EVALUATION OF MIXED LEAF MEAL SUPPLEMENTED WITH SYNTHETIC AMINO ACIDS AND EXOGENOUS ENZYMES IN THE DIET OF *LABEO ROHITA* (HAMILTON, 1822) FINGERLING

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

M.F.Sc. (Fish Nutrition and Feed Technology)
by

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JULY, 2019

Anakhy Mondal, 2019., Evaluation of mixed leaf meal supplemented with synthetic amino acids and exogenous enzymes in the diet of *Labeo rohita* (Hamilton, 1822) fingerling, M.F.Sc. Dissertation, ICAR-Central Institute Of Fisheries Education (University Established Under Section 3 of UGC Act 1956) Panch Marg, Off Yari Road, Versova, Andheri (W), Mumbai – 400 061

DEDICATED TO MY BELOVED PARENTS

Dated: 30th June, 2019

CERTIFICATE

Certified that the dissertation entitled **"**Evaluation of mixed leaf meal supplemented with synthetic amino acids and exogenous enzymes in the diet of *Labeo rohita* (Hamilton, 1822) fingerling**"** is a bonafide record of independent research work carried out by **Ms. Anakhy Mondal** during the period of study from August 2018 to June 2019 under our supervision and guidance for the degree of **Master of Fishery Science (Fish Nutrition and Feed Technology)** and that the dissertation has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title.

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DECLARATION

I hereby declare that the dissertation entitled **"EVALUATION OF MIXED LEAF MEAL SUPPLEMENTED WITH SYNTHETIC AMINO ACIDS AND EXOGENOUS ENZYMES IN THE DIET OF *LABEO ROHITA* (HAMILTON,1822) FINGERLING"** 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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ACKNOWLEDGEMENT

I am thankful to almighty God for showering blessings to me always for success of my dissertation and till this phase of life.

I am overwhelmed with rejoice to avail this rare opportunity to evince my profound sense of reverence and gratitude to ***Dr. N.P. Sahu****, H.O.D. & Principal Scientist, Fish Nutrition, Biochemistry and Physiology Division, CIFE, Mumbai and Chairman of my Advisory Committee for his inspiring guidance, constant encouragement, untried co-operation, constructive criticism and soothing affection during the course of investigation and preparation of this manuscript.*

I express my deepest gratitude and veneration to the esteemed members of my Advisory Committee, ***Dr. Parimal Sardar****, Principal Scientist, Fish Nutrition, Biochemistry and Physiology Division, CIFE, Mumbai;* ***Dr.Shamna N****, Scientist, Fish Nutrition, Biochemistry and Physiology Division, CIFE, Mumbai and* ***Mr. Manish Jayant****, Scientist, Fish Nutrition, Biochemistry and Physiology Division, CIFE, Mumbai for their constructive and valuable suggestions and guidance during the whole course of this investigation and preparation of this manuscript.*

I express my sincere thanks to Dr. Ashutosh D. Deo, Principal Scientist, Fish Nutrition, Biochemistry and Physiology Division, CIFE, Dr. P. P. Srivastava, Principal Scientist, Dr. Subodh Gupta, Principal Scientist, Dr. Tincy vargese, Scientist, Dr. Sikendra Kumar, Scientist for their co-operation, kind support and timely help during the course of my research work.

I am thankful to ***Dr. Gopal krishna,*** *Director, CIFE, Mumbai for providing necessary facilities during the investigation and completion of my research.*

I extend my sincere thanks to Prof. ***T, S. Nagesh, Dr. Somen Sahu, Prof. T.J Abraham*** *and* ***Dr. N.A Talwar*** *of W.B.U.F.S. who were not with me during the course of investigation but encouragement and constant help during the course of investigation.*

At this juncture of time my heart is full, mouth is dump and I feel short of words at my command to express my regards and respect to my beloved parents ***Dr. Anandamoy Mondal*** *and Mrs. Manju Mondal.*

It is indeed a pleasure to acknowledge the love, affection, inspiration, encouragement, selfless help and cheerful company by my friends ***Debayan, Susmita, Triparna, Sangita, Sahana, Neli, , Deeksha, Atul, Ramjan, Neha, Kuntal, Subhra.***

I would like to express my sincere thanks to my seniors ***Dilip sir, Mritunjoy da, Prasanta da, Manas da, Munish sir, Gyandeep sir, Shradhanjali di, Sajina di, Nuzaiba di, Sutanu da, Sanchita di, Kasturi di*** *who encouraged me always and helped in all possible ways.*

I would like to thank my beloved batchmates ***Gopika, Krishnapada, Hussain, Chinmay, Thiru, Ifra, Banani, Avilipsha, Vikram, Susmita, Selvi f****or their tireless help and support.*

I express my love to my juniors for their sincere help ***Monalisha, Nisha, Subham, Asha, Rinkesh, Tanmoy, Somnath.***

I extend my respect and love towards my beloved ***Didin and Dadan*** *, who are though not with me at this point of life but had encouraged and loved me a lot throughout my childhood .*

I extend my sincere thanks to ***Mr. Kaushik, Mr. Ujjal, Mr. Tarak, Mr. Nirmalya, Mr. Shankar*** *for their selfless help for collection of leaves and effortless help towards completion of my dissertation.*

I also express my whole hearted thanks to my relatives ***Mamu, Mamima, Boro masi and meso, Mejo masi and meso, Choto masi and meso, Ranjana kakima*** *for their continuous support, cordial assistance and guidance in course of the study. I express my love towards my little sisters Diya and Goody for cherishing me always.*

I also wish to record my sincere thanks to all the faculty and non-teaching staff of the Fish Nutrition, Biochemistry and Physiology Division, CIFE, Mumbai for their kind cooperation and help extended during the period of research work.

I gratefully acknowledge the financial help I received from ICAR, New Delhi during my entire period of my post-graduation. I am grateful to the all staff members of the CIFE for their substantial assistance.

There are so many others whom I may have inadvertently left out. I sincerely thank them for their help.

CIFE, Mumbai **(Anakhy Mondal)**
July, 2019 **Author**

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I hereby declare that the dissertation entitled **"Evaluation of mixed leaf meal supplemented with synthetic amino acids and exogenous enzymes in the diet of *Labeo rohita* (Hamilton, 1822) fingerling"** 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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ABSTRACT

A 60-day experiment was carried out to study the effect of dietary mixed leaf meal (MLM) on growth performance, feed utilization and physio-metabolic responses in *Labeo rohita* fingerlings. Mixed leaf meal was prepared by mixing *Vigna mungo* (Black gram)*, Ipomea aquatic* (Water spinach) and *Hygrophila spinosa* (Kulekhara) leaf meal in 1:1:1 ratio. Six iso-nitrogenous (30% crude protein) and iso-caloric (350 Kcal DE/100g) experimental diets were prepared *viz*. control (0% MLM, 40% DORB), T1 (20% MLM, 20% DORB), T2 (40% MLM, 0% DORB), T3 (Control + 0.1% exogenous enzyme (xylanase & cellulase) + 0.14%, DL-methionine & 1.04%, L-lysine;), T4 (T1 + 0.1% exogenous enzyme (xylanase & cellulase) + 0.94%, L-lysine), T5 (T2 + 0.1% exogenous enzyme (xylanase & cellulase) + 1.04%, L-lysine). Two hundred sixteen fingerlings (average body weight 3.41±0.01g) were randomly distributed in eighteen experimental tanks following a completely randomized design and fed to satiation level for 60 days. A significantly higher ($p<0.05$) weight gain%, SGR growth performance and nutrient utilization (PER) were observed in *L. rohita* fingerlings fed with 20% MLM supplemented with 0.1% exogenous enzyme and 0.94% L-lysine than control group. Significantly higher ($P<0.05$) protease activity was found in T1 and T4 groups, whereas amylase activity was found to be significantly higher ($P<0.05$) in fish of T4 group. Lipase activity was found to be independent of dietary treatments. Supplementation of exogenous enzymes exhibited increased muscle alanine aminotransferase and aspartate aminotransferase activities in T4 group, while the muscle lactate dehydrogenase and malate dehydrogenase activities were significantly lower in that group. Oxidative enzyme like superoxide dismutase activity was found to be affected significantly in gill and liver of *L. rohita* fingerlings, whereas the catalase activity did not vary among the treatments. DORB based control diet was found to be deficient in lysine and metheonine. However, mixed leaf meal was not deficient in methionine. The final composition of MLM showed reduction in fibre content (11.82%) and 11.86% in total tannin, 28.42% in alkaloids content, 4.87% in total oxalates and around 2% in phytic acid composition than that of the average anti-nutritional values found in the individual leaves. Therefore, it is concluded that the mixed leaf meal *(Vigna mungo, Ipomea aquatica* and *Hygrophila spinosa* leaf meal in 1:1:1 ratio) at 20% inclusion level supplemented with 0.1% exogenous enzyme and 0.94% L- lysine in the diet of *Labeo rohita* fingerling showed significant higher growth than control but 40% MLM inclusion level supplemented with 0.1% exogenous enzyme and 1.04% lysine can successfully replace 100% DORB in the diet of *Labeo rohita* fingerlings.

2. REVIEW OF LITERATURE

2.1. Aquaculture

Indian aquaculture is an important food production sector, providing nutritional security to the food basket of the country and also greatly contributing to the agricultural exports. In world, aquaculture is the fastest growing food producing sector and contributes around 50% of fish and shellfish production. Indian fisheries occupy a second position in global fish production and 3rd position in aquaculture in the world. The annual growth rate of aquaculture is 8.2% per year and higher than other food producing sectors viz. 1.3% in capture fisheries and 2.6% for meat production (FAO, 2013). Indian aquaculture has demonstrated a six and half fold growth over the last two decades, with freshwater aquaculture contributing over 95% of the total aquaculture production (Jayasankar, 2018). India is bestowed with 3.15 million ha of reservoirs, 2.36 million ha of ponds and tanks as well as 0.19 million ha of rivers and canals. Freshwater aquaculture with a share of 34% in inland fisheries in mid 1980s has increased to about 80% in recent years (DAHDF, 2017).

2.2. *Labeo rohita* (rohu) culture

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). Rohu is the natural resident of the riverine system of northern and central India, and the rivers of Bangladesh, Pakistan and Myanmar. However, due to its feasibility for pond culture, it has spread to many parts of the world. Rohu grows faster than mrigal but slower than catla in pond conditions and can attain a size of 500-1000g in one year under good farm conditions. India was the biggest contributor in the global rohu production with a share of 44.1% (FAO, 2011). Rohu, being a column feeder, grows well in deeper ponds (2-3 m water depth), which are rather uncommon; in shallower ponds it does not attain its optimum growth potential. Nowadays, the culture of rohu 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, Japan, China, Philippines, Malaysia, Nepal and some countries of Africa. As its important role in Indian aquaculture, several efforts has been made to improve its growth rate of which development of a new strain i.e. genetically improved rohu (popularly known as “Jayanti rohu” through selective breeding by ICAR- Central Institute of Freshwater Aquaculture, Bhubaneswar, India.

The ability to use dietary carbohydrates as an energy source differs among fish species. Most fresh and warm water fish, including carp, can use much higher levels of dietary carbohydrates than Coldwater or marine fish. This may be attributed to the fact that warm water fish has a much higher intestinal amylase activity than Coldwater species (Wilson 1994). The polysaccharides dextrin and starch are well utilized by carp (Wilson & Poe, 1987).The Carp feeds contains adequate amounts of grain or grain by-products that are rich in carbohydrates. Inclusion of carbohydrates is crucial to provide metabolic intermediates for the synthesis of other biologically important compounds (Wilson *et al.,* 1994). Thus, it is important to provide appropriate level of carbohydrate in the carp feed, to spare protein energy.

2.3. Replacement of conventional aquafeed ingredients

Several studies have been carried out to make supplementary feeding of fish cost-effective and have been directed to substitute the costly fish meal with less expensive protein sources. This aspect of feed development research is solely based on the search for inexpensive, readily available and nutritious plant protein sources that can supply all the nutritional benefits to the fish. Fish meal along with other conventional aquafeed ingredients tends to show lower availability nowadays along with increase in its price which calls for its replacement with other plant based ingredients. The main reason for the rise in the price of fish feed is due to the rise in price of fish meal, which is an important protein ingredient of aquafeed. Extensive studies have been done for replacing and reducing the use of fish meal in aquafeed

and many recommendations have come out (Mahanta *et al.,* 2007). Several studies have shown that the success of the aquaculture industry lies in the replacement of fish-based protein by plant alternatives and even holds true for high-valued carnivorous species (Cho and Bureau, 2002).

2.4. Rice bran

The conventional fish feed in use are mainly based on rice bran as the dominant energy source. The rice bran is one of the ingredients commonly incorporated in aquaculture feed formulation and act as a carbohydrate source in providing energy in the diet and also provide a substantial part of the essential amino acids (Tsvetanov and Duneva, 1990). Rice bran is the outer layer of rice kernel and is a by-product of rice milling which constitutes 8% of whole rice grain. It consists of pericarp, aleurone, subaluerone layer, seed coat, nucellus, part of the germ and small part of starchy endosperm (Hargrove *et al.,* 1994). Rice bran is light in colour, sweet in taste, moderately oily and has a slightly toasted nutty flavour (Hu *et al.,*1996). Due to its good nutritional profile, rice bran is one of the most common agricultural byproducts used to feed fish. It contains appreciable amount of nutrients like protein, fat and dietary fibre. Rice bran also contains phytochemicals with promising health benefits (Jariwalla 2001). Presence of antioxidants like tocopherol, tocotrienols and ϒ - oryzanol brighten prospects of rice bran's utilization for humans for health benefit. The nutraceuticals developed from the soluble and fibre fractions of rice bran control both type I and type II Diabetes Mellitus in human (Qureshi *et al.,* 2002).

In Southeast Asia, rice bran is one of the most common by-products used to feed fish (Jhingran and Pullin, 1985). Even in farm made feed major part is contributed by the rice bran (50-80%) with other grain, oilcakes and other byproducts of agriculture. It contains appreciable amount of nutrients like protein, fat and dietary fibre. However the price of DORB is eventually increasing day by day and need to be replaced by other low cost plant based ingredients. Even the proper utilization of DORB to reduce FCR is required which will bring down the demand and supply gap of DORB (Ranjan *et al.,* 2018). Fermented sweet potato leaf meal can replace 100%

DORB in the diet of *Labeo rohita* with almost no side effect on growth performance. (Meshram *et al.,* 2018).It has been reported that 33% dietary inclusion level of de-oiled rice bran with 30% dietary crude protein and 6% dietary lipid is optimum for better feed utilization and growth performance of *Labeo rohita* (Kumar *et al.,* 2017).

2.5. Leaf meal as an aquafeed ingredient

Leaf can be invariably obtained from both terrestrial and aquatic plants for preparation of leaf meal.The use of plant protein in fish feed formulation has been attempted for various commercial fish based on their specific requirements (Dorothy *et al.,* 2018).The sweet potato leaf meal can be used as a protein source in the diet of *Tilapia zilli* to an extent of 15% inclusion level without effecting its growth (Adewolu, 2008).

Diet with 12.5% replacement of ground nut oil cake (GNOC) by *Moringa oleifera* leaf meal resulted in the better growth and feed conversion in *Clarias gariepinus* fingerling (Olaniyi *et al.,* 2013). Feeding of 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 *Labeo rohita* fingerlings without any adverse effect on growth of the fish, thus aided to produce cost effective formulated fish feed (Saha *et al.,* 2011).

2.6. ***Vigna mungo*** (Black gram)

Scientific classification

Kingdom: Plantae

Order: Fabales

Family: Fabaceae

Subfamily: Faboideae

Genus: Vigna

Species: Mungo

Scientific name: *Vigna Mungo*

V. mungo is an important pulse crop belonging to the family Fabaceae. Mung bean is primarily used for food purpose. It is a rich source of protein and amino acid especially lysine and thus can supplement cereal-based human diets. (Achakzai *et al*, 2012). In the previous study it has been reported that *Vigna mungo* can give 100% DORB replacement, however, significantly higher growth rate result was shown at 30% inclusion level. (Sahoo, 2018)

2.7. *Ipomea aquatica* (water spinach)

Scientific classification

Kingdom: Plantae

Clade: Angiosperms

Order: Solanales

Family: Convolvulaceae

Genus: Ipomoea

Species: Aquatica

Scientific name: *Ipomoea aquatica*

Ipomea aquatica (commonly called water spinach) is an emergent aquatic plant belongs to the family Convolvulaceae. It is cultivating commercially as an edible green leafy vegetable in Hong Kong, Taiwan and China. It is a tender, trailing or floating perennial aquatic plantfound on moist soil along the margins of fresh water and in ditches, marshes and wet rice fields. It is also commonly used as a green leafy vegetable in rural India for its nutritional profiling (Prasad *et al.,* 2005). Studies have

shown that these leaves have higher amount of calcium in it i.e. 110mg/ gram of leaf (Mukherjee *et al*, 2010).

Studies have shown the composition of the *Ipomea aquatica* after fermentation at optimal conditions by the fish gut isolate *Bacillus subtilis* revealed that there was marginal increase in the contents of protein, lipid, and minerals and fermentation was effective in reducing the crude fiber content and the antinutritional factors such as tannins, phytic acid, and trypsin inhibitor, and enhancing available free amino acids and fatty acids. (Khan *et al.,* 2013)

2.8. *Hygrophila spinosa* (kulekhara)

Scientific classification

Kingdom: Plantae

Clade: Angiosperms

Family: Acanthaceae

Genus: Hygrophila

Species: Spinosa

Scientific name: *Hygrophila spinosa*

Aquatic weed that remain unutilized and often cause deterioration of water quality may be converted into valuable fish flesh through their incorporation in carp diets as a feedstuff. *Hygrophila spinosa* belongs to Acathaceae family. Previous studies reveals that these leaves are rich in vitamin-c, iron, potassium, sodium and copper and good source of riboflavin and can be used to improve the iron status of the human beings. (Mukherjee *et al*, 2015)

From the previous study, it has been reported that the percentage weight gain (WG%), SGR and FCR values were significantly higher in the *Labeo rohita* fed with 10% *Hygrophila spinosa* leaf meal, whereas,30% *Hygrophila spinosa* leaf meal

can be use to replace DORB at same inclusion level (Maiti, 2018). Rajeswari *et al.* (2012) have observed a significantly higher growth rate in *Fenneropenaeus indicus* by incorporation of *H. spinosa* leaf extract and its active immune-stimulants 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.

2.9 Strategies to improve the utilization of leaf meal in fish diet

Leafy shrubs and aquatic weeds grow 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 various anti-nutritional factors like phytate, trypsin inhibitor, alkaloid, oxalate, tannin and cyanide as a defense mechanism of the plants. However, these ANFs can be eliminated or reduced to the tolerable limit of fish by following different strategies.

Moist heat treatments of plant based ingredients to reduce or destroy trypsin inhibitor, oxalates lectins and proteinaceous anti-nutritional factors. Soaking of plant based ingredients is known to help in reduction of anti-nutritional factors. Nowadays, fermentation of ingredients is reported to be an innovative approach, which can improve nutrient digestibility and bio-availability (Kim *et al*., 1999). Mixing the plant based ingredients could be a strategy to improve the nutritional value, digestibility and reduction of anti-nutritional factors. The drawbacks of using plant sourced ingredients in fish feed formulation are the presence of various anti-nutritional factors, varying amino acid contents etc. which can be minimized by implying various strategies such as soaking of the ingredients, heating, drying, breaking down in to finer particles or conversion of plant protein to concentrates and mixing the plant based ingredients (Dorothy *et al.,* 2018).

2.9.1. Mixed leaves

Plant-derived products are known to contain a variety of phytochemicals such as phenolic acids, flavonoids, tannins, lignin and other minor compounds.

Eventually, these compounds may possess numerous health-related effects such as antibacterial, antimutagenic, anticarcinogenic, antioxidant, antithrombotic and vasodilatory activities (Wendakoon *et al.,* 2012). Unconventional plant ingredients (oilcake and leaf meals) comprise various anti-nutritional factors viz. tannin, phytic acid, trypsin inhibitors, alkaloid, oxalates, saponin, cyanogens, and other factors. These factors not only binds with other nutrients to reduce their bio-availability but also interfere with feed intake and digestion in fish or animals which adversely affect the growth performances of fish or animals.Leaf meal has been a promising commodity in the replacement of de-oiled rice bran and other conventional aqua–feed ingredients but different leaves have their own pros and cons. Mixing of leaves with each other may serve following purposes: Anti–nutritional factors present in different leaves in various concentrations would get diluted by mixing them together. The wider availability would be ensured with mixed leaf approach instead of only depending on one kind of leaf. The amino acid deficiency of one leaf could be overcome by mixing it with other non–deficient leaf. It has been reported that better growth and feed conversion in *O. niloticus* can be achieved by substituting 20% fish meal with mixed plant sources *viz*. maize gluten meal, wheat gluten meal and bagasse kenna mix (Thobaiti *et. al.,* 2018).

2.10. Effect of leaf meal based diet on growth, nutrient utilization and on physio-metabolic parameters in fish

Singh *et al*. (2016) used jute (*Corchorus olitorius*) leaf powder, as a dietary protein source for Labeo 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. In a different study Ali *et al.* (2018) evaluated that fermented *Ipomea* leaf meal has potential to replace fish meal up to 25% in *Labeo rohita* diets without adverse effect on growth performance, daily protein retention and nutrient diposition. They concluded that *L. rohita* could utilize limited amount of carbohydrate in the feed, there by sparing protein to render better growth of the fish. It was found by Mondal *et.al*. (2012) that the nutrient utilization and growth performance significantly increased in *Labeo bata* fed with 65% of mulberry leaf meal containing diet replacing 50% of fish meal.

The hematological parameters are indicators of the different response of the animal to the environment. The changes in hematological and blood biochemical values often reflect alteration of physiological state of fish (Osman *et al*, 2010). Blood parameters can be useful for the measurement of physiological disturbances in stressed fish and thus provide information about the level of damage in the fish. Kang *et al.* (2013) has observed the potential application of enzymes complex made up of xylanase, beta-glucanase and cellulose, in the corn-paddy-soybean diet, can improve performance and nutrition digestibility in meat-type duck. Quince extract protects the red blood cells from UVA damage. Quince leaf extract has the ability to prevent hemato-toxic stress induced by UVA and results in enhancement of the immune system of catfish (Osman *et al*, 2010). Plant based root-powder has been shown to positively influence the hematological profile of fish (Adegbesan *et al.,* 2007).

2.11. Exogenous enzymes

The digestibility of nutrients in the feed can affect the aquaculture production efficiency and impact the environment. An ingredient used in aqua-feed is known by its digestibility or how well it is being utilized by the cultured organism. Digestion or digestibility is always related to digestive enzymes like amylase, protease and lipase present in the stomach and intestine.The strategy like exogenous enzyme supplementation in plant based diets of *Labeo rohita* can improves the growth performance and nutrient utilization significantly (Ranjan *et al*., 2017). Supplementation of the 0.01% exogenous enzyme in DORB based diet results a significantly higher weight gain, SGR, PER and lower FCR as compared to the non-supplemented DORB based diet (Ranjan *et al*., 2017).

The dietary supplementation of all kinds of exogenous enzymes in Japanese seabass has shown higher SGR compared to of the non-supplemented feed (Qinghui Ai *et al,*. 2007). Leaf meal based diet is known to have higher amount of indigestible fibre hindering in the digestion process in the gastro–intestinal tract of fishes and thereby increasing the need to incorporate exogenous enzyme in their diet. Dietary

exogenous enzyme increases the digestion capacity and improves the health status of fish (Wenk, 1998).

2.12. Synthetic Amino Acids

Amino acids are the building blocks of protein which can be further classified into two major categories, i.e. essential amino acids and non-essential amino acids. Deficiency of the essential amino acids in diet may lead to many nutritional deficiency diseases in fish. The inclusion of total aromatic amino acid (phenylalanine+tyrosine) in the range of 2.16% of the dry diet (corresponding to 5.40% of protein) to 2.22% of the dry diet (corresponding to 5.55% of the dietary protein) is required in developing total aromatic amino acid balanced diets for intensive culture of *L. rohita* fry (Khan *et al.,* 2007). Abidi *et al*. (2011) has determined the inclusion of TSAA (total sulphur amino acid) in the range of 25.2–31.31 g/kg of protein as optimum level of which 33–39% could be spared by cystine in rohu fry. It has been suggested that rohu fingerlings can effectively utilize the supplemented amino acids and sesame seed meal protein can replace up to 50% of fish meal protein in the diets for rohu through the proper processing, fermentation and supplementation of deficient amino acids of sesame seed meal (Mukhopadhyay *et al*., 1999).

Among these, lysine and methionine are most limiting amino acids in plant protein based fish feed (Small and Soares, 2000). Furthermore, all finfish species require lysine and methionine as essential dietary component, especially when alternative plant protein sources are used instead of fishmeal. It has also been observed that many fish skeletons contain highest lysine concentrations (Ahmed and Khan, 2004). In addition, fishes fed with essential amino acid deficient diets showed reduced growth and higher mortality rates (Ketolea, 1983). Moreover, lysine is the precursor in carnitine synthesis in skeletal muscle cells and liver and it has an important role in in transportation of long chain fatty acids into mitochondria for beta-oxidation to produce energy (Walton *et al.,* 1984). The lysine concentration (in percentage) is required in fish feed varies between 3.2 to 6.2% of the total dietary protein for different fishes (Wilson, 2002).

1. INTRODUCTION

Aquaculture is considered as one of the major food producing sectors in India, providing nutritional security to the ever increasing human population, supporting domestic market demand and also contributing to the exports. The country has shown continuous increments in fish production since independence and it is contributing around 1.1% of the total GDP and 5.23% of the agricultural GDP (NFDB, 2018). The major cultured species in India includes carps, catfishes, tilapia, shrimps etc., in which *Labeo rohita* is the most important cultivable species with high consumer demand due to its taste. This increase in demand ultimately resulted in an increase in fed aquaculture production. Feeding can increase the growth rate and total yield of *Labeo rohita* (Maity & Patra, 2008; Meshram *et al*., 2018).Traditionally, in India, carps especially rohu is fed with rice bran and groundnut oil-cake at 1:1 ratio. However, commercial feed for fishes is prepared from different plant and animal based ingredients like soybean meal, rice bran, various oil seed cakes, fish meal, shrimp meal etc. Presently there is a huge competition between various food and feed industries for ingredients like soybean meal and DORB. Moreover, the production of rice bran is insufficient to meet the requirement of feed industry. Hence, market price of this ingredient is increasing due to high demand and non-availability. Therefore, there is an urgent need to replace the de-oiled rice bran in aquafeed with suitable, easily available and lower cost plant protein to sustain the feed based aquaculture.

There are several studies to replace conventional ingredients by alternate feed ingredients in formulation and preparation of aquafeed. Among the unconventional sources of ingredients, leaf meal is one of the potential and cheapest sources of protein (Adewolu, 2008). A huge variety of leaf meals obtained from both aquatic and terrestrial sources have been utilized successfully in the fish diet by several researchers (Osman et al., 1996; Sheeno and Sahu, 2006; Bairagi *et al*., 2002; Adewolu, 2008; Mondal *et al*., 2012; Meshram *et al*., 2018).India, the land of agriculture has several seasonal crops, which can be a potential source of leaf meal. After collection of leaves, leaf meal can be easily prepared by drying and grinding into fine powder.

Detailed study of nutritive and anti-nutritive components of leaf meal can be helpful in selecting suitable leaf meal to replace protein and energy sources for animal feed (Osman, 2007; Vhanalakar & Muley, 2014).Though research is under progress, leaf meal based feed has been proved to be growth promoting and leaf meal is able to replace conventional aqua feed ingredients up to some extent. There are some preliminary studies on individual leaf meal like *Vigna mungo, Ipomea aquatica* and *Hygrophila spinosa*, which have been proved to be potential ingredients in aqua feed at different inclusion levels (Sahoo, 2018; Gurung, 2018; Maiti, 2018). However, leaf meal contains several anti nutritional factors and is also deficient of few essential amino acids which can be a cause of poor nutrient utilization, amino acid imbalance, poor growth along with some nutritional deficiency diseases. Several methods have been used to remove the ANFs from leaf meals and plant based ingredients (Campbell & Bedford, 1992; Mwachireya, Beames, Higgs, & Dosanjh, 1999; Almazan, 1995; Kumar et al., 2012; Shamna et al., 2015). But, most of the methods are tedious, costly and lab based. Moreover, leaf meals may be deficient in some essential amino acids like methionine, lysine etc (George *et al*., 2001). A better growth in fishes is ensured by incorporation of the quality protein ingredient in the feed which supplies the required essential amino acids to the fishes. Mixing of different leaf meals can be a feasible and potential strategy to reduce anti-nutritional factors and to compensate the deficient amino acids in one leaf meal by the other one, which is rich of that very amino acid. The supplementation of diet with synthetic amino acids can be a strategy to improve the quality of leaf meal in case of any deficiency of the essential amino acids.

Digestibility is an important parameter to decide the quality of an ingredient as digestibility tells how better the fishes are able to digest and absorb the nutrients from the given feed. A suitable ingredient for fish feed should have higher digestibility and acceptability by fishes. The leaves are known to contain higher amount of fibre. Digestibility of fibre can be improved by adding exogenous enzymes in the diet. The optimum utilization of leaf based ingredient through exogenous enzyme supplementation in fish feed is still a researchable issue in the aqua-feed industry.

Studies have been carried out in our lab to optimize the inclusion level of different leaf meals indicated that *Vigna mungo* (Black gram) up to 30%, *Ipomea aquatica* (Water spinach) up to 20% and *Hygrophila spinosa* (Gokulakanta) up to 10% (Sahoo, 2018; Gurung, 2018; Maiti, 2018) increased the growth rate without causing any detrimental effect in fish. Mixing of leaves can ensure wider availability and dilution of ANFs which can make it more appropriate as an aquafeed ingredient. Hence, the present study was planned and aimed to find out the effect of feeding the mixture (1:1:1 ratio) of these three leaves with or without supplementation of exogenous enzyme and synthetic amino acid on the growth and physio- metabolic condition of *Labeo rohita*. The objectives of the study were as follows:

- To evaluate the growth & nutrient utilization in *Labeo rohita* fingerlings fed with mixed leaf meal supplemented with synthetic amino acids and exogenous enzymes

- To asses the physio-metabolic changes in *Labeo rohita* fingerlings fed with mixed leaf meal supplemented with synthetic amino acids and exogenous enzymes

3. MATERIAL AND METHODS

3.1. Site of the experiment

The experimental setup was kept for 60 days from 1st March to 29thApril in wet lab of ICAR- Central Institute of Fisheries Education (CIFE), Mumbai. The various physio-chemical and biochemical analysis were done at Fish Nutrition, Biochemistry and Physiology division laboratory, ICAR- Central Institute of Fisheries Education (CIFE), Mumbai.

3.2. Chemicals and glass wares

The glass wares used throughout the experiment were neutral glass of Borosil make. Chemicals of various companies viz. Sigma, SRL, HI-media, Qualichem, Innoline and Merck etc. were used.

3.3. Collection of leaves

The leaves of *Hygrophila spinosa*, *Vigna mungo* and *Ipomea aquatica* were collected partly from the Pursurah village, Hoogly district, West Bengal and Howrah district, West Bengal.

3.4. Preparation of leaf meal

Collected leaves were washed thoroughly in water anddried in sunlight. Then the leaves were brought to the lab and again dried in oven at 45^{o}C for overnight. The dried leaves were ground, sieved with 50 µm mesh and stored in air tight container.

3.5. Preparation of mixed leaf meal (MLM)

The three leaves were mixed thoroughly with each other in the ratio of 1:1:1 for the preparation of mixed leaf meal.

Hygrophila spinosa (Kulekhara)

Ipomea aquatica (Water spinach)

Vigna mungo (Mung bean)

PLATE 1: Leaves used for Mixed Leaf Meal(MLM) preparation

3.6. Amino acid analysis

The instrument and software used for the amino acid analysis are Agilent 1100 HP-HPLC and Chem-station software. It was done in Shankara Nethralaya, Chennai Lab with the following procedure:

Before starting, the instrument is calibrated using 1.0 nm, 500 pm, 250 pm standards individually. Then 10μl of the standard in 60μl borate buffer and 10 μl of OPA reagent were mixed in dilution vial and cyclomix it. From this mixture 50 μl is injected in the HPLC using Hamilton syringes. Each standard was individually run in the gradient program and the chromatogram for them was obtained. Two consecutive runs that have the same retention time, there average was taken which used for plotting the graphs in the calibration table. The procedure is termed as calibration and the curve obtained for the same is calibration curve.

Further for sample analysis10μl of the sample in 60μl borate buffer and 10 μl of OPA reagent were mixed in dilution vial and cyclomix it. From this mixture 50 μl is injected in the HPLC using Hamilton syringes. After the sample is run the chromatogram is obtained for the same. From the chromatogram the area of the peaks will be recorded and calibrated along with the standards and used for calculation.

3.7. *In vitro* protein digestibility of mixed leaf meal

In vitro protein digestibility study was carried out using the method of Ali *et al*. (2009). Fresh tissue of alimentary canal of the fish was homogenized under cold condition and diluted with distilled water (1:10 w/v). The enzyme was extracted by centrifuging it at 12000 rpm for 15 min at 4°C. An equivalent amount of finely powdered MLM that provided 160mg of crude protein was weighed and mixed with20 ml of distilled water and 2ml of enzyme to obtain 8mg crude protein per milliliter and the pH was adjusted to 8 (Eutop pH tutor, Thermo Fisher Scientific, Singapore).The pH drop was recorded at every minute interval for 10 min and casein was used as the reference protein. Relative Protein Digestibility was calculated using the following formula.

Relative Protein Digestibility (RPD %) = (-ΔpH of ingredients/-ΔpH of casein) × 100.

3.8. Experimental Design

Completely randomized design was followed for the experiment (Table 1). It was comprised of total 5 treatments and one control with 3 replications in each group. Control group was fed with basal diet without mixed leaf meal, enzyme or synthetic amino acids. T1- 20% MLM; T2- 40% MLM; T3- Control + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.14% methionine &1.04% lysine; T4- 20% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.94% of lysine; T5 - 40% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 1.04% of lysine. Twelve number of homogenous rohu fingerlings were randomly distributed among all the treatments.

Table1: Experimental design

Treatments	Condition	R1	R2	R3
C	Basal diet without any leaf meal			
T1	20% mixed leaf (1:1:1) containing diet			
T2	40% mixed leaf (1:1:1) containing diet			
T3	Basal diet but with 0.1 % exogenous enzyme & synthetic amino acids			
T4	20% mixed leaf (1:1:1) containing diet with 0.1 % exogenous enzyme & synthetic amino acids			
T5	40% mixed leaf (1:1:1) containing diet with 0.1 % exogenous enzyme & synthetic amino acids			

3.9. Preparation of experimental diets

For experimental purpose, six iso-nitrogenous (30%crude protein) and iso-caloric (350 Kcal/100g DE) practical diets were prepared with graded level of MLM and other ingredients (Table 2). The ingredients such as de-fatted soybean meal (DSBM),groundnut oil cake (GNOC), wheat flour, de-oiled rice bran (DORB), mixed leaf meal (MLM), cod liver oil, sunflower oil, carboxymethyl cellulose, vitamin and mineral

mixture (Agrimin Forte), BHT, choline chloride and liquid exogenous enzyme mixture (Cellulase and Xylanase) were used for the feed formulation. Six different diets with different composition were prepared to replace DORB by using graded level of mixed leaf meal (MLM) supplemented with/without exogenous enzyme (EE) and synthetic amino acids (SAA).

The amino acid analysis of mixed leaf meal and feed were done in Shankara Nethralaya Lab and supplementation of deficient amino acid was done with synthetic amino acids as per the requirement of *L. rohita* fingerlings following FAO manual(FAO, 2016).

All the ingredients along with MLM were weighed properly as per the formulation and kept in a plastic tray. The ingredients were then mixed properly to form dough with the addition of adequate quantity of water. The dough was transferred to a heat resistant plastic bag and placed in a pressure cooker for cooking/steaming for 20 minutes. After that pre-cooked dough was allowed to cool in room temperature. The pressure cooker was then removed from the flame and kept aside for cooling. When the steamed dough was completely cooled, the calculated proportions of the oils, chromium oxide, vitamins and minerals mixture, choline chloride, BHT, CMC, liquid exogenous enzymes and synthetic amino acids were incorporated in it and mixed well. The adequately mixed dough was pressed through a pelletizer to get uniform sized pellets which were spreaded on a sheet of paper and allowed to dry in air. After drying the pellets were packed in polythene bags, sealed airtight and labeled according to the treatments and stored in frozen (-20^{0}C) condition until fed.

Table:2 Formulation of the different experimental diets

	Treatments[1]					
Ingredients (g/kg)	**C**	**T1**	**T2**	**T3**	**T4**	**T5**
DSBM	195	157.5	120	200	161	125
GNOC	300	300	300	300	300	300
Wheat flour	23.8	61.3	98.8	6	47	82.4
DORB	400	200	0	400	200	0
MLM	0	200	400	0	200	400
Sunflower oil	20	20	20	20	20	20
Fish oil	20	20	20	20	20	20
Betaine	5	5	5	5	5	5
Vitamin-mineral mix	20	20	20	20	20	20
Choline chloride	1	1	1	1	1	1
CMC	15	15	15	15	15	15
BHT	0.2	0.2	0.2	0.2	0.2	0.2
Exogenous enzyme	0	0	0	1	1	1
Methionine	0	0	0	1.4	0	0
Lysine	0	0	0	10.4	9.8	10.4
Total	1000	1000	1000	1000	1000	1000

DSBM: De-oiled soybean meal, GNOC=Groundnut oil cake, DORB=De-oiled rice bran, MLM= Mixed leaf meal, CMC=Carboxymethyl cellulose, BHT=Butylated Hydroxytoluene.

[1]C-Control; T1- 20% MLM; T2- 40% MLM; T3- Control + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.14% methionine &1.04% lysine; T4- 20% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.94% of lysine; T5 - 40% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 1.04% of lysine

Composition of vitamin mineral mix (AGRIMIN FORTE) (quantity kg^{-1}) Vitamin A, 7,00,000 IU; Vitamin D_3, 70,000 IU; Vitamin E, 250 mg; Nicotinamide, 1000 mg; Cobalt 150 mg; Copper 1200 mg; Iodine 325 mg; Iron 1500 mg; Mg 6000 mg; Mn 1500 mg; K 100 mg; Na 5.9 mg; S 0.72%; Zn 9600 mg; Ca 25.5%; P 12.75%.

Exogenous enzyme from *Trichodermareesei* (Cellulase-10000 ECU/g; Xylanase- 350000 BXU/g) was supplied by AB Vista, Wilshire, UK.

20% MIXED LEAF MEAL BASED FEED

40% MIXED LEAF MEAL BASED FEED

PLATE 2: MIXED LEAF MEAL BASED FEED

3.10. Anti-nutritional factors (ANFs) of leaf meals and experimental diets

3.10.1. Phytic acid

Phytic acid of leaf meals and diets estimation was carried out following the spectrophotometric procedure of Vaintraub and Lapteva (1988). Five hundred mg of finely ground leaf and feed sample was taken in an Erlenmeyer flask of 50mLand 3% trichloroacetic acid (TCA) solution was added. After shaking for 30 min the mixture was centrifuged at 3000g for 10 min. Four ml of $FeCl_3$ solution was added rapidly to an aliquot of 10ml. This was kept in a water bath at boiling temperature for 45 min. The solution was centrifuged at 3000g for 10–15 min. After washing with 3% TCA, the precipitate was dispersed in few ml of distilled water and 3ml of 1.5 N NaOH. The solution was made up to 30ml and filtered through a Whatman No. 2 filter paper and the precipitate was dissolved in 100ml volumetric flask containing 40mLhot 3.2N HNO_3. After cooling, the volume was made to 100ml with distilled water. From this, 5ml aliquot was made to 100ml using 20ml 1.5M KSCN and distilled water. The reading was measured at 480 nm in UV-visible spectrophotometer (Shimadzu, UV1800, Kyoto, Japan) and a blank with each sample was run.

3.10.2. Total tannin

Total tannin was estimated using spectrophotometric method as described by Makkar *et al.* (2007). The reagent used was Folin-Ciocalteu reagent and the results were expressed as tannic acid equivalents. The total tannin content was calculated from the calibration curve of tannic acid standard and expressed as milligrams/100g of tannin. All the extractions were carried out in triplicates.

3.10.3. Oxalates

The titration method as described by Day and Underwood (1986) was followed. One gram of the sample was weighed into 100ml conical flask. Seventy-five ml of 3M H_2SO_4 was added and stirred for 1hr with a magnetic stirrer. This was filtered using

a Whatman No 1 filter paper. About 25ml of the filtrate was then taken and titrated while hot against 0.05M $KMnO_4$ solution until a faint pink colour persisted for at least 30 Sec. The oxalate content was then calculated by taking 1ml of 0.05m $KMnO_4$ as equivalent to 2.2mg oxalate (Chinma and Igyor,2007).

3.10.4. Total alkaloid

The alkaloid content was determined gravimetrically (Haborne and Green, 1980).Briefly, 5g of each sample was weighed using a weighing balance and dispersed into 50ml of 10% acetic acid solution in ethanol. The mixture was well shaken and then allowed to stand for about 4h before it is filtered. The filtrate was then evaporated to one quarter of its original volume on a hot plate. Concentrated ammonium hydroxide was added drop wise in order to precipitate the alkaloids. A pre-weighed filter paper was used to filter off the precipitate and it was then washed with 1% ammonium hydroxide solution. The filter paper containing the precipitate was dried in an oven at 60^{o}C for 30 min, transferred into desiccators to cool and then reweighed until a constant weight was obtained. The constant weight was recorded. The weight of the alkaloid was determined by weight difference of the filter paper and expressed as a percentage of the sample weight analyses. The experiment was repeated thrice for each feed and leaf sample and the reading recorded as the average of three replicates.

3.11. Experimental set-up

The fishes used for the experimental purpose were fingerlings of *Labeo rohita* with an average initial weight of 3.41 ±0.01 g. The fishes were procured from Prem fish farm, Ankleshwar, Gujrat. The fishes were transported in plastic bags filled with 1/3 water and 2/3 oxygen. Later, they were transferred to a circular fiber tank containing treated water with adequate aeration. Fishes were treated with mild salt and KMnO4 (4 mgL-1) solution on the next day to reduce handling stress. The stock was acclimatized for 15 days under aerated condition. Two hundred and sixteen fingerlings of *Labeo rohita* were randomly distributed in six distinct experimental groups in triplicates following a completely randomized design. The experiment was conducted for a period of 60days in 18 plastic rectangular tubs (200L capacity) covered with perforated lids. The tubs were

initially washed and filled with KMnO4 solution (4 mg/L). The tubs were flushed out on the next day and were thoroughly washed with clean water. Twelve fishes with initial average weight 3.41 ±0.01gwerestocked in each plastic tub filled with chlorine free bore-well water. The total volume of the water in each tub was maintained throughout the experimental period with round the clock aeration.

3.12. Rearing

The fishes maintained in tubs were fed with a control diet before the commencement of the experiment. No measures were taken to stimulate or control the environmental condition. The experimental conditions were kept same throughout the experimental period. The body weight was measured at 15 days interval to assess the growth. The fishes were starved overnight every time before taking the body weight.

3.13. Feeding

Feeding was done twice a day to satiation throughout the 60 days feeding trial. The daily ration was divided into two equal parts and fed at 10:00 am in the morning and 17:00 pm in the evening.

3.14. Cleaning and siphoning

The experimental tubs were cleaned manually and siphoning was done every day in order to remove the fecal matter and debris. An equal volume of clean bore well water replaced the siphoned water throughout the experimental period.

3.15. 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.

3.15.1. Temperature

The water temperature of all the experimental tubs was recorded using thermometer (MERCK, Germany).

3.15.2. pH

The pH was measured by a digital pH meter (LABINDLA) from all the experimental tubs.

3.15.3. Dissolved oxygen

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

3.15.4. Free carbon dioxide

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

CO_2 (mg/L) = A x N x 44 x 1000/Volume of sample (ml)

Where, A = Volume of titrant (NaOH)

N = Normality of titrant (N/44)

3.15.5. Carbonate hardness

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

3.15.6. Ammonia

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

3.15.7. Nitrite-N

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

3.16. *In vivo* digestibility trials for experimental diets

A digestibility trial was conducted for a period of 10 days after the feeding trial was over. The digestibility study was carried out by indirect method using the chromic oxide (Cr_2O_3) marker (Fawole *et al.,* 2016). The experimental diets were prepared as specified earlier (table 1) by incorporating0.5% chromic oxide (Cr_2O_3). The fishes were fed once daily at 10:00 AM. The unconsumed feeds were removed 1 hr after daily feeding. Fecal matters were collected once a day at about 17.00 PM. The digestibility trial was conducted for 10 days to collect enough faecal samples, using a modified method described by Shiau and Liang (1994). Faecal samples were collected by siphoning from the bottom of each tank. The faeces were then dried and stored at -20^0C until analysis (Shiau and Liang, 1994; Usmani *et al.,* 2003). The faecal samples collected were analyzed for crude protein and lipid by following methods of AOAC (1995). Quantification of chromium oxide content of feed and faecal matters were carried out by the method of Furukawa and Tsukahara (1966).The apparent digestibility coefficients (ADCs) of dry matter, protein and lipid were calculated as follows (Cho and Singer, 1979):

$$\text{ADC of dry matter (\%)} = 100X\left(1-\frac{\%\text{of Chromium oxide in diet}}{\%\text{ of Chromium oxide in feces}}\right)$$

$$\text{ADC of dry matter (\%)} = 100X\left(1-\frac{\%\text{of Chromium oxide in diet X \% of nutrient in feces}}{\%\text{ of Chromium oxide in feces X \%of nutrient in feed}}\right)$$

3.17. Proximate analysis of feed ingredients, diets, fish tissues and fecal matter

The fishes were collected from the experimental tubs and the biomasses of the respective tubs were noted down. The samples were then stored at 0°C for conducting the proximate analysis. It was done by prescribed method (AOAC, 1995) in the Nutrition Laboratory of CIFE. Similarly, the proximate composition of all the feed ingredients, experimental diets and fecal matter was also done.

3.17.1. Moisture

The moisture content of the diets and animal tissue were determined by taking a known weight of the sample in the petridis and dried it in hot air oven at 100-105°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:

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

3.17.2. Crude protein (CP)

The nitrogen content of the ingredients, experimental diets and whole body tissue dried samples were estimated quantitatively by Kjeltec semi-automated method (2200 Kjeltec Auto Distillation, Foss Tecator, Sweden) using titration as the means for determining the nitrogen percentage. The crude protein percentage was calculated by multiplying the nitrogen percentage by a factor of 6.25.

$$\text{Crude protein}\ (\%) = \text{N}\ (\%) \times 6.25$$

3.17.3. Ether extracts (EE)

Ether extract of dried ingredients, experimental diets and carcass tissue samples was estimated by Soxhtech apparatus using diethyl ether (boiling point 55ºC ±5) as the solvent.

The calculation was made as follows.

$$\text{EE}\ (\%) = \frac{\text{Initial weight of sample (g)} - \text{Final weight of sample (g)}}{\text{Initial weight of sample (g)}} \times 100$$

3.17.4. Total ash

Ash content was estimated by taking a known weight of sample in a silica crucible and placing it in a muffle furnace at 600°C for 6 hrs. The calculation was done as follows.

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

3.17.5. Nitrogen free extract (NFE)

Nitrogen free extract from feed was calculated by subtracting the sumpercentage of crude protein, crude fiber, ether extract and total ash from 100.

NFE (%) =100 – (CP % + CF % + EE % + Total ash %)

3.17.6. Total carbohydrate (TC)

The total carbohydrate (TC) from whole tissue was calculated bysubtracting the percentage of other nutrients from 100 (Hasting, 1969)

TC (%) =100 — (Moisture % + CP % + EE % + Total Ash %)

3.17.7. Crude fibre (CF)

Crude fibre content of different test diets (fat-free samples) were determined by acid (1.25% HCL) and alkali digestion (1.25% NaOH) using FibroTRON (Tulin equipments, India) followed by drying (100ºC±2) and incineration (in muffle furnace at 550ºC for 4hrs) of the samples. The calculation was made as follows.

$$\text{Crude fibre } (\%) = \frac{\text{Weight of dried sample} - \text{Weight of ash (g)}}{\text{Initial weight of sample (g)}} \times 100$$

3.17.8. Digestible energy (DE)

The digestible energy value of the experimental diets was calculated on the basis of standard physiological values (Halver, 1976) as per the following formula.

Digestible energy (kcal/100g) = [CP (%) x 4 + EE (%) x 9 + NFE (%) x 4]

3.18. Growth parameters

Samplings were done at intervals of 15 days to assess the bodyweight of the fishes. Fishes were starved overnight before taking the weight. The weight was taken on an electronic weighing balance.

3.18.1. Percentage weight gain

The percentage weight gain was calculated using the following formula.

$$\text{Weight gain } (\%) = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \text{X}100$$

3.18.2. Specific growth rate (SGR)

The specific growth rate was calculated by the following formula:

$$\text{SGR } (\%) = \frac{\text{Ln (final weight)} - \text{Ln (initial weight)}}{\text{Duration of feeding}} \times 100$$

3.18.3. Feed conversion ratio (FCR)

The Feed Conversion Ratio was calculated by the following formula:

$$\text{FCR} = \frac{\text{Feed intake (g dry weight)}}{\text{Body weight gain (g wet weight)}}$$

3.18.4. Protein efficiency ratio (PER)

The protein efficiency ratio was calculated by the following formula:

$$\text{PER} = \frac{\text{Net weight gain (g)}}{\text{Protein fed (g)}}$$

3.19. Survival rate

At the end of the experiment, all the experimental tubs were dewatered and the number of the experimental animals in each tub was counted and the survival rate (%) was calculated by the following formula

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

3.20. Body Indices

3.20.1. Hepato-somatic Index (HSI)

The liver weights of fishes of different treatment groups were recorded and the hepato-somatic index was calculated as

$$\text{HSI} = \frac{\text{Liver weight (g)}}{\text{The weight of fish (g)}} \times 100$$

3.20.2. Intestinal somatic Index (ISI)

The gastrointestinal tract of different treatment groups was recorded and the intestinal somatic index was calculated as follow

$$\text{ISI} = \frac{\text{Intestine weight (g)}}{\text{weight of fish (g)}} \times 100$$

3.21. Enzyme Assays

3.21.1. Tissue homogenate preparation

The muscle, liver, gill and intestine of the fishes were removed carefully and were weighed. It was homogenized with chilled sucrose solution (0.25M) in a glass tube using tissue homogenizer. The tube was continuously kept in ice to avoid heating. The homogenate was centrifuged at 5000rpm for 10 min at 4˚C. The supernatant was stored at 4˚C until use. A 5% tissue homogenate was prepared for muscle, liver, gill and intestine.

3.21.2. Estimation of tissue protein

Quantification of protein in the different tissue homogenate was carried out by Lowry's method (Lowry *et al*., 1951). The resultant tissue protein value was used for estimating the enzyme activities in respective samples.

Tissue homogenate (0.1ml) were precipitated using 1ml of 10% Trichloroacetic acid (TCA) in 1.5ml Eppendorf tubes. The protein precipitate was obtained by discarding the supernatant produced after centrifugation at 5000 rpm for 20 minutes. The residue was dissolved in 0.5ml of 0.1N NaOH by vortexing and from this 0.1ml of the dissolved sample containing tissue protein was used for further analysis. Alkaline copper sulphate (5 ml) was added to dissolve protein residue and left for 10 minutes. Subsequently, 0.5ml of 1N Folin's reagent was added and incubated for 30 minutes in the dark. After that the optical density was read at 660nm against blank. Bovine serum albumin was used as standard to prepare the standard curve. From the standard curve, the protein concentration of different tissue samples was estimated.

3.21.3. Digestive enzymes

3.21.3.1. Protease activity

Protease activity was determined by the casein digestion method (Drapeau, 1974). One unit of enzyme activity was measured as the amount of enzyme needed to release acid soluble fragments equivalent to Δ0.001 at 280 nm per min at 37°C and pH 7.8.

3.21.3.2. Amylase activity

Amylase activity was estimated as the reducing sugars produced due to the action of gluco-amylase and α-amylase on carbohydrate using di-nitrosalicylic-acid (DNS) method (Rick and Stegbauer, 1974). Specific amylase activity was expressed as mole of maltose released from starch per min at 37°C.

3.21.3.3. Lipase activity

Titrimetric determination of lipase activity was done according to procedure described by Cherry and Crandall (1932) in this procedure where native substrates (triacylglycerols) are hydrolyzed to yield fatty acids. Subsamples were withdrawn from reactive mixtures at predetermined intervals, and reactivity was quenched by the addition of ethanol. The amount of fatty acids released during the reaction was determined by direct titration with NaOH to a phenolphthalein end point.

3.21.4. Enzymes of protein metabolism

3.21.4.1. Aspartate aminotransferase (AST)

AST activity from different tissues of *Labeo rohita* 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°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 min with occasional shaking. Then 5ml of 0.4ml NaOH solution was added and the contents were thoroughly mixed. After 10 min, the OD was recorded at 540nm against the blank. The AST activity was expressed as nanomoles oxaloacetate formed/mg protein/min at 37°C.

3.21.4.2. Alanine aminotransferase (ALT)

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 formed / mg protein / min at 37°C.

3.21.5. Enzymes of carbohydrate metabolism

3.21.5.1. Lactate dehydrogenase (LDH)

The LDH activity was assayed in different tissues by the method of Wroblewski and Ladue (1955). Total 3ml of the 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 was started after addition of substrate sodium pyruvate. The OD was

recorded at 340nm at 30 sec interval for 2 min. The enzyme activity was expressed as units/ mg protein/ min at 25 °C where 1 unit was equal to Δ 0.01 OD/ min at 37°C.

3.21.5.2. Malate dehydrogenase (MDH)

The MDH activity was assayed in different tissues by the method of Ochoa (1955). Total 3ml of the reaction mixture comprised of 2.7ml of 0.1M phosphate buffer (pH 7.5), 0.1ml of NADH solution (2 mg NADH dissolved in 1 ml of phosphate buffer solution), 0.1ml of tissue homogenate and 0.1 ml of freshly prepared oxaloacetate solution (2 mg oxaloacetate dissolved in 2 ml chilled distilled water). The reaction was started after addition of oxaloacetate solution as substrate. The OD was recorded at 340nm at 30 sec interval for 2 min. The enzyme activity was expressed as units/mg protein/min at 25°C where 1 unit was equal to Δ 0.01 OD/ min at 37°C.

3.21.6. Enzymes of oxidative stress

3.21.6.1. Superoxide dismutase (SOD)

Superoxide dismutase was assayed according to the method described by Mishra and Fridovich (1972) based on the oxidation of epinephrineadrenochrome 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 EDTA (pH 10.2) and 0.5 ml epinephrine (3mM) was added and mixed well. Change in optical density at 480 nm was recorded immediately for 3 min in a spectrophotometer. One unit of SOD activity was the amount of protein required to give 50% inhibition of epinephrine auto oxidation. SOD expressed as unit activity (amount of protein required to give 50% inhibition of epinephrine auto oxidation).

3.21.6.2. 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 240nm at 15sec

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 nanomolesH_2O_2decomposed / min / mg protein at 37°C.

3.22. Haemato-Immunological Parameters

3.22.1. Collection of blood

Each fish was anesthetized with clove oil (50µl of clove oil per liter of water) before taking blood from fish. Blood was taken from a caudal vein using a medical syringe which was previously rinsed with 2.7% EDTA solution. Blood collected was then transferred immediately to test tube containing a thin layer of EDTA powder (as an anticoagulant) and stirred well in order to prevent hemolysis of blood.

3.22.2. Collection of serum

Each fish was anesthetized with clove oil (50µl of clove oil per liter of water) before taking blood from fish. Blood was taken from a caudal vein using a medical syringe without using 2.7% EDTA solution. 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, which allows the blood to clot. After clotting the blood, the yellow straw color serum was carefully collected and transferred to another Eppendorf tube.

3.22.3. Nitroblue tetrazolium (NBT) assay

Nitroblue tetrazolium assay was done by the method of Stasiack and Baumann (1996). Blood collected from the fish by severing caudal vein in a test tube containing 2.7% EDTA as anticoagulant. 100 µl of blood was placed into the wells of 'U' bottom microtitre plates and incubated at 37ºC for 1hr to facilitate adhesion of cells. Then the supernatant was removed and the located wells were washed three times with PBS. After washing, 100 µl of 0.2% NBT (Sigma, USA) was added and incubated for further 1 hr. The cells were then fixed with 100% methanol for 2-3 minutes and again washed with 70% methanol thrice. The plates were then air dried. 120 µl of 2N potassium hydroxide

(KOH) and 140 µl of dimethyl sulphoxide (DMSO) were added into each well to dissolve the formazan blue precipitate formed. The OD of the turquoise blue coloured solution was then read in ELISA reader at 620 nm.

3.23. Blood parameters

The various blood parameters like hemoglobin; RBC & WBC was analyzed by Suburban Diagnostics Lab following standard protocol.

3.24. Serum glucose

Serum glucose was estimated by using Trinder's method using ERBAKIT. Glucose in the sample was oxidized to yield gluconic acid and hydrogen peroxide in the presence of glucose oxidase. The enzyme peroxidase catalyzes the oxidative coupling of 4-aminoantipyrine with phenol to yield a coloured quinonemine complex, with absorbance proportional to the concentration of glucose in sample.

3.24.1. Serum total protein

Plasma protein was estimated by biuret method (Reinhold, 1953) using kit (ERBA). Proteins present in the plasma binds with copper ions in an alkaline medium of the biuret reagent and produce a purple coloured complex, whose absorbance is proportional to the protein concentration. Three test tubes labelled as Blank (B), Standard (S) and Test (T) were taken. Into all the tubes, 1mlof 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 test. It was then mixed well and incubated at 37◦ C for 10min. 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:

Concentration of standard=6g/dl

$$serum\ protein\ (g\%) = \frac{concentration\ of\ standard * absorbance\ of\ test\ (T)}{absorbance\ of\ standard}$$

3.24.2. Serum albumin

Albumin was estimated by bromocresol green binding method (Doumas *et al*., 1971) by using ERBA KIT. Albumin in a buffered medium binds with bromocresol green (BCG) and produces a green color whose absorbance 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 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 in to the test tube labelled as test. It was then mixed well and incubated at 37^{o}C for 10min. 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:

$$serum\ albumin\ (g\%) = \frac{concentration\ of\ standard * absorbance\ of\ test\ (T)}{absorbance\ of\ standard}$$

3.24.3. Serum globulin

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

Globulin (g%) = Total protein (g %) – Albumin (g%)

3.24.4. Albumin-globulin ratio

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

3.25. Statistical analysis

Statistical significance of different study parameters was analyzed by one-way analysis of variance (ANOVA) using a SPSS 22.0 for Windows. Duncan's multiple range test was used for post hoc comparison of mean ($P<0.05$) between different experimental groups. All data presented in the text, figures and tables are mean ± standard error (SE) and statistical significance for all statistical tests were set at 5% probability level ($P< 0.05$).

4. RESULTS

4.1. Physico-Chemical Parameters of Water

Physico-chemical parameters of water such as temperature (^{o}C), dissolved oxygen (mg/l), pH, free carbon dioxide (mg/l), total hardness (mg/l), ammonia (mg/l), and nitrite - N (mg/l) were estimated and average values of all the treatments are presented in Table 3.

4.1.1. Temperature

The water temperature of the different experimental treatment ranged from 28.5^{o}C to 32.9^{o}C during the 60 days of experimental period.

4.1.2. Dissolved oxygen

The dissolved oxygen concentration of all the experimental tubs was recorded within the range of 6 to 7.9mg/l during the entire experimental period.

4.1.3. pH

The pH values were recorded within the range of 7.2 to 8.5.

4.1.4. Total hardness

The carbonate hardness was found to be 231 - 244 mg/l during the experimental period.

4.1.5. Free carbon dioxide

The free carbon dioxide in water was recorded to be negligible during the experimental period.

4.1.6. Ammonia

The total ammonia content of all the experimental tubs were recorded before water exchange and were found to be in the range of 0.04 to 0.10 mg/l.

4.1.7. Nitrite

The nitrite-N content was found to be in the range of 0.04 to 0.09mg/l.

Table 3: Physico-chemical parameters of water during the experimental period of 60 days for different experimental groups

Treatments[1]	Temperature (^{0}C)	Dissolved oxygen(mg /l)	pH	Free CO_2 (mg/l)	Total hardness (mg/l)	Ammonia (mg/l)	Nitrite (mg/l)
C	28.7-32.5	6.0-7.1	7.4-8.2	ND	231-242	0.05-0.09	0.05-0.07
T1	28.5-32.2	6.2-7.5	7.6-8.2	ND	233-241	0.04-0.08	0.04-0.08
T2	28.6-32.7	6.0-7.7	7.3-8.1	ND	234-242	0.05-0.08	0.06-0.09
T3	29.4-32.4	6.1-7.9	7.2-7.9	ND	233-241	0.06-0.10	0.06-0.09
T4	29.0-32.8	6.3-7.8	7.3-8.0	ND	233-239	0.04-0.07	0.05-0.09
T5	29.1-32.9	6.0-7.4	7.6-8.2	ND	236-242	0.06-0.09	0.04-0.08

ND: Not Detected

[1]C-Control; T1- 20% MLM; T2- 40% MLM; T3- Control + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.14% methionine & 1.04% lysine; T4- 20% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.94% of lysine; T5 - 40% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 1.04% of lysine

4.2. Proximate Composition of the feed ingredients

The proximate composition of the different feed ingredients used for preparation of experimental diets is given in the Table 4.

4.3. Proximate Composition of the Experimental Diets

The proximate composition of the different experimental diets is given in the Table: 4. The crude protein in the diets varied from 30.10 to 31.00%, whereas, the ether extract level varied from 5.50 to 6.19%.The ash content of the diets was between 7.47 and 8.24%. The crude fibre of the diets was estimated in the range of 5.10 to 7.98%. The nitrogen-free extract was calculated within the range of 48.21 and 50.57%. The estimated digestible energy (DE) varied from 342.43to 349.57 kcal/100 g of feed.

4.4. Whole body composition of *Labeo rohita* fingerlings

The proximate composition of the fish tissues of different experiment group is shown in Table 5.There was no significant difference ($p>0.05$) found in moisture content, crude protein, ether extract, total carbohydrate and total ash content (%) of fish tissue after feeding with different experimental diets for the period of 60 days.

4.5. Proximate composition of different leaf meal

The proximate composition of the leaves is given in Table 7.

Table 4: Proximate composition of different feed ingredients (on % dry matter basis) used for the preparation of experimental diet

Variables	Ingredients[1]				
	MLM	DORB	SBM	GNOC	Wheat flour
Dry matter	94.26±0.17	93.82±0.10	94.08±0.16	94.44±0.28	94.98±1.06
Crude Protein	23.54±0.17	16.00±0.20	52.23±0.18	43.64±0.30	12.02±0.10
Ether Extract	2.30±0.07	1.20±0.90	1.50±0.40	3.56±0.20	2.54±0.22
Crude Fibre	16.77±0.29	13.73±0.26	4.10±0.90	4.87±0.14	0.56±0.03
Nitrogen free extract	37.86±0.22	50.02±0.28	28.96±0.18	35.96±0.38	80.24±0.37
Total Ash	13.74±0.09	12.20±0.27	7.24±0.08	6.57±0.10	1.56±0.06
Gross Energy	373±4.2	386±6.06	435±5.1	419±5.85	399±5.2

All values are expressed as Mean ± SE (n=3)
[1]MLM= Mixed leaf meal, DORB= De-oiled rice bran, SBM: Soybean meal, GNOC= Groundnut oil cake

Gross Energy is expressed in (kca/100gram)

Table 5: Proximate composition of different experimental diets (on % dry matter basis) fed to *Labeo rohita* fingerlings during the experimental period of 60 days

Variables	Treatments[1]					
	C	T1	T2	T3	T4	T5
Dry matter	91.18	91.15	90.23	90.72	90.49	90.19
Crude Protein	30.34	30.10	30.35	30.63	31.00	30.52
Ether Extract	5.50	5.83	6.19	5.51	5.82	6.07
Crude Fibre	6.67	5.80	5.10	6.40	5.93	6.69
Nitrogen free extract	50.02	50.53	50.57	49.22	49.33	48.21
Total Ash	7.47	7.74	7.79	8.24	7.92	8.50
DE	349.57	342.43	343.29	344.27	346.99	348.55

C-Control; T1- 20% MLM; T2- 40% MLM; T3- Control + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.14% methionine & 1.04% lysine; T4- 20% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.94% of lysine; T5 - 40% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 1.04% of lysine

[2]DE (kca/100g) Digestible energy= (Crude Protein × 4) + (Ether Extract × 9) + (Nitrogen Free Extract ×4) (Halver, 1976)

Table 6: Proximate composition (on %wet weight basis) of the whole body of *Labeo rohita* fingerlings fed with different experimental diets for the experimental period of 60 days

Treatments[1]	Moisture	Protein	Ether extract	Total carbohydrate	Total ash
C	74.99±0.17	15.41±0.23	4.07±0.40	2.06±0.43	3.48±0.08
T1	74.64±0.33	14.99±0.35	4.19±0.25	2.85±0.11	3.32±0.07
T2	74.63±0.49	15.03±0.61	4.60±0.34	2.36±0.58	3.37±0.09
T3	75.07±0.40	14.80±0.71	5.03±0.24	1.62±0.40	3.52±0.06
T4	75.25±0.30	14.11±0.67	3.75±0.59	3.23±0.21	3.64±0.07
T5	75.10±0.47	15.15±1.32	4.05±0.04	3.16±0.55	3.63±0.13
P-value	0.799	0.864	0.215	0.101	0.079

All values are expressed as Mean ± SE (n=3).

[1]C-Control; T1- 20% MLM; T2- 40% MLM; T3- Control + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.14% methionine &1.04% lysine; T4- 20% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.94% of lysine; T5 - 40% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 1.04% of lysine

Table 7: Proximate composition (on % dry matter basis) of different leaf meal and mixed leaf meal (MLM)

	Vigna mungo	*Ipomea aquatica*	*Hygrophilla spinosa*	Mixed leaf meal
Dry matter	91.71±0.08	93.79±0.16	92.38±0.08	94.26±0.17
Crude Protein	23.85±0.17	23.12±0.12	22.19±0.15	23.54±0.17
Ether Extract	2.43±0.03	2.14±0.09	2.09±0.12	2.30±0.07
Crude Fibre	16.02±0.05	16.21±0.11	16.60±0.08	16.77±0.29
Nitrogen free extract	45.62±0.06	45.41±0.06	44.92±0.04	43.65±0.22
Total Ash	12.08±0.04	13.12±0.13	14.20±0.04	13.74±0.09

4.6. Anti-nutritional Factors (ANFs) of Mixed Leaf meal (MLM)

The results for anti-nutrient composition in mixed leaf meal (Table 8) showed low values of total tannins (0.52±0.07/100gm), phytic acid (3.43±0.03 mg /100g), total oxalate (0.39±0.06 mg/100g) and alkaloid (0.68±0.04 g/100g). The mixing of three leaves together has diluted the concentration of ANFs than that of in the individual leaves.

4.6.1. Anti-nutritional Factors (ANFs) of experimental diets

The results for anti-nutrient composition of the different experimental diets formulated from the mixed leaf meal is given in Table 9.

Table 8: Anti-nutritional factors (ANFs) of different leaf meal

Anti-nutrients	*Vigna mungo*	*Ipomea aquatic*	*Hygrophilla spinosa*	Average values	Mixed Leaf Meal	% dilution
Total tannins*	0.36 ± 0.08	0.94±0.12	0.49±0.14	0.59	0.52±0.07	11.86
Alkaloid*	0.31 ± 0.01	0.73±0.01	1.82±0.10	0.95	0.68±0.04	28.42
Total oxalate*	0.005±0.00	0.83±0.02	0.41±0.02	0.41	0.39±0.06	4.87
Phytic acid**	0.06±0.02	4.63±0.10	5.81±0.15	3.5	3.43±0.03	2.00

All values are expressed as Mean ± SE (n=3).
*Expressed in g/100g
**Expressed in mg/100g

Table 9: Anti-nutritional factors (ANFs) of different experimental diets fed to *Labeo rohita* fingerlings for the experimental period of 60 days

Anti-nutrients	C	T1	T2	Tolerable limit	Reference
Total tannins*	0.07±0.02	0.22±0.04	0.32±0.05	<2	Francis *et al.* (2001)
Alkaloid*	0.11±0.05	0.33±0.01	0.42±0.02	<0.02	Francis *et al.* (2001)
Total oxalate*	0.06±0.07	0.13±0.03	0.19±0.03	<0.5	Rahman *et al.* (2013)
Phytic acid**	0.05±0.10	1.83±0.11	2.81±0.06	<500	Francis *et al.* (2001)

All values are expressed as Mean ± SE (n=3).
*Expressed in g/100g
**Expressed in mg/100g

4.7. Amino acid composition (% of protein)

The amino acid composition of the DORB, mixed leaf meal and the experimental diets are given in Table 10.

Table 10: Amino acid composition (% of protein)

Amino acids	Mixed leaf meal	DORB (Ranjan *et al*,2018)	Control[1]	T1[2]	T2[3]
Essential Amino Acids					
Arginine	13.36	12.82	6.40	8.40	6.30
Histidine	2.89	2.65	3.00	3.20	3.00
Isoleucine	4.82	4.45	3.80	3.90	3.40
Lysine	3.53	2.81	2.20	2.40	2.20
Leucine	8.18	8.70	3.70	4.30	3.50
Metheonine	4.60	2.17	2.40	5.80	5.00
Phenylalanine	5.30	5.67	4.20	4.40	4.10
Threonine	6.17	6.92	4.70	4.70	4.60
Valine	5.91	5.75	4.20	4.20	4.50
Non- Essential Amino Acids					
Alanine	5.30	9.12	5.79	2.70	3.78
Glycine	15.13	9.05	7.08	8.40	1.68
Aspartic acid	2.54	14.63	2.70	1.50	4.98
Glutamic acid	2.64	8.27	6.18	6.09	6.60
Serine	2.80	4.07	6.60	3.48	2.19
Tyrosine	4.71	2.85	5.28	8.40	2.70

[1]Control -0% leaf meal ,[2]T1 -20% leaf meal, [3]T2 – 40% leaf meal

4.8. *In-vitro* Relative Protein Digestibility of MLM

The crude protein content of MLM is 23.54 ± 0.17% and relative protein digestibility was found to be 28.42 ± 0.26% compared to casein.

4.9. *In vivo* digestibility of experimental diets

There were significant difference observed in apparent digestibility coefficient (ADCs) of dry matter ($p<0.05$) among the treatment groups (Table 11). The enzyme supplemented groups showed significantly higher digestibility than that of the non-supplemented group. T3, T4 and T5 groups showed significantly higher results compared with control, T1 and T2. Apparent protein and lipid digestibility (%) did not significantly differ ($p>0.05$) among the treatment groups and ranged from 77.62 to 82.56 and 93.45 to 94.59 respectively.

Table 11: *In vitro* and *In vivo* apparent digestibility coefficients of nutrients in different experimental diets

Treatments[1]	ADMDC[2] (%)	ACPDC[3] (%)	IVPD[4] (%)	ALDC[5](%)
MLM	-	-	28.42 ± 0.26	-
C	60.39^{a}±0.18	77.62±1.37		94.25±0.15
T1	62.80^{b}±0.27	80.25±0.61	-	94.34±0.30
T2	64.24^{c}±0.53	79.23±1.52	-	94.59±0.35
T3	65.10^{cd}±0.13	78.99±0.93	-	93.45±0.23
T4	68.10^{e}±0.49	82.56±0.35	-	94.15±0.35
T5	65.55^{d}±0.36	80.55±0.84	-	94.34±0.40
P-value	0.00	0.167		0.359

All values are expressed as Mean ± SE (n=3). Mean values in the same column with different superscript differ significantly ($p<0.05$)

[1]Treatments-C-Control; T1- 20% MLM; T2- 40% MLM; T3- Control + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.14% methionine &1.04% lysine; T4- 20% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.94% of lysine; T5 - 40% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 1.04% of lysine, [2] ADMDC- Apparent dry matter digestibility coefficient, [3]ACPDC- Apparent crude protein digestibility coefficient, [4]IVPDC- In vitro protein digestibility coefficient, [5]ALDC- Apparent lipid digestibility coefficient

4.10. Growth Performance and Nutrient Utilization

4.10.1. Weight gain (%)

The weight gain % of *Labeo rohita* fingerlings were significantly ($p<0.05$) different among the treatments (Table 12; Fig 1). The T4 group (20% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.94% of lysine) showed significantly higher ($p<0.05$) weight gain % compared to all other groups, whereas control and T2 (40% MLM) showed significantly lower weight gain % than other treatments.

4.10.2. Specific growth rate (SGR)

The SGR of *Labeo rohita* fingerlings of the different experimental groups ($p<0.05$) is shown in the Table 12; Fig 2. The highest value was found in T4 (20% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.94% of lysine) and lower values were observed in control and T2 (40% MLM) groups.

4.10.3. Feed conversion ratio (FCR)

The FCR of *Labeo rohita* fingerlings of different treatment groups is given in Table 12; Fig 3. The mean values of FCR among the different experimental group varied significantly ($P<0.05$). FCR value of T4 (20% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.94% of lysine) group was significantly ($P<0.05$) lower compared to other groups whereas control showed a significantly higher value.

4.10.4. Protein efficiency ratio (PER)

The PER value of *Labeo rohita* fingerlings of different treatment groups is given in Table 12; Fig 4. The mean values of PER among the different experimental group varied significantly ($P<0.05$). The highest PER was recorded in T4 (20% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.94% of lysine) group, while the lower values were observed in Control, T2 (40% MLM) and T3 (Control + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.14% methionine &1.04% lysine).

4.10.5. Survival Rate (%)

There was no mortality of *Labeo rohita* fingerlings was observed in all the experimental groups during the experimental period of 60 day.

Table 12: Growth parameters of the different experimental groups fed with different experimental diets at the end of the experiment

Treatments[1]	In. wt.(g)[2]	Fn. wt.(g)[3]	WG%[4]	SGR[5]	FCR[6]	PER[7]	Survival %
C	3.40±0.00	5.57^{a}±0.04	68.05^{a}±1.16	0.82^{a}±0.01	2.30^{e}±0.03	1.39^{a}±0.02	100
T1	3.41±0.01	5.93^{bc}±0.05	74.22^{b}±1.08	0.93^{c}±0.01	2.10^{c}±0.01	1.54^{b}±0.01	100
T2	3.41±0.01	5.59^{a}±0.07	67.27^{a}±1.00	0.82^{a}±0.02	2.20^{d}±0.01	1.40^{a}±0.01	100
T3	3.41±0.01	5.77^{b}±0.05	70.27^{a}±0.80	0.88^{b}±0.01	2.14^{c}±0.02	1.43^{a}±0.01	100
T4	3.39±0.01	6.40^{d}±0.08	88.65^{c}±1.97	1.06^{d}±0.02	1.90^{a}±0.02	1.60^{c}±0.02	100
T5	3.40±0.01	6.05^{c}±0.04	76.17^{b}±0.30	0.96^{c}±0.02	2.01^{b}±0.02	1.54^{b}±0.01	100
P- value	0.840	0.00	0.00	0.00	0.00	0.00	

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

[1]Treatments**-** C-Control; T1- 20% MLM; T2- 40% MLM; T3- Control + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.14% methionine &1.04% lysine; T4- 20% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.94% of lysine; T5 - 40% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 1.04% of lysine

[2]In. wt.= Initial weight, [3]Fn. wt.= Final weight, [4]WG%= Weight gain %, [5]SGR= Specific growth rate, [6]FCR= Feed conversion ratio, [7]PER= Protein efficiency ratio

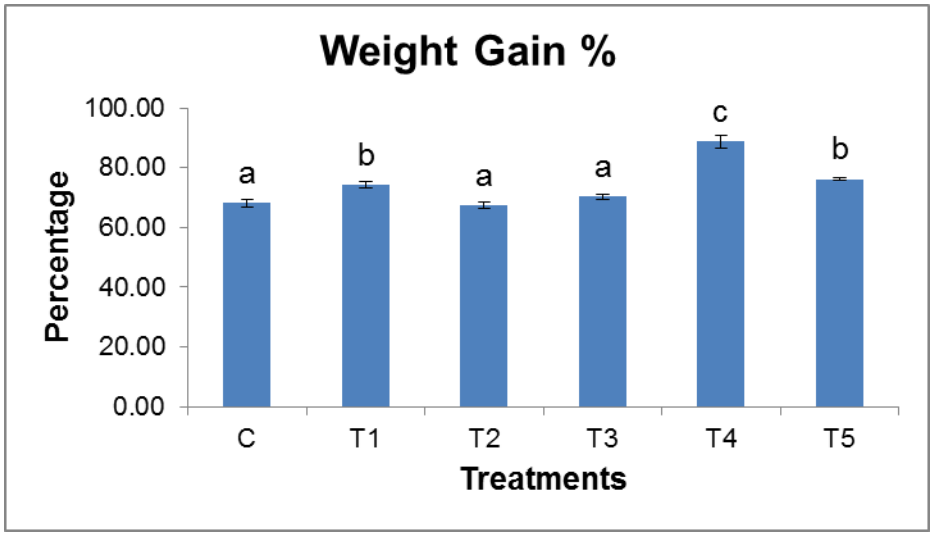

Fig 1: Weight gain percentage of *Labeo rohita* fingerlings fed with different experimental diets for 60 days; Values are expressed as mean ± SE, n = 3; Bars with different superscript on the top differ significantly (P<0.05)

Treatments -C-Control; T1- 20% MLM; T2- 40% MLM; T3- Control + 0.1% Exogenous enzyme (xylanase&cellulase) + 0.14% methionine &1.04% lysine; T4- 20% MLM + 0.1% Exogenous enzyme (xylanase&cellulase) + 0.94% of lysine; T5 - 40% MLM + 0.1% Exogenous enzyme (xylanase&cellulase) + 1.04% of lysine

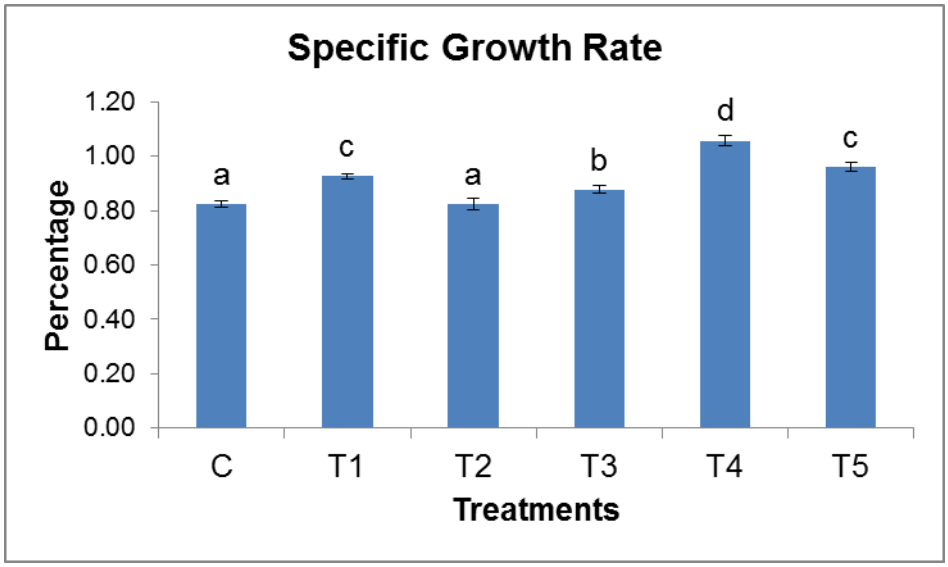

Fig 2: Specific growth rate of *Labeo rohita* fingerlings fed with different experimental diets; Values are expressed as mean ± SE, n = 3; Bars with different superscript on the top differ significantly (P<0.05)

Treatments -C-Control; T1- 20% MLM; T2- 40% MLM; T3- Control + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.14% methionine &1.04% lysine; T4- 20% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.94% of lysine; T5 - 40% MLM + 0.1% Exogenous enzyme (xylanase&cellulase) + 1.04% of lysine

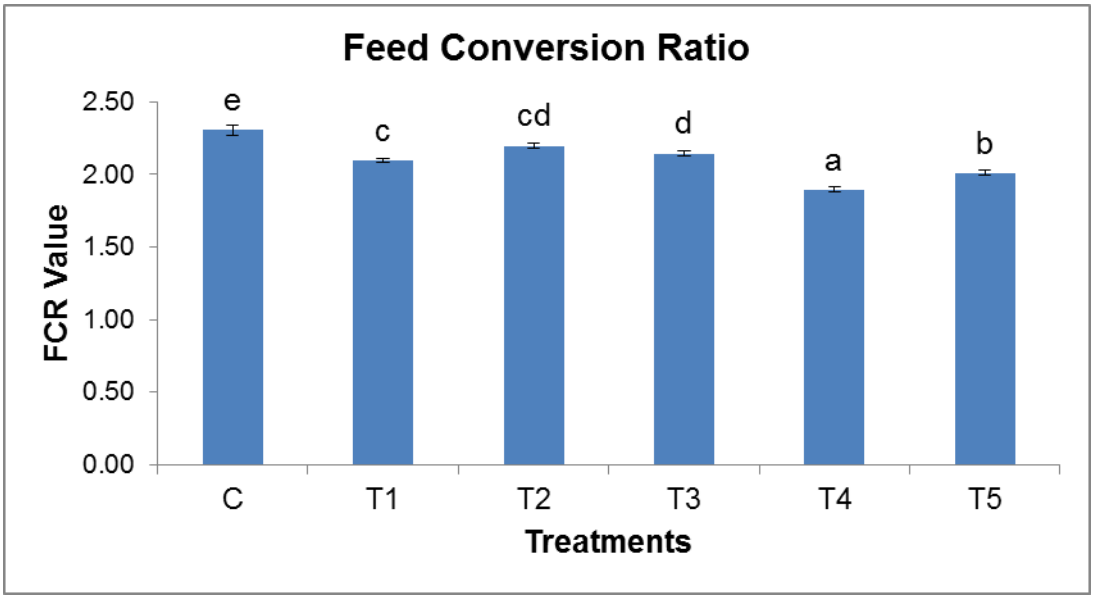

Fig 3: Feed conversion ratio of *Labeo rohita* fingerlings fed with different experimental diets; Bars with different superscript on the top differ significantly (P<0.05); Values are expressed as mean ± SE, n = 3

Treatments -C-Control; T1- 20% MLM; T2- 40% MLM; T3- Control + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.14% methionine &1.04% lysine; T4- 20% MLM + 0.1% Exogenous enzyme (xylanase&cellulase) + 0.94% of lysine; T5 - 40% MLM + 0.1% Exogenous enzyme (xylanase&cellulase) + 1.04% of lysine

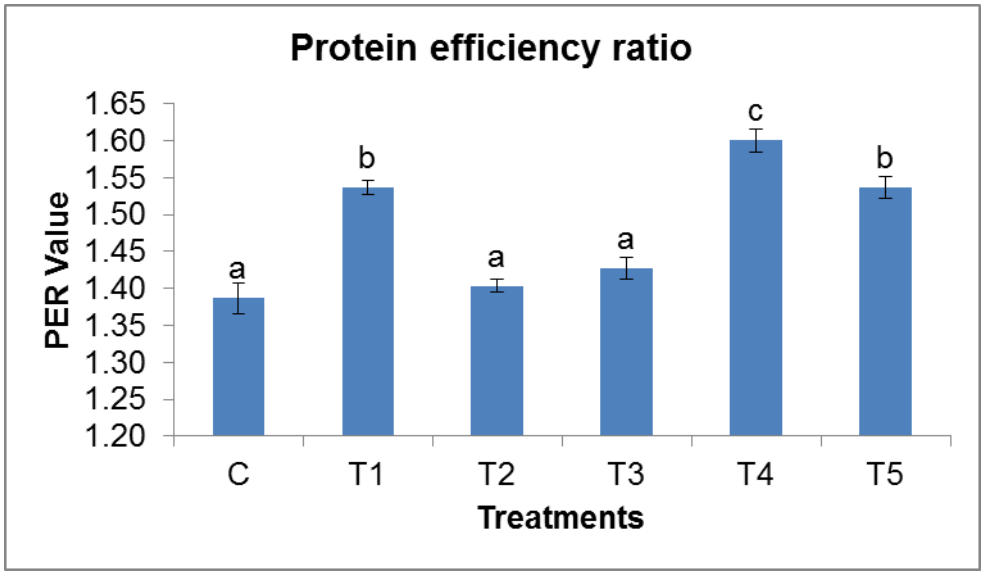

Fig 4: Protein efficiency ratio of *Labeorohita*fingerlings fed with different experimental diets. Barswith different superscript on the top differ significantly (P<0.05). Values are expressed as mean ± SE, n = 3

Treatments -C-Control; T1- 20% MLM; T2- 40% MLM; T3- Control + 0.1% Exogenous enzyme (xylanase&cellulase) + 0.14% methionine &1.04% lysine; T4- 20% MLM + 0.1% Exogenous enzyme (xylanase&cellulase) + 0.94% of lysine; T5 - 40% MLM + 0.1% Exogenous enzyme (xylanase&cellulase) + 1.04% of lysine

4.11. Body Indices

4.11.1. Hepatosomatic index (HSI)

The HSI (%) of *Labeo rohita* fingerlings of different treatments are given in Table 13. The HSI value of *Labeo rohita* fingerlings of different treatment was not significantly different (P>0.05).

4.11.2. Intestinal somatic index (ISI)

The ISI (%) of *Labeo rohita* fingerlings of different treatments are given in Table 13. The ISI among the experimental groups vary significantly (P>0.05). Control, T1 and T2 showed significantly higher values which was not different from T4 group. Lower ISI values were observed in T3 and T5 groups.

Table 13: HSI and ISI of different experimental groups fed with different experimental diets at the end of the experiment

Treatments[1]	HSI[2] (%)	ISI[3] (%)
C	1.36±0.21	4.17^{b}±0.13
T1	1.11±0.11	4.32^{b}±0.24
T2	1.16±0.14	4.34^{b}±0.16
T3	1.22±0.08	3.50^{a}±0.20
T4	0.93±0.09	3.74^{ab}±0.24
T5	1.04±0.12	3.30^{a}±0.20
P- Value	0.305	0.001

Data expressed as Mean ± SE n=3.

[1]Treatments**-** C-Control; T1- 20% MLM; T2- 40% MLM; T3- Control + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.14% methionine &1.04% lysine; T4- 20% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.94% of lysine; T5 - 40% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 1.04% of lysine
[2]HSI– Hepato somatic index, [3]ISI– Intestinal somatic index

4.12. Digestive Enzyme Activities

The digestive enzyme activity of protease, amylase and lipase in the intestine of *Labeo rohita* fingerlings of different treatment groups are given in Table 14.

4.12.1. Amylase

The mean values of amylase enzyme activity of *Labeo rohita* fingerlings among the experimental groups varied significantly ($P<0.05$) (Fig 6). The amylase activity was significantly higher in the intestine of T4 - 20% MLM + 0.1% exogenous enzyme (xylanase & cellulase) + 0.94% of lysine group of fishes. There was no significant difference ($P>0.05$) among the control, T2, T3 and T5 groups.

4.12.2. Protease

The protease activity of *Labeo rohita* fingerlings was found significantly higher in the T1, T2, T4 and T5 as compared to control group.

4.12.3. Lipase

There was no significant variation found ($P>0.05$) in lipase activities of *Labeo rohita* fingerlings among the different treatment and control groups.

Table 14: Digestive enzyme activities in the intestine of *Labeo rohita* fingerlings fed with different experimental diet for the experimental period of 60 days

Treatment[1]	Protease[2]	Amylase[3]	Lipase[4]
C	11.81^{a}±0.63	4.98^{a}±0.16	0.21±0.02
T1	18.09^{b}±0.90	6.20^{b}±0.38	0.22±0.01
T2	17.42^{b}±0.47	4.98^{a}±0.32	0.18±0.01
T3	11.56^{a}±0.81	4.96^{a}±0.11	0.18±0.01
T4	19.28^{b}±0.83	8.88^{c}±0.25	0.23±0.01
T5	17.15^{b}±0.93	5.56^{ab}±0.15	0.21±0.02
P-value	0.00	0.00	0.113

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

[1]Treatments**-** C-Control; T1- 20% MLM; T2- 40% MLM; T3- Control + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.14% methionine &1.04% lysine; T4- 20% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.94% of lysine; T5 - 40% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 1.04% of lysine

[2]Protease activity is expressed in millimole of casein released/min/mg protein at 37°C

[3]Amylase activity is expressed in micromole maltose released /min/mg protein at 37°C

[4]Lipase activity is expressed in units/hour/mg protein at 37°C

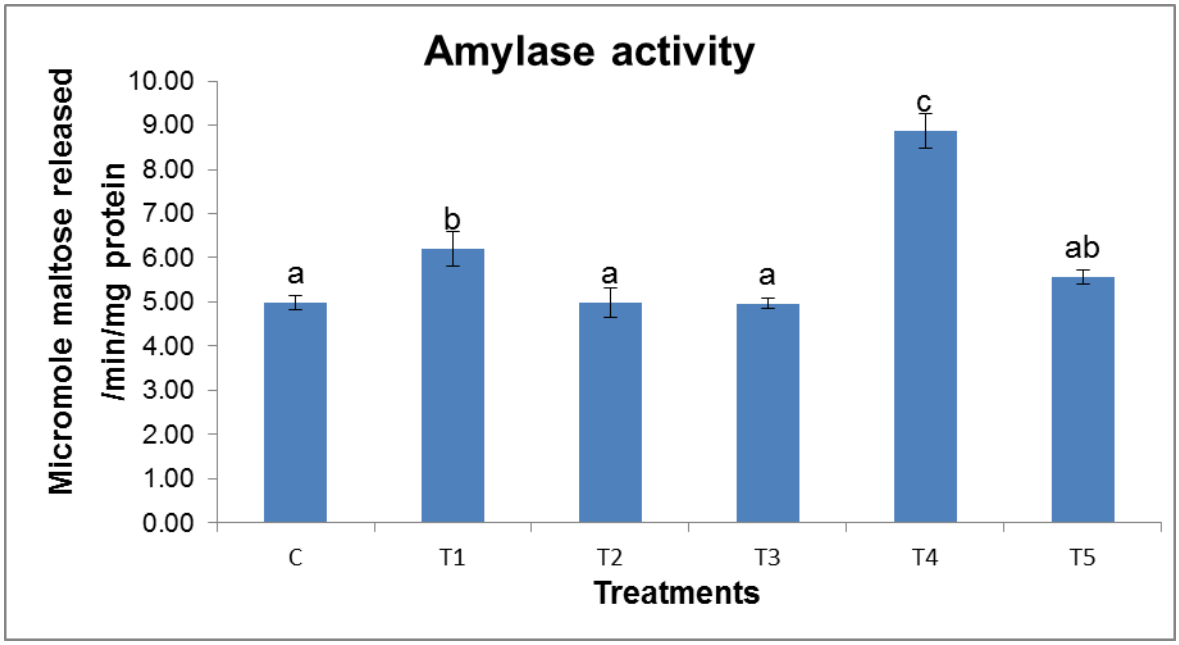

Fig 5: Amylase activity in *Labeo rohita* fingerlings fed with different experimental diets; Values are expressed as mean ± SE, n = 6; Bars with different superscript on the top differ significantly (P<0.05)

[1]Treatments- C-Control; T1- 20% MLM; T2- 40% MLM; T3- Control + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.14% methionine &1.04% lysine; T4- 20% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.94% of lysine; T5 - 40% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 1.04% of lysine

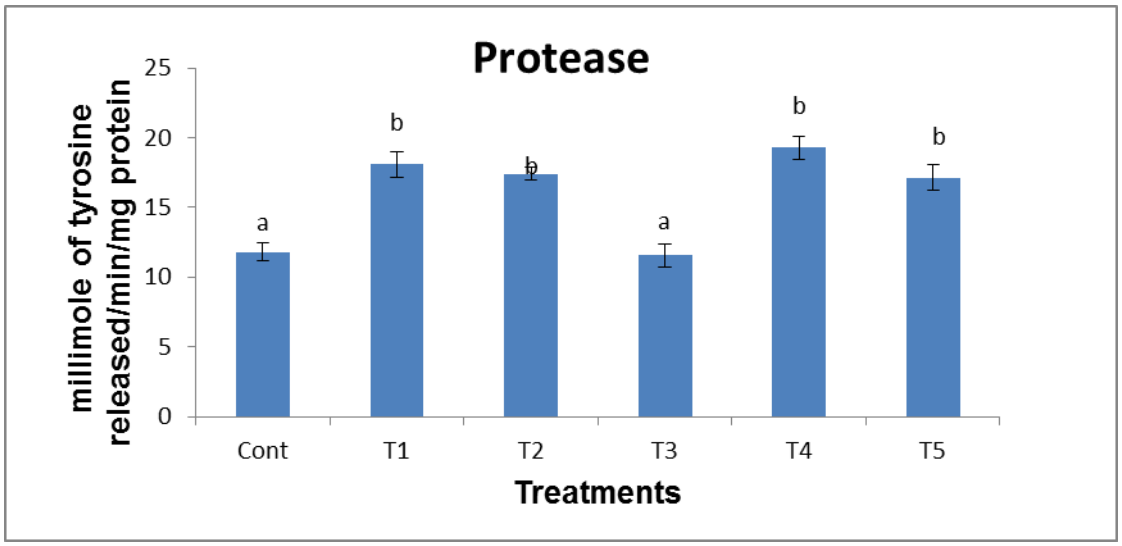

Fig 6: Protease activity in *Labeo rohita* fingerlings fed with different experimental diets. Values are expressed as mean ± SE, n = 6; Bars with different superscript on the top differ significantly (P<0.05)

[1]Treatments- C-Control; T1- 20% MLM; T2- 40% MLM; T3- Control + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.14% methionine &1.04% lysine; T4- 20% MLM + 0.1%Exogenous enzyme (xylanase & cellulase) + 0.94% of lysine; T5 - 40% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 1.04% of lysine

4.13. Enzymes of Protein Metabolism

4.13.1. Aspartate amino-transferase (AST) activity

The AST activity of *Labeo rohita* fingerlings fed with different experimental diet is shown in Table 15 and Fig 7.In the liver significantly higher ($P<0.05$) activity was observed in T4 group of fishes.

The activity of AST enzyme in muscle of *Labeo rohita* fingerlings differs significantly ($P<0.05$).In the muscle, the highest AST activity was observed inT4 group of fishes and the lower activity was recorded in all other treatment group.

4.13.2. Alanine amino-transferase (ALT) activity

The ALT activity of *Labeo rohita* fingerlings fed with different experimental diet is shown in Table 15 and Fig 8.The activity of ALT enzyme in liver differs significantly ($P<0.05$) among the groups where T4 group had significantly higher activity than all other groups.

The activity of ALT enzyme in muscle of *Labeo rohita* fingerlings differs significantly ($P<0.05$). In the muscle, the highest ALT activity was observed in T4 group and in the entire enzyme supplemented groups, whereas the lowest activity was recorded in the control group.

Table 15: Protein metabolism enzymes activity in *Labeo rohita* fingerlings fed with different experimental diets for the experimental period of 60 days

Treatments[1]	AST[2]		ALT[3]	
	Liver	Muscle	Liver	Muscle
Control (C)	12.26^{a}±0.51	14.17^{a}±0.32	12.25^{a}±0.51	15.42^{a}±0.29
T1	13.34^{b}±0.26	15.87^{b}±0.57	14.21^{b}±0.32	18.06^{b}±0.46
T2	15.85^{c}±0.10	18.61^{c}±0.64	16.05^{c}±0.34	20.38^{c}±0.50
T3	14.07^{b}±0.17	14.25^{a}±0.34	14.96^{bc}±0.34	21.25^{c}±0.51
T4	17.64^{d}±0.27	24.56^{e}±0.65	21.29^{e}±0.25	25.82^{d}±0.57
T5	13.21^{ab}±0.38	22.63^{d}±0.50	18.49^{d}±0.31	24.88^{d}±0.43
P-value	<0.05	<0.05	<0.05	<0.05

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

[1]Treatments**-** C-Control; T1- 20% MLM; T2- 40% MLM; T3- Control + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.14% methionine &1.04% lysine; T4- 20% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.94% of lysine; T5 - 40% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 1.04% of lysine

[2]AST (Aspartate amino-transferase) - specific activities expressed as nanomoles of oxaloacetate released/min/mg protein at 37^{0}c.
[3]ALT (Alanine amino-transferase) - specific activities expressed as nanomoles of sodium pyruvate released/min/mg protein at 37^{0}c.

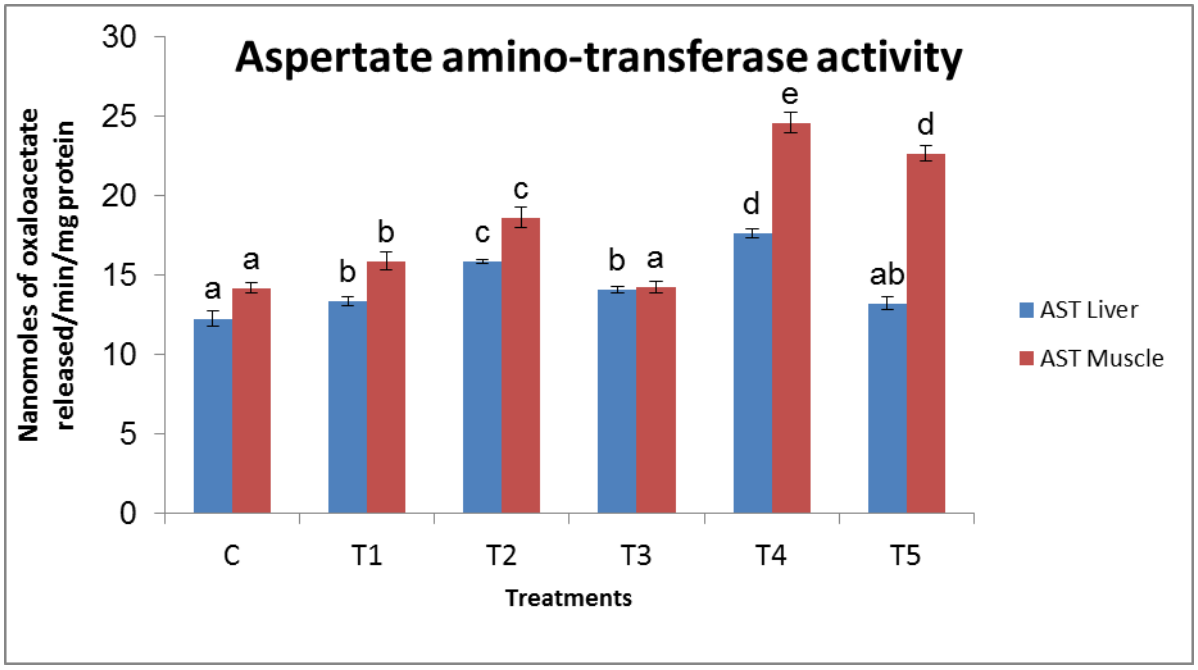

Fig 7: AST activity in muscle and liver of *Labeo rohita* fingerlings fed with different experiment diets; Values are expressed as mean ± SE, n = 6; Bars with different superscript on the top differ significantly (P<0.05)

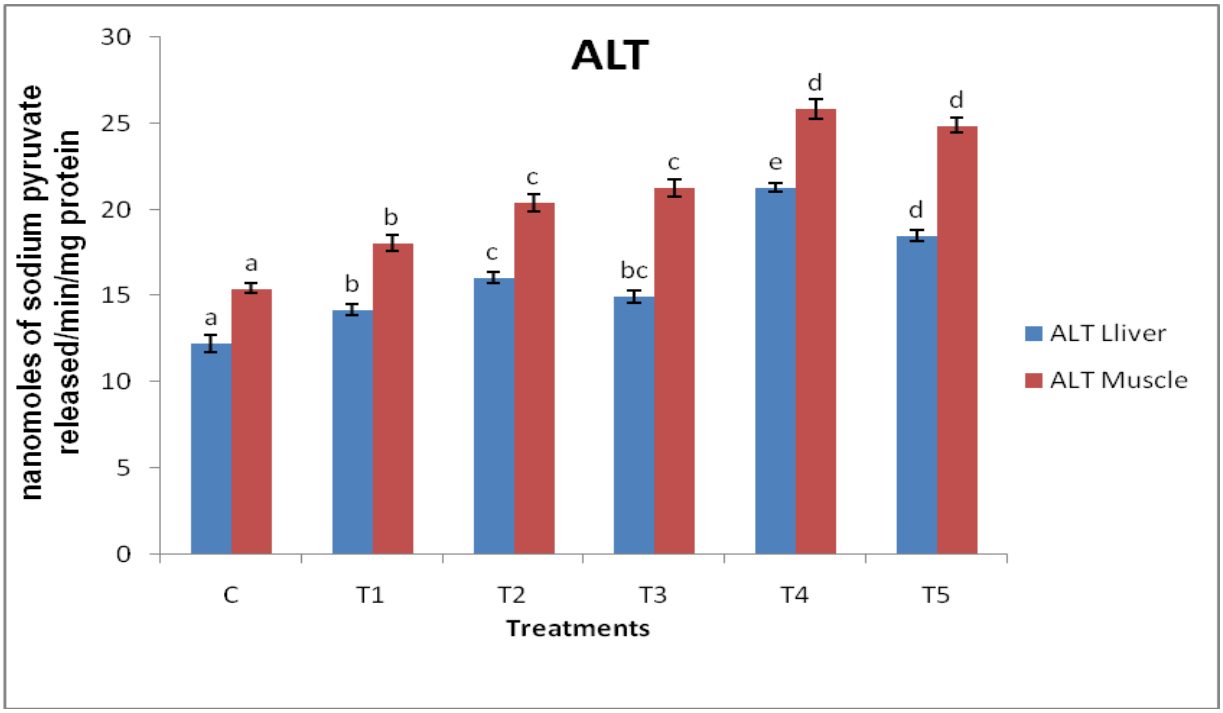

Fig 8: ALT activity in muscle and liver of *Labeo rohita* fingerlings fed with different experiment diets; Values are expressed as mean ± SE, n = 6; Bars with different superscript on the top differ significantly (P<0.05)

4.14. Enzymes of Carbohydrate Metabolism

4.14.1. Lactate dehydrogenase (LDH)

The LDH activity in the liver and muscle of *Labeo rohita* fingerlings of the different experimental groups are presented in Table 16 and Fig 9. A significant difference ($P<0.05$) among the various treatment groups were recorded. Liver LDH activity was significantly ($p<0.05$) higher in T1 i.e. fishes fed with 20% mixed leaf meal than all other treatments.

Moreover, in the of *Labeo rohita* fingerlings muscle significantly higher ($p<0.05$) LDH activity was observed in T1 i.e. fishes fed with 20% mixed leaf meal than all other treatments.

4.14.2. Malate dehydrogenase (MDH)

The MDH activity in liver and muscle of *Labeo rohita* fingerlings presented in Table 16 and Fig 10. T3 and T4 of MDH liver values were lower than other treatments.

Labeo rohita fingerlings fed with mixed leaf meal supplemented with exogenous enzyme and synthetic amino acids group, showed significant lower value ($p<0.05$) of MDH muscle in comparison with other treatment groups.

Table 16: Carbohydrate metabolism enzymes activity in *L. rohita* fingerlings fed with different experimental diets for the experimental period of 60 days

Treatments[1]	LDH[2]		MDH[3]	
	Liver	Muscle	Liver	Muscle
Control (C)	2.56^{a}±0.23	5.82^{b}±0.08	0.27^{c}±0.01	0.20^{c}±0.01
T1	6.13^{d}±0.33	9.62^{d}±0.27	0.25^{c}±0.01	0.18^{b}±0.01
T2	4.78^{c}±0.37	7.07^{c}±0.36	0.16^{a}±0.01	0.17^{b}±0.00
T3	3.41^{b}±0.21	5.38^{a}±0.22	0.18^{ab}±0.02	0.18^{b}±0.01
T4	3.20^{ab}±0.10	6.04^{ab}±0.03	0.20^{b}±0.01	0.11^{a}±0.00
T5	3.70^{b}±0.18	6.71^{bc}±0.34	0.26^{c}±0.01	0.12^{a}±0.00
P-value	0.00	0.00	0.00	0.00

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

[1]Treatments**-** C-Control; T1- 20% MLM; T2- 40% MLM; T3- Control + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.14% methionine &1.04% lysine; T4- 20% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.94% of lysine; T5 - 40% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 1.04% of lysine

[2]LDH (lactate dehydrogenase) activity expressed in Unit/mg protein/min at 37^{0}C

[3]MDH (malate dehydrogenase) activity expressed in Unit/mg protein/min at 37^{0}C

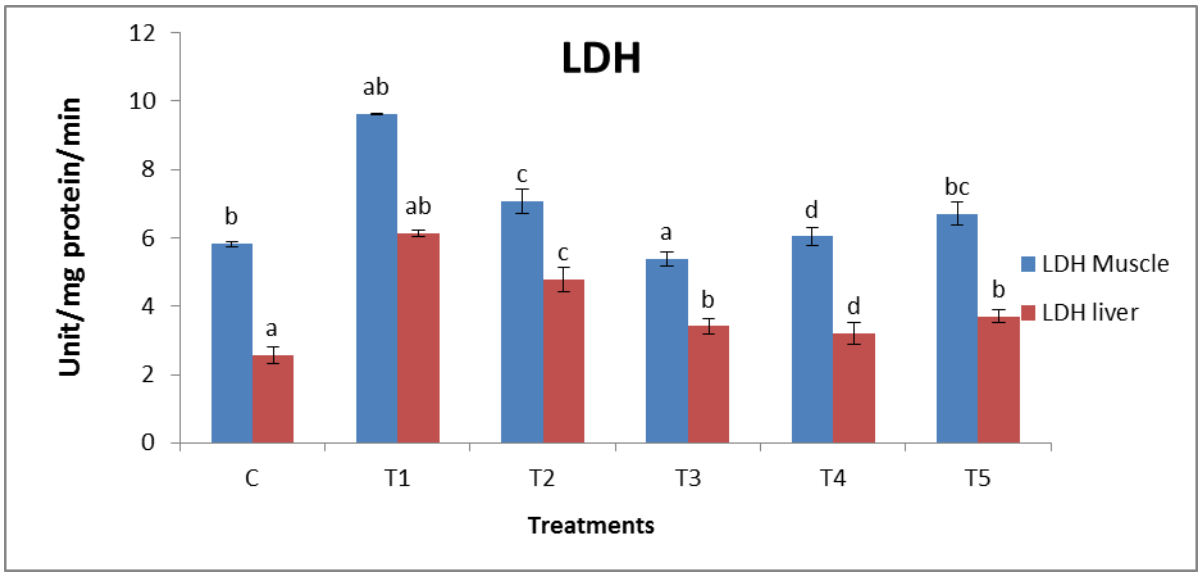

Fig 9: LDH activity in muscle and liver of *Labeo rohita* fingerlings fed with different experiment diets; Values are expressed as mean ± SE, n = 6; Bars with different superscript on the top differ significantly (P<0.05)

[1]Treatments- C-Control; T1- 20% MLM; T2- 40% MLM; T3- Control + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.14% methionine &1.04% lysine; T4- 20% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.94% of lysine; T5 - 40% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 1.04% of lysine

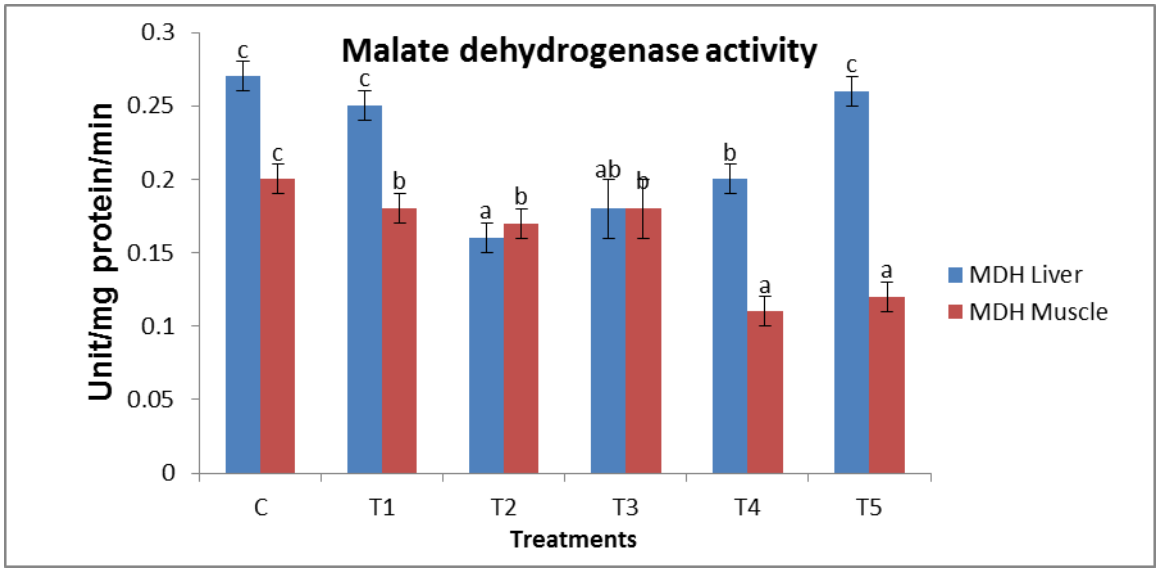

Fig 10: MDH activity in muscle and liver of *Labeo rohita* fingerlings fed with different experimental diets; Values are expressed as mean ± SE, n = 6; Bars with different superscript on the top differ significantly (P<0.05)

[1]Treatments- C-Control; T1- 20% MLM; T2- 40% MLM; T3- Control + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.14% methionine &1.04% lysine; T4- 20% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.94% of lysine; T5 - 40% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 1.04% of lysine

4.15. Enzyme of Oxidative Stress

4.15.1. Superoxide dismutase (SOD)

The SOD activity in gill and liver of *Labeo rohita* fingerlings fed with different experimental diet is shown in Table 17 and Fig 11. The activity of the SOD enzyme in liver differs significantly ($P<0.05$). In the liver, control and T5 group were higher over the entire treatment group, and the lowest activity was found in the T4 (20% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.94% of lysine) group. Similarly, SOD activity in gill was also found lowest in the T4 group and T1 group.

4.15.2. Catalase

There was no significant variation ($P>0.05$) in the gill and liver catalase enzyme activities among *Labeo rohita* fingerlings of the different treatment groups.

Table 17: Oxidative stress enzymes activity in *Labeo rohita* fingerlings fed with different experimental diets for the experimental of 60 days

Treatments[1]	SOD[2]		CATALASE[3]	
	Liver	Gill	Liver	Gill
C	20.49^{d}±0.36	25.81^{bc}±0.88	2.60±0.58	0.34±0.01
T1	19.41^{cd}±0.20	20.62^{a}±0.68	2.44±0.56	1.39±0.50
T2	18.34^{bc}±0.25	20.94^{a}±0.74	1.74±0.56	2.83±0.12
T3	17.06^{b}±0.50	27.48^{c}±0.17	2.85±27	1.94±0.68
T4	15.10^{a}±0.08	21.80^{a}±0.95	2.78±34	1.66±0.55
T5	19.01^{c}±0.74	25.10^{b}±0.26	2.68±57	1.59±0.72
P-value	0.00	0.00	0.651	0.087

Data are expressed as mean ±SE, (n=6). Data Values in the same row with different superscript differ significantly (P<0.05).

[1]Treatments**-** C-Control; T1- 20% MLM; T2- 40% MLM; T3- Control + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.14% methionine &1.04% lysine; T4- 20% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.94% of lysine; T5 - 40% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 1.04% of lysine

[2]SOD activity is expressed as 50% inhibition of epinephrine auto-oxidation/mg protein/min

[3]Catalase activity expressed as nanomoles H_2O_2 decomposed/min/mg protein

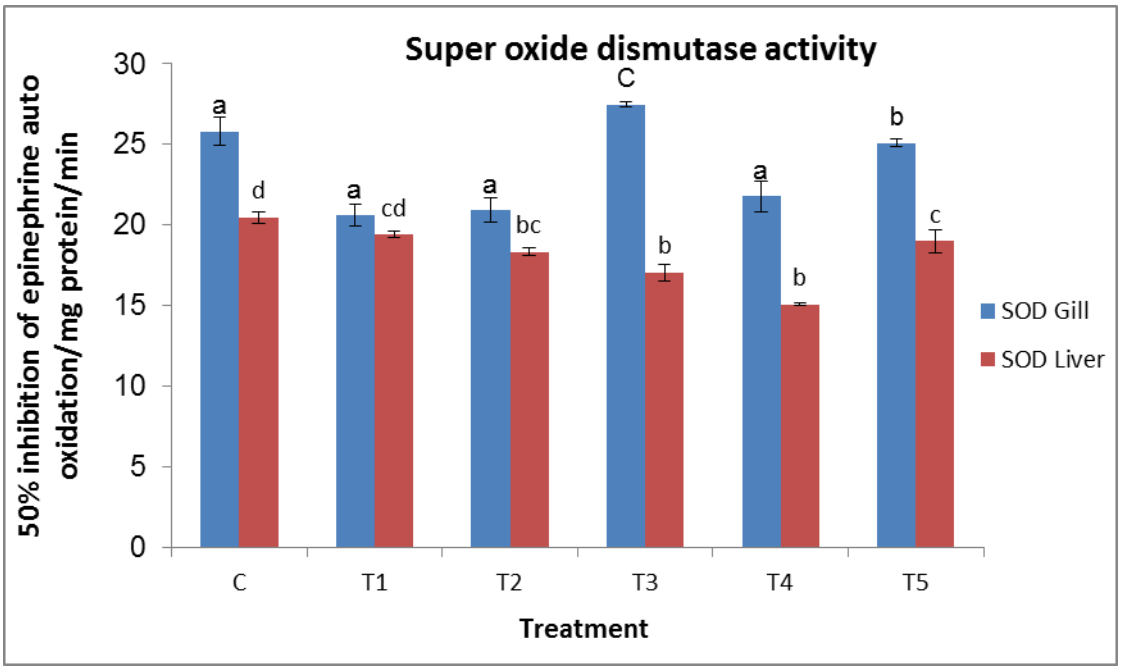

Fig 11: SOD activity in gill and liver of *Labeo rohita* fingerlings fed with different experimental diets; Values are expressed as mean ± SE, n = 6;Bars with different superscript on the top differ significantly (P<0.05)

[1]Treatments- C-Control; T1- 20% MLM; T2- 40% MLM; T3- Control + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.14% methionine &1.04% lysine; T4- 20% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.94% of lysine; T5 - 40% MLM + 0.1% Exogenous enzyme (xylanase&cellulase) + 1.04% of lysine

4.16. Haemato-Immunological Parameters

4.16.1. Serum Glucose

The glucose level of *Labeo rohita* fingerlings of the different experimental groups was estimated and is shown in Table 18 and Fig 12. There was significant difference (P<0.05) observed in the serum glucose level of *Labeo rohita* fingerlings of the different experimental groups. The significantly higher glucose level was recorded in the T4 and T5 group but it was significantly similar in dietary fed group T3 followed by T2 and lowest glucose level was found in control groups.

4.16.2. Total serum protein, albumin, globulin and albumin/globulin ratio

The serum protein levels of *Labeo rohita* fingerlings are given in Table19.The serum protein levels of different experimental groups did not varied significantly (P>0.05).

The serum albumin and serum globulin values of *Labeo rohita* fingerlings of the different experimental groups did not differ significantly (P>0.05) and given in Table 19.

The albumin/globulin ratio of *Labeo rohita* fingerlings is shown in Table 19. There was no significant (P>0.05) difference between the groups.

Table 18: Serum glucose level of the different experimental groups of *Labeo rohita* fingerlings for the experimental period of 60 days

Treatments[1]	Glucose (mg/dl)
C	86.55^{a}±0.71
T1	90.63^{bc}±0.22
T2	89.32^{b}±0.70
T3	90.95^{cd}±0.37
T4	92.05^{d}±0.29
T5	92.26^{d}±0.43
P-value	0.00

Data are expressed as Mean ±SE, (n=3); Data Values in the same row with different superscript differ significantly (P<0.05).

[1]Treatments**-** C-Control; T1- 20% MLM; T2- 40% MLM; T3- Control + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.14% methionine &1.04% lysine; T4- 20% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.94% of lysine; T5 - 40% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 1.04% of lysine

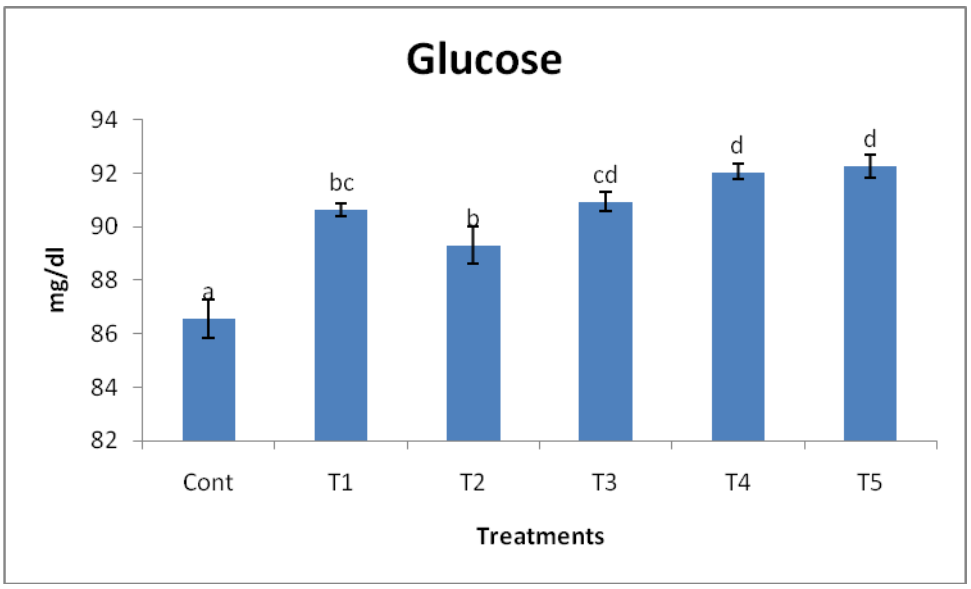

Fig 12: Serum glucose level in *Labeo rohita* fingerlings fed with different experimental diets; Bars with different superscript on the top differ significantly (P<0.05); Values are expressed as mean ± SE, n = 3

[1]Treatments- C-Control; T1- 20% MLM; T2- 40% MLM; T3- Control + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.14% methionine &1.04% lysine; T4- 20% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.94% of lysine; T5 - 40% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 1.04% of lysine

Table 19: Serum protein, albumin (A), globulin (G) and A:G ratio of the different experimental groups for the experimental of 60 days

Treatments[1]	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	A:G ratio
C	3.89±0.09	1.35±0.01	2.53±0.10	0.54±0.03
T1	3.87±0.09	1.39±0.03	2.49±0.10	0.56±0.03
T2	3.81±0.04	1.40±0.03	2.42±0.03	0.58±0.01
T3	3.85±0.06	1.38±0.03	2.47±0.05	0.56±0.01
T4	3.99±0.02	1.44±0.04	2.55±0.05	0.56±0.03
T5	4.01±0.02	1.37±0.05	2.64±0.05	0.52±0.03
P-value	0.20	0.58	0.37	0.62

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

[1]Treatments**-** C-Control; T1- 20% MLM; T2- 40% MLM; T3- Control + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.14% methionine &1.04% lysine; T4- 20% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.94% of lysine; T5 - 40% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 1.04% of lysine

4.16.3. Nitro blue tetrazolium assay (NBT)

A significant difference (P<0.05) in respiratory burst activity among *Labeo rohita* fingerlings of the various treatment groups were noticed (Table 20 and fig 14). The control group and T1 exhibited the least NBT activity. The highest activity was found in the T4 (20% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.94% of lysine) and T5 (40% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 1.04% of lysine) groups.

4.16.4. Total Haemoglobin

The blood haemoglobin values of *Labeo rohita* fingerlings o fdifferent treatments are shown in Table 20. No significant difference (P>0.05) was found among all the groups of fishes.

4.16.5. Total Erythrocyte Count

The total erythrocyte count of *Labeo rohita* fingerlings of the different treatment groups is given in Table 20. There was no significant difference (P>0.05) was found among all the groups of fishes.

4.16.6. WBC Count

The WBC of the different treatment groups of *Labeo rohita* fingerlings is given in the Table 20 and Fig 15. There was a significant difference (p<0.05) among the various treatments groups. The highest count was recorded in T2 and T4 and the lowest count was observed in T3 group.

Table 20: Blood parameters of *Labeo rohita* different experimental groups for the experimental of 60 days

Treatments[1]	NBT[2]	Hemoglobin (g/dl)	RBC[3] (mil/cm)	WBC[4] (no×10^6/cm)
Control (C)	0.24^{a}±0.00	6.47±0.09	1.70±0.01	0.23^{b}±0.00
T1	0.27^{a}±0.02	6.13±0.15	1.60±0.03	0.22^{b}±0.02
T2	0.36^{b}±0.01	7.30±0.44	1.86±0.08	0.25^{c}±0.01
T3	0.37^{b}±0.03	5.77±0.24	1.49±0.04	0.18^{a}±0.00
T4	0.43^{c}±0.01	6.60±0.85	1.74±0.20	0.26^{c}±0.01
T5	0.39^{bc}±0.00	6.57±0.12	1.75±0.01	0.22^{b}±0.00
P-value	0.00	0.246	0.157	0.001

Data Expressed as Mean ± SE (n=3). Mean values in the same column with different superscript differ significantly ($p<0.05$).

[1]Treatments**-** C-Control; T1- 20% MLM; T2- 40% MLM; T3- Control + 0.1% Exogenous enzyme (xylanase&cellulase) + 0.14% methionine &1.04% lysine; T4- 20% MLM + 0.1% Exogenous enzyme (xylanase&cellulase) + 0.94% of lysine; T5 - 40% MLM + 0.1% Exogenous enzyme (xylanase&cellulase) + 1.04% of lysine

[2]NBT=Nitro blue tetrazolium

[3]RBC= Red Blood Cells

[4]WBC= White Blood Cells

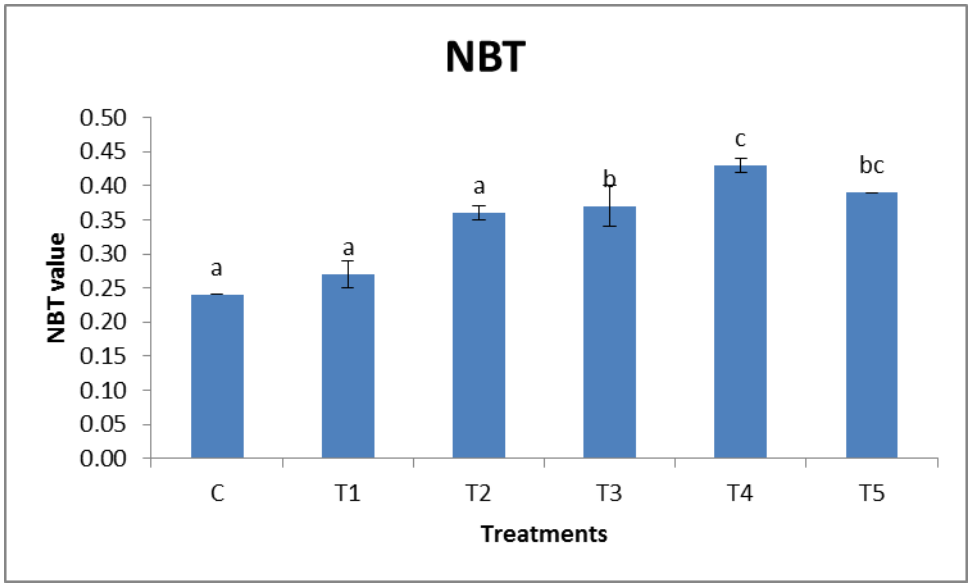

Fig 13: NBT Value in the blood of *Labeo rohita* fingerlings fed with different experimental diets; Values are expressed as mean ± SE, n = 3; Bars with different superscript on the top differ significantly (P<0.05)

[1]Treatments- C-Control; T1- 20% MLM; T2- 40% MLM; T3- Control + 0.1% Exogenous enzyme (xylanase&cellulase) + 0.14% methionine &1.04% lysine; T4- 20% MLM + 0.1% Exogenous enzyme (xylanase&cellulase) + 0.94% of lysine; T5 - 40% MLM + 0.1% Exogenous enzyme (xylanase&cellulase) + 1.04% of lysine

5. DISCUSSION

A feeding trial was conducted to see the effect of inclusion of mixed leaf meal in the diet of *Labeo rohita* fingerlings through studying the growth, physio-metabolic and biochemical parameters and finding out an effective feeding strategy to enhance the utilization efficiency of mixed leaf meal based diet in *Labeo rohita*. A mixed leaf meal was prepared by mixing three leaves like *Hygrophilla spinosa* (Kulekhara), *Vigna mungo* (Mung bean) and *Ipomea aquatica* (Water spinach). Mixed leaf meal containing of 23.54% crude protein has been included in the diet of *Labeo rohita* in two inclusion levels, *i.e.* 20 and 40% and conducted a feeding trial for 60 days following a CRD design. Growth parameters like final weight gain, weight gain %, SGR, FCR and PER, digestive enzymes like protease, amylase and lipase, metabolic enzymes like GOT, GPT, LDH and MDH, stress related enzymes like SOD and catalase and haemato-immunological and parameters were studied to understand the optimum inclusion level of mixed leaf meal in rohu diet. The feeding trial was conducted for 60 days and the sampling done in 15 days interval as well as at the end of the experiment. Growth parameters like final weight gain, weight gain % , SGR, FCR and PER, digestive enzymes like protease, amylase and lipase, metabolic enzymes like AST, ALT, LDH and MDH, stress related enzymes like SOD and catalase and haemato-immunological parameters were studied following the feeding trial.

5.1. Physico-chemical Parameters of Water

All the physico-chemical parameters of water such as temperature, pH, dissolved oxygen, free carbon dioxide, total hardness, ammonia, and nitrite-N were observed to be within the optimum range as suggested by many authors. Indian major carps can thrive well at a temperature range of 18-38^{0}C (Jhingran, 1991), which supports the range observed in the present study (28.5^{o}C to 32.9^{o}C). Similarly, the pH of water in all the experimental groups ranged from 7.2 to 8.5, which was within the acceptable range (6.5-9.0) as suggested by Swingle (1967). The dissolved oxygen levels in different experimental tubs were recorded within the range of 6 to 7.9 mg/l. This level was found

to be within the optimum range (6-7 mg/l) for cyprinids suggested by Huet (1975). This was achieved with proper aeration throughout the experimental period. The carbon dioxide concentration was found to be negligible, as the biomass was optimum and water exchange was done regularly. Hence, it did not have any adverse effect on the survival and growth performance of the *Labeo rohita* fingerlings. The total hardness was found to be within 231 - 244 mg/l during the experimental period**.** Schaperclaus (1933) had suggested that water having a hardness of 250 mg/l or above as satisfactory for the growth of carps**.** The suggested value of ammonia nitrogen in water ranges from 0 to 0.1 mg/l (Jhingran, 1991) and this support the range (0.04 to 0.10mg/l) in the present study. Nitrite concentration was recorded in the range of 0.04 to 0.09mg/l which was well within the permissible range for pond aquaculture (Boyd and Tucker, 1998).

5.3. Proximate composition of different feed ingredients

The major ingredients used for feed formulation were de-oiled rice bran, de-fatted soybean meal, groundnut oil cake and wheat flour having crude protein % of 16.00%, 52.23%, 43.64 %, 12.02 %, respectively. The crude fat content were estimated from these ingredients and found to be 1.2%, 1.5%, 3.56% and 2.54%, respectively. According to the findings of Kumar *et.al.* (2018), the crude protein content of DORB, Defatted soybean meal, GNOC and wheat flour were 15-16%, 42-45%, 48-52% and 12-14%. The values observed in the present study were within the above limit.

5.4. Proximate composition of individual leaf meal and mixed leaf meal

In the present study the proximate composition of the both individual and mixed leaf meal was done. Mixed leaf meal has crude protein around 23.54%. Crude lipid, crude fibre and total ash content in mixed leaf meal found to be 2.30%, 16.77%, 13.74%, respectively. The calculated nitrogen free extract was found to be 37.86%.

5.5. Proximate composition of experimental diets

There were total six iso-caloric and iso-nitrogenous diets were prepared in the present study containing 30 % crude protein and 5.50 to 6.19% ether extract. Jafri

and Anwar (2001) suggested that optimum protein and lipid requirement of rohu ranges between 30-35% and 5%, respectively. The Digestible energy content of the experimental diet was found to be within the range of 342.43- 349.57 Kcal/100g.

5.6. Proximate composition of the whole body

Proximate composition of whole body of *Labeo rohita* did not differ significantly ($p>0.05$) among the experimental groups during the period of 60 days. The carcass protein was same among all the fishes of the experimental groups fed with the different experimental diets. It had also been reported by Adewolu (2008) that the carcass protein content was not affected due to inclusion of sweet potato leaf meal up to the level of 20% in the diet of Tilapia zilli fingerlings.

The lipid content in the fish tissue remained same in all the fishes of different experimental tubs. It had also been observed by Bairagi *et.al.* (2004) that the lipid content in whole body of rohu did not vary significantly ($p<0.05$) among *Leucanea leucocephala* leaf meal incorporated diets. Similarly, the values of total carbohydrate as well as of total ash did not show any significant difference among the various experimental groups.

5.7. Amino acid composition (% protein basis)

In the present study, amino acid analysis of the mixed leaf meal and formulated experimental diets were done. The dietary proteins should provide essential amino acids (EAA) in sufficient amounts to meet the EAA requirement of a given animal and it also varies from species to species. Mohanty *et. al.*(1991). Those formulated diet which were found to be deficient in few particular essential amino acids were supplemented were accordingly supplemented by synthetic amino acids. Abidi *et.al.* (2007)

5.8. Anti-nutritional factors (ANFs) of mixed leaf meal

Vigna mungo leaf meal contains ANFs like total tannins (0.36 g/100gm), phytic acid (0.06 mg/kg), total oxalate (0.005 g/100g) and alkaloid (0.31 g/100g). *Ipomea aquatica* is known to have total tannins (0.94 g/100gm), phytic acid (4.63mg/kg), total oxalate (0.63 g/100g) and alkaloid (0.73 g/100g). In *Hygrophila spinosa* leaf meal total tannins (0.49 g/100gm), phytic acid (5.81 mg/kg), total oxalate (0.41 g/100g) and alkaloid (1.82 g/100g) are present. The combination of these leaves at 1:1:1 ratio positively resulted in a decrease in total anti-nutritional profile than that of the individual leaves. In the mixed leaf meal the anti nutritional factors were found to have total tannins (0.52 g/100gm), phytic acid (3.43 mg/kg), total oxalate (0.39 g/100g) and alkaloid (0.68 g/100g). The mixing of leaves in the ratio of 1:1:1 has shown 11.86% dilution in total tannin, 28.42% in alkaloids content, 4.87% in total oxalate and around 2% reduction in phytic acid composition than that of the average anti-nutritional values found in the individual leaves.

The bioavailability of protein and minerals of feed ingredients is known to be inhibited by the presence of tannins (Makkar *et al.,* 1993) and in the present study, by mixing of leaves ensured there was no effect of tannins in the nutrient utilization by the fishes as we got less tannin value in all the formulated feed which is within the tolerance level of fishes i.e. < 2g/100g (Francis *et al.,*2001).

The Anti-nutritional factors (ANFs) of different experimental diets were found within the tolerable limits of the fishes as stated by Francis *et al.* (2001) except the alkaloids which may limit the higher inclusion of mixed leaf meal in the diet of fishes. However, with the best of our knowledge no detailed anti-nutritional profiling of these three mixed leaf meal has been conducted till date.

5.9. *In-vitro* relative protein digestibility of mixed leaf meal

The digestibility of nutrients is one of the most important criteria for evaluating the suitability of feed ingredients to be used in feed formulation and varies from species to species. The *in vitro* protein digestibility of MLM which was found to be 28.42±0.26% which was found to be similar to that of *in vivo* apparent protein digestibility of rice bran (29.95%) reported by Udo and Umoren (2011) in *Clarias gariepinus.*, which proves MLM as a potential ingredient in fish feed.

5.10. *In-vivo* digestibility of experimental diets

It has been reported by Ahmad *et al.,* (2014) that the apparent dry matter digestibility coefficient of sweet potato leaf meal was 58.02%. Similarly Maiti *et al.,* (2017) 63% apparent dry matter digestibility coefficient of *Hygrophila spinosa* leaf meal. In the present study it has been found that mixed leaf meal had higher apparent dry matter digestibility coefficient than individual leaf meal and increased with exogenous enzyme supplementation and balancing of essential amino acids in the diet of *Labeo rohita.*

5.11. Growth Performance and Nutrient Utilization

The growth parameter such as percentage weight gain, SGR and PER showed a significant difference between the control and treatment groups. The highest growth rate was observed in 50% DORB replaced group with amino acid balanced and enzyme supplemented mixed leaf meal. The significantly higher value in 20% MLM group and 40% MLM group with enzyme supplementation indicate a better growth in these groups. 40% MLM fed group showed a statistically similar value as of control group suggest that 100% replacement of DORB is possible with MLM without adverse effect on growth. Lower FCR and higher PER values in the groups fed with 20% mixed leaf meal supplemented with Exogenous enzyme (xylanase & cellulase) + 0.14% methionine &1.04% lysine support the growth data. Up to date there is no published information on the incorporation of mixed leaf meal in fish diets as a replacer of any ingredients. However, available information on individual leaf meal as a replacer of

DORB reveal that *Hygrophila spinosa* leaf meal fed at the rate of 10% shown significantly higher growth and better nutrient utilization in *Labeo rohita* fingerlings (Maiti, 2018). Similarly, Sahoo (2018) also found that 30% of *Vigna mungo* leaf meal is able to replace 100% DORB without having any adverse effect of the *Labeo rohita* fingerlings. Gurung, 2018 also found similar result with 20% inclusion of *Ipomea aquatica* leaf meal. Other reports showed that *Leucaema leucocephala* leaf meal in the diets of *Oreochromis niloticus* at 12.5% inclusion did not adversely affect the growth (Wee *et al.*, 1987). Meshram *et al.* (2018) has observed that the fermented sweet potato leaf meal can replace 100% DORB in the diet of *Labeo rohita* without any detrimental effect on growth performance and nutrient utilization.

The present study also showed that exogenous supplementation of enzymes has shown significantly higher growth and improved nutrient utilization which is also supported by the following findings. The 0.01% exogenous enzyme supplementation to the DORB based diet led to significantly higher weight gain, SGR, PER and lower FCR as compared to the DORB based diet in which exogenous enzyme was not supplemented Ranjan *et al.* (2017).

5.12. Survival rate

The 100% survival rate in the present study indicates that the inclusion of MLM and exogenous enzyme in the diet of *Labeo rohita* had no adverse effect on fish. Previous reports by Meshram *et al.* (2017), Sahoo (2018), Maiti (2018), Gurung (2018), and Ahmad *et al.* (2019) also observed 100% survival on feeding leaf meal in the diet of rohu fingerlings.

5.13. Hepato-somatic index (HSI) and Intestinal-somatic index (ISI)

The study of intestinal-somatic and hepato-somatic indices has an important role in the metabolism of fishes, related to digestion and absorption of nutrients, synthesis and secretion of digestive enzymes and carbohydrate metabolism (Ighwela *et al.*, 2014). In the present study, the HSI values of different experimental

groups did not vary significantly. However, the ISI values were lower in the enzyme supplemented groups as compared to control. Ricther *et al.,* (2013) found that the higher inclusion of moringa leaf meal in the diets of *Oreochromis niloticus* significantly reduced the ISI levels compared to control groups.

5.14. Enzyme assays

5.14.1. Digestive enzymes

The protease, amylase and lipase are the digestive enzymes which help in breaking down macro nutrients like protein, carbohydrates and fat, respectively from the feed given to the fish. In the present study, irrespective of enzyme supplementation, protease activity was increased in mixed leaf meal fed groups.

Amylase activities varied significantly ($P<0.05$) among different the treatments and feeding 20% MLM supplemented with 0.1% xylanase and cellulose increased the amylase activity. This might be due to improved carbohydrate availability by the externally supplemented carbohydrases. Xavier et al. (2012) had found that cellulase supplementation increases the amylase activity of *Labeo rohita*. The 20% MLM fed group and 40% MLM supplemented with 0.1% xylanase and cellulose also showed a higher amylase activity. Mondal *et.al*. (2012) found that the α-amylase activity significantly increased in *Labeo bata* fed with 65% of mulberry leaf meal containing diet.

Lipase activity showed no significant ($P>0.05$) difference among the treatments. It has been observed by Debnath *et al*. (2007) that lipase activity of *Labeo rohita* fingerlings was not altered with respect to the varied dietary composition of the feed.

5.14.2. Enzymes of protein metabolism

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are important aminotransferase enzyme in the liver and muscle of fishes that evidently supports in protein synthesis and growth (Jiang *et.al.,* 2015). Apart from this, the amino acids are deaminated to produce TCA cycle intermediates and also help in energy

production in the form of ATP (De Silva and Anderson, 1995). In the present study, both ALT and AST were studied in the muscle and liver of fishes. AST activity in liver has showed a significantly higher values among the treatment groups than control and highest activity was found in T4 and AST activity in muscle also showed a similar trend.

In liver both AST and ALT activity are significantly higher in T4 groups which are in similar trend with weight gain percentage of the fishes. This might be due to increased utilization of dietary protein for synthesis of other amino acids and gluconeogenesis. The increased muscle AST and ALT activity in T4 group might be due to the better utilization of the glucose derived TCA intermediates for protein synthesis and hence significantly higher growth.

5.14.3. Enzymes of carbohydrate metabolism

Lactate dehydrogenase is the terminal enzyme of the glycolytic pathway. LDH converts lactate to pyruvate in the presence of coenzyme NADH, which is converted to NAD+. Thus, lactate dehydrogenase helps in maintaining the glycolysis cycle by supplying NAD+. In the presence of enough oxygen pyruvate enters the Krebs's cycle, but when there is an oxygen shortage in the tissue, pyruvate is converted to lactate (Murray et al., 2000).

In the present study, LDH activities in liver and muscle were found to be higher when fishes were fed with graded level of mixed leaf meal without exogenous enzyme supplementation. This can be due to higher energy demand for utilization of leafmeal diet which is not supplemented with exogenous enzymes. In the present study, enzyme supplemented groups were showing a significantly lower LDH activity. Similar results were observed by Maiti *et al.* (2018), who observed a lower LDH activity in both liver and muscle when fed with carbohydrase supplemented *Hygrophila spinosa* leaf meal in rohu diet.

Malate dehydrogenase (MDH) is an enzyme of the citric acid cycle catalyzes the conversion of malate into oxaloacetate and vice versa. In the present study, fishes fed with mixed leaf meal supplemented with exogenous enzyme and

synthetic amino acids group, showed significant lower value ($p<0.05$) in liver and muscle than other treatment groups and it is in agreement with results reported by Ahmad *et al.* (2014). The author reported that lower activity of MDH in liver and muscle in *L. rohita* fingerlings had been related with reduced stress as MDH activity is related with energy demand. In present study it was found that the group with significantly higher growth showed relatively less MDH activity as compared to the control group of fishes.

5.14.4. Enzyme of Oxidative Stress

Oxidative stress arises when the organism has elevated levels of reactive oxygen species (ROS), but normal or low amounts of antioxidant defenses system activity (Schieber and Chandel, 2014). ROS are byproducts of aerobic metabolism, including immune system processes, and are chemically reactive for different biological targets. Living organisms are protected from the ROS by several defence mechanisms, including antioxidant enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase. As such, they are an important antioxidant defense in nearly all cells exposed to oxygen. The activity of superoxide dismutase (SOD) comprises the dismutation of superoxide into oxygen and hydrogen peroxide. Catalase is also an endogenous antioxidant enzyme found in nearly all living organisms that are exposed to oxygen, where it catalyzes the decomposition of hydrogen peroxide to water and oxygen. When acclimating to increased levels of oxidative stress, SOD and catalase concentrations typically increase with the degree of stress conditions. In the present study, significantly lower SOD activities in liver and gill were observed in fish fed with MLM than control. This indicates that the free radicals were effectively scavenged by the multiple compound present in MLM. It may be due to the presence of anti-oxidative compounds like phenolic compounds, flavonoid and tannins and these compounds had been shown to scavenge free radicals and protect the lipid membrane and other compounds being oxidized or destroyed (Popeskovic *et al.* 1980).

The catalase activity was found to be non-significant among the control and treatment groups of fishes.

5.15. Hemato-Immunological Parameters

Glucose is one of the important source of energy for the animal and it has been studied as an indicator of stress caused by physical factors (Demeal, 1978; Manush *et al*., 2004). Nakono and Tomison, (1967) observed that secretion of catecholamine increased during stressed condition, leading to breakdown of glycogen and enhances blood glucose level. Serum glucose concentration again depends on intestinal absorption, hepatic production, and tissue uptake of glucose. Blood glucose concentrations are maintained through hepatic glycogenolysis, glycolysis, and gluconeogenesis (Guo *et al.,* 2012). In the present study, the serum glucose concentrations in *L. rohita* fingerlings were affected by dietary MLM. Significantly higher values were recorded in *L. rohita* fingerlings fed with dietary raw MLM than control group but dietary supplementation of exogenous enzymes in MLM mixed diet did not affect the serum glucose level in *L. rohita* fingerlings. Jahanbakhshi *et al.* (2013) reported that plasma glucose concentration in great sturgeon, *Huso huso* was increased during substitution of fishmeal with plant origin protein. Whereas, the supplementation of exogenous enzymes with plant-based diet significantly reduces serum glucose level than control fish meal based diet (Mahmoud *et al.*, 2014).

Serum protein level is an essential indicator of the non-specific immune system and health status of fish species. Serum proteins are synthesized in liver and play an important role in the immune response. It comprises two major proteins viz. the albumins and globulins. Of which, albumins play an important role in homeostasis; it creates an osmotic force that maintains the fluid volume of the vascular space. The serum globulins comprised of alpha, beta and gamma globulins. The gamma globulin fraction is shown to have immune functions in blood and essential for the functional immune status of the fish. In the present study, the serum protein, albumin and globulin were determined and are found to be non-significant in *L. rohita* fingerlings fed with dietary MLM.

5.16. NBT Activity

The phenomenon known as respiratory burst activity, gives a measure of oxygen-dependent defence mechanism in vertebrate phagocytic cells. There is a generation of reactive oxygen intermediates with powerful microbicidal activity in phagocytic cells (Babior, 2005). In mammals, stimulation of the phagocytic cell membrane, and thereby activation of the membrane-associated NADPH oxidase, initiates increased oxygen consumption and the production of reactive oxygen species (ROS) with microbial activity in a process termed the respiratory burst (Allen *et al.,* 1972). In the present study, NBT values in experimental groups were measured by reduction of nitrobluetetrazolium (NBT) by intracellular superoxide radicals produced by leucocytes. It was observed that NBT values were tended to increase in fish fed with increasing dietary MLM and found significantly higher in fish fed with 20% dietary MLM supplemented with exogenous enzymes than other fed groups. The results were in agreement with Kumar *et al.* (2005) results, who reported that exogenous carbohydrases improved NBT activity when supplemented with non-gelatinized starch.

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APPENDIX

Abbreviation:

ADC : Apparent digestibility coefficients

ALT : Alanine aminotransferase

ANOVA : Analysis of variance

AST : Aspartate aminotransferase

BHT : Butylated hydroxyl toluene

CAT : Catalase

CMC : Carboxy methyl cellulose

CP : Crude protein

DM : Dry matter

DORB : De-oiled rice bran

EE : Exogenous enzyme

FCR : Feed conversion ratio

g : Gram

DE : Digestible energy

DORB : De-oiled rice bran

DSBM : De-fatted soybean meal

Hb : Hemoglobin

Hr : Hour

Hrs : Hours

HSI : Hepato-somatic index

ISI : Intestinal-somatic index

Kg : Kilogram

L : Litre

LDH : Lactate dehydrogenase

MDH : Malate dehydrogenase

mg : Milligram

Mg/L : Milligram per liter

MLM : Mixed leaf meal

n mole : Nano mole

NBT : Nitro blue tetrazolium

NFE : Nitrogen free extract

PER : Protein efficiency ratio

pg : Picogram

RBC : Red blood cells

SAA: Synthetic amino acids

SGR : Specific growth rate

SOD : Superoxide dismutase

TA : Total ash

WG% : Percent weight gain

µg : Microgram

µl : Microliter

µM : Micromole

6. SUMMARY

Aquaculture is expanding exponentially and providing nutritional security to the ever increasing population of the world. It remains the fastest growing food-producing sector in the world and can eliminate future global supply-demand gap for aquatic food among the consumer and producer. The total fish production of India during 2017-18 is estimated to be 12.60 million metric tonnes, of which nearly 65% is from inland sector and about 50% of the total production is from culture fisheries, and constitutes about 6.3% of the global fish production. The future expansion of aquaculture and production performance lies in the hands of aquafeed industries. Traditionally de-oiled rice bran (DORB) is the major ingredient in fish feed used by the farmers in India mainly to feed the Indian Major Carps (IMCs). DORB production in India is presently 9.0 MMT but shows an increasing cost and lower availability due to its higher demand and competition with other animal feed producing sector. Therefore, there is an urgent need to find cost-effective, readily available and good quality alternative sources of plant based fish feed ingredient for the partial or complete replacement of the DORB. Various studies and research have been invariably carried out to utilize the terrestrial and aquatic plants as a fish feed ingredients in order to replace the use of DORB. The large scale application of using plant based ingredients in fish feed formulation is limited due to the presence of high anti-nutritional factors, crude fibre, and imbalance of the essential amino acids. However, the supplementation of exogenous enzymes and balancing the essential amino acids may overcome the drawbacks of these ingredients and maximise its utilisation in aquafeed formulation.

Leaf meal had been a promising commodity in fish feed formulation, which can be used as a replacer of DORB. An innovative approach of mixing of leaves would dilute the anti-nutritional factors present in individual leaves and would ensure its wider availability round the year. Studies have already been carried out to optimize the utilization of the three individual leaves namely *Vigna mungo, Ipomea aquatica* and *Hygrophila spinosa* in 1:1:1 ratio. Keeping this in view, the present study was aimed to optimise the utilisation of these three leaves mixed in the ratio of 1:1:1 in the diet of

Labeo rohita to replace the DORB. The final mixed leaf composition exhibited reduction in fibre content (11.82%) and antinutritional factors like total tannin (11.86%), alkaloids content (28.42%), total oxalate (4.87%) and around 2% reduction in phytic acid composition than that of the average anti-nutritional values found in the individual leaves.

In the present study, two hundred sixteen fingerlings (216) of *Labeo rohita* were randomly distributed in six treatments in triplicates. Six iso nitrogenous and isocaloric practical diets were prepared with either 20% or 40% mixed leaf meal and with or without supplementation of the exogenous enzyme and synthetic amino acids as per the requirement of *Labeo rohita*. MLM contain 23.54% crude protein, which is higher than the DORB but contain considerable amount of fibre (16.77%). Control group was fed with basal diet without mixed leaf meal, enzyme or synthetic amino acids. T1- 20% MLM; T2- 40% MLM; T3- Control + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.14% methionine &1.04% lysine; T4- 20% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 0.94% of lysine; T5 - 40% MLM + 0.1% Exogenous enzyme (xylanase & cellulase) + 1.04% of lysine.

The experiment was conducted for 60 days in the wet lab of ICAR-CIFE Mumbai. Feeding was done up to satiation of *Labeo rohita* fingerlings at 10:00 AM and 5:00 PM daily. The initial body weight and final body weight of fingerlings were recorded to assess the growth. The proximate analysis of feed confirmed that the nutrient contents were as per the requirement of *L. rohita* fingerlings. The amylase enzyme activity was higher due to exogenous enzyme supplementation. The stress marker enzymes like SOD and LDH activities were low in T4.

From this experiment, it can be concluded that MLM can successfully replace 100% DORB in the diet of *Labeo rohita* fingerlings. The 20% inclusion of MLM with 0.1% exogenous enzyme supplementation and 0.94% L-lysine in the diet of *Labeo rohita* revealed significantly higher growth rate than the control group. However, 40% MLM feed supplemented with 0.1% exogenous enzyme and 1.04% L-lysine can successfully replace 100% DORB in the diet of *Labeo rohita* fingerlings.

Future Recommendations:

- Mixing of other leaves may also be tried in some other ratios to determine the best inclusion.
- Long-term feeding trial is suggested to find the safe level of inclusion in the diet.