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Effects of dietary *Moringa oleifera* leaf extract on growth performance, enzyme activity and non-specific immune parameters in rohu *Labeo rohita* (Hamilton, 1822)

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Abstract

A 60-day study was conducted to evaluate the effect of dietary *Moringa oleifera* leaf extract (MLE) on growth, enzyme activity and non-specific immune parameters in rohu *Labeo rohita*. To ascertain this, 180 fingerlings of *L. rohita* (average weight 12.24±0.5 g) were randomly distributed into four groups based on the level of inclusion of *M. oleifera* leaf extract *viz.,* control (C): basal diet without extract, T1: 2.5 g (0.25%), T2: 5 g (0.5%) and T3: 10 g (1%) extract per kg of basal feed per day respectively. After 60 days, results revealed that experimental fish significantly ($p<0.05$) improved growth, digestive enzymes *viz.,* amylase and total protease activity in all the treatments compared to the control with the highest performance in T3. In addition, serum antioxidative enzymes *viz.,* superoxide dismutase and catalase activity were also significantly ($p<0.05$) enhanced in the treatment groups. Myeloperoxidase, respiratory burst activity, alternative complement activity and total serum protein were significant ($p<0.05$) in fish administered 10 g of *M. oleifera* leaf extract *via* basal diet. Conclusively, dietary supplementation of *M. oleifera* leaf extract at 0.5% ~1% through basal feed significantly improved growth, enzyme activity and important immunological parameters in *L. rohita*.

Introduction

Immunomodulation is the key to sustainable aquaculture and a cost-effective intervention in the present aquaculture scenario. Super-intensive nature of farming systems poses several challenges, including environmental issues such as poor water quality, rise in opportunistic pathogenic microorganisms and increased stress conditions. These factors can negatively impact fish growth and health, rendering them more susceptible to infections and diseases, which often result in mortality and substantial economic losses (Bulfon *et. al.,* 2015; Holmes *et. al.,* 2016). A range of chemicals/formulations and selected antibiotics are used by farmers during the culture period to combat such situations (Cabello 2006; Ng and Koh, 2016; Mishra *et al*., 2017). To address such incidences, the use of natural alternatives or immunostimulants is strongly encouraged. Indigenous medicinal plants, recognised for their potential as growth promoters and immunomodulatory effects, have shown impressive results. The extracts from various parts of these plants, including the bark, stem and leaves are found to enhance both non-specific and specific defense mechanisms in fish and they offer a promising, sustainable and safe alternative to antibiotics and other chemotherapeutic agents (Evans, 1994). Moreover, herbal-based components offer numerous benefits, including being cost-effective, easily biodegradable, eco-friendly, economical, non-resistance forming, non-habit forming, non-narcotic and free of side effects for both the host fish and the environment (Chakraborty and Hancz, 2011). Hence, it is imperative to adapt sustainable fish farming practices that are both environmentally and economically sound. Use of natural immunostimulants such as medicinal plants or herbs can effectively strengthen the immune systems

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of fish, helping to prevent recurring diseases and infections caused by the same pathogens (García-Beltran *et al.,* 2020). In addition, herbal supplementation is both affordable and accessible to small and large-scale fish farmers alike. It effectively reduces disease-associated mortality while enhancing fish growth and feeding efficiency (Awad and Awaad, 2017; Harikrishnan *et al.,* 2021).

Moringa oleifera, commonly known as *"The miracle tree," "Horseradish-tree,"* or *"Ben oil tree"* (Mehdi *et al*., 2016) is a fast-growing, widely distributed plant in tropical and subtropical countries including India, with significant industrial and medicinal applications. The leaf extracts of *M. oleifera* has antioxidant and hypo-cholesterolaemic activities (Chumark *et al.,* 2008). It contains antioxidants, which can nullify harmful free radicals produced during normal cellular activities and during different traumatic conditions (Rapatsa and Moyo, 2014). The leaves of *M. oleifera*, contain flavonoids and phenolic compounds which have various biological activities, including anticarcinogenic, antioxidant, immunomodulatory and hepatoprotective properties (Sherif *et al.,* 2014) and contains essential amino acids *viz*., methionine, cysteine, tryptophan and lysine (Abdulkarim *et al*., 2005; Sherif *et al.,* 2014). The crude protein content of *M. oleifera* leaves is estimated to be approximately 260 g kg^{-1} (Hiam *et al.,* 2019) making it a potential alternative plant-based protein source for aquaculture (Elabd *et al.,* 2019). With this background information, the present study was intended to evaluate the potential effect of dietary supplementation of hydro-methanolic moringa leaf extract in *Labeo rohita* focusing on growth, enzymatic activity and selected immunological parameters.

Materials and methods

All applicable international, national and institutional guidelines for the care and use of animals were followed. Further, the study has been approved by the institutional ethical committee and the research advisory committee of ICAR-Central Institute of Freshwater Aquaculture (ICAR-CIFA), Bhubaneswar, India.

Fish and experimental conditions

One hundred and eightly *L. rohita* fingerlings (average weight 12.24±0.5 g) with no previous record of disease and infections were procured from the carp culture unit of ICAR-CIFA, Bhubaneswar, India. The fish were acclimatised for three weeks in 500 l fibreglass reinforced plastic (FRP) tanks with gentle aeration and the fish were fed with a commercial feed comprising 24% protein at 3% body weight daily twice. Upon acclimatisation, the healthy active fish were randomly distributed into twelve separate tanks (FRP) containing 15 fish in each tank containing 400 l of fresh aerated water. Dietary treatments were designed as control (C): basal diet without extract; T1: 2.5 g (0.25%) extract kg^{-1} of basal feed; T2: 5 g (0.5%) extract kg^{-1} of basal feed and T3: 10 g (1%) extract kg^{-1} of basal feed. The ration was split into two equal daily amounts (10:00 hrs and 16:00 hrs) for 60 days experimental period. The experiment was performed in triplicate and ~30% of tank water was exchanged regularly to maintain optimum water quality. The important water quality parameters like dissolved oxygen, total alkalinity, temperature and total ammonia nitrogen were recorded every 7 days and found to be in the optimal ranges throughout the feeding trial.

Plant material and leaf extract preparation

Fresh *M. oleifera* leaves were collected from ICAR-CIFA, Bhubaneswar campus and identified by the plant taxonomy department of Odisha University of Agriculture and Technology (OUAT), Odisha. To process for extraction, the leaves were cleaned thoroughly and dried in an oven at 40°C for 24 h, cut into small pieces to powder them (mechanically) and subsequently, 50% methanol was added at the ratio of 1:10 (W: V). The mixture was thoroughly mixed by a magnetic stirrer at 120 rpm and kept at room temperature for 48 h. To remove all fine particles, the extract was filtered through a Whatman filter paper (No. 1, 42 µm pore size) using an autoclaved funnel. The remaining alcohol in the filtered mixture was removed using a rotary evaporator at 60°C (El-Refai *et al.,* 2018). The powdered extract was stored at -20°C inside airtight glass container until further use.

Preparation of experimental diet and feeding

Four experimental diets (Table 1a) were prepared with graded levels of leaf extract as described earlier. Inclusion levels were decided based on the studies conducted in another fish (Elabd *et al.,* 2019). Locally available feed ingredients were used to prepare the basal feed. The ingredients were ground, weighed and made into a dough to prepare the feed pellet. All prepared pellets of different experimental and control feed were dried overnight, crushed to a 2 mm diameter pellet and finally stored at 4°C until fed to the fishes.

Table 1a. Ingredients (g) used for 1 kg of feed in the basal and experimental diet of rohu fingerlings

Feed ingredients (g)	Control (g kg^{-1} of feed)	0.25% MLE (g kg^{-1} of feed)	0.5% MLE (g kg^{-1} of feed)	1.0% MLE (g kg^{-1} of feed)
Fish meal	50	50	50	50
Soy meal	280	277.5	275	270
Ground nut oil cake (GNOC)	260	260	260	260
Rice bran	200	200	200	200
Corn flour	150	150	150	150
Oil mix (ml)	40	40	40	40
Vitamin and mineral mixture[a]	20	20	20	20
Extract (MLE)	0	2.5	5	10

a Composition of vitamin-mineral premix (PREEMIX PLUS) (quantity per 2.5 kg).
Vitamin A - 5500000 IU; Vitamin D3 - 1100000 IU; Vitamin B2 - 2000 mg; Vitamin E - 750 mg; Vitamin K - 1000 mg; Vitamin B6 - 1000 mg; Vitamin B12 - 6 mcg; Calcium Pantothenate - 2500 mg; Nicotinamide - 10 g; Choline Chloride - 150 g; Mn- - 27,000 mg; I - 1000 mg; Fe - 7500 mg; Zn - 5000 mg; Cu - 2000 mg; Co - 450 mg; L-lysine - 10 g; DL - Methionine, 10 g; Selenium - 50 ppm.

Both experimental and control fish were fed at 3% body weight daily for 60 days. The proximate composition of the experimental diet (Table 1b) was determined following AOAC (1995).

Sampling of fish

Five fish were randomly sampled from each tank. Blood samples were collected from the caudal vein using a 2.0 ml hypodermal sterile syringe after anaesthetising fish with clove oil (Himedia, India) at 50 µl l^{-1} of water. The collected blood from each fish was immediately divided into two microcentrifuge tubes, where one vial was coated with a thin layer of anticoagulant EDTA and the other one without EDTA for serum collection. Vials having EDTA coats were shaken gently to prevent haemolysis and clotting of blood. Serum was collected by the method mentioned by Das *et al*. (2021) and stored at −20°C until use.

Three fish per tank were sampled randomly for the collection of digestive tissues. The intestine of each fish was excised and the adipose tissue was carefully cleaned. Further, tissue samples were rinsed and homogenised with chilled 50 mM tris-HCl (pH 8.1) solution using an electric blender operating at 8000 rpm for 30 s. The homogenate was centrifuged at 10,000 *g*, (4°C) for 20 min. to eliminate tissue debris and lipids. The supernatant of the tissue extract was gently pipetted into 1.5 ml Eppendorf tubes and frozen at −20°C until performing the enzymatic assays. Furthermore, three additional fish were randomly sampled from each tank, minced, pooled and frozen at −20°C for whole-body proximate composition analysis.

Growth indices

On termination of the experimental period (60 days), four fish from all the treatment groups and control were harvested and subjected to estimation of growth indices as follows:

Weight gain (%) (WGP) = (Final weight - Initial weight) X 100 / Initial weight,

Feed conversion ratio; FCR = Feed given (DW) / Body weight gain (WW),

Feed efficiency ratio; FER = 1 / FCR,

Specific growth rate; SGR (%) = [ln (FW) - ln (IW)/N] × 100

where, FW = Final weight, IW = Initial weight, DW = Dry weight, WW = Wet weight, ln = Natural log and N = Number of culture days.

Enzyme assays

The antioxidant enzyme activities *viz*., superoxide dismutase (SOD) and catalase activity in the serum was measured using a commercial kit (Caymans Chemical, USA) according to the instructions of the manufacturer.

Estimation of non-specific immune parameters

The serum lysozyme assay was determined following the methods of Ellis (1990). Serum myeloperoxidase (MPO) activity was carried out as per Quade and Roth (1997) and the respiratory burst (RBA) activity of the collected blood was measured according to Anderson and Siwicki (1994) within 1 h of blood collection.

The total serum protein was estimated using a commercial kit (Merck, India) following the manufacturer's instructions , while total serum albumin was determined by the bromocresol green binding method (Doumas *et al*., 1971) using albumin kit (Merck, India). The serum globulin content was measured by subtraction of total protein value with respective serum albumin values.

Alternate complement activities (ACH50) in serum were determined following the method of Yano (1992). The serum volume causing 50% haemolysis of rabbit red blood cells (RaRBC) was determined spectrophotometrically at 414 nm and ACH50 activities were expressed as unit ml^{-1}.

Data analysis

Data were tested for normality (Shapiro-Wilk test) and homogeneity of variance by Levene's test (Zar, 1999), then subjected to one-way analysis of variance (ANOVA) using SPSS ver. 14.0 (IBM, USA) and Duncan's multiple range test (DMRT) was used to determine the significant differences between the means. Data are presented as mean±standard error (SE) and $p<0.05$ was considered statistically significant.

Results

Growth performance and digestive enzyme activities

The growth performance of *L. rohita* fingerlings fed different doses of *M. oleifera* leaf extract for 60 days is presented in Table 2. There was a significant difference ($p<0.05$) between the control group and *M. oleifera* leaf extract-fed groups in terms of final weight, WGP, SGR, FCR and PER ($p<0.05$). WGP and SGR were highest in 1% *M. oleifera* leaf extract-supplemented groups with the lowest being recorded in the control group. The highest WGP, SGR, PER and lowest FCR obtained in the treatment group fed with 1% *M. oleifera* leaf extract. Digestive enzymes like amylase and total protease activities in intestinal tissues of the treatment and control groups are shown in Table 3. From Table 3, it is evident that incorporating *M. oleifera* leaf extract in the basal diet significantly ($p<0.05$) improved intestinal amylase and total protease activities compared to the control group.

Effects of *M. oleifera* leaf extract on non-specific immune parameters

Serum total protein, albumin, globulin and A/G ratio in treatment and control groups are presented in Table 4. Dietary supplementation at 0.5-1% *M. oleifera* leaf extract in all three treatments showed a significant effect on these parameters compared to the control ($p<0.05$).

Serum antioxidative enzymes *i.e.* SOD and catalase activities are shown in Fig 1. The highest activities of these two enzymes

Table 1b. Proximate composition of diets. Data expressed as mean±SE (n = 3)

Moisture (%)	Protein (%)	EE (%)	Ash (%)	Crude fibre (%)	NFE (%)	Energy (kcal per 100 g)
8.05±0.05	29.25±0.03	7.99± 058	8.51±1.13	4.95±0.31	39.29±1.00	422.921±0.69

Table 2. Growth performance and feed utilisation of *L. rohita* fed with different levels of *M. oleifera* leaf extract (MLE) for 60 days

Growth indices	Control	0.25% MLE	0.5 % MLE	1.0%MLE
Initial weight (g)	12.03±0.055	12.28±0.164	12.71±0.107	12.23±0.176
Final weight (g)	16.3±0.088^{a}	17.9±0.053^{b}	18.9±0.059^{c}	19.8±0.39^{d}
% Weight gain	35.3±0.85^{a}	46.3±2.65^{b}	48.7±1.64^{b}	61.8±2.44^{c}
SGR (% day^{-1})	0.5±0.012^{a}	0.64±0.03^{b}	0.66±0.021^{b}	0.8±.026^{c}
FCR	5.1±0.13^{c}	3.9±0.23^{b}	3.7±0.12^{b}	2.9±0.12^{a}
PER	0.7± 0.018^{a}	0.92±0.053^{b}	0.97±0.033^{b}	1.23±0.05^{c}

IW: Initial weight; FW: Final weight; WG: Weight gain; SGR: Specific growth rate; FCR: Feed conversion ratio; PER: Protein efficiency ratio. Different letters (a–d) in the same row indicate significant differences (p<0.05).

Table 3. Digestive enzyme activities in *L. rohita* fingerlings in different treatments after 60 days of feeding

Digestive enzyme	Control	0.25% MLE	0.5 % MLE	1.0%MLE
Amylase*	0.19±0.005^{a}	0.23±0.021^{b}	0.28±0.024^{c}	0.32± 0.03^{d}
Total protease*	0.58±0.012^{a}	0.66±0.017^{b}	0.88±0.089^{c}	0.96±0.12^{d}

Different letters (a-d) in the same row indicate significant differences (p<0.05).
*Amylase activity expressed as micromoles of maltose released min^{-1} g^{-1} protein; Protease activity expressed as micromoles of tyrosine released min^{-1} g^{-1} (n=3)

were recorded in 1% fed treatment groups as compared to other treatments and control groups (p<0.05).

Serum lysozyme, MPO and RBA (blood) and alternative complement (ACH50) activities are presented in Fig 2. It has been observed that, the inclusion of ≥1% *M. oleifera* leaf extract significantly (p<0.05) enhanced serum lysozyme, MPO and RBA (blood) as well as ACH50 activities as compared to control.

Discussion

Immunostimulants are valuable inputs for intensive or super-intensive aquaculture practices, offering significant potential to enhance both the immunity and growth of farmed fish when administered at appropriate dose and duration. Further, researchers consistently strive to assess parameters related to immunity, enzymes activity and intestinal health to derive definitive conclusions.

In this study, the incorporation of *M. oleifera* leaf extract in the basal feed at 0.5-1%, improved the growth performance which corroborates the findings of Faheem *et al.* (2020) and Garcia-Beltran *et al*. (2020). In concurrence with the present results, enhanced growth of Nile tilapia and scampi was obtained upon feeding of 0.15-0.25 and 0.25-0.5% *M. Oleifera* leaf extract respectively in their diets as compared to control (Kaleo *et al.*, 2019; Monir *et al*., 2020). Earlier studies by Gopalakrishnan *et al*. (2016) also suggested that more than 40% essential amino acids (histidine, tryptophan, valine and arginine) are present in *M. oleifera* leaf and these are very useful in improving the growth of fish, which ultimately enhances the overall health status of fish. Al-Khalaifah *et al*. (2020) reported that, in African catfish (*Clarias gariepinus*), feeding Doum Palm fruit powder incorporated (at 5, 10, or 15 g kg^{-1} of basal feed) diets for a period of 70 improved growth parameters, including final body weight, body weight gain (WG), SGR, FCR and PER, which are in line with our findings. Likewise, Yousefi *et al*. (2021), while administering Afsanteen (*Artemisia absinthium*) aqueous extract (AE) at 0.5-1% of basal feed for 60 days found that supplementation of AE improved growth performance, innate immunity and oxidative status of common carp, *Cyprinus carpio* significantly which corroborate our findings.

Serum biochemical parameters have been recognised as valuable tools for monitoring the metabolic and physiological status of fish (Mondal *et. al*., 2020). Likewise, in this study, we have explored some of the important enzyme parameters (digestive enzymes and antioxidants) which ultimately infer the health conditions of experimental fish before and after being fed with *M. oleifera* leaf extract. In the present study, the activities of selected digestive enzymes such as total protease and amylase activity were also recorded and found to be highest in 1% *M. oleifera* leaf extract fed fish than control fish. The increased activity of the above digestive enzymes could be attributed to the efficient utilisation of feed and absorption of nutrients thereby ultimately enhancing the growth indices in *L. rohita*. Antioxidant defence systems are the main reason behind quenching of oxygen free radicals under a natural physiological state. *Moringa* leaf has outstanding antioxidant properties due to its inherently present bioactive compounds mainly ascorbic acid, folic acid and other organic acids (linolenic, linoleic, and decanoic acids) which helps in the heavy metal chelating properties and amelioration of oxidative stress (Paikra, 2017; Kou *et al*., 2018) thereby increasing its therapeutic effects. Moreover, the antioxidant activity of *Moringa* leaf is found to surpass that of synthetic compounds such as butylated hydroxy toluene, rutin and vitamin C. Further, the aqueous or alcoholic extracts of *Moringa* leaf have demonstrated the ability to scavenge and neutralise free radicals, as well as break down peroxidases. This is attributed to the strong electron-donating properties of phenolic compounds

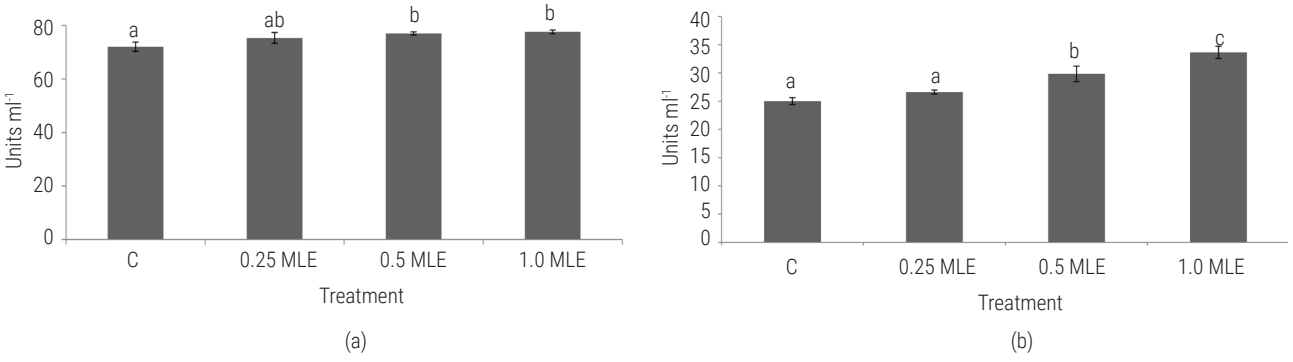

Fig 1. Serum antioxidants enzyme (SOD and catalase) activities in *L. rohita* fingerlings after 60 days of feeding with graded levels of *M. oleifera* extract (MLE). Different letters (a–d) in the same row indicate significant differences (p<0.05)

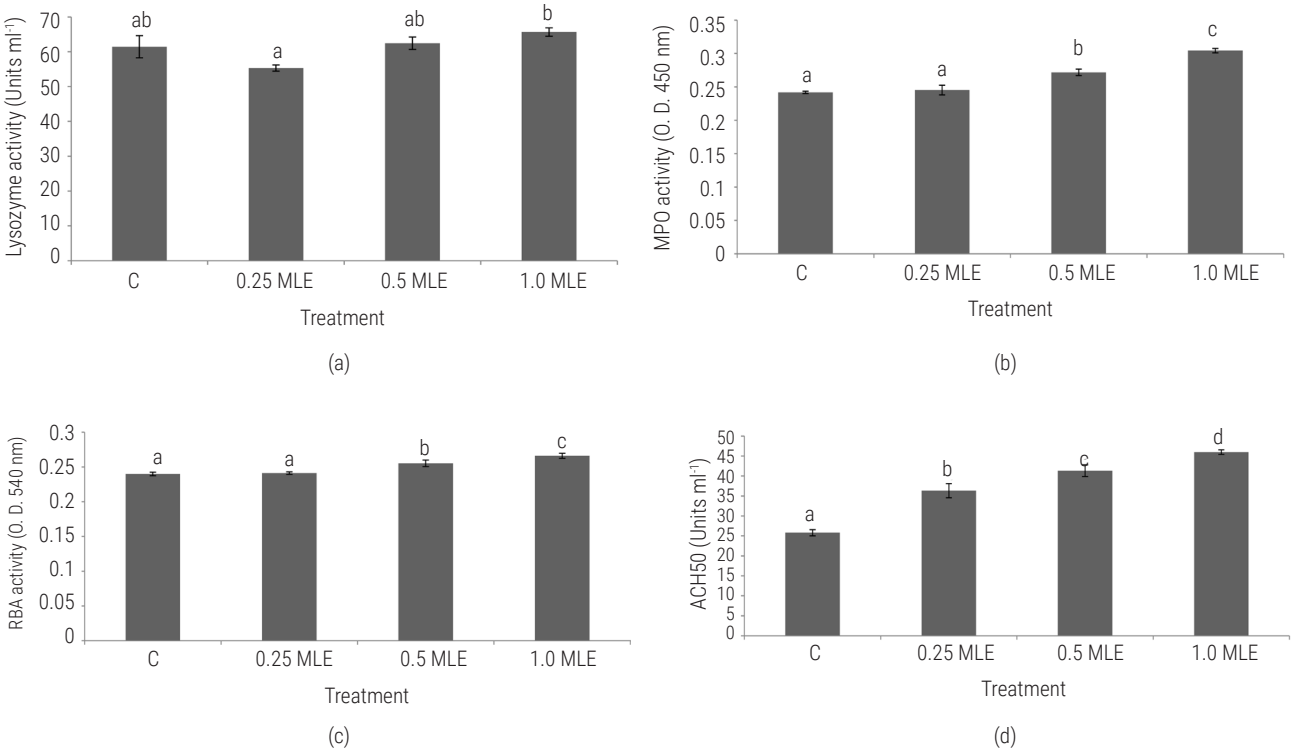

Fig 2. Immunological indices of fish under different treatments. Serum lysozyme, myeloperoxidase (MPO), respiratory burst activity (RBA) and alternative complement activities (ACH_{50}) in *L. rohita* fingerlings after 60 days of feeding with graded levels of *M. oleifera* extract (MLE). Different letters (a–d) in the same row indicate significant differences ($p<0.05$)

which ultimately inhibit the formation of hydroxyl radicals. In the present investigation, the dietary inclusion of *M. oleifera* leaf extract at a dose of 0.25 to 1 g per kg of feed has resulted in increased activity of SOD and catalase antioxidant enzyme activities which was more pronounced in the case of serum catalase activity. These changes in the antioxidant activities may be the reflection of the direct role of important phytochemicals of *M. oleifera* leaf extract, mainly vitamin C and rutin (Falowo *et al*., 2018) which pose strong antioxidant actions. SOD, CAT and GPX enzymes are the main players of antioxidant actions and are involved in the protection of fish tissues from the harmful effects of lipid peroxidation (Ray and Husain, 2002). SOD protects from superoxide radical toxicity by catalysing the conversion of superoxide radicals into hydrogen peroxide and oxygen. On the other hand, CAT and GPX facilitate the break down of H_2O_2 into water and oxygen. Therefore, the elevated activities of serum SOD and CAT observed in the present study indicates enhanced protection against cellular free radical damage and reduced severity of cellular impairment. In concurrence with the present investigation, Mansour *et al*. (2020) also reported that dietary supplementation of 1-5% of *M. oliefera* leaf to gilthead seabream diet upregulated the expression of SOD and CAT genes in skin and gills in a dose dependent manner. The antioxidant properties of aqueous and ethanolic extracts of *M. oliefera* leaf powder have been extensively investigated by Garcia-Beltran *et al*. (2020) at different concentrations. These properties were attributed to the presence of high amounts of phenolic compounds in the leaf extracts.

In addition to enhanced antioxidant defences, the present study also documented enhanced immune functions indicating improved immune systems in the treated fishes. The non-specific immune parameters play an important role in fighting against a wide range of pathogens. To boost the immunity of farmed fish, it is always advisable to target the non-specific immunity. Respiratory burst activity, myeloperoxidase and lysozyme are the immune parameters that are regarded as the humoral components of innate immunity and are the first to get activated and act on infectious microbes instantly (Magnadottir *et al*., 2005). The present study has also explored selected non-specific immune parameters after feeding with *M. oleifera* leaf powder. The findings indicated better immune responses in rohu fingerlings fed with *M. oleifera* leaf extract incorporated feeds. Lysozyme is an important component of the innate immune system and plays a vital role in the defence mechanism (Jolles and Jolles, 1984). Abdel-Tawwab *et al*. (2018) also found that dietary inclusion of ethanolic extract of clove basil leaves (CBLE) enhanced respiratory burst and lysozyme activity after 12 weeks. In agreement with the present findings Mansour *et al*. (2018) reported that the inclusion of Moringa leaf meal at 5-10% level resulted in enhanced humoral components such as serum lysozyme, and peroxidases in seabream. Similarly increment in serum lysozyme was observed after feeding gibel carp with fermented Moringa leaf at different levels (Zhang *et al*., 2020). Several recent studies have highlighted immunostimulant potential of Moringa leaves in diverse aquatic animals when incorporated into their diets as Moringa leaf meal or Moringa leaf extract at different concentrations (Kaleo *et al*., 2019; Faheem *et al*., 2020; Mansour *et al*., 2020).

In the current study, *M. oliefera*-extract incorporated diet fed groups demonstrated significant improvements in serum total protein, albumin and globulins. Increased levels of serum total protein, albumin and globulins suggest enhancement of innate immune response in fish (Wiegertjes *et. al*., 1996). Activation of complement system is the reflection of an activated and potent immune system primed to encounter invading foreign pathogens. Increased activities of ACH50 was observed in the present study which is in agreement with enhanced ACH50 reported in seabream upon feeding of 5% Moringa leaf meal supplemented feed (Mansour *et al*., 2018). Dawood *et al*. (2018) also reported that herbal compounds sufficiently stimulate the complement system in fish.

Supplementing the diet of *L. rohita* with *M. oleifera* leaf extract at a concentrations of 0.5-1% of the basal feed, significantly improved the growth performance, digestive enzyme activity, antioxidant enzyme activity and selected non-specific immunological parameters. These findings suggest that administering an appropriate dosage (0.5-1.0%) of *M. oleifera* leaf extract to *L. rohita* fingerlings serves both as a growth enhancer and an immunostimulant. Future research could explore its effects on the gut microbiome, immune gene expression and intestinal histology paving the way for broader applications as a potential feed supplement in carp aquaculture.

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