et al*. (1994) who reported that lysine was the most deficient amino acid in castor meal and castor seed protein isolate. Generally, plant proteins are deficient in lysine and sulphur-containing amino acid (Devappa and Swamylingappa, 2008; Caire-Juvera *et al*., 2013; Fawole *et al*., 2016a). The calculated essential amino acid index (EAAI) of DCKM and CPI were 60.44% and 82.50%, respectively. The EAAI values in CPI are similar to the EAAI values reported in castor seed protein isolate (79%, Puttaraj *et al*.,1994) and jatropha protein isolates (82.0%, Devappa and Swamylingappa, 2008) while it was higher than rubber protein isolates (69.7%,

Fawole *et al*., 2016a). Nutritional index (NI) value of CPI (72.39) was higher than the DCKM (35.43) and it indicates good quality of the protein isolate than the meal. In this study, nutritional index values of CPI were higher than the values previously reported for *Acipenser persicus* fillet (19.1), milk (25), chicken (43) and beef (59) (Crisan and Sands 1978; Jannat Alipour *et al*., 2010) and was similar to the CPI (68, Puttaraj *et al*.,1994). The predicted-PER values of DCKM and CPI were 0.39 and 1.7, respectively. The improvement in P-PER might be attributed to better digestibility as a result of removal of toxic and antinutritional constituents (Devappa and Swamylingappa, 2008). The P-PER values of CPI is similar to those reported for pigeon pea (0.7-1.8) but lower than casein (2.5), soybean (0.7-2.4), and rice bran concentrate (2.0-2.5, Kinsella and Srinivasan 1981; Wang *et al.,* 1999) canola protein (3.29; El-Medany and El-Reffaei, 2015) and rubber protein isolates (2.09, Fawole *et al*., 2016a). Relative protein digestibility is an indicator of protein quality of an ingredient and protein quality of ingredients is dependent on its composition and availability of amino acids along with better digestibility (Caire-Juvera *et al.,* 2013). The CPI exhibited higher in-vitro digestibility (94.47%) than the rapeseed protein isolates (83%, Savoie *et al.,* 1988; 97.6%, Mwachireya *et al*., 1999), and jatropha protein isolates (88.5 - 90.6%; Devappa and Swamylingappa, 2008 but lower than casein and rubber protein isolate (97%, Fawole *et al.,* 2016a). In the present study, the *in-vitro* digestibility found to be higher in CPI than DCKM and increase in digestibility may be attributed to high nutritional value of CPI (high protein content with reduced fibre, and availability of amino acids, Devappa and Swamylingappa, 2008).

In conclusion, the overall results of the present study revealed that isolating protein from defatted castor kernel meal could significantly increase the protein content (>90 %) with a concomitant reduction in the antinutritional and toxic factors. Further, the amino acid composition, *in-vitro* digestibility value and the calculated nutritional indices indicated that castor seed protein isolate could be a promising source of protein for fish feed preparation but deficient in lysine and sulphur containing amino acids. Hence, supplementations of deficient amino acids are required.

Experiment II

The stagnation or declining trend of capture fisheries demands the aquaculture system to be more intensified and feed-based to meet the growing demand of the animal protein. As competition is increasing for the same feed ingredients from the entire animal production sector, there is a high demand for the common feed ingredients such as soybean meal, fish meal, rice bran and oilcakes. In this context several alternate plant proteins have been tested in the diets for aquatic species but found not fit to use in diets as they contains different antinutritional factors. Thus, the quest for alternate protein sources has become a necessity for the sustainable aquaculture production. There is a need to discover the good quality, cheaper and readily available alternative resources i.e. agro-industrial wastes and by-products to replace the costly ingredients in the fish feed. Castor seed meal or cake is a by-product of castor oil extraction industry and could be used in animal feed but presence of antinutritional factors limits its use in animal feed. Ricin is the major toxin present in the cake (Darby *et al*., 2001; Olsnes, 2004; Kozlov *et al.,* 2006; El-Nikhely *et al*., 2007). Several efforts have been made to utilize the castor seed meal/ cake in animal feeds (Mustapha *et al.,* 2015; Nagalakshmi and Dhanalakshmi, 2015); Santos *et al*., 2015); Borja *et al.,* 2017) and different processing methods have been used to detoxify the castor seed meal (Anandan *et al*., 2005; Diniz *et al.,* 2010; Santos *et al.,* 2015) and microbial fermentation (Madeira *et al*., 2011; Akande *et al*., 2011; Akande *et al*., 2016). Because toxins and anti-nutrients, many plant protein sources i.e. unconventional plant protein sources are not viable substitutes for the existing feed ingredients (Glencross *et al*., 2005; Kumar *et al*., 2011b). Modern processing technologies have overcome many of these hindrances, not only by denaturing antinutritional factors and solvent extracting much of the unsuitable lipid, but also by removing a large part of the undesirable carbohydrate and other components (Oliva-Teles *et al*., 1994; Nepal *et al*., 2010). Protein isolate from soybean, pea and canola meals are commercially available and currently used in fish feed production; but to the best of our knowledge little information is available on protein isolate from castor kernel meal. Therefore, the present study was designed to evaluate the nutritional potential of castor seed protein isolate (CPI) prepared from defatted castor kernel

meal in fish feed. In order to accomplish this objective, an attempt was made to study the effects of CPI on growth performance, nutrient utilization and digestibility; haemato-biochemical, digestive enzymes and metabolic responses; immunological and histological changes. Soybean protein isolate (SPI) based diet served as the reference diet.

5.5 Growth Performance, Nutrient Utilization and Body Indices

In the present study, the highest weight gain was recorded in the control, which was not statistically significant from CPI_{25}, CPI_{50}, and CPI_{75} groups while lowest values were observed in CPI_{100} group, which was significantly different from other groups. Growth rate i.e. weight gain (%) and specific growth rate (SGR) was found to be affected by dietary inclusion level of CPI. Highest growth rate was recorded in control and CPI_{75} group, followed by CPI_{25} & CPI_{50} groups and lowest in CPI_{100} group, which was significantly different from the other groups. It indicates that 75% substitution of soybean protein isolate is effectively possible with CPI without any adverse effect on the growth performance. But, the growth reduction in CPI_{100} group might be attributed to the severe deficiency of amino acids (L-lysine and DL-methionine) in the experimental diets. It has been reported that growth reduction in an animal might be attributed to imbalance of nutrients (essential amino acids), high indigestible material, and presence of ANFs in plant protein based diets (Francis *et al.,* 2001). Puttaraj *et al*. (1994) concluded that from the nutritional point of view, the detoxified castor protein isolate could not be recommended as the sole source of protein due to inherent amino acid imbalances (lysine and sulphur containing amino acids). However, in the present study the crude fibre content was optimal to the requirement of *L. rohita* fingerlings and anti-nutritional factors values also ranged as per the tolerance level of the fish. Medale *et al*. (1998) reported that total replacement of fish meal by soy protein concentrate led to a significant decrease in feed intake and resulted in poor growth and it was attributed to methionine deficiency in the soy protein concentrate based diet. Similarly, Fawole *et al*. (2018) reported that the

dietary jatropha protein isolate (JPI) affects the growth, feed conversion and nutrient utilization in *L. rohita* fingerlings as the growth rates and feed conversion were similar ($P>0.05$) at 25%, 50% and 75% substitution level of JPI with control. The author concluded that 75% substitution of soybean protein isolate (SPI) is possible with JPI and with 0.5% L-lysine supplementation up to 100% replacement of SPI. The presence of anti-nutritional factors such as cyanide, which apart also causes depletion of body amino acid (Deng *et al.*, 2015) and it was reported that cyanide concentration in diets is leading to amino acids imbalance particularly, sulphur containing amino acids (Okolie and Osagie, 1999; Okafor and Anyanwu, 2006).

Conversely, former studies indicate that feeding detoxified castor seed meal above 5% inclusion level causes depression in growth performance, feed conversion and phosphorus availability of juvenile grass carp, *Ctenopharyngodon idellus* (Cai *et al.*, 2005). It has been reported that poultry can utilize detoxified castor meal in their diets and is resistant to ricin toxicity as compared to other animals. The dietary inclusion of castor seed meal @ 5 to 20% is possible in the diet of poultry if detoxified properly. Different researchers has reported different processing methods and inclusion of CSM in poultry diets [soaking and fermentation (5% in starter male chicks, Oso *et al*. (2011); cooked (10–15% in broiler starter, Ani and Okorie, 2005), Ani and Okorie, 2009); sodium hydroxide treated (10% in broilers, Akande *et al.*, 2013); boiling and sodium hydroxide treatment (15% in broilers, Akande and Odunsi, 2012); boiling and β-xylanase treatment (15% in broiler finisher, Babalola *et al.*, 2006); methionine supplementation (15% in broilers, Ani and Okorie, 2013; and 10-20% in broilers, Ani and Okorie, 2005; Ani and Okorie, 2009); boiled for 40 min (complete replacement for soybean meal in layers, Nsa *et al.*, 2013); recovered with alcohol at 110 ºC for 15 min and dried at 110 ºC (200 g kg^{-1} in quail hens, Santos *et al.*, 2015)].

There was no significant difference found in feed intake in *L. rohita*. It signifies that feed is well consumed by fish and palatability and acceptability of the feed was good, no residue of feed was observed in experimental tanks. The acceptance of feed was not affected due to low level of tannin in diet (0.84-0.90 g kg^{-}

^{1}). These findings are in contrast to those reported by others who observed a decline in feed intake of soy and lupin protein isolates fed at higher than 40% inclusion levels to common carp and rainbow trout (Escaffre *et al*., 1997; Glencross *et al*., 2005). High tannin concentration in diets leads to decline in feed intake (Makkar *et al*. 2007b). Latif *et al.* (2015) observed normal fish behavior and voluntary feed intake when detoxified jatropha protein isolates (50% and 75%) substituted for fishmeal protein in common carp diets. Feed conversion, nutrient utilization and protein retention indices were affected by dietary CPI inclusion levels. Highest feed conversion and nutrient utilization indices were observed in control and CPI_{75} group while lowest in CPI_{100} group. It is suggested that *L. rohita* fingerlings were able to digest, and utilize the nutrient of CPI_{75} and control diet and it can be attributed to high nutritional values and low anti-nutrients present in the diets. Several authors have tried to evaluate the protein isolates obtained from different oilseeds in fish diets and find out the possible replacement of fish meal or soybean protein isolate (SPI) such as 75% fish meal with detoxified jatropha protein isolate in common carp (Kumar *et al.,* 2012); 100% fish meal with canola protein isolate in rainbow trout (Slawski *et al*., 2013); 50% fish meal with detoxified jatropha protein isolate in common carp (Latif *et al*., 2015); 100% soybean protein concentrate with fermented jatropha protein concentrate in rohu (Shamna *et al*., 2015); 100% SPI with rubber protein isolate supplemented with DL-methionine in rohu (Fawole *et al*., 2016b); 75% SPI with detoxified jatropha protein isolate supplemented with L-lysine in rohu (Fawole *et al.,* 2018).

Both the HSI and VSI values showed no significant difference among the experimental groups. It indicates that the digestive physiology of the intestine was not affected after feeding dietary CPI. The present results are in agreement with other researchers (Kumar *et al.,* 2012; Latif *et al*., 2015; Shamna *et al*., 2015*;* Fawole *et al*., 2016b, 2018). As reported that ricin is most lethal toxin present on the earth and LD_{50} value for animals were reported by different researchers such as as in pigs, 13-65 mg kg^{-1} body weight; in rabbits, 9–45 mg kg^{-1} body weight; in horses, from 1 to 5 mg kg^{-1} body weight; and in chickens, 140–170 mg kg^{-1} body weight (Garland and Bailey, 2006). The survival rate did not vary among different dietary treatments and it indicates that there was no toxicity of ricin in *L. rohita* fingerlings.

5.6 Whole Body Composition

Data pertaining to the biochemical composition of the experimental fish is given in Table 19. Whole body composition of *L. rohita* fingerlings *viz*. moisture, crude protein, ether extract, total carbohydrate and total ash content did not vary among the treatments. It indicates that CPI-based experimental diets contain optimum digestible energy and protein required for rohu fingerlings and did not contain ANFs beyond tolerance level. Protein isolates obtained from different oilseeds meal/cakes did not affect the whole body composition of different fish (soybean protein concentrate in rainbow trout (Mambrini *et al.,* 1999); detoxified jatropha protein isolate in common carp (Kumar *et al.,* 2012); canola protein isolate in rainbow trout (Slawski *et al*., 2013); detoxified jatropha protein isolate in common carp (Latif *et al*., 2015); fermented jatropha protein concentrate in rohu (Shamna *et al*., 2015); rubber protein isolate supplemented with DL-methionine in rohu (Fawole *et al*., 2016b); detoxified jatropha protein isolate supplemented with L-lysine in rohu (Fawole *et al.,* 2018). In the present study, a negative correlation was observed between the lipid and moisture contents (r= -0.72) in rohu fingerlings and it was supported by Kumar *et al*. (2012) and Fawole *et al*. (2016b, 2018).

5.7 Apparent Digestibility Coefficients (ADCs)

ADC of protein is a good indicator to evaluate the protein quality. In the present study, apparent digestibility coefficients (ADC) of dry matter and nutrients (protein and lipid) were not affected by dietary treatments. ADC of DM, protein, and lipid values ranged from 68.73% to 70.71%, 79.44% to 84.02%, and 86.16% to 87.54%, respectively. ADC of protein in our study compares well with other reports such as for canola protein in rainbow trout, 57.0–76.8% (Mwachireya *et al*., 1999); wheat gluten and soybean meal in turbot, 83.8–86.3% (Bonaldo *et al*., 2011); corn gluten in turbot, 73.5–88.5% (Regost *et al*., 1999); 78.83 – 84.23 % in common carp (Suprayudi *et al.,* 2015); and 73.90 – 82.13 % in *L. rohita* (Baruah *et al.,* 2007); rubber

protein isoate in rohu, 78.59-86.24% (Fawole *et al.*, 2016b); Jatropha protein isolate in rohu, 76.72-89.00%, (Fawole *et al.*, 2018). No significant difference in ADC of lipid was found among the various groups, which is in agreement with the findings of Kumar *et al*. (2012).

5.8 Digestive Enzyme Activities

Digestive enzyme plays an important role in the breakdown of complex nutrients of feed into simple absorbable form in order to facilitate their absorption by the intestine. Now-a-days, the incorporation of plant protein ingredients in fish feed has been practised and these plant ingredients contain anti-nutritional factors. However, negative physiological and morphological effects have been reported with the intake of antinutritional factors in fish species (Chikwati *et al*., 2012; Knudsen *et al*., 2008; Kortner *et al*., 2012; Yamamoto *et al*., 2012). ANFs affect the digestion process either by inhibiting the activities of digestive enzymes or by interaction with nutrients and reducing their bioavailability. In the present study, dietary CPI did not affect the digestive enzyme activities such as protease, amylase and lipase. It indicates that experimental diets contained anti-nutritional factors below the tolerable range and did not exhibit any inhibitory effects on the digestion process. The present results are in line with the findings of other researches (Kumar *et al*., 2012; Luo *et al*., 2012; Fawole *et al*., 2016b). Conversely, Shamna *et al.* (2015) observed a significant decline in intestinal protease enzyme activity in *L. rohita* fingerlings fed jatropha protein concentrate and might be due to the presence of anti-nutritional factors.

5.9 Metabolic Enzyme Activities

5.9.1 Aminotransferase enzyme activity

Among the transaminases, the most studied ones are aspartate aminotransferase and alanine amino transferase and a higher activity of these

enzymes were observed during an elevated protein catabolism and gluconeogenesis (Das, 2002), which are also considered as good indicators of liver damage when detected in serum or plasma (Oluah, 1999). In the present study, the AST activity in liver and muscle and ALT activity in liver was affected significantly by dietary CPI levels while ALT activity in muscle did not vary among different dietary treatments. In liver and muscle, AST activity increased with the dietary CPI levels and was found to be highest in CPI_{75} group and decreased afterwards. ALT activity in liver was found to decrease with the dietary CPI levels up to 75% replacement of SPI in CPI_{75} group and then increased afterwards. AST and ALT activities in CPI_{75} group are similar to control group, which supported the growth performance and protein retention in the present study. Increased AST activity in muscle in CPI fed groups may suggest that increase in the efficiency of the enzyme to synthesize non-essential amino acids for building a new protein as experimental diets were deficient in L-lysine and DL-methionine (Dalal *et al.,* 2001). Based on the results of aminotransferase it can be inferred that experimental diets were able to fulfill the nutritional requirement of *L. rohita* and present results are supported by the other researchers (Luo *et al*., 2012). Several researchers did not find any variations among the aminotransferase activities in fish when fed with protein isolates of oil seeds (Deng *et al*., 2015; Fawole *et al*., 2016b). While feeding Jatropha protein concentrate to rohu fingerlings exhibited decreased ALT and AST enzymes activities in both the muscle and liver (Racicot *et al*., 1975; Shamna *et al*., 2015).

5.9.2 Lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) enzyme activity

In the present study, LDH activity in liver was affected by the dietary CPI levels while in muscle, LDH activity did not vary among the various treatment groups. The activities of LDH enzyme in both the liver (except CPI_{100} group) and muscle showed no significant variation compared to the control group. Murray *et al.,* (2000) reported that an increase in tissue level LDH activity reflects the animal is under stress. Similarly, Shamna *et al.* (2015) reported that feeding detoxified jatropha protein concentrate to rohu led to increase in LDH activity in liver and muscle. The

results of present study reveal that fish are not under oxygen debt or hypoxic stress and this is well supported by Fawole *et al*. (2016b and 2018).

Malate dehydrogenase (MDH) is an enzyme of the citric acid cycle which catalyzes the conversion of malate into oxaloacetate and vice-versa and increased activity of MDH indicates the energy demand of the tissue. In the present study, MDH activity in liver and muscle tissue did not change with the dietary CPI levels. It indicates that there is no stress in rohu fingerlings, and energy demand could have been fulfilled with the experimental diets only. Shamna *et al*. (2015) reported a higher MDH activity when fed with dietary jatropha protein isolate (DJPI) than dietary fermented jatropha protein isolate levels. An increase in MDH activity might be due to the stress caused by anti-nutritional factors present in DJPI which lead to increased energy demand of tissue. While fermentation of the DJPI with *Aspergillus niger* causes reduction in anti-nutritional factors and thus resulted in reduction of MDH activities in liver and muscle tissue of rohu fingerlings. This finding is supported by the findings of Das *et al*. (2006), where elevated MDH activity in fishes acclimated at higher temperature was observed.

5.10 Antioxidant Enzyme and Oxidative Status

Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase constitute the first line of the enzymatic defense mechanism against the damaging effects of free radicals induced by oxidative stress (Deng *et al.,* 2015; Fawole *et al.,* 2015). To evaluate the animal/ fish health, the quantification of anti-oxidant enzymes can be used as stress and immune-response biomarkers (Sagstad *et al.,* 2007; Tovar-Ramirez *et al.,* 2010; Tancredo *et al.,* 2015; Fawole *et al.,* 2015). In the present study, the SOD activity in liver varied significantly with dietary CPI levels and the activity in CPI_{25}, CPI_{50} and CPI_{75} was found similar to control and highest ($P<0.05$) activity was observed in CPI_{100} group. The higher activity might be attributed to the presence of anti-nutritional factors and deficiency of amino acids and it is in agreement with the findings of Deng *et al*. (2015). While the SOD

activity in gills and catalase activity in liver and gills did not vary in rohu when fed with dietary CPI and these results are supported by Fawole *et al*. (2016b).

5.11 Biochemical and Haemato-Immunological Parameters

5.11.1 Serum protein biochemistry

In a previous study, Babalola *et al*. (2006) observed that serum total protein, albumin and globulin concentrations were decreased in pullet chicks when fed with increasing dietary boiled castor seed meal and it might be due to the presence of ricin content in experimental diet which causes impaired protein metabolism in the liver. In the present study, dietary CPI levels did not affect the serum protein biochemistry (except serum total protein). Maximum serum total protein concentration was found in rohu fingerlings in CPI_{100} and CPI_{25} groups and minimum in control, CPI_{50} and CPI_{75} groups. However, CPI_{75} and control diet exhibited similar nutrient utilization therefore, it can be inferred that there would not be any impaired protein metabolism in liver. Nevertheless, the globulin concentration in rohu fingerlings did not vary with dietary CPI levels and it signifies that the dietary CPI does not suppress the immune system as supported by other researchers (Wiegertjes *et al*., 1996; Latif *et al*., 2015; Kumar *et al*., 2010a; Fawole *et al*., 2017).

5.11.2 Haemato-immunological parameters

The activity of aminotransferase enzymes (ALT and AST) are mainly localized in the liver, and become an indicator of liver damage in animal when detected at higher concentration in the bloodstream (Goel *et al.,* 1984; Xu *et al.,* 2012; Li *et al.,* 2015). In the present study, the serum ALT activity tended to increase with dietary CPI level and highest activity was observed in CPI_{100}. The ALT activity in CPI_{75} and control was not differed significantly and it was supported by the best growth and nutrient utilization. The detection in higher concentration reveals liver damage in CPI_{100} but it was not supported by changes in ultrastructure of liver and

insignificant AST activity. The present results are in agreement with other researchers (Kumar *et al.*, 2010b; Fawole *et al.*, 2017, 2018).

The respiratory burst activity (RBA) as measured by NBT reduction forms the basis of a highly potent antibacterial system, which means an increased RBA can be correlated with increased activity of phagocytic cells and better immunity (Sharp and Secombes, 1993; Anderson, 1994). In the present study, dietary CPI affected respiratory burst activity and it is reflected in an increase in NBT activity in rohu fingerlings with CPI levels. Fawole *et al.* (2017) observed no significant difference found in NBT activity in rohu when fed with dietary rubber protein isolate.

Elevated blood glucose level has been reported in many fish species fed plant protein based diets due to the higher contents of carbohydrates (Kikuchi *et al.,* 1999; Kumar *et al.,* 2010b; Akinleye *et al.,* 2012). In the current study, no significant difference was observed in the serum glucose concentration among the various experimental fed groups. This is in concurrence with the findings of Slawski *et al.* (2012) and Fawole *et al.* (2018).

5.12 Haematological Parameters

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 (Roberts, 1989; NRC 1993; Houston, 1997; Santhakumar *et al.,* 1999; Whyte, 2007). In a previous study, it was observed that feeding detoxified castor meal (DCM) to pullet chicks greatly affected the haematological parameters. Akande and Odunsii (2012) revealed that all haematological parameters were shown to decrease with the increasing dietary (DCM) levels and it might be due to the presence of lectins (ricin) and ricinine. In the current study, all the haematological parameters except RBC and haemoglobin, showed no significant difference between the control and the treatment groups,

suggesting that the dietary CPI did not hinder the erythropoiesis process in rohu. Maximum total erythrocyte counts (RBC) was reported in CPI_{75} group, while CPI_{25}, CPI_{50}, and CPI_{100} groups did not show any variation with the control group. The higher concentration of haemoglobin was observed in control, CPI_{50}, and CPI_{75} while least in CPI_{25}, and CPI_{100} groups. It indicates that there is no toxicity of ricin, HCN and ricinine (RCA) in rohu fingerlings up to CPI_{75} level as it causes high haemagglutination of red blood cells and affects blood parameters (Sehgal *et al.*, 2011). Ricinine exhibits more agglutination properties than ricin and is concentrated in seed coat and the hull was removed during preparation of protein isolate. The results of the hematological indices are in agreement with the findings of other scholars (Sun *et al.,* 1995; Kumar *et al.,* 2010; Akinleye *et al.,* 2012; Slawski *et al.*, 2012) and are in disagreement with the findings of Soltan *et al.* (2008) and Shamna *et al.* (2015). Therefore, it can be inferred that *L. rohita* fingerlings fed different experimental diets were in good and or equal nutritional and health status, and the tested ingredients (CPI) had no detrimental effect on the fish health.

5.13 Histopathological Changes

The liver and intestine are the most important organs in digestion and absorption of nutrients, hence, it is important to examine these organs when diet is changed (Hall and Bellwood, 1995; Caballero *et al.,* 2003). In the present study, the histological examination of the liver revealed polygonal hepatocytes and prominent nuclei and nucleoli, and granular cytoplasm in the control group. No remarkable changes such as necrosis, cellular degeneration, steatosis or hepatic lipidosis were observed in liver of *L. rohita* fingerlings fed with different level of CPI. In a laboratory experiment where fishmeal was replaced with soybean meal, a pyknosis of liver nuclei as well as smaller nuclear area was observed (Raskovic *et al.,* 2009), and the authors stated that the reduction in nuclear size may be a sign of malnourishment. Histological examination of fish organ is important in understanding the pathological alteration related to nutritional sources (Gargiulo *et al.,* 1998). The histological

examination of the intestine exhibited cellular changes in all dietary treatment and control groups. The noticeable changes were increased fusion of mucosal folds, reduced number of supra-nuclear absorptive vacuoles in enterocytes and increased width and intracellular infiltration in the lamina propria of intestine with gradual increase in the dietary CPI level. It might be due to the imbalance of amino acids (L-lysine and DL-methionine) or presence of anti-nutritional factors such as hydrogen cyanide. In experimental diets, the deficiency of L-lysine and DL-methionine increases with the dietary CPI levels concomitant with increased concentration of HCN in diet. Similarly, Puttaraj *et al.* (1994) reported that dietary CPI influenced the histological changes in liver and kidney of rat and these cellular changes may be attributed to the deficiency of sulphur amino acids in castor protein isolate. In a related experiment, severe abnormalities such as cellular infiltration, desquamation of mucosa and necrosis were profoundly noticed in the proximal and distal intestine of common carp juvenile fed with unprocessed rubber seed meal (Suprayudi *et al.,* 2015). The authors described these severe changes to the chronic exposure of the fish to cyanide through the diet. However, Nagel *et al.* (2012) and Bonaldo *et al.* (2011) observed no histo-pathological alterations in the mid-gut of turbot *Psetta maxima* fed rapeseed protein isolate and mixture of plant proteins, respectively. Like-wise, Fawole *et al.*, 2017 also observed no significant changes in histological examination of intestine when fed with rubber protein isolate. Similar observation was also made in gilthead sea bream (*Sparus aurata*) fed bioprocessed soybean meal as a substitute for fishmeal (Kokou *et al.,* 2012).

Experiment III

Soybean meal or cake is the most commonly used feed ingredient and also has competition with human food industries (Yue and Zhou, 2008). In response to it, several plant proteins were tested in animal feed but these did not fit as they contained huge indigestible portion and inherited several anti-nutritional factors, which limit their use in animal feed (Glencross *et al*., 2005; Kumar *et al*., 2011a). Hence, the necessity arises to discover good quality, cheaper and readily available alternative resources i.e. agro-industrial waste and by-products to replace the costly ingredients in the fish feed. Non-edible seed cake could serve as an alternate feed ingredient in animal feed if they do not contain anti-nutritional factors (Glencross *et al*., 2005; Kumar *et al*., 2011b; Shamna *et al*, 2015; Fawole *et al*., 2016a). Castor seed meal or cake is a by-product of castor oil extraction refineries and also contains ANFs. Several efforts have been made to utilize the castor seed meal/ cake in animal feeds (Mustapha *et al.,* 2015); Nagalakshmi and Dhanalakshmi, 2015); Santos *et al*., 2015); Borja *et al.,* 2017). The modern processing technologies have overcome many of these hindrances, not only by denaturing antinutritional factors and solvent extracting much of the unsuitable lipid, but also by removing a large part of the undesirable carbohydrate and other components (Oliva-Teles *et al*., 1994; Nepal *et al*., 2010).

In experiment II, amino acid profile indicated that L-lysine and DL-methionine were deficient in all the experimental diets (Table 5). The amino acid requirement of *L. rohita* fingerlings was fulfilled by supplementing the deficient amino acids as suggested by NRC (2011). Therefore, the present experiment was designed to evaluate the nutritional potential of protein isolates prepared from defatted castor kernel meal (CPI) supplemented with deficient L-lysine and DL-methionine in *Labeo rohita* fingerlings. In order to achieve this objective, an attempt was made to examine the effects of CPI on growth performance, nutrient utilization and digestibility; haemato-biochemical, digestive enzymes and metabolic responses; immunological and histological changes. Soybean protein isolate (SPI) served as the reference diet.

5.14 Growth Performance, Nutrient Utilization and Body Indices

In experiment II, the growth performance, feed conversion and nutrient utilization indices exhibited that castor seed protein isolate possesses good nutritional value as a dietary protein source for *Labeo rohita* fingerlings and can replace soybean protein isolate up to 75%. But the deficiency of L-lysine and DL-methionine were reported in all experimental diets. In this experiment, the deficiencies of L-lysine and DL-methionine were fulfilled and fed to *L. rohita* fingerlings. The growth performance, feed conversion and nutrient utilization indices revealed that complete substitution of soybean protein isolate is possible with dietary castor protein isolate. The improvement in growth and nutrient utilization indices observed in the present experiment indicate that the depression in growth response noticed in experiment II might be attributed to the amino acid imbalances. Many researchers evaluated the possible replacement of fish meal and/or soybean protein isolates in fish feeds such as 75% fish meal with detoxified jatropha protein isolate in common carp (Kumar *et al.,* 2012); 100% fish meal with canola protein isolate in rainbow trout (Slawski *et al.*, 2013); 50% fish meal with detoxified jatropha protein isolate in common carp (Latif *et al.*, 2015); 100% soybean protein concentrate with fermented jatropha protein concentrate in rohu (Shamna *et al.*, 2015); 100% SPI with rubber protein isolate supplemented with DL-methionine in rohu (Fawole *et al.*, 2016b); 75% SPI with detoxified jatropha protein isolate supplemented with L-lysine in rohu (Fawole *et al.,* 2018). These studies suggested that protein isolates did not cause any adverse effects on growth performance, feed conversion, and nutrient utilization. In the current study, the highest weight gain and growth rates i.e. weight gain (%) and specific growth rate (SGR) were observed in *L. rohita* fingerlings in $ACPI_{50}$ and $ACPI_{75}$ groups, while similar values were observed in control, $ACPI_{25}$**,** and $ACPI_{100}$ groups. It signifies that growth rate at 50% and 75% substitution level was best but 25% and 100% replacement levels exhibited similar growth with control (100% SPI). Feed conversion efficiency, protein efficiency ratio and protein productive values were also follow the same trends as growth and were affected by the dietary CPI levels. Feed intake was found to be similar in different treatments and control and it might be due to lower concentration of anti-nutritional factors in experimental diets (Kumar *et*

al., 2012; Latif *et al.,* 2015). Both the HSI & VSI values and survival rate did not differ among different experimental groups (P>0.05).

5.15 Whole Body Composition

Chemical composition of *L. rohita* fingerlings was found to be independent of dietary CPI levels (P>0.05) except crude protein content. The crude protein content in rohu fingerlings did not exhibit significant difference in control (100% SPI) and 100% CPI substitution level and higher values were observed at 25%, 50% and 75% substitution level. Similar results were obtained in previous studies in fishes fed with protein isolates *viz*. soybean protein concentrate in rainbow trout (Mambrini *et al.,* 1999); detoxified jatropha protein isolate in common carp (Kumar *et al.,* 2012); canola protein isolate in rainbow trout (Slawski *et al*., 2013); detoxified jatropha protein isolate in common carp (Latif *et al*., 2015); fermented jatropha protein concentrate in rohu (Shamna *et al*., 2015); rubber protein isolate supplemented with DL-methionine in rohu (Fawole *et al*., 2016b); detoxified jatropha protein isolate supplemented with L-lysine in rohu (Fawole *et al.,* 2018).

5.16 Digestive Enzyme Activities

Digestive enzyme activities are the indicator of nutritional status and digestive capacity of the fish (Eroldogan *et al*., 2008) and the activities can be hindered by different ANFs present in plant based diets. ANFs such as trypsin inhibitors inhibit the activities of digestive enzymes like trypsin, and chymotrypsin (Alarcon *et al.,* 1999), and hindering digestion of proteins (Bajpai *et al*., 2005; Guillamon *et al*., 2008). Whereas, phytates bind with divalent minerals and form a complex with protein thereby, reduces the bioavailability of minerals and protein required for muscle growth (Sugiura *et al.,* 1999; Kumar *et al*., 2012). In the current study, it was noticed that the supplementation of deficient amino acids (L-lysine and

DL-methionine) and lower concentration of ANFs in experimental diet support better growth and nutrient utilization in rohu without any adverse effects on digestive physiology. These results are in agreement with the previous findings reported by other researchers (Luo *et al*., 2012; Kumar *et al*., 2012; Fawole *et al*., 2018). On the other hand, the changes in digestive enzymes activities were reported by Saha and Ghosh (2013) and Shamna *et al.* (2015).

5.17 Metabolic Enzyme Activities

5.17.1 Aminotransferase enzyme activity

In the present study, the AST activity in liver and muscle and ALT activity in muscle was affected significantly by the dietary CPI levels while ALT activity in liver did not vary among the different dietary treatments. In liver and muscle, maximum AST activity was found in *L. rohita* fingerlings fed with 25% and 50% CPI substitution level and exhibited a decreasing trend in liver and muscle (up to 75% replacement of SPI). In muscle, maximum ALT activity was recorded in control and ACP_{100} groups and showed a decreasing trend up to 75% replacement of SPI. No significant differences were recorded in AST and ALT activities in liver with control which is in agreement with the growth performance and protein retention in the present study. The study indicates that the dietary protein met the requirement of the fish and it was adequately utilized for muscle growth. Although, the ALT activity in the muscle was found to be higher in fish fed control and $ACPI_{100}$, but did not indicate that amino acids been utilized for glucose production since the activity in liver showed no significant variation. Similarly, Deng *et al*. (2015) observed that rubber seed meal did not affect the hepatic ALT and AST activities in tilapia. Conversely, Luo *et al*. (2012) and Shamna *et al*. (2015) reported a decrease in AST and ALT activity in juvenile cobia and rohu fingerlings.

5.17.2 Lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) enzyme activity

In the presence of enough oxygen, pyruvate enters the Kreb's cycle, but when there is an oxygen debt in the tissue, pyruvate is converted to lactate by LDH and NADH which is converted to NAD^+ to maintain redox-potential in the tissue (Murray *et al*. 2000). Thus, lactate dehydrogenase helps in maintaining the glycolysis cycle by supplying NAD^+. In the present study, LDH activities in liver and muscle were affected by the dietary CPI levels and showed a decreasing trend with the dietary CPI levels. LDH activity in rohu fingerlings in 100% substitution level of CPI was similar to control group which indicates that there is no condition of hypoxic stress in rohu fingerlings. Fawole *et al*. (2016b and 2018) did not found any hypoxic stress condition arises because LDH activity did not exhibit any variation. Whereas, Shamna *et al.* (2015) revealed the increased activity of LDH enzymes in rohu when fed with jatropha protein concentrate.

Malate dehydrogenase (MDH) catalyzes the inter-conversion of L-malate and oxaloacetate using nicotinamide adenine dinucleotide (NAD) as a coenzyme. An increase in MDH activity correlates with raising energy demand of the tissue. Present study exhibited that MDH activity in liver did not change with the dietary CPI levels while in muscle, it was significant with dietary CPI. However, MDH activities observed in rohu fingerlings in 75% and 100% substitution of SPI level and control group were similar. It reveals that there is no stress condition in rohu and the results are contradictory to the findings of Shamna *et al*. (2015) who found increase levels of MDH when fed with jatropha protein isolate but not with fermented jatropha protein isolate.

5.18 Antioxidant Enzyme and Oxidative Status

SOD, CAT and GPx are considered as major antioxidant enzymes in fish (Furne *et al*., 2009). They exhibit the natural defense against peroxidation of the

lipid (Zimmermann *et al*., 1973). These enzymes scavenge the free radicals which are produced during stress and ameliorate the stress. SOD converts the ROS produced during stress to H_2O_2 while CAT and GPx detoxify the H_2O_2 (Slaninova *et al*., 2012). In the present study, the SOD and CAT activities were not affected by the dietary CPI levels, suggesting that no oxidative-induced stress occurred a result of feeding CPI even at higher inclusion levels. The similar SOD activity observed among the experimental groups was in consonant with the findings of Deng *et al*. (2015), Fawole *et al*. (2016b and 2018) while contradictory to findings of Shamna *et al.* (2017) and Zheng *et al.* (2012).

5.19 Biochemical and Haemato-Immunological Parameters

5.19.1 Serum protein biochemistry

Serum total protein is another important parameter showing the nutritional and health status of fish (Martinez, 1976). Albumin and globulin are important component of serum protein, whose functions in the immune response of fish have been documented (Kumar *et al.,* 2010b; Makkar *et al.,* 2011; Fawole *et al.,* 2015). Results of present study revealed that serum total proteins, albumin, globulin and albumin-globulin ratio were not significantly different in rohu when fed dietary CPI. Study indicates that fish are in good health condition and dietary CPI did not elicit any detrimental effect on fish health. The present results are in agreement with the findings of other researchers (Kumar *et al*., 2010b; Latif *et al*., 2015; Fawole *et al*., 2017) and contradictory to the findings of Babalola *et al*. (2006) who observed decreased serum total protein, albumin and globulin concentrations in pullet chicks and it was attributed to presence of antinutritional factors in castor seed meal.

5.19.2 Haemato-immunological parameters

Aminotransferase enzymes (ALT and AST) are present mainly in the liver, but can be released into the bloodstream when there is damage to the liver cells (Racicot *et al.,* 1975; Goel *et al.,* 1984; Li *et al.,* 2015). Hence, the increased activity

in serum or plasma is often considered as an indicator of the liver impairment caused by the action of toxins or pathogen (Burtis and Ashwood, 1996; Xu *et al.,* 2012). In the present study, no significant variations were observed in the serum ALT and AST activities in rohu fingerlings among the various groups and these results are in agreement with Sanden *et al.* (2006), Makkar *et al.* (2011) and Fawole *et al*. (2017). Conversely, Deng *et al.* (2015) observed significant changes in plasma ALT levels in hybrid tilapia when fed with rubber seed meal. The respiratory burst activity (RBA) as measured by NBT reduction forms the basis of a highly potent antibacterial system, which means an increased RBA can be correlated with increased activity of phagocytic cells and better immunity (Sharp and Secombes, 1993; Anderson, 1994). However, in the present study, effects of CPI were examined on cellular (respiratory burst activity) and it was found that higher RBA activity was observed in dietary treatments than control group (except $ACPI_{75}$ group) in rohu. Fawole *et al*. (2017) observed no significance difference in NBT activity in rohu when fed with dietary rubber protein isolate. Kumar *et al*. (2012) observed that blood glucose level was depended on the dietary carbohydrate sources and incorporation of plant protein based diets in fish species interfere with carbohydrate metabolism (Kikuchi *et al.,* 1999; Kumar *et al.,* 2010). In the present study, feeding dietary CPI to rohu fingerlings did not affect the serum glucose concentration among the various treatment groups. This is in concurrence with the findings of Slawski *et al.* (2012) and Fawole *et al*. (2018).

5.20 Haematological Parameters

The level of stress in fish can be assessed by changes in various biochemical and physiological parameters and their return to normal conditions (Hoseini, 2011). Haematological changes are considered as stress biomarker (Barcellos *et al*., 2004; Jahanbakhshi *et al.,* 2013). In the current study, all the haematological parameters except haemoglobin, exhibited no significant difference among different dietary treatments in rohu. The highest concentration of haemoglobin

was observed in rohu fingerlings at control, and 75% substitution level of SPI but hemoglobin concentration at 100% substitution level of SPI did not show any discrepancy. It indicates that dietary CPI supplemented with L-lysine and DL-methinine did not elicit any adverse effect on erythropoiesis process and are in agreement with Slawski *et al.* (2012), Fawole *et al*. (2017 and 2018) whereas these results are contrary to Soltan *et al.* (2008) and Shamna *et al.* (2015). Therefore, it can be said that *L. rohita* fingerlings fed different levels of CPI were in good and or equal nutritional and health status, and the tested ingredients (CPI) had no detrimental effect on the fish health.

5.21 Histopathological Changes

Plant-based fish feeds exhibited imbalanced indispensible amino acid composition, primarily methionine and lysine (Kaushik, 1995; Watanabe, 2002). Inclusion of plant protein sources in the diet of aquatic fish species affects the growth performance, nutrient utilization as they inherit several ANFs. These ANFs affects the digestion and utilization of nutrients in fishes and chronic feeding lead to changes at cellular levels in digestive organ and gland (Van den Ingh *et al*., 1991; Storebakken *et al*., 2000; Caballero *et al.,* 2003; Krogdahl *et al*., 2003). Histological examination of liver exhibits no significant changes as was observed in experiment II. Dietary CPI did not elicit any adverse change at cellular level in liver of *L. rohita* fingerlings. Dietary CPI supplemented with deficient L-lysine and DL-methionine did not reveal changes in intestinal histological structure of rohu fingerlings. Ultrastructure of the intestine exhibited improved structural integrity in the intestine of *L. rohita* fingerlings when fed with dietary CPI supplemented with deficient amino acids (L-lysine and DL-methionine). The noticeable changes were less fusion of mucosal folds, increased number of vacuoles in enterocytes and less intracellular infiltration in the lamina propria of intestine in comparison to the results of experiment II. Dietary inclusion of CPI with supplementation of L-lysine and DL-methionine did not affect digestive physiology; this fact was supported by the insignificant digestive enzyme activities

and histological examination of liver and intestine. No histopathological alterations were observed in fishes when fed with protein isolates and concentrates and are in agreement with the findings of Bonaldo *et al.*, (2011), Nagel *et al.*, (2012), and Fawole *et al*. (2017). Conversely, feeding castor meal to lambs exhibited severe cellular changes in intestine, liver and kidney and the noticeable changes were necrosis, congested blood vessels with increased kuffer cell activity in liver and swollen kidney tubules with increase in leukocyte cell infiltration with swollen lamina propria, and decreased goblet cell activity.

Experiment IV

Major feed ingredients used for fish feed include soybean meal, groundnut oil cake, mustard oil cake, cotton oilcake, de-oiled rice bran, wheat bran, and corn flour etc. depending on the availability and price. Among the most promising plant protein ingredients researched, soybean meal, appeared to be the best plant protein source used in aquafeed (Yue and Zhou, 2008). While, rice bran as agriculture byproduct is abundantly available locally and is being used as an energy source in fish feed. But, the competition from other animal feed industry for common feed ingredients such as soybean meal, fish meal, rice bran and oilcakes limit their availability. This has resulted in surging the price of fish feed and thereby low returns affecting sustainability of this enterprise. Thus the expedition for alternate protein sources has become a necessity for the sustainable aquaculture production. Several attempts have been made for incorporation of leaf meal in fish feed (Osman, 2007; Vhanalakar and Muley, 2014; Meshram *et al*., 2018; Ahmad *et al*., 2019). Among leaf meals, sweet potato (*Ipomoea batatas*) leaf can serves as an efficient protein and energy source for the preparation of fish diets as it contains 23-33% crude protein with the high amino acid score but presence of high crude fibre and anti-nutritional factors (ANFs) confines its use in animal feed. Several methods such as moist heat treatments, water soaking, and fermentation etc. have been used and documented to neutralize the ANFs from leaf meal. Fermentation with micro-organism could be an innovative approach to minimize the ANFs concentration as well as digestion of crude fibre in SPLM (Kim *et al*., 1999; Mahesh and Mohini, 2013; Keishing *et al*., 2015).

Nonedible oil seed i.e. castor seed was evaluated in present study and prepared protein isolates obtained from defatted meal has shown promising results and could be used as protein source in rohu, *L. rohita* diet with supplementation of deficient amino acids. Therefore, the present experiment was designed to examine the effects of CPI and FSPLM on growth performance, nutrient utilization and digestibility; haemato-biochemical, digestive enzymes and metabolic responses; immunological and histological changes. Further, four iso-nitrogenous (35.13±0.09% crude protein) and iso-caloric (420.95±0.46 kcal $100g^{-1}$) experimental diets were

formulated and prepared (Table 8) and fed to *L. rohita* fingerlings twice daily till satiation level for 60 days. Growth performance, survival, nutrient utilization, feed conversion, body indices, digestive enzymes and physio-metabolic changes were measured at the end of experiment and used as response parameters. Soybean protein isolate (SPI) and de-oiled rice-bran (DORB) served as the reference diet.

5.22 Proximate Composition and Anti-nutritional Factors of Sweet Potato Leafmeal (SPLM) and Fermented Sweet Potato Leafmeal (FSPLM)

Ample availability of sweet potato leaf meal projects it as a good alternative source of ingredient for animal feed in African and South-east Asian countries but high fibre content and presence of different anti-nutritional factors limits its use in fish and animal feed. Several attempts have been made to evaluate the nutritional value of SPLM in fish and animal feed (Adewolu, 2008; Abonyi *et al.*, 2012; Yuan *et al.*, 2013; Meshram *et al.*, 2018; Ahamd *et al.*, 2019). Many researchers have tried for removal of anti-nutritional factors in SPLM (Almazan, 1995; Kim *et al.*, 1999; Mwachireya *et al.,* 1999; Adewolu, 2008; Meshram *et al.*, 2018). In the present study, solid-state fermentation of SPLM with *Chaetomium globosum* for 120 hr was carried out to improve the nutritional value of SPLM (in terms of crude protein and crude fibre) and to minimize the anti-nutritional factors. The results of solid-state fermentation of SPLM revealed that the crude protein content had increased from 21.47% to 31.20% (45.32% increment) while crude fibre content decreased from 19.43% to 7.22% (62.84% reduction). It indicates that solid state fermentation improves the nutritional values of SPLM (Ramachandran *et al.*, 2005; Yuan *et al.*, 2013; Meshram *et al.,* 2018). Since, SPLM contains high crude fibre, and excessive fibre content leads to digestion problems in monogastric animals. Therefore, enzymatic hydrolysis of crude fibres especially cellulose by *C. globosum* is probably responsible for the reduction of the crude fibre content (Onyimba *et al.,* 2015). Present results were in agreement with those reported by Hassan *et al.* (2015),

reported solid state fermentation with *Saccharomyces cerevisiae* in soyabean meal increased protein content significantly. Onyimba *et al*. (2015) observed increased protein content in SPLM when subjected to submersed fermentation with *Chaetomium globosum*. The present study results are in agreement with the results of other researchers (Ogbonna and Popoola, 1997; Meshram *et al*., 2018) who observed that solid-state fermentation of SPLM resulted in a reduction in anti-nutritional factors such as tannin, phytates, trypsin inhibitor, oxalates, alkaloid and HCN up to 64.95%, 57.51%, 15.31%, 37.32%, 50.00% and 61.70%, respectively. Ramachandran *et al*. (2005) observed that solid state fermentation improves nutritional quality by reducing anti-nutritional factors thus, enhances the utilization of nutrients and growth performance of the animal. The results of present study are in agreement with the results of Makkar and Becker (2008); Kumar *et al*. (2010a); Shamna *et al.* (2016). The improvement of nutritional value of fermented plant-based ingredients have been observed by several researches (cotton seed meal, Sun *et al*., 2015; sesame seed meal, Bairagi *et al.,* 2002; and palm kernel meal, Ng and Wee, 1989).

5.23 Growth Performance, Nutrient Utilization and Body Indices

The current experiments, experimental diets were analyzed for amino acid profiling and concluded that the diets were deficient in L-lysine and DL-methionine. The deficiencies of L-lysine and DL-methionine were fulfilled as per the requirement of *L. rohita* fingerlings. The growth performance i.e. weight gain (%) and specific growth rate (SGR), feed conversion (FCR, FER, & PER) and nutrient utilization (ANPU) in *Labeo rohita* fingerlings exhibited no significant difference among the dietary treatment groups. It indicates that *L. rohita* fingerlings were able to utilize castor seed protein isolate (CPI) as the main source of protein and fermented sweet potato leaf meal (FSPLM) as main source of energy. However, in this study, all the diets were well accepted by the fish, and feed intake was similar among the groups. It indicates that preparation of protein isolates and solid-state fermentation of

SPLM resulted in reduction of anti-nutritional factors, thererby feed intake was not affected (Makkar *et al.,* 2007a; Kumar *et al.*, 2012; Latif *et al.,* 2015). Fawole *et al.* (2016b, 2018) observed that complete substitution of soybean protein isolate is possible with dietary rubber protein isolate and jatropha protein isolate (supplemented with 0.5% L-lysine) in rohu. Likewise, Slawski *et al.* (2013) did not observe any adverse effect on growth, nutrient utilization and feed intake in rainbow trout fed with canola protein isolate and 100% replacement of fish meal was possible in rainbow trout. Meshram *et al*. (2018) observed significantly higher growth performance in *L. rohita* fingerlings fed with 30% fermented SPLM than other treatment groups and control. The reason for this might be in the fact that that fermented SPLM found to be richer in essential amino acid concentration than SPLM (Onyimba *et al*., 2015). Both the HSI and VSI values showed no significant difference among the experimental groups. This was in agreement with the results of Shamna *et al.* (2015) and Fawole *et al*. (2018) who found no marked variation in the HSI and VSI values of *L. rohita* fed fermented jatropha protein concentrate and jatropha protein isolate, respectively.The present results are in agreement with Ahmad *et al*. (2019) and Meshram *et al*. (2018), the authors concluded that HSI and VSI in *L. rohita* fingerlings were not affected by feeding SPLM. The survival rate did not change significantly among different dietary treatments and better survival between 93.33±3.33 and 100.00±0.00 % were reported in the current study which indicates that there was no toxicity caused by anti-nutritional factors. This result is in agreement with Fawole *et al*. (2016b), Shamna *et al*. (2015), Fawole *et al*. (2018), Meshram *et al*. (2018), and Ahmad *et al*. (2019).

5.24 Whole Body Composition

Whole body composition of *L. rohita* fingerlings *viz*. moisture, ether extract, total carbohydrate and total ash contents did not exhibit any marked variation and it is in concomitant with the results of other researchers (Mambrini *et al*., 1999; El-Saidy and Gaber, 2003; Latif *et al*., 2015; Fawole *et al*., 2016b, 2018). Feeding leafmeal did not cause any changes in body composition and these results agreed

with the results reported in rohu, *L. rohita* fingerlings (Ahmad *et al*., 2019), *Pleurotus florida* (Muin *et al*. 2013), and *Tilapia zilli* fingerlings (Adewolu, 2008). The negative correlation observed between the lipid and moisture contents (r= -0.91) of the whole body in the current study were in agreement with those of Kumar *et al*. (2012).

5.25 Digestive Enzyme Activities

An improvement in the growth performance of animals is a function of the digestive enzyme influence on the digestion processes (Lemieux *et al.,* 1999). No variations were observed in the digestive enzymes activities such as protease, amylase and lipase in the present study. It indicates that dietary CPI and FSPLM did not elicit any inhibitory effect on the digestion process due to lower concentration of anti-nutritional factors in the experimental diets. This could be in earlier studies that feeding protein isolates did not alter the digestive enzymes activities in different fishes such as in common carp (Kumar *et al*., 2012) and in rohu (Fawole *et al*., 2016b, 2018). Feeding raw SPLM affected the digestive enzymes activity in rohu fingerlings (Meshram*,* 2017; Ahmad *et al*., 2019) might be due to the presence of high crude fibre and anti-nutritional factors. These observations were in corroboration with the findings of Santigosa *et al*. (2008) and Krogdahl *et al*. (1994) in trout. Author revealed that the digestive enzyme activity in FSPLM at 30% inclusion level was similar to DORB fed group (Meshram, 2017).

5.26 Metabolic Enzyme Activities

5.26.1 Aminotransferase enzyme activity

In the present study, the AST and ALT activities in liver and muscle was not affected significantly by dietary CPI and FSPLM levels. It indicates that the dietary protein met the requirement of the fish and adequately utilized it for muscle growth. Similar results have been reported in fishes when fed with protein isolates (Latif *et al*.,

2015; Fawole *et al*., 2017, 2018) whereas, Luo *et al*. (2012) and Shamna *et al*. (2015) reported that the activities of AST and ALT enzymes declined when fed with protein isolates. Ahmad *et al*. (2019) reported that feeding SPLM to rohu affects the transaminase activity and similarly, Meshram (2017) reported that higher AST activity in muscle was observed in 30% dietary inclusion of FSPLM than other groups. It was suggested that higher activity of AST and ALT enzymes in the muscle indicates the synthesis of pyruvate and oxaloacetate, which are involved in the synthesis of non-essential amino acids required for growth (Ahmad, 2016; Meshram, 2017).

5.26.2 Lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) enzyme activity

In the present study, LDH activities in liver was affected by dietary CPI and FSPLM levels. While in muscle, it did not vary among different dietary treatments. Rohu fingerlings fed with dietary FSPLM exhibited higher LDH activity in liver than DORB fed groups and this might be due to the presence of ANFs in SPLM (Meshram *et al*., 2018 and Ahmad *et al*., 2019). It indicates that fish fed with dietary FSPLM were under stress but since other indices were not affected by dietary CPI and FSPLM therefore, it signifies that the fish are not under oxygen debt or hypoxic stress. Likewise, Fawole *et al*. (2016b and 2018) revealed that LDH activity in rohu fingerlings did not vary when fed with dietary rubber protein isolate and jatropha protein isolate. In contrast to our findings, Shamna *et al.* (2015) observed an increased tissue level activity of LDH enzymes in *L. rohita* fingerlings when fed with jatropha protein concentrates.

Significant variations in MDH activity was reported in fishes when fed with plant protein isolates and authors revealed that an increase in MDH activity might be due to the stress caused by anti-nutritional factors leading to increased energy demand of tissue (Shamna *et al*., *2015*). In the present study, MDH activity did not vary among different dietary treatments and this is in agreement with other findings in fishes fed with protein isolates (Das *et al*., 2006; Shamna *et al*., 2015) and leafmeal (Ahmad, 2016; Meshram, 2017).

5.27 Antioxidant Enzyme and Oxidative Status

Oxidative stress results when the antioxidant defenses are overcome by pro-oxidant forces and reactive oxygen species are not removed adequately (Sies *et al*. 1986). Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase constitute the first line of the enzymatic defense mechanism against the damaging effects of free radicals induced by oxidative stress (Deng *et al.,* 2015; Fawole *et al.,* 2015). In the present study, the SOD and CAT activities did not vary in rohu fingerlings among different treatments and are in consonant with the findings of Deng *et al*. (2015) and Fawole *et al*. (2016b and 2018). Our results are in contrast to the findings of Shamna et al. (2017) who reported that feeding jatropha protein concentrate (20% JPC) containing high level of phorbol esters to *L. rohita* fingerlings resulted in a decreased hepatic SOD activity. Ahmad (2016) reported that the SOD activity decreased with dietary SPLM levels and indicates that the free radicals were effectively scavenged by the multiple compounds present in SPLM. Reduced activity might be due to the presence of phenolic compounds, flavonoid and tannins, which act as a powerful antioxidant and had been shown to scavenge free radicals and protect the lipid membrane and other compounds being oxidized or destroyed (Popeskovic *et al*., 1980). Meshram (2017) observed no difference in SOD and CAT activities in rohu when fed with dietary FSPLM among different treatments and control.

5.28 Biochemical and Haemato-Immunological Parameters

5.28.1 Serum protein biochemistry

There were no significant differences observed in the total protein, albumin, globulin concentrations and albumin-globulin ratio in *L. rohita* fingerlings when fed with dietary CPI and FSPLM. Similar findings were also reported by several researchers in common carp fed with detoxified Jatropha protein isolate (Latif *et al*., 2015) and detoxified jatropha kernel meal (Kumar *et al*., 2010b), in rohu fed with

rubber protein isolate and jatropha protein isolate (Fawole *et al*., 2017, 2018). Likewise, serum protein indices were found to be independent of dietary SPLM in rohu fingerlings (Ahmad, 2016; Meshram, 2017).

5.28.2 Haemato-immunological parameters

Aminotransferase enzymes (ALT and AST) are present mainly in the liver, but can be released into the bloodstream when there is damage to the liver cells (Racicot *et al.,* 1975; Goel *et al.,* 1984; Li *et al.,* 2015). In the present study, no significant variations were observed in the serum ALT and AST activities in rohu fingerlings among various groups. Similar result were also reported in Nile tilapia, Atlantic salmon and rohu fed *Jatropha platyphylla* kernel meal, soybean meal (SBM), and rubber protein isolate, respectively (Sanden *et al.,* 2006; Makkar *et al.,* 2011; Fawole *et al*., 2017). Ahmad (2016) reported that no significant increase in serum ALT and AST were seen in rohu fingerlings fed with dietary SPLM and it indicates that no hepatic cellular damage was noticeable in dietary treatments and control in their study.

It is well known that fish phagocytes are able to generate superoxide anions and its reactive derivatives during a period of intense oxygen intake (Secombes and Fietcher, 1992; Secombes, 1996). This is an immunological defense mechanism of the animals against external pathogens. In the present study, the dietary CPI and FSPLM based diets did not provoke any significant change in respiratory bust activity among dietary treatments and control. Fawole *et al.* (2017) observed no significance difference in NBT activity in rohu when fed with dietary rubber protein isolate. Similarly, Ahmad (2016) and Meshram (2017) also observed that RBA activity did not change in rohu fingerlings when fed with SPLM and FSPLM, respectively.

High blood glucose concentration in fish has been considered as secondary level stress indicator (Hattingh, 1977) and also indicates high energy demand of tissues (Manush *et al.,* 2004). It is reported that during all types of stress catecholamine secretion increases, which in turn elevate the glucose level in blood for

energy production to mitigate the stressful condition (Nakano and Tomlinson, 1967). In the present study, feeding dietary CPI and FSPLM to rohu fingerlings did not affect the serum glucose concentration among the various treatment groups. This is in concurrence with the findings of Slawski *et al.* (2012) and Fawole *et al*. (2018). Similarly, Ahmad (2016) and Meshram (2017) also observed that serum glucose concentration did not vary significantly in rohu fingerlings when fed with SPLM and FSPLM, respectively.

5.29 Haematological Parameters

Haematological indices are considered as indicators of animal health status (NRC, 1993; Sun *et al.,* 1995; Kumar *et al.,* 2010b; Akinleye *et al.,* 2012). Results of haematological parameters (RBC, Hb, WBC, Hct, MCV, MCH, and MCHC) exhibited no significant difference between the control and the dietary treatment groups and are in agreement with the findings of Slawski *et al.* (2012) and Fawole *et al*. (2017, 2018). Conversely, Shamna *et al.* (2015) recorded a decreased RBC and Hb levels in *L. rohita* fingerlings and Soltan *et al.* (2008) observed that fishmeal protein replaced by mixture of plant proteins in tilapia diets lead to a reduction in haematocrit value. Ahmad (2016) reported that haematological parameters viz. RBC count, Hb concentration and WBC count were not affected by dietary SPLM in rohu fingerlings. Therefore, it can be inferred that in our study, *L. rohita* fingerlings fed different experimental diets were in good and or equal nutritional and health status.

6. SUMMARY

The emergent demand for feed due to rapid growth of aquaculture enterprise and the higher cost of conventional feed ingredient has encouraged researchers to quest for alternative feed resources especially from plant materials or by-products of agro-industries to meet the need of conventional sources. This search has been directed by most nutritionist towards ingredient that do not conflict with human food security, of which non-edible oils seeds or by-products of non-edible oil seeds resources became most prominent and preferred choice. However, many of these non-edible oil seeds contain inherent toxic component, antinutritional factors, and high level of indigestible materials which limit their incorporation in animal/fish feed as alternative protein source. Simultaneously, leaf meal could serve as an efficient energy source for the preparation of animal/fish feed.

Castor seed cake, the by-product of castor oil extraction process, could serve as protein source in animal/fish feed but occurrence of antinutrients restricts its use in animal feed. However, with adequate processing methodology their nutritional potential can be enhanced. Preparation of high-value protein isolates could be an alternative approach to improve the nutritional potential and utilization of non-edible oil seed by-products in aquafeed preparation.

Similarly leaf meals, especially sweet potato (*Ipomoea batatas*) leaves comprise of high protein and good nutritional profile but presence of high crude fibre and anti-nutritional factors (ANFs) limits its use in animal/ fish feed. Fermentation with micro-organism could be an ideal approach to minimize the ANFs concentration as well as digestion of crude fibre in SPLM. Solid-state fermentation (SSF) is a process of biological detoxification of agro-industrial by-products and plant ingredients with different microorganisms. Keeping this in view, the present study was aimed to isolate the protein from castor seed cake; evaluate its nutritional values for use in aquafeed development *in-vitro*; and in leafmeal based diet of *Labeo rohita*.

To achieve broad objectives, four experiments were performed in the present study. In the first experiments (experiment I), the conditions for the preparation of protein isolate using the principle of iso-electric precipitation was optimized, determined the anti-nutrient contents and evaluated its nutritional quality in terms of the amino acid composition, amino acid score, predicted protein efficiency ratio (P-PER), essential amino acid index (EAAI), predicted biological value (P-BV), nutritional index (NI) and *in vitro* digestibility. The results of this experiment revealed higher protein content between 81.52% and 92.55% under different extraction conditions (pH combination). The maximum value of protein was observed in pH combination of 12.0/5.0 followed by 12.0/4.0 and 12.5/5.0, while pH combination of 10.0/4.0 registered the lowest protein content. The dry matter and protein recoveries ranged from 31.12% to 49.83% and 43.31% to 78.68%, respectively. Based on the dry matter & protein recoveries, and protein content of CPI, the pH combination of 12.0 and 5.0 was used for extraction and precipitation of proteins. This combination exhibited most significant ($P<0.05$) results than other pH combinations. The toxic components such as hydrogen cyanide and other ANFs were found to decrease after protein extraction to a safe level. The amino acid compositions of protein isolates were found to be comparable to soybean protein isolate and FAO/WHO reference protein. However, lysine was recorded to be the first limiting amino acid in both the protein isolates and kernel meal. Other calculated nutritional quality indices indicate that protein isolates are of good quality and could be a promising protein source for fish feed preparation.

The second experiment (experiment II) was carried out to evaluate the nutritional value of protein isolates prepared from defatted castor kernel meal (CPI) in the diet of *Labeo rohita* fingerlings. One hundred fifty fingerlings of *L. rohita* (initial average weight, 3.33±0.04 g) were randomly distributed into five distinct experimental groups in triplicates following a completely randomized design. Fish were fed with five iso-nitrogenous (34.31±0.04 % crude protein) and isocaloric (421.76± 0.24 kcal 100 g^{-1}) diets, with control diet containing soybean protein isolate (SPI) as the major protein source. The SPI protein was progressively replaced at 25%, 50%, 75% and 100% with CPI and designated as CPI_{25}, CPI_{50}, CPI_{75}, and CPI_{100}, respectively. The feeding

trial lasted for 60 days. The results showed that the growth performance indices such as % weight gain, and specific growth rate were higher in control but not statistically different (P>0.05) from CPI_{50}, and CPI_{75} groups but a decrease in growth performance was observed in CPI_{100} group. The nutrient utilization parameters like protein efficiency ratio (PER), feed conversion and protein retention (PR) values among various groups exhibited the same trends as growth performance. The feed intake, body indices and survival rate did not vary among different treatment groups. The apparent digestibility coefficient values of dry matter and nutrients (protein and lipid), whole body composition and digestive enzymes activities of protease, amylase, and lipase were not affected as a result of feeding CPI in replacement for SPI protein in rohu diets. The activity of AST in liver and muscle, ALT, LDH and SOD in liver exhibited a significant difference in rohu fingerlings while, ALT and LDH in muscle, MDH in liver and muscle, SOD in gill and CAT in liver and gill did not vary significantly with the inclusion level of dietary CPI. The total protein concentration exhibited significant variation while, other indices such as albumin, globulin and albumin globulin ratio were not affected (P>0.05) by dietary CPI levels. No difference was observed (P>0.05) in the serum glucose concentrations. Serum enzyme ALT showed significant difference (P<0.05) but AST did not exhibit any change (P>0.05) in rohu fingerlings. NBT activity was shown to increase with the dietary CPI inclusion level in the feed and maximum activity was observed in CPI_{100} group. Dietary inclusion of CPI as replacement for SPI had no significant effects on the haematological parameters like WBC, Hct, MCV, MCH, and MCHC (except haemoglobin and RBC). Histological study revealed no remarkable changes in the liver while intestine exhibited cellular changes in all dietary treatment and control groups. The noticeable changes were increased fusion of mucosal folds, reduced number of supranuclear absorptive vacuoles in enterocytes and increased width and intracellular infiltration in the lamina propria of intestine.

The third experiment (experiment III) was carried out to evaluate the nutritional value of castor seed protein isolate (CPI) supplemented with deficient L-lysine and DL-methionine in the diet of *L. rohita* fingerlings. One hundred fifty fingerlings of *L. rohita* (initial average weight, 7.17±0.02 g) were randomly distributed

into five distinct experimental groups in triplicates following a completely randomized design. Fish were fed with five iso-nitrogenous (35.45±0.14 % crude protein) and isocaloric (421.82±0.16 kcal $100g^{-1}$) diets, with control diet containing soybean protein isolate (SPI) as the major protein source. The SPI protein was progressively replaced at 25%, 50%, 75% and 100% with CPI and designated as $ACPI_{25}$, $ACPI_{50}$, $ACPI_{75}$, and $ACPI_{100,}$ respectively. In experiment II, amino acid profile indicated that L-lysine and DL-methionine were deficient in all the experimental diets. Therefore, the amino acid requirement of *L. rohita* fingerlings was fulfilled by supplementing the deficient amino acids. The feeding trial lasted for 60 days. The results showed that the growth performance indices such as % weight gain, and specific growth rate were higher in $ACPI_{50}$ and $ACPI_{75}$ groups but growth rates in *L. rohita* fingerlings of $ACPI_{25}$ and $ACPI_{100}$ groups was found to be similar (P>0.05) with the control group. The nutrient utilization parameters like protein efficiency ratio (PER), feed conversion and protein retention (PR) values among the various groups exhibited the same trends as growth performance. The feed intake, body indices and survival rate did not vary among the different treatment groups. The whole body composition (except crude protein) and digestive enzyme activities of protease, amylase, and lipase were not affected as a result of feeding CPI as replacement for SPI protein in rohu diets. The activity of AST and LDH in liver and muscle, ALT and MDH in muscle exhibited a significant difference in rohu fingerlings while ALT and MDH in liver, SOD and CAT in liver and gill did not vary significantly with the dietary CPI. The serum protein biochemistry indices such as total protein, albumin, globulin and albumin globulin ratio were not affected (P>0.05) by feeding different levels of CPI. No difference was observed in the serum glucose concentrations. Serum enzyme ALT and AST did not exhibit any significant change in rohu fingerlings. Dietary inclusion of CPI affected the NBT activity significantly in rohu fingerlings. Dietary inclusion of CPI as replacement for SPI had no significant effects on the haematological parameters like RBC, WBC, Hct, MCV, MCH, and MCHC (except haemoglobin). Histological study revealed no remarkable changes in the liver while reduced intracellular infiltration, improved villi health and reduced fusion of mucosal folds was observed in the intestinal architecture of *L. rohita* fingerlings.

The fourth experiment (experiment IV) was carried out to evaluate the nutritional value of castor seed protein isolate (CPI) supplemented with deficient L-lysine and DL-methionine in the fermented sweet potato leaf meal (FSPLM) based diet of *L. rohita* fingerlings. Solid-state fermentation (SSF) of SPLM with *Chaetomium globosum* for 120 hr was carried out to improve the nutritional value of SPLM (in terms of high crude protein and low crude fibre) and to minimize the anti-nutritional factors. The results of SSF of SPLM revealed that the crude protein content had increased from 21.47% to 31.20% (45.32% increment) while crude fibre content decreased from 19.43% to 7.22% (62.84% reduction). While, SSF of SPLM also resulted in a reduction in anti-nutritional factors such as tannin, phytates, trypsin inhibitor, oxalates, alkaloid and HCN up to 64.95%, 57.51%, 15.31%, 37.32%, 50.00% and 61.70%, respectively. One hundred twenty fingerlings of *L. rohita* (initial average weight, 9.25±0.02 g) were randomly distributed into five distinct experimental groups in triplicates following a completely randomized design. The amino acid profiles of prepared experimental diets revealed that diets are deficient in L-lysine and DL-methionine and amino acid requirement of *L. rohita* fingerlings was fulfilled by supplementing the deficient amino acids. Four iso-nitrogenous (35.45±0.14 % crude protein) and isocaloric (420.95±0.46 kcal $100g^{-1}$) experimental diets were formulated, prepared and fed to *L. rohita* fingerlings twice daily till satiation level for 60 days. Growth performance (weight gain (%), and specific growth rate), feed conversion and nutrient utilization parameters [protein efficiency ratio (PER), and apparent net protein utilization (ANPU)] were not affected in *L. rohita* fingerlings fed with dietary CPI and FSPLM. The feed intake, body indices and survival rate did not vary among the different treatment groups. The whole body composition and digestive enzymes activities of protease, amylase, and lipase were not affected as a result of feeding CPI and FSPLM. The activity of AST, ALT, LDH and MDH in liver and muscle in rohu fingerlings while, ALT and MDH in liver, SOD and CAT in liver and gils did not vary significantly with dietary CPI and FSPLM. The serum protein biochemistry indices such as total protein, albumin, globulin and albumin globulin ratio were not affected ($P>0.05$) by dietary CPI and FSPLM levels. No difference was observed in the serum glucose concentrations. Serum enzyme ALT and AST did not exhibit any significant

change in rohu fingerlings. Dietary inclusion of CPI and FSPLM did not affect the NBT activity significantly in rohu fingerlings. Dietary inclusion of CPI and FSPLM in replacement for SPI and DORB had no significant effect on the haematological parameters like RBC,Hb, WBC, Hct, MCV, MCH, and MCHC.

Conclusion

The results of the current study showed that protein isolates prepared from defatted castor kernel meals could serve as a potential replacer for soybean protein isolates in rohu diets. Processing strategy for preparation of protein isolate aided in reducing the inherent toxic factors and ANFs present in defatted castor kernel meal to a level below tolerance limits, thereby raising its nutritional and feeding value. Based on dry matter & protein recovery and protein content of CPI, it can be concluded that pH combination of 12.0/5.0 was the best for mass scale production. The feeding trial results of second experiment revealed that 75% substitution of dietary SPI is possible with CPI in the diet of *L. rohita* fingerlings without any adverse effect on growth performance, feed conversion, nutrient utilization, haemato-biochemical indices, and non-specific immune response of the fish. Though intestinal architecture exhibited gradual intracellular infiltration, shortening and widening of mucosal folds, and reduced number of absorptive vacuoles with dietary CPI levels. Nevertheless, the experimental diets were deficient in L-lysine and DL-methionine. In third experiment, the deficient amino acids were supplemented to fulfill the amino acid requirement of L-lysine and DL-methionine in *L. rohita*. The feeding trial results revealed that CPI fed fish exhibited comparable growth performance and nutrient utilization to SPI-fed control without any detrimental effects on haemato-biochemical indices, non-specific immune response and organ integrity of the fish. Experiment IV results revealed that CPI and FSPLM can serve as protein and energy source and can replace 100% SPI and 100% DORB in the diet of *L. rohita*. Therefore, it can be concluded that production of high-value protein isolates could be an alternative approach to make use of non-edible seed cakes or meals for aquafeed production.

This may lead to use of alternate plant protein sources that can be used in aquafeed to replace heavily-dependent soybean meal and open a new market avenue for their use as a new feed resource. There is a scope of exploring option for more recovery of dry matter and protein from these non-edible seeds by applying different strategies. During the extraction process, there is wastage of organic and inorganic nutrients, further research is needed to make use of these nutrients and also to make use of these nutrients and also to make the extraction more efficient.

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ABBREVIATIONS

%	:	Percent
°C	:	Degree centigrade
ALT	:	Aspartate aminotransferase
ANOVA	:	Analysis of variance
ANPU	:	Apparent net protein utilization
AST	:	Alanine aminotransferase
BHT	:	Butylated hydroxytoluene
CAT	:	Catalase
CKM	:	Castor kernel meal
cm	:	Centimetres
CP	:	Crude protein
CPI	:	Castor seed protein isolate
DCKM	:	Defatted castor kernel meal
DE	:	Digestible energy
DM	:	Dry matter
DO	:	Dissolved oxygen
DORB	:	De-oiled rice bran
EAA	:	Essential amino acid
EE	:	Ether extract
FCR	:	Feed conversion ratio
FER	:	Feed efficiency ratio
FSPLM	:	Fermented sweet potato leaf meal
g	:	Gram

GE	:	Gross energy
Hb	:	Hemoglobin
Hct	:	Hematocrit
hrs	:	Hours
HSI	:	Hepatosomatic Index
Kcal	:	Kilocalorie
kg	:	Kilogram
L	:	Liter
LDH	:	Lactate dehydrogenase
MCH	:	Mean cell hemoglobin
MCHC	:	Mean cell hemoglobin concentration
MCV	:	Mean cell volume
MDH	:	Malate dehydrogenase
mg	:	Milligram
min	:	Minutes
mm	:	Millimeters
n mole	:	Nano mole
NBT	:	Nitroblue tetrazolium
NEAA	:	Nonessential amino acid
NFE	:	Nitrogen free extract
PER	:	Protein efficiency ratio
psi	:	Pound -force per square inch
RBA	:	Respiratory burst activity
RBC	:	Red blood cells

SBM	:	Soybean meal
SGR	:	Specific growth rate
SOD	:	Superoxide dismutase
SPI	:	Soybean protein isolate
SPLM	:	Sweet potato leaf meal
TC	:	Total carbohydrate
VSI	:	Vicerosomatic index
WBC	:	White blood cells
WG	:	Weight gain
WG (%)	:	Weight gain percentage
µg	:	Microgram
µl	:	Microlitre
µM	:	Micromole