Effect of Parsley (*Petroselinum crispum*) Leaves and Seed Oil on Growth Performance and Haemato-Immunological Indices of *Labeo rohita* Fingerlings

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ISLAMABAD
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MASTER IN PHILOSOPHY
IN
FISHERIES AND AQUACULTURE

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"In the Name of ALLAH, the most Beneficent, the most Merciful"

CERTIFICATE

This dissertation "Effect of Parsley (*Petroselinum crispum*) leaves and seed oil on growth performance and haemato-immunological indices of *Labeo rohita* fingerlings" submitted by **Ms. Sadia Meo,** is accepted in its present form by the Department of Zoology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad as satisfying the thesis requirement for the degree of Master of Philosophy in Fisheries and Aquaculture.

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DECLARATION

I hereby declare that the work presented in the following thesis is my own effort, except where otherwise acknowledged, and that the thesis is my own composition. No part of this thesis has been previously presented for any other degree.

SADIA MEO

DEDICATION

This thesis is Dedicated to my loving Parents, respected Supervisor, siblings & beloved friends for their advice, patience and faith in me

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List of Abbreviations used

ANOVA	Analysis of variance
ADWG	Average daily weight gain
BSA	Bovine serum albumin
°C	Celsius
$CuSO_4$	Copper sulphate
µL	Microliter
µM	Micromolar
CAT	Catalase
cm	Centimeter
D-H_2O	Distilled water
EDTA	Ethylenediaminetetraacetic acid
FCR	Feed conversion ratio
g	Gram
g/ml	Gram per milliliter
H_2O	Water
H_2O_2	Hydrogen peroxidase
HGB	Hemoglobin
HCT	Hematocrit
IgM	Immunoglobulin M
Kg	Kilogram
L	Liter
L. rohita	*Labeo rohita*
LPO	Lipid peroxidation
LSD	Least significant difference
M	Molarity

List of Abbreviations used

M cm	Molar coefficient
m/v	Mass per volume
Mg	Milligram
mg/L	Milligram per liter
MCH	Mean corpuscular hemoglobin
MCV	Mean corpuscular volume
MCHC	Mean corpuscular hemoglobin concentration
Min	Minute
mM	Millimolar
mm	Millimeter
Na_2CO_3	Sodium carbonate
$NADH^+$	Nicotinamide adenine dinucleotide hydrogen
NBT	Nitro blue tetrazolium
nmol	Nanomole
O_2	Oxygen
OE	Edema
P	Probability
pH	Power of hydrogen
POD	Peroxidase
%	Percentage
QAU	Quaid-i-azam University
Rpm	Revolution per minute
RBC	Red blood cell
SE	Standard error
SGR	Survival growth rate

List of Abbreviations used

SOD	Superoxide dismutase
S. aureus	*Stapyhlococcus aureus*
TBARS	Thio-barbituric acid reactive substances
U/mg	Unit per milligram
U/L	Units per liter
UV	Ultra-violet
WBC	White blood cell
Wf	Final body weight
Wi	Initial body weight
WG	Weight gain

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ABSTRACT

Aquaculture is the leading industry playing significant role in enhancement of food quality and security. The use of herbs in formulated feed opened new doors for controlling infectious diseases. Parsley *(Petroselinum crispum*) is a dietary herb with high concentration of bioactive compounds, with antimicrobial and antifungal properties. For investigation of the effect of *P. crispum* leaves and oil, a 75-day feeding trial was conducted in triplicate on *Labeo rohita* fingerlings in semi-static conditions to find its effect on growth parameters, haemato-immunological indices and digestive enzymes. About 210 *L rohita* fingerlings of uniform size with an average weight (4.1± 0.02g) were distributed in 21 glass aquaria and randomly divided into seven groups. Control group were fed diets without parsley supplements. $PL_{5,}$ PL_{10} and PL_{15} were fed diets supplemented with graded levels of leaves powder (5g, 10g and 15g respectively). Similarly, PO_1 PO_2 and PO_3 were fed diet supplemented with 1ml, 2ml and 3ml of parsley essential oil. The results indicated significant ($p<0.05$) increase in weight gain%, SGR% in dose-dependent manner in parsley leave fed groups but, in oil fed groups highest weight gain and SGR% was observed in PO_2 (diet with 2ml parsley oil). An increase in haematological indices was also seen among parsley fed groups compared to control. The antioxidant activity (SOD, POD, CAT) and LPO activity was decreased in parsley supplemented groups in dose dependent manner among all the parsley supplemented groups. The parsley leaves and oil supplements also showed dose dependent positive effect on the immunological indices (Albumin, globulin, Total protein, IgM level, albumin and globulin ratio, lysozyme activity, phagocytic activity and respiratory burst activity). The digestive enzyme activity showed variable results, i.e., protease and amylase showed dose dependent increase in activity compared to lipase that showed decreasing trend in response to parsley leaves supplement. However, in response to Parsley seed oil supplement, protease and lipase showed dose dependent increasing trend compared to amylase that showed decreasing trend. It is concluded that parsley leaves at the rate of 15g/kg diet or 2ml/kg diet parsley seed oil, could be used to improve the growth performance, health status and immunity of *Labeo rohita.*

INTRODUCTION

Aquaculture is the controlled or semi-controlled rearing of aquatic organisms. In recent decades, aquaculture has experienced the greatest rate of growth among all food production technologies. The availability of seafood has been transformed by increased aquaculture output, which is also elevating seafood's status as a major worldwide food source (Garlock *et al.,* 2020). Approximately 50% of fish consumed by humans today is produced by aquaculture. Fish is a well-known source of omega-3 fatty acids and proteins, which are thought to give consumers good health advantages (Ansari *et al*., 2021). Aquaculture is a significant food business that guarantees the provision of nutritional needs of consumers worldwide (Dawood *et al*., 2021).

Aquaculture and fishing provide employment and income for millions of people in developing countries (FAO, 2020). According to Naylor *et al*. (2021) by 2050, the demand for fish and other aquatic foods is expected to double globally due to population growth, economic growth, globalization, urbanization in emerging countries, and dietary shifts in developed countries. While rapid growth in demand offers opportunities for aquaculture expansion in poor and middle-income nations, restrictions such as rising resource supply and quality prices (Kong *et al*., 2020), infectious diseases and rising labor costs may hamper economic progress (Nwuba *et al.,* 2022). To meet customer demands, intensive farming methods are proposed; however, the viability of these systems depends on the maintenance of water quality and nutritional diets (Dawood *et al*., 2021).

Aquaculture is a crucial activity for promoting food security and enhancing fish farmer's income, especially in developing regions. Furthermore, due to the rapid growth of the human population, the rise in the demand for cheap sources of protein, and the loss in fish captures from inland natural lakes prompted the need of urgent development of aquaculture (Bostock *et al*., 2010). Because aquaculture is the most potential industry for supplying the world's food demand, governments and development organizations have recently given fish farming more attention (Dawood *et al*., 2021). Aquaculture is expanding faster than any other technique of producing food, and this expansion will be essential for battling food insufficiency and malnutrition and supplying the world's rising food demand (El-Saadony *et al*., 2021). The body's fundamental nutritional demands are satisfied by the high protein foods found in fisheries resources, which also contain polyunsaturated fatty acids, one of the most crucial nutrients for preserving a healthy life due to its favorable effects on human physiology and metabolic processes (FAO, 2018).

Overall, at global level, people obtain 25% of protein from fish and shellfish. Surveys of 2004 inform us about utilization of almost 75% of fish produced in the world by humans (Lauria *et al*., 2018). The world's total fisheries and aquaculture production, excluding algae, increased by 41% between 2000 and 2020, reaching a record-breaking 178 million tonnes in 2020, just a little less than the record-breaking 179 million tonnes of 2018. Comparing this we can say that from 2000, there has been an overall increase of 52 million tonnes (FAO, 2022). The overall production of fisheries and aquaculture (excluding algae) increased slightly in 2020 compared to 2019, although it decreased (-0.7%) from the record high set in 2018. Capture fisheries output decreased from a peak of 96.5 million tonnes in 2018 to 90.3 million tonnes in 2020, accounting for 51% of total output (FAO, 2022).

Asia contributed significantly to the entire increase in fisheries and aquaculture production, accounting for around 70 percent of the total in 2020 compared to 57% in 2000 (FAO, 2020). Aquaculture also plays a significant part in the region's economies, ensuring food security, employment, income, eradicating poverty, and, more broadly, promoting social and economic stability in rural areas. China is the world's top producer of both capture fisheries and aquaculture with a share of 36% of global output in 2020 compared to 30% in 2000 (FAO, 2020). The viability of water quality, which is necessary for the growth, reproduction, survival, and output of aquaculture species, has been compromised recently owing to a variety of environmental issues brought on by population increase and industrial activity. (Shelton, 2014b; Hadyait *et al*., 2020; Mehmood *et al*., 2020b)

About two-thirds of Pakistan's population, i.e., 135 million people are dependent on agriculture, either directly or indirectly, as the nation is mostly an agricultural one (Noman *et al*., 2019). Fisheries, a subsector of agriculture, is important to Pakistan's national economy and helps ensure the country's food security (Mohsin *et al*., 2017a). Even while fishing only makes up around 1% of Pakistan's GDP, or roughly 4% of the country's GDP from agriculture, it nevertheless has a substantial impact on the country's economic growth by giving work to a sizable portion of the population living in underdeveloped Balochistan and Sindh areas (Khan, 2020). About 600,000 people work in auxiliary sectors in addition to the approximately 400,000 fishermen who are employed directly by the fishing sector (Nazir *et al*., 2017).

In addition to being a significant source of food and revenue for the nation, fishing provides a means of subsistence for Pakistan's coastal residents (Siddiqi *et al*., 1992). Fish is most affordable and valuable source of animal protein for humans (Ali *et al*., 2020). In Pakistan, fish is usually consumed throughout winter starting from October till April. Fish consumption in Pakistan is low. The fact that most fish produced is exported is a key factor in the low consumption. Official data and records indicate that the majority of fish harvested in Pakistan comes from marine sources, accounting for 70% of the country's total fish exports (Rehman *et al*., 2019).

Pakistan exports fish, but barely 0.25 percent of all exports worldwide, mostly to European, US-Japanese, and Middle Eastern nations (Khan *et al*., 2018). The overall export potential from the currently available natural resources has been calculated to be around $1 billion USD (Baset *et al*., 2020). In Pakistan, the fisheries for shrimp are a valuable resource. Through its export, this fishery resource has been essential for generating income. The largest new export market for Pakistani shrimp aquaculture is China. China receives over 75% of the items made from shrimp. This fishery resource has enormous export potential, which may be further expanded by increasing output and putting in place trade-promoting regulations (Mehak *et al*., 2020).

According to World Bank Survey Report, the aquaculture production of Pakistan was estimated to be 162462 metric tonnes and total fisheries production was 655245 metric tonnes in 2020. The productivity of capture fisheries in Pakistan's marine waters is declining over time. This is a result of ongoing overfishing in Pakistani maritime waterways (Chandio *et al*., 2020). The maritime industry is subject to an open access policy as a result of a lack of planning, attention, and policy execution. As a result, overfishing must be prevented in order to maintain fisheries resources and there must be a decent alternative to generate fish meals for human use. Marine resources are now under stress (Mohsin *et al*., 2017c).

The most significant species that are commercially grown throughout the country as the most preferred culturable fish species in polyculture practice of carp are the Indian major carps (IMCs), which comprises of *Cyprinus carpio* (Common carp), Catla (*Catla catla*), Mrigal (*Cirrhinus mrigala*), and *Labeo rohita* (commonly known as rohu) (Murthy *et al*., 2002). *Labeo Rohita* is common in North India, Pakistan, Orissa and Bengal and is called Rohu but in Assam it is known as Rohiti. This specie is extensively spread in South Asia, South-East Asia, Sri Lanka, the former USSR, Japan, China, the Philippines, Malaysia, Nepal, and certain African nations (FAO, 2006).

Rohu is a zooplankton- and phytoplankton-feeding column feeder. It expands quickly and gains more than 1 kilogram in a year. With a terminal mouth and fringed lips, its head is smaller than that of Catla (*Catla catla*). The body is a little bit pinker and longer than Catla (*Catla catla*). Rohu is a native of freshwater rivers in Pakistan, India, Nepal, Bangladesh, Burma, and Sri Lanka. Under culture conditions, highly variable growth is seen by different researchers (Jhingran *et al*., 1985).

Traditionally and commercially, rohu are given a 30-35% protein diet consisting of rice bran and oil cake. Rohu contributes 15% of the world's production of freshwater aquaculture and 4% of the principal species produced at the global level of aquaculture (FAO, 2018). Although Pakistan, Bangladesh, Myanmar, and Thailand are all important producers of rohu but India is the largest rohu producer in Asia (FAO, 2006). It contributes to roughly 35% of the overall output of big carp in India (FAO, 2001). The development of rohu using protein-rich supplemental meals has received the most attention due to its high protein content.

Rohu, *Labeo rohita* generated more than 0.95 million tonnes of production in 2006, making it a considerable prospective species for aquaculture in Pakistan and other south-east Asian countries. The Indian major carp*, Labeo rohita*, is the most major commercial fish in India and has the highest market demand and consumer acceptance as food due to its flavor and flesh. (FAO, 2006).

Despite the aquaculture industry's rapid development, satisfying the worldwide demand for standard quality fish provision is a challenge for people associated with this industry. Many health experts see fish as a healthy primary dietary supply of omega-3 fatty acids and proteins, which might possibly benefit human health by lowering the risk of heart disease and cancer and promoting brain growth and function (Ruxton *et al.*, 2007; Tacon *et al*., 2020). Another factor that contributes for increased fish production and intake of animal and fish proteins is heightened health consciousness (Yaakob *et al*., 2014).

Fish nutrition management is required in sustainable aquaculture practice to decrease costs and enhance growth performance. For instance, an ideal feed rate, or the quantity of nutrition taken daily by fish, is required to handle fish nutrition for the greatest potential development performance and fish health (Ahmad *et al*., 2018). Almost half of all aquaculture investment output expenses are attributed to feed and feeding, which are regarded as the major contributors to aquaculture

production costs (El-Sayed *et al*.,1999). Therefore, proper feeding rates and feeding management are essential for successful cultivation of marine and freshwater fish. Knowledge of feeding levels is vital to achieve optimal levels of growth and feed efficiency and to reduces water quality significant damage to overfeeding. Furthermore, the optimal feeding level greatly lowers feed costs (Davies *et al*., 2006). Numerous factors, such as fish size, species, age, feed quality, and circumstances during rearing, have an impact on the appropriate feeding quantity (Gupta, 2020).

Formulated feed is required for roughly 70% of global aquaculture output, and feed costs between 50% and 70% of total production expenses (Gong *et al*., 2019). Formulated diets are made up of a variety of substances, such as proteins, oils, essential amino acids, carbohydrates, vitamins, minerals, and colors, which come from a number of sources (Miranda *et al*., 2020). Depending on the type of fish and its stage of growth, different amounts of each component are included. When compared to grower and finisher stages, the protein demand is higher during early development (e.g., larval or juvenile) because of higher metabolic growth. Ingredients that produce protein are a significant cost component of all feed components (Stickney, 2002).

Besides ensuring adequate growth rates, high-quality fish feed also benefits fish physiologically. Because of this, it is essential to make sure that those feed elements are pertinent (Kiron, 2012). Incorrect practices can seriously affect fish growth and survival as well as influence the efficiency of fish digestion and nutrient retention (Gilannejad *et al*., 2019). Because feed limitation can increase feed utilization while lowering feed expenditures, it has been utilized as a dietary feeding approach. The evaluation of the best feeding approach is crucial for determining fish performance overall, physiological reaction, and digestive efficiency (Pedrosa *et al*., 2019).

Recently, both researchers and feed production companies have shown an increasing interest in incorporating herbs in animal diets. Herbs have been utilized to successfully manage shrimp and fish infections in a number of nations, including Mexico, India, Thailand, and Japan. There has been comparatively little study on the medicinal plants that can be used to treat fish ailments, despite the fact that herbal treatments have been utilized for human therapy for thousands of years (Pandey *et al*., 2012; Dugenci *et al*., 2003).

Herbal medicines can be used as preventatives for infections, growth promoters, and stress-resistance boosters in addition to being treatments. Because they are affordable, environmentally

friendly, and have few side effects, herbal medications are therefore becoming more and more successful in the management of disease (Pandey *et al*., 2012). Due to its potential health advantages and manageable side effects, traditional medicines (such as herbal and algae extracts) are increasingly being used in place of chemical pharmaceuticals as a trend in recent years. Consequently, combining nutritional techniques and functional feed additives like natural immunostimulants to boost culture productivity can enhance animal health and lower the likelihood of output loss (Hoseinifar *et al*.,2020).

Phytogenic compounds made from plants, are added to feed to enhance animal performance or feed palatability. These bioactive compounds have a variety of beneficial effects on organisms, such as enhancing feed efficiency and digestion, lowering nitrogen excretion, and enhancing gut flora and overall health. Phytogenic feed additives can be obtained from the leaves, roots and fruits of herbs, spices, or other plants. They can be used in feed as extracts or essential oils, as well as in solid, dried, or crushed form (Yang *et al.,* 2015).

Aquaculture has been using medicinal plants (including spices, herbs, and their extracts) more and more because to their low cost and ease of application. Medicinal plants have a wide range of benefits for farmed fish, including improved growth, immunity, antibacterial and anti-stress properties, as well as defence to infections. Generally, the quantity of important active components such alkaloids, quinones, lectins, steroids, phenolic compounds, tannins, terpenoids, saponins, and flavonoids is directly correlated with the effectiveness of medicinal plants and their derivatives especially, extracts and essential oils (Orso *et al*., 2022). Scientific interest in aromatic plants, both as extracts and essential oils, is developing among the therapeutic plants used a fish feed additive (Sutili *et al*., 2018).

Petroselinum crispum belongs to the family Umbeliferae is a bright green plant that is cultivated widely in the tropic, subtropic, and temperate regions. It is a biennial plant that is sometimes grown as an annual (Mehmood *et al*., 2014). The herb is frequently employed as a flavor and spice in food (Eddouks *et al*., 2017). Because of its high boron and fluorine content as well as the presence of iron and calcium*, P. crispum* serves as a vital dietary component for bone health. It is also rich in vitamins B, C, and -carotene (Daradkeh & Essa, 2016).

P. crispum seeds are used for treatment of various gastro-intestinal diseases, inflammation, halitosis, kidney stones, and amenorrhea and have been reported to be antibacterial, antiseptic, astringent, gastrotonic, antidote, antispasmodic, carminative, digestive, and sedative (Farzaei *et al*., 2013). Parsley plant parts (the leaf, stem and root) are rich sources of bioactive compounds such as furanocoumarins (e.g., xanthoxin, trioxalen and angelicin), essentials oils (e.g., sesquiterpene hydrocarbons, monoterpene hydrocarbons and alcohols, furanocoumarins, aldehydes and aromatic compounds), flavonoids (e.g., quercetin, apiol, myristicin, apigenin, luteolin and their glycosides), carotenoids (e.g., neoxanthin, β-carotene, lutein and violaxanthin), vitamins (e.g., tocopherols and A, C and B complexes), minerals (e.g., iron, zinc, calcium and phosphorous) and fatty acids (e.g., linolenic and palmitic acid) (Liberal *et al*., 2020).There have been reports of the essential oil of parsley having antibacterial, diuretic, and modest antioxidant properties. The parsley essential oil (PEO) contains a variety of bioactive substances, including alpha-pinene, D-limonene, myristicin, and oleic acid. Myristicin is a powerful cancer chemoprotective agent (Farag *et al*., 2021).

The understanding of the regulation of embryonic and postnatal skeletal muscle growth and development is extremely important to completely understand mechanisms that control cell specification and differentiation during embryogenesis (Chen *et al*., 2000). Studies show that members of the MyoD family (MyoD, myf-5, myogenin and MRF4) play pivotal role in myogenic regulation at molecular level and are expressed throughout the development and maturation of skeletal muscles in animals (Sengupta *et al*., 2014). Fish have an ability to recruit new skeletal muscle fibers throughout the larval life and even during juvenile and adult life. Both hyperplasia and hypertrophy occur during myogenesis in larval and adult muscle growth of fish which reach a large adult size (Johnston *et al*., 1998). Parsley leaves can be used as growth stimulators

An experimental study on common carp juveniles given parsley leave supplementation at four graded levels 0.1,0.25,0.5 and 1% illustrated that fish fed with parsley showed enhanced growth performance. The results revealed that highest weight gain, specific growth rate and lowest FCR was observed in treatment fed with 0.5% parsley in diet. To conclude that 0.5% parsley can be used as growth stimulator (Mooraki *et al*., 2014).

An experiment was conducted on broiler chicken with parsley as feed additive to assess the effects of parsley on chick's performance. In this study, chicks were divided into five groups T1, T2, T3, T4 and T5 with supplementation of parsley at 0,3,6,9 and 12g/kg respectively. The group

fed with 9g/kg parsley had significant effects on body weight and weight gain. The feed intake and feed conversion ratio also improved compared to control (Majeed *et al*., 2021). In another experiment, fenugreek, parsley and sweet basil seeds were used as feed additive on growth performance of broiler chicken. The results indicated that dietary parsley fed to chicken resulted in significantly improved body weight, feed efficiency and feed intake in broiler chicken (Abbas *et al*., 2010).

Parsley is rich in iron and vitamin C. Due to its high iron content, it builds up the blood (Duke *et al*., 2009). An experiment was conducted by using fresh parsley leaves as feed additive in diet of local Iraqi geese to check the hematological traits. About 24 local geese of about 1 year of age were used in experiment. The birds were allocated in four different groups. T1 fed with parsley free diet, T2 fed with 80g/day parsley, T3 fed with 160g/day parsley and T4 fed with 2400g/day parsley. The results indicated that supplementing the geese with fresh parsley leave diet at different levels showed significant improvement in most hematological characteristics (Al-Daraji *et al*., 2012).

In a 60 days experiment, *O. niloticus* was exposed to BF at 0.68 µg/L for 60 days to evaluate the hematological, biochemical and immunological responses. Additionally, the potential of parsley essential oil to ameliorate BF-induced toxic effects was also explored by co-supplementation at two different levels. The results indicated that dietary co-supplementation of PEO with concomitant BF exposure alleviated the adverse effects of insecticide in dose-dependent manner. Thus, conforming the protective role of parsley and applicability as promising feed supplement to fish (Farag *et al*., 2021).

HYPOTHESIS

It is predicted that supplementation of parsley in fish diet positively enhances the growth rate, antioxidant status, hematological and immunological indices. So, the current research will focus on following aim and objectives.

AIM AND OBJECTIVES

The main aim of current study is to determine the potential growth- stimulating & immuno-modulatory effects of parsley leave powder & essential oil on *L rohita* fingerlings.

The main objectives are to assess the effects of parsley-leave powder and essential oil on:

- Growth performance of *L. rohita* fingerlings.
- Hematological indices (RBCs, WBCs, Hb and hematocrit.
- RBC related indices (MCV, MCH, MCHC).
- Antioxidant enzymes (SOD, POD, CAT) and LPO activity.
- Immunological indices (IgM, Total protein estimation, Albumin, Globulin, A/G ratio, respiratory burst, lysozyme and phagocytic activities).
- Intestinal enzyme (protease, amylase and lipase) activity.

MATERIALS AND METHODS

Collection of Fish

About 200 *Labeo rohita* fingerlings were live transported in well-aerated polythene bags from the Nursery Unit, Peshawar Hatchery to Fisheries Research Center, Department of Zoology, Quaid-i-azam University, Islamabad.

Acclimatization of Fish Stock

Fish were transferred to raceways at Fisheries research center, QAU for acclimatization of 2 week. Fish was fed twice daily with 35% protein basal diet at the rate of 5% body weight. After two weeks, active, healthy and uniformed size *L rohita* fingerlings with average body weight 4.0±0.02g were shifted to well-aerated glass aquaria with maintained temperature, after giving them salt bath, to avoid the microbial growth and acclimatized for another week. During the experiment, the unused feed was siphoned out and 25% of water was changed regularly on alternate days. Fish were given prepared experimental feeds with 35% CP twice a day at the rate of 5% of their body weight. The research work was carried out at Fisheries and Aquaculture Lab, Department of Zoology, Quaid-i-azam University, Islamabad.

Feed Formulation:

Parsley (*Petroselinum crispum*) was purchased from market in two forms i.e., dried parsley leaves and parsley essential oil. Dry parsley leaves were crushed to make powder. For preparation of feed with 35% protein, all the dry ingredients were crushed and added in a predetermined ratio calculated for feed formulation (Table 4). After mixing all the ingredients, they were grinded and water was added in an amount so that feed was minced easily through feed mill to form pellets. Furthermore, feed was oven dried at 60°C for 24 hours and kept in airtight containers to store at room temperature for daily use. The preparation of feed was done at Fisheries and aquaculture Center, QAU Islamabad.

Parsley Supplementation

Parsley (*P. crispum*) was supplemented in two forms in 35% protein diet for *L rohita*. Parsley leaves and oil were supplemented at concentrations 0,5g/kg,10g/kg,15g/kg and 1ml/kg,2ml/kg and 3ml/kg respectively.

Experimental Design

The experiment was performed in a controlled condition with maintained aeration, dissolved oxygen, pH and temperature in glass aquaria (60x30x30cm^3). About 210 *L rohita* fingerlings of uniform size with an average weight (4.1± 0.02g) were distributed in 21 glass aquaria and randomly divided into seven groups (a feeding trial was conducted in triplicate). Fish were distributed in each glass aquaria at the stocking density of 1.5gL^{-1} (n=10). All of the glass aquaria were covered with net and secured with clips in order to avoid any damage due to jumping behavior of *L rohita* fingerlings. feeding trial was conducted in triplicate. During the experiment, the unused feed was siphoned out and 25% of water was changed regularly at alternate days. *L rohita* fingerlings were provided with parsley supplemented diets twice a day at the rate of 5% of the total body weight.

Growth Performance

After 75 days of feeding, fish from each aquarium were captured and quickly weighed using weighing balance. Other factors that contribute to growth performance were calculated using formulae.

Weight gain (g) = Wf -Wi

WG% = Wf – Wi /Wi × 100

SGR% = *ln* Wf - *ln* Wi / Number of days of experiment ×100

ADWG (g)= Wf – Wi /Number of experimental days

FCR= Total feed consumed (g) / Total wet weight gain (g)

(Where Wf is Final Body Weight, Wi is Initial Body Weight, ADWG is Average Daily Weight Gain SGR is Specific Growth Rate & FCR is Feed Conversion Ratio)

Fish Dissection and Sampling

After 75 days of feeding trial, fingerlings were starved 24 hours before sampling. For different parameters to be measured, sampling was done.

Hematological Parameters

The blood samples from caudal vein were taken using 1ml heparinized syringes and collected in EDTA VACUETTE® K3 tubes from control and all treated groups of *L. rohita* and were analyzed on Automatic analyzer for determination of RBCs, WBCs, Hematocrit, Hemoglobin, Mean corpuscular Volume, Mean corpuscular hemoglobin concentration and Mean corpuscular hemoglobin. Pool the blood of 2 fishes from each aquarium to analyze the complete hematological indices.

Respiratory Burst Activity

Respiratory burst activity was measured using fresh heparinized blood by following Anderson and Siwicki (1995) method. This method is colorimetric determination of free oxygen radicals or ROSs, produced by leukocytes. In this activity, the nitro blue tetrazolium reduces into dark blue precipitate with in the phagocyte, known as formazan granules. Take 0.1ml fresh blood and mix it with 0.1ml NBT solution (0.2%), then incubate the mixture at 25°C for 30 minutes. Pipette out 50ul incubated NBT and blood suspension and pour it into fresh test tube containing 1000 µL(1mL) of NN Dimethyl Formamide followed by the centrifugation of test tubes at 3000×g for 5 minutes. After centrifuge, supernatant was collected and by shifting it in glass cuvette, the absorbance was checked at 540 nm using spectrophotometer. During this procedure, the blank test tube consists of same components and steps except the blood, which was replaced by distilled water was used as reference.

Total Proteins Determination

To determine the total protein in blood plasma of experimental feeding groups of experiment, Lowery *et al.,* (1951) method was adopted. For this purpose, Standard proteins BSA (bovine serum albumin1:1) solution preparation was done by dissolving 1mg of BSA in 1 ml of D-H2O in fresh and clear test tube. Standards were prepared from the stock solution by making dilutions with distilled water so that the volume of every solution was reached to 5 ml in each test tube. After the preparation of various dilutions, 0.2 ml (2000 µl) proteins were pipetted out to different test tubes and 0.2 ml (2000 µl) of Alkaline CuSO4 reagent was added to test tubes, mixed by shaking gently and incubation of tubes was done at 25°C for 10 minutes. Then 0.2 ml (200 µl) of Folin-Ciocaltaeu's reagent solution was added in each tube and again incubated at 25°C for 30

minutes. After second incubation absorbance of the mixture was recorded at 660 nm by spectrophotometer. Then standard calibration curve of absorbance values was plotted against proteins concentration. After that unknown sample absorbance was checked using UV-Visible spectrophotometer and concentration of total proteins in the unknown sample was determined using a standard curve plot.

Total serum proteins (%) = Absorbance of sample / Absorbance of standard×6

Albumin, Globulin, and A: G ratio Determination

The albumin content in the blood was measured following the protocol mentioned by bromocresol methodology using the commercial kit Lab test Diagnóstica 3.A (Lagonis, Minas Gerais, Brazil). Total globulin from serum was obtained by subtracing total albumin from total protein. The albumin to globulin (A: G) ratio was also determined.

Plasma Immunoglobulin IgM

Blood plasma was used for the determination of plasma immunoglobulin level. Using sterilized syringe, blood was taken and collected in EDTA tubes. Then, blood in the EDTA tubes was centrifuged at 1500 x g for 10 minutes. The resultant supernatant (cells) was removed and then centrifuged at 2000x g for 15 minutes to deplete the platelets in the plasma sample. The resulting supernatant were considered as blood plasma. For determination of the immunoglobulin level in plasma, we adopted Anderson and Siwicki (1995) methodology. Mix 100ul (0.1ml) of plasma with 100ul (0.1ml) of 12% polyethylene glycol (1:1) for separation of immunoglobulin by precipitation from plasma. The solutions were incubated at 25°C on incubator shaker under constant shaking for 2 hours and then centrifuged at 7000×g for 10 minutes. OD of proteins contents was measured at 660 nm using spectrophotometer. The total IgM level was calculated by subtracting proteins of the supernatant from the total proteins in the plasma.

Total IgM = Proteins content in plasma - Protein contents in supernatant

Immunological Parameters

For immunological tests blood of 3-4 fingerlings from each feeding group was collected in SST (Serum Separation Tubes) tubes. The blood was allowed to rest for 30 minutes in these tubes to clot at room temperature. Then these tubes were centrifuged at 1000-2000 g for 10 minutes at

cold temperature. Then the resultant supernatant, serum was collected in separate Eppendorf tubes and stored at 4°C, which is used for different immunological tests.

Lysozyme Activity

Using blood serum, the lysozyme activity was determined by following modified Anderson and Siwicki (1995) method. Briefly 100 µL of serum was mixed in fresh and clear test tubes with 900 µL of Micrococcus lysideikticus suspended in phosphate buffer saline (6.2 pH) (Sigma, St Louis, MO, USA). The 900 µL of bacterial was taken from the stock suspension containing 750 µg/ml Micrococcus lysodeikticus. After mixing of serum with bacteria, absorbance change of the reaction was measured at 450 nm via spectrophotometer after 1minute interval for 10-minutes. Activity of lysozyme was calculated by the, use of standard hen egg white lysozyme (Sigma-Aldrich).

Phagocytic Activity

Nitro blue-tetrazolium dye (NBT) method, described by (Anderson & Siwicki, 1995) was used for the determination of free oxygen radicals produced from the phagocytes present in the blood. Briefly, for the determination of phagocytic activity, S. aureus (Sigma, St Louis, MO, USA) was purchased from a local supplier.

The assay was conducted by pipetting 100 µL heparinized blood in a microwell plate, subsequently added 100 µL of killed S. aureus 1×107 cells along with 100 µL PBS (pH 7.2) in each well. After well mixing the solution was left for 30 min at room temperature. Afterward, 5 µL solution of each well was used for making smear on a clean glass slide. The smear was air- dried and mixed in 95% ethanol for 5 minutes and again air-dried. Two smears per sample were prepared and stained with 7% Giemsa stain. The prepared slides were observed under a microscope with oil emersion and enumerated phagocytes, phagocytic cells with ingested bacteria. For the determination of Phagocytic activity (PA) and Phagocytic index (PI), 100 phagocytes per slide were observed and the following standard formulas were adopted.

Phagocytic ratio (PR) = No. of phagocytic cells with engulfed bacteria /No of phagocytes

Phagocytic Index (PI) =No. of engulfed bacteria / Phagocytic cells

Antioxidants Enzymes Determination

For determination of antioxidant enzymes in gills and liver tissues, was homogenized by Dounce manual homogenizer (Sigma, Aldrich) in 100 mmol potassium phosphate buffer containing 1 mmol EDTA. Homogenate was centrifuged at 12000×g for 30 minutes at 4°C. Pellets were discarded, supernatant was collected, and different aliquots were formed in Eppendorf tubes and subsequently stored at -20°C until antioxidants enzymes determination.

Superoxide Dismutase (SOD) Assay

The SOD activity was determined by using modified Kakkar *et al*., (1984) method. Reaction contains 0.3 ml supernatant, 1.2 mL (0.052 mM) sodium pyro-phosphate buffer (pH=7.0)] and 0.1 mL (186 µM) phenazine methosulphate solution. Reaction was started by addition of 0.2 mL (780 µM) NADH+ solution and stopped after 1 minute by the addition of 1 mL of glacial acetic acid. The resulting chromogen was measured by spectrophotometer at 560 nm absorbance wavelength.

One unit of SOD is the quantity of enzymes per protein mg which inhabits the quercetin oxidation reaction by 50% of maximal inhibition. The results of SOD activity were expressed in µmoles/ min/ mg protein using 6.22×103 / M cm as a molar coefficient.

Catalase (CAT) Assay

The catalase activity was determined following Chance and Maehly method (1955). 0.1 ml (100 µl) 0f supernatant, 2.5 ml of 50 mM phosphate buffer (pH=5.0) and 0.4 ml (400 µl) of 5.9 mM hydrogen peroxides were mixed. Absorbance in reaction mixture was observed via spectrophotometer set at 240 nm wavelength after 1minute interval.

The result of CAT was expressed as nmol/ min/ mg protein using 43.6/ M cm as a molar coefficient.

Per-oxidase (POD) assay

POD activity was determined using Chance and Maehly method (1955) Followed by Bibi (2012). 2.5 ml of 50 mM PBS (pH 5.0), 0.3 ml of 40 mM H2O2, 0.1 ml of 20 mM Guaiacol and 0.1 ml of supernatant were mixed for reaction. Absorbance was recorded at 470 nm after 1 min by spectrophotometer.

Activity of POD was measured as nmol min-1mg-1 protein using 2.66×104 / M cm as a molar coefficient.

Lipid peroxidation (LPO/ TBARS) Assay

LPO activity was determined by following Wright *et al*., (1981) methodology. 1.0 ml reaction mixture was prepared by 0.2 ml (200µl) supernatant, 0.58 ml (580µl) phosphate buffer (0.1 M, pH 7.4), 0.02 ml (20 µl) ferric chloride (100mM) and 0.2 ml (200 µl) ascorbic acid (100 mM) mixing. Then water bath solution was incubated for one hour at 37°C and then 1.0 ml of trichloroacetic acid 10% was added for stopping reaction. Then, 1.0 ml of thio-barbituric acid (TBA) was added, in water bath all the tubes were boiled for 20 mins. Then the tubes were cooled subsequently in cooled ice bath and centrifuged at 2500×g for 10 min. Absorbance at 535 nm wavelength via spectrophotometer was recorded after 1 min interval.

LPO activity was expressed as nM TBARS/ min/ mg tissue at 37°C using 1.56× 105 / M cm as a molar extinction coefficient.

Enzyme Analysis

After 75 days, six fish from each aquarium were collected, anaesthetized immediately with clove oil and dissected on the ice pad and intestine of each fish removed for digestive enzyme analysis. Using hand held glass homogenizer, the intestinal contents (1g) suspended in 10 mL phosphate buffer (pH 7.5) were homogenized. Then centrifugation of the homogenate was done at 15000 rpm for 15 minutes at 4ºC. The resultant supernatant was collected in falcon tubes and stored at 4ºC till further analysis.

Determination of Protease Activity:

By dissolving 0.65 g of casein in water and incubating it for 5 minutes at 37 °C, a solution of 0.65% casein was prepared in order to test the protease activity. The casein solution was then mixed with 1 mL of the enzyme solution and heated at 37 °C for an additional 10 minutes. The addition of 5 mL of a 110 mM TCA solution lead to termination of the reaction. The mixture was then allowed to cool at room temperature for 30 minutes before being filtered through a Whatmann filter paper. A 10 mL test tube containing 2 mL of the filtered solution, 1 mL of 0.5 mM Folin-

Ciolcaltea's reagent, and 5 mL of 500 mM Na_2CO_3 was incubated for 30 minutes at 37°C. After cooling of mixture, the absorbance was checked at 660 nm using a UV-Visible spectrophotometer.

Determination of Amylase Activity:

The amylase activity was assessed using the 3,5-Dinitrosalicylic acid (DNS) method, which was modified from the method developed by Bernfeld (1955) and adopted by Areekijseree *et al*. (2004). The evaluation of reducing sugars was done by measuring the absorbance at 560 nm using maltose as the standard. After that, 1 mL of DNS reagent was added, and the mixture was incubated for around 5 minutes in a boiling water bath. 10 mL of reagent grade water was added once the solution had cooled to room temperature, and the absorbance at 540 nm was measured using a spectrophotometer. The quantity of enzyme in one milli-litre of filtrate that releases one microgram of reducing sugar per minute is known as an amylase unit.

Lipase Activity

The approach described by (Hayashi & Topple, 1970) was used to measure the specific activity of lipase. A mixture of 0.25 ml of supernatant, 1 ml of phosphate buffer (pH 7), and 0.25 ml of olive oil was incubated for 15 min at 30 °C. After the reaction mixture had stood for 10 minutes, it was well shaken before 2 ml of ATC reagent and 10 ml of chloroform were added. Then, a 2ml pipette of the chloroform layer was taken out. One ml of the lipase coloring agent was added. The color's wavelength was 55 nm.

Gene Expression Study

For evaluating the expression of genes related to the growth and immunity, the separated 4 fish per group were dissected and approximately 50 mg liver tissues per fish was taken aseptically in RNA latter.

RNA Extraction

Liver tissue samples were taken out of RNA latter and thawed on ice for 10-15 min. About 50 mg of sample was homogenized in microcentrifuge tubes with the help of pellet pestles and added 0.5 mL of chilled Trizol Reagent. The homogenate was then incubated at room temperature for 5 minutes. The homogenate was shaken vigorously for 15 seconds after adding 0.1 mL chloroform. The sample was incubated for 5 min at room temperature before being centrifuged at

12,000g for 15 minutes at 4°C. The upper colorless aqueous layer was separated after centrifugation with wide diameter micro-tips and transferred to a new microcentrifuge tube. To the separated aqueous phase, 0.25 mL of chilled isopropyl alcohol (absolute) was added and vortex briefly. The samples were incubated at room temperature for 10 min before being centrifuged at 12,000g at 4°C for 10 min. The liquid phase was removed, and the pellets were rinsed twice with 0.5 mL of 75% ethanol (prepared in DEPC treated water). After air-drying the pellets, they were dissolved in 30 µL of nuclease-free water. The extracted RNA was kept at -80°C until it was needed.

RNA Quantification

The quality i.e., purity of isolated RNA and quantity of RNA in the sample were assessed TM by using Nano-Drop (ND-1000, UV-visible spectrophotometer, Thermo-Scientific, USA). For quantification, Nano-Drop was set at X 260nm and sample absorbance was noted, while for checking the purity of samples two readings one at X 260 nm and other at 7. 280 nm were taken and then the ratio of OD at both wavelengths (260 nm and 280 nm) was calculated for observing the quality of RNA in the sample with expected values between 1.9 and 2.0.

cDNA Construction

The isolated RNA of each sample was reverse transcribed to cDNA by adopting *Murtaza et al.* (2020). For each individual sample, 8 µL RNA, 4 µL Buffer, 1 µL dNTPs, 0.5 µL RNase inhibitor, 2.5 µL Random primer, 1 µL MMLV-RT and 3 µL H2O were added making the final volume of 20 µL. After adding these chemicals, samples were kept in the water bath at 37°C for one hour and after that kept at 55°C in the PCR machine (BIO-RAD T100TM thermal cycle) for 5 minutes. After which samples were taken to nano drop and were checked for the concentration and quality of cDNA and stored at -20°C.

Primer Designing

For the quantitative analysis of genes, specific primer pairs of each gene (Table 1) were designed through Oligo Explorer 1.1.2 software using an mRNA sequence of respective genes from NCBI (http://www.ncbi.nlm.nih.gov) and manufactured from Humanizing Genomics Macrogen (Ahmad *et al*., 2020). *β*-actin was used for the normalization of the sample and as a positive control. The quality of cDNA and its compatibility with primers were tested by performing PCR of each

sample in duplicate at least two independent runs, while the specificity of each primer was confirmed by raining a simple PCR reaction and performing gel electrophoresis.

For amplification reaction, 15 µL reaction mixture was made by combining 0.5 µL of cDNA, 0.3 µL of forward and reverse primers, 0.3 µL of DNTPs, 0.09 µL of Taq polymerase, 1.5 µL of 10x PCR buffer, and 12 µL of PCR water (Sure Bio-Diagnostic and Pharmaceutical) (Table 2). A gradient thermal cycler PCR was used to perform the PCR reaction (BIO-RAD TTM thermal cycler). The PCR process consisted of initial denaturation at 94°C for 15 min was followed by 35 PCR cycles at 94°C for 30 sec, then annealing at a lower temperature, i.e., at 55°C for 30 sec; followed by extension at 72°C for 2 min.

RT-qPCR

Quantitative real-time qPCR was utilized to investigate the relative expression of the myogenic regulatory factor Myogenin genes in the muscle. Myogenin primers were used to amplify cDNA. For each studied gene, PCR conditions and cycle numbers were optimized. Each RT-qPCR reaction was carried out in a 20 µl reaction mixture containing 1.6 µL of diluted cDNA, 0.4 µL of forward and reverse primers, 10 µL of SYBER green, and 7.6 µL of water (Table 3). As a positive control, B-actin was used for normalization of the sample. A standard of 10 µL was prepared for each PCR reaction. Initial denaturation at 94°C for 1 min was followed by 40 cycles at 95°C for 15 seconds and then 62°C for 15 seconds in the RT-qPCR conditions.

Table 1: Target genes and sequences of primers

Gene	Primer Sequences	TM	GC%	Accession No.
B-actin	F<AAGGGAGGTATTGTGGGTAAAC	57.69	45.45	XM-051108628.1
	R<GTTGTCCTGGCACTCAATCT	57.81	50	
Myogenin	F<TGAGGTCCCTGACGTCTATT	57.47	50	XM-051122763.1
	R<CCATCACCCTCCTCGTTTATTT	58.11	45.45	

Table 2: Reaction mixture for simple polymerase chain reaction

Reagents	Volume (µL)
cDNA	0.5
Forward primer	0.3
Reverse primer	0.3
10x PCR buffer	1.5
DNTPs	0.3
Taq Polymerase	0.09
PCR water	12
Total reaction Volume	**15**

Validation of qPCR Primers

A PCR reaction was performed in duplicate to check the specificity of each gene's primer and a standard curve was constructed using a dilution series. Three dilutions (1/10, 1/100, and 1/1000) were made using pooled cDNA (cDNA from all samples), and each dilution was conducted in triplicate.

The efficiency of primer was calculated using the equation below.

$$E\ (\%) = (10^{-1/slope} - 1) \times 100$$

The conventional AACT method (Pfaff, 2001) was used to calculate the relative variation in gene expression.

Table 3: Reaction mixture for RT-qPCR

Reagents	**Volume (µL)**
Diluted cDNA	1.6
Forward primers	0.4
Reverse primers	0.4
SYBER green	10
Nuclease free water	7.6
Total volume	**20**

Statistical analysis

Before performing statistical analysis, all the data was normalized. Growth performance, hematological indices, immunological parameters, antioxidants and intestinal enzymes results were expressed as Mean ± SEM. All the experimental data was analyzed by One-way ANOVA followed by the LSD post hoc test through IBM SPSS Statistics 21 and Statistics Version 8.1 software. GraphPad Prism software version 8.0.2 was used to plot the graphs.

RESULTS

Growth Performance

Parsley leaves supplemented diets showed a significant effect on the growth performance of advanced fry of *Labeo rohita* as shown in Table 3. Statistical analysis of results by adopting One-way ANOVA indicated a significant effect of parsley supplemented diets at different dosage level on final body weight (n=3, $F_{3,8}$= 14.05; P<0.001), %WG (n=3, $F_{3,8}$ = 11.7; P<0.002), %SGR (n=3, $F_{3,8}$ =12.5; P<0.002) and ADWG (n=3, $F_{3,8}$ =14.03; P<0.001) among all experimental groups of fish (Table 6). Pair-wise comparison of results indicated significantly highest %WG in PL_{15} supplemented group of fish followed by PL_{10} while lowest in control group of fish. Moreover, a significant effect was observed on the growth performance of fish reared on diets supplemented with seed oil extract of parsley at different dosage levels on final body weight (n=3, $F_{3,8}$ =15.2; P<0.001), %WG (n=3, $F_{3,8}$ = 17.5; P<0.001), %SGR (n=3, $F_{3,8}$ =19.5; P<0.001) and ADWG (n=3, $F_{3,8}$ =15.24; P<0.001) among all experimental groups. Pair-wise comparison of results showed highest %WG in PO2 supplemented group of fish followed by PO3 while lowest in control group of fish (Table 7).

Furthermore, the statistical analysis using one-way ANOVA showed a significant difference on FCR of leave supplemented fish (n=3, $F_{3,8}$=15.14, P<0.001) among all experimental groups. The pair-wise comparison of results indicated significantly lowest and best FCR in PL_{15} supplemented group of fish followed by PL_{10} and PL_5 supplemented groups, while highest in control group of fish (Table 6). Similarly, significant FCR (n=3, $F_{3,8}$=15.95, P<0.001) was observed in oil supplemented fish among all experimental groups. The pair-wise comparison showed lowest and best FCR in PO_2 supplemented group followed by PO_3 and PO_1 supplemented groups, while highest in control group of fish (Table 7).

Overall comparison among all the parsley diet groups using one-way ANOVA revealed a significant difference in weight gain (n=3, $F_{6,14}$=9.06; P<0.0004) among all the experimental groups of fish. The pair-wise comparison showed highest weight gain in PL_{15} as compared to other diet groups (Figure 1).

Hematological Indices

Dietary parsley supplementation showed significant effect on hematological indices of *L. rohita* fingerlings. Statistical analysis of results by adopting one-way ANOVA indicated a significant effect of parsley leave supplemented diets at different dosage level on RBC (n=9, $F_{3,32}$=230; P<0.000), WBCs count (n=9, $F_{3,32}$=19.5; P<0.001), HCT% (n=9, $F_{3,32}$ = 184; P<0.000) and levels of HGB (n=9, $F_{3,32}$ =89.8; P<0.000), MCV (n=9, $F_{3,32}$ =184; P<0.000), MCH (n=9, $F_{3,32}$ =95.5; P<0.000) and MCHC (n=9, $F_{3,32}$=46.9; P<0.001) among all parsley leave diet groups of fish. The pair-wise comparison showed the highest values of hematological indices in PL_{15} supplemented fish followed by PL_{10} supplemented fish while lowest in control group of fish (Table 8). Moreover, a significant effect was observed on the hematological indices of fish reared on diets supplemented with seed oil extract of parsley at different dosage levels on RBC count (n=9, $F_{3,32}$=272; P<0.000), WBCs count (n=9, $F_{3,32}$=8.74; P<0.002), HCT% (n=9, $F_{3,32}$ = 205 ; P<0.000) and levels of HGB (n=9, $F_{3,32}$ =67.4; P<0.000), MCV (n=9, $F_{3,32}$ =245; P<0.000), MCH (n=9, $F_{3,32}$=210 ; P<0.001) and MCHC (n=9, $F_{3,32}$ =25.6 ; P<0.000) among all experimental groups of fish. The pair-wise comparison showed the highest values of hematological indices in PO_2 group as compared to other diet groups (Table 9).

Comparison between all parsley leave and oil supplemented groups showed significant difference in RBC count (n=9, $F_{6,56}$=174; P<0.000). The pair-wise comparison showed the highest RBC count in PL_{15} supplemented group of fish (Figure 2).

Non-Specific Immunological Indices

Dietary parsley supplementation considerably affected the non-specific immunological indices of *L rohita* fingerlings. Statistical analysis of results by adopting One-way ANOVA indicated a significant effect of parsley leave supplemented diets at different dosage level on plasma protein (n=9, $F_{3,32}$ =81.72; P<0.000), IgM level (n=9, $F_{3,32}$ =282.04; P<0.001), Albumin (n=9, $F_{3,32}$ =84.85; P<0.000), Globulin (n=9, $F_{3,32}$ =73.95 ; P<0.000), serum lysozyme activity (n=9, $F_{3,32}$ =90.71; P<0.000), Phagocytic activity (n=9, $F_{3,20}$=21.48; P<0.001), and NBT activity (n=9, $F_{3,32}$ =17.89; P<0.001) among all parsley leave supplemented groups of fish. The pair-wise comparison showed highest values of non-specific immunological indices in PL_{15} compared to other supplemented and control groups of fish (Table 10).

Moreover, a significant effect was observed on the non-specific immunological indices of fish reared on diets supplemented with seed oil extract of parsley at different dosage levels on plasma protein (n=9, $F_{3,32}$ =30.21; P<0.002), IgM level (n=9, $F_{3,32}$ =60.3; P<0.000), Albumin (n=9, $F_{3,32}$ =84.8; P<0.000), Globulin (n=9, $F_{3,32}$ =27.8; P<0.001), serum lysozyme activity (n=9, $F_{3,32}$ =22.4; P<0.001), Phagocytic activity (n=9, $F_{3,32}$ =45.8; P<0.000) and NBT activity n=9, $F_{3,32}$ =261.3; P<0.001). The pair-wise comparison showed highest values of non-specific immunological indices in PO_3 (Table 11).

Comparison between all parsley leave and oil supplemented groups showed significant difference in respiratory burst activity (n=9, $F_{6,56}$=178.9; P<0.000). The pair-wise comparison showed the highest respiratory burst activity in PO_2 supplemented group of fish (Figure 2).

Antioxidant Activity

Dietary parsley supplementation showed a significant effect on the liver antioxidant enzyme activity of *L. rohita* fingerlings. Statistical analysis of results by adopting One-way ANOVA indicated a significant effect of parsley leave supplemented diets at different dosage level on SOD (n=3, $F_{3,8}$ =85.92; P<0.000), POD (n=3, $F_{3,8}$ =111.4; P<0.000), CAT (n=3, $F_{3,8}$ =195.8; P<0.000) and LPO (n=3, $F_{3,8}$ =376.2; P<0.000). Pair-wise comparison showed significantly high SOD, POD, CAT activity and LPO activity in control group of fish followed by PL_5 and PL_{10} supplemented groups while low in PL_{15} supplemented group of fish (Table 12).

Moreover, a significant effect was observed on the antioxidant activity of fish reared on diets supplemented with seed oil extract of parsley at different dosage levels on SOD (n=3, $F_{3,8}$=58.02; P<0.001), POD (n=3, $F_{3,8}$=99.1; P<0.000), CAT (n=3, $F_{3,8}$=70.5; P<0.001) and LPO (n=3, $F_{3,8}$=257.6, P<0.000). Pair-wise comparison showed significantly high SOD, POD, CAT activity and LPO activity in control group of fish followed by PO_1 and PO_3 supplemented groups while low in PO_2 supplemented group of fish (Table 13).

Intestinal Enzyme Analysis

Dietary parsley supplementation showed a significant effect on the intestinal protease, lipase and amylase activities of *L. rohita* fingerlings. One-way ANOVA showed significant differences in protease (n=9, $F_{3,32}$=336.9 P<0.001), lipase (n=9, $F_{3,32}$=60.6; P<0.001) and amylase activity (n=9, $F_{3,32}$=135.3; P<0.001) among all parsley leave supplemented groups of fish. A pair-wise comparison

indicated the highest protease and amylase and lowest lipase activity in a PL_{15} group of fish (Table 14). Moreover, a significant effect was observed on the enzyme activity of fish reared on diets supplemented with seed oil extract of parsley at different dosage levels on protease (n=9, $F_{3,32}$=511.1; P<0.000), lipase (n=9, $F_{3,32}$=135.6; P<0.001) and amylase activity (n=9, $F_{3,32}$=5.13; P<0.005) among all experimental groups of fish. Pair-wise comparison showed highest protease and lipase activity in PO_3 supplemented group whereas amylase activity was noted to be non-significant in oil supplemented groups (Table 15).

Gene Expression

The mRNA expression level of Myogenin gene in the liver tissues of *L. rohita* fingerlings fed parsley supplemented diets for 75 days are shown in (Figure 4&5). Statistical analysis by adopting One way ANOVA showed significant differences (P<0.05) in expression level of Myogenin gene among all parsley- supplemented experimental groups of fish. Pair-wise comparison showed significant upregulation in relative expression of Myogenin gene compared to the house-keeping B-actin gene in PL_{15} group of fish followed by PL_{10}, PL_5 and control group of fish (Figure 4). Similar to it, a pair-wise comparison showed significant upregulation in relative expression of Myogenin gene compared to the house-keeping B-actin gene in PO_2 group of fish followed by PO_3, PO_1 and control group of fish (Figure 5).

Table 4: Formulation of 35% CP diet for *Labeo rohita* fingerlings with Parsley (*Petroselinum crispum*) supplementation at different dosage levels

Feed ingredients	Control	PL_5	PL_{10}	PL_{15}	PO_1	PO_2	PO_3
Fish meal	22	22	22	22	22	22	22
Soybean meal	15	15	15	15	15	15	15
Rice bran	14	14	14	13.5	14	13	13
Wheat bran	15	14.5	14	14	14	14	13
Corn gluten	13	13	13	13	13	13	13
Sunflower meal	11	11	11	11	11	11	11
Fish oil	5	5	5	5	5	5	5
Mineral premix [a]	1	1	1	1	1	1	1
Vitamin premix [b]	1	1	1	1	1	1	1
Vitamin C	1	1	1	1	1	1	1
CMC	2	2	2	2	2	2	2
Parsley leave	0	0.5	1	1.5	0	0	0
Parsley essential oil	0	0	0	0	1	2	3
Total	100	100	100	100	100	100	100

[a] Composition of mineral premix /kg: manganese, 53 g; zinc, 40 g; iron, 20 g; copper, 2.7 g; iodine, 0.34 g; selenium, 70 mg; cobalt, 70 mg and calcium carbonate as carrier up to 1 kg.
[b] Composition of vitamin premix /kg: vitamin A, 8,000,000 IU; vitamin D3, 2,000,000 IU; vitamin E, 7000 mg; vitamin K3, 1500 mg; vitamin B1, 700 mg; vitamin B2, 3500 mg; vitamin B6, 1000 mg; vitamin B12, 7 mg; biotin, 50 mg; folic acid, 700 mg; nicotinic, 20,000 mg; pantothenic acid, 7000 mg.
Control = 35% CP diet without parsley supplementation, **PL_5** = 5g/kg parsley leave powder in diet, **PL_{10}**= 10g/kg parsley leave powder in diet, **PL_{15}** = 15g/kg parsley leave powder in diet, **PO_1** = 1ml/kg parsley oil in diet, **PO_2** = 2ml/kg parsley oil in diet and **PO_3** = 3ml/kg parsley oil in diet.

Table 5: Proximate composition of different diets fed to *Labeo rohita* fingerlings

Proximate composition (%)							
Parameters	**Control**	**PL_5**	**PL_{10}**	**PL_{15}**	**PO_1**	**PO_2**	**PO_3**
Crude protein	35.14	35.11	35.08	35.06	35.08	35.04	34.98
Lipid	5.1	5.1	5.1	5.1	5.9	6.1	6.3
Fiber	5.7	6.2	6.8	7.2	5.8	5.8	5.8
Ash	7.2	7.5	7.7	7.9	7.3	7.3	7.3
NFE [a]	46.86	46.09	46.12	44.74	45.92	45.76	45.62

[a] Nitrogen free extract = 100 – (crude protein + lipid + fiber + ash)

Control = 35% CP diet without parsley supplementation, **PL_5** = 5g/kg parsley leave powder in diet, **PL_{10}**= 10g/kg parsley leave powder in diet, **PL_{15}** = 15g/kg parsley leave powder in diet, **PO_1** = 1ml/kg parsley oil in diet, **PO_2** = 2ml/kg parsley oil in diet and **PO_3** = 3ml/kg parsley oil in diet.

Table 6: Growth performance of *Labeo rohita* fingerlings after 75 days feeding of basal & experimental diets supplemented with graded levels of Parsley (*Petroselinum crispum*) leave powder

Parameters	Control	PL_5	PL_{10}	PL_{15}	F value	P value
IBW (g)	4.08 ± 0.02^{a}	4.08 ± 0.02^{a}	4.12 ± 0.02^{a}	4.09 ± 0.02^{a}	0.42	0.746
F BW (g)	7.06 ± 0.3^{c}	8.61 ± 0.4^{b}	9.16 ± 0.4^{ab}	9.66 ± 0.3^{a}	14.0	0.001
WG (g)	3.0 ± 0.2^{b}	4.53 ± 0.4^{a}	5.03 ± 0.4^{a}	5.56 ± 0.3^{a}	13.1	0.002
WG%	73.1 ± 4.7^{b}	110.8 ± 5.17^{a}	122 ± 8.7^{a}	136 ± 6.28^{a}	11.7	0.003
SGR%	0.10 ± 0.02^{b}	0.27 ± 0.02^{a}	0.32 ± 0.02^{a}	0.38 ± 0.02^{a}	12.5	0.002
ADWG (g)	0.039 ± 0.002^{b}	0.061 ± 0.002^{a}	0.067 ± 0.004^{a}	0.074 ± 0.003^{a}	14	0.001
FCR	1.95 ± 0.13^{a}	1.27 ± 0.05^{b}	1.15 ± 0.07^{b}	1.04 ± 0.05^{b}	15.14	0.001

Data is presented as Mean ± SEM (n=3) One-way ANOVA followed by LSD post hoc test shows a comparison between growth performance of fish fed parsley leave powder supplements at four graded levels. Superscript letters show a comparison between groups. Means sharing different letters are significantly different from each other ($P < 0.05$).
Control = 35% CP diet without parsley supplementation, **PL_5** = 5g/kg parsley leave powder in diet, **PL_{10}** = 10g/kg parsley leave powder in diet, **PL_{15}** = 15g/kg parsley leave powder in diet.
IBW= Initial Body Weight, **FBW**= Final Body Weight, **WG**= Weight Gain, **WG%**= Weight Gain Percent, **SGR**= Specific Growth Rate, **ADWG**= Average Daily Weight Gain, **FCR**= Feed Conversion Ratio.

Table 7: Growth performance of *Labeo rohita* fingerlings after 75 days feeding of basal & experimental diet supplemented with graded levels of Parsley (*Petroselinum crispum*) oil

Parameters	Control	PO_1	PO_2	PO_3	F value	P value
IBW (g)	4.08±0.02^{a}	4.12±0.02^{a}	4.12±0.02^{a}	4.11±0.02^{a}	0.42	0.745
FBW (g)	7.06±0.3^{c}	8.53±0.3^{b}	9.61±0.3^{a}	9.19±0.2^{ab}	15.2	0.001
WG (g)	3.0±0.2^{c}	4.41±0.3^{b}	5.49±0.3^{a}	5.08±0.2^{ab}	16.5	0.001
WG%	73.1±4.7^{c}	107±5.58^{b}	133.4±4.68^{a}	115.1±5.53^{ab}	17.5	0.001
SGR%	0.10±0.02^{c}	0.25±0.02^{b}	0.38±0.02^{b}	0.33±0.02^{ab}	19.5	0.001
ADWG(g)	0.039±0.002^{c}	0.058±0.003^{b}	0.067±0.002^{a}	0.073±0.003^{ab}	16.46	0.001
FCR	1.94±0.13^{a}	1.31±0.06^{b}	1.05±0.03^{b}	1.13±0.05^{b}	15.95	0.001

Data is presented as Mean ± SEM (n=3) One-way ANOVA followed by LSD post hoc test shows a comparison between growth performance of parsley oil supplements at four different dosage levels. Superscript letters show a comparison between groups. Means sharing different letters are significantly different from each other ($P < 0.05$).

Control = 35% CP diet without parsley supplementation, **PO_1** = 1ml/kg parsley oil in diet, **PO_2** = 2ml/kg parsley oil in diet and **PO_3** = 3ml/kg parsley oil in diet.

IBW= Initial Body Weight, **FBW**= Final Body Weight, **WG**= Weight Gain, **WG%**= Weight Gain Percent, **SGR**= Specific Growth Rate, **ADWG**= Average Daily Weight Gain, **FCR**= Feed Conversion Ratio.

Table 8: Hematological Indices of *Labeo rohita* fingerlings after 75 days feeding of basal & experimental diets supplemented with graded levels of Parsley (*Petroselinum crispum*) leaves powder

Parameters	Control	PL_5	PL_{10}	PL_{15}	F value	P value
RBC ($\times10^6$/µL)	1.21 ± 0.01^d	1.32 ± 0.01^c	1.38 ± 0.01^b	1.48 ± 0.01^a	230	0.001
WBC ($\times10^3$ /µL)	112.6 ± 0.6^b	113.3 ± 0.3^b	115 ± 0.3^a	116.1 ± 0.1^a	19.5	0.000
HGB (g/dl)	6.49 ± 0.01^d	6.7 ± 0.01^c	6.8 ± 0.01^b	6.97 ± 0.01^a	89.8	0.001
HCT (%)	16.4 ± 0.01^d	16.5 ± 0.01^c	16.7 ± 0.01^b	16.8 ± 0.01^a	184	0.001
MCV (fL)	135.6 ± 0.3^a	125.5 ± 0.3^b	120.7 ± 0.1^c	114 ± 0.2^d	184	0.001
MCH (Pg)	53.6 ± 0.1^a	50.8 ± 0.1^b	49.1 ± 0.06^c	47.1 ± 0.1^d	95.5	0.001
MCHC (g/dl)	39.5 ± 0.02^d	40.4 ± 0.02^c	40.6 ± 0.01^b	41.3 ± 0.02^a	46.9	0.001

Data is presented as Mean ± SEM (n=9) One-way ANOVA followed by LSD post hoc test shows a comparison between hematological indices of parsley leave supplemented diet on fish at four graded levels. Superscript letters show a comparison between groups. Means sharing different letters are significantly different from each other ($P < 0.05$).

Control = 35% CP diet without parsley supplementation, **PL_5** = 5g/kg parsley leave powder in diet, **PL_{10}** = 10g/kg parsley leave powder in diet, **PL_{15}** = 15g/kg parsley leave powder in diet.

RBC (Red Blood Cell), **WBC** (White Blood Cell), **HGB** (Haemoglobin), **HCT%** (Hematocrit), **MCV** (Mean Corpuscular Volume), **MCH** (Mean Corpuscular Hemoglobin) & **MCHC** (Mean Corpuscular Hemoglobin Concentration).

Table 9: Hematological Indices of Labeo rohita fingerlings after 75 days feeding of basal & experimental diets supplemented with graded levels of Parsley (*Petroselinum crispum*) oil

Parameters	Control	PO_1	PO_2	PO_3	F value	P value
RBC ($\times 10^6$ / µL)	1.21 ± 0.01^{d}	1.34 ± 0.01^{c}	1.47 ± 0.01^{a}	1.41 ± 0.01^{b}	272	0.001
WBC ($\times 10^3$ /µL)	112.6 ± 0.6^{b}	113.6 ± 0.5^{ab}	115.6 ± 0.5^{ab}	114.6 ± 0.8^{a}	8.74	0.04
HGB (g/dl)	6.49 ± 0.01^{d}	6.6 ± 0.01^{c}	6.9 ± 0.01^{a}	6.7 ± 0.01^{b}	67.4	0.001
HCT (%)	16.4 ± 0.01^{d}	16.5 ± 0.01^{c}	16.9 ± 0.01^{a}	16.8 ± 0.01^{b}	205	0.001
MCV (fL)	135.6 ± 0.3^{a}	123.7 ± 0.3^{b}	114.7 ± 0.3^{d}	119.2 ± 0.1^{c}	245	0.001
MCH (Pg)	53.6 ± 0.1^{a}	50 ± 0.1^{b}	46.8 ± 0.1^{d}	47.7 ± 0.09^{c}	210	0.001
MCHC (g/dl)	39.5 ± 0.02^{d}	40.4 ± 0.02^{b}	40.8 ± 0.02^{a}	40.1 ± 0.02^{c}	25.6	0.001

Data is presented as Mean ± SE (n=9) One-way ANOVA followed by LSD post hoc test shows a comparison of hematological indices between dietary levels of parsley oil at four different dosage levels. Superscript letters show a comparison between groups. Means sharing different letters are significantly different from each other ($P < 0.05$).

Control = 35% CP diet without parsley supplementation, **PO_1** = 1ml/kg parsley oil in diet, **PO_2** = 2ml/kg parsley oil in diet and **PO_3** = 3ml/kg parsley oil in diet.

RBC (Red Blood Cell), **WBC** (White Blood Cell), **HGB** (Haemoglobin), **HCT%** (Hematocrit), **MCV** (Mean Corpuscular Volume), **MCH** (Mean Corpuscular Hemoglobin) & **MCHC** (Mean Corpuscular Hemoglobin Concentration).

Table 10: Immunological indices of Labeo rohita fingerlings after 75 days feeding of basal & experimental diets supplemented with graded levels of Parsley (*Petroselinum crispum*) leave powder

Parameters	Control	PL_5	PL_{10}	PL_{15}	F value	P value
Total protein (mg/ml)	5.10 ± 0.01^{d}	6.59 ± 0.01^{c}	7.32 ± 0.01^{b}	8.47 ± 0.01^{a}	81.72	0.000
Albumin (mg/ml)	3.39 ± 0.004^{d}	4.39 ± 0.004^{c}	4.88 ± 0.004^{b}	5.64 ± 0.004^{a}	84.8	0.000
Globulin (mg/ml)	1.70 ± 0.002^{d}	2.19 ± 0.002^{c}	2.44 ± 0.002^{b}	2.82 ± 0.002^{a}	73.95	0.000
Respiratory Burst	0.303 ± 0.001^{d}	0.362 ± 0.001^{c}	0.625 ± 0.001^{b}	0.63 ± 0.001^{a}	17.89	0.000
IgM (mg/ml)	0.911 ± 0.001^{d}	1.21 ± 0.001^{c}	1.25 ± 0.001^{b}	1.39 ± 0.001^{a}	28.2	0.000
Lysozyme activity	7.26 ± 0.12^{d}	7.86 ± 0.17^{c}	8.4 ± 0.15^{b}	8.9 ± 0.11^{a}	90.71	0.000
Phagocytic activity	22.3 ± 1.1^{d}	25.3 ± 1.1^{c}	27.6 ± 1.1^{b}	30.6 ± 1.1^{a}	21.48	0.000

Data is presented as Mean ± SEM (n=9) One-way ANOVA followed by LSD post hoc test shows a comparison between immunological indices of fish supplemented with different dietary levels of parsley leave powder. Superscript letters show a comparison between groups. Means sharing different letters are significantly different from each other ($P < 0.05$).
Control = 35% CP diet without parsley supplementation, **PL_5** = 5g/kg parsley leave powder in diet, **PL_{10}** = 10g/kg parsley leave powder in diet, **PL_{15}** = 15g/kg parsley leave powder in diet.
IgM is Immunoglobulin

Table 11: Immunological indices of Labeo rohita fingerlings after 75 days feeding of basal & experimental diets supplemented with graded levels of Parsley (*Petroselinum crispum*) oil

Parameters	Control	PO_1	PO_2	PO_3	F value	P value
Total protein (mg/ml)	5.10 ± 0.01^{d}	6.83 ± 0.01^{c}	8.76 ± 0.01^{b}	11.398 ± 0.01^{a}	30.2	0.000
Albumin (mg/ml)	3.39 ± 0.004^{d}	4.55 ± 0.004^{c}	5.84 ± 0.004^{b}	7.59 ± 0.004^{a}	84.85	0.000
Globulin (mg/ml)	1.70 ± 0.002^{d}	2.27 ± 0.002^{c}	2.92 ± 0.002^{b}	3.80 ± 0.002^{a}	27.81	0.000
Respiratory Burst	0.303 ± 0.001^{d}	0.472 ± 0.001^{c}	0.727 ± 0.001^{b}	0.80 ± 0.001^{a}	261.3	0.000
IgM (mg/ml)	0.911 ± 0.001^{d}	1.15 ± 0.001^{c}	1.36 ± 0.001^{b}	1.91 ± 0.001^{a}	60.3	0.000
Lysozyme activity	7.26 ± 0.1^{d}	8.2 ± 0.1^{c}	8.9 ± 0.1^{b}	10.6 ± 0.1^{a}	22.4	0.000
Phagocytic activity	22.3 ± 1.1^{c}	27.3 ± 0.9^{b}	30.6 ± 0.9^{a}	32.6 ± 0.9^{a}	45.8	0.000

Data is presented as Mean ± SEM (n=9) One-way ANOVA followed by LSD post hoc test shows a comparison between dietary levels of parsley leaves at four dosage levels. Superscript letters show a comparison between groups. Means sharing different letters are significantly different from each other ($P < 0.05$).

Control = 35% CP diet without parsley supplementation, **PO_1** = 1ml/kg parsley oil in diet, **PO_2** = 2ml/kg parsley oil in diet and **PO_3** = 3ml/kg parsley oil in diet.

IgM is Immunoglobulin

Table 12: Antioxidant activity of *Labeo rohita* fingerlings after 75 days feeding of basal & experimental diets supplemented with graded levels of Parsley (*Petroselinum crispum*) leave powder

Parameters	Control	$PL_{0.5}$	PL_1	$PL_{1.5}$	F value	P value
SOD(µmol/min/mg protein)	43.5±0.2[a]	42.1±0.15[b]	41.1±0.02[c]	39.7±0.02[d]	85.91	0.000
CAT(µmol/min/mg protein)	124.6±0.1[a]	123.4±0.04[b]	122.6±0.1[c]	121.7±0.005[d]	195.8	0.000
POD(µmol/min/mg protein)	79.6±0.04[a]	78.4±0.1[b]	77.5±2.04[c]	73.7±0.05[d]	111.4	0.000
LPO(µmol/min/mg protein)	8.47±0.004[a]	7.82±0.004[b]	7.25±0.004[c]	6.23±0.004[d]	376.2	0.000

Data is presented as Mean ± SE. (n=3) One-way ANOVA followed by LSD post hoc test shows a comparison between antioxidant activity of parsley leave supplemented fish at different dosage levels. Superscript letters show a comparison between groups. Means sharing different letters are significantly different from each other ($P < 0.05$).
Control = 35% CP diet without parsley supplementation, **PL_5** = 5g/kg parsley leave powder in diet, **PL_{10}** = 10g/kg parsley leave powder in diet, **PL_{15}** = 15g/kg parsley leave powder in diet.
SOD = Superoxide Dismutase, **CAT** =Catalase, **POD** = Peroxidase, **LPO** = Lipid Peroxidation.

Table 13: Antioxidant activity of *Labeo rohita* fingerlings after 75 days feeding of basal & experimental diets supplemented with graded levels of Parsley (*Petroselinum crispum*) oil

Parameters	Control	PO_1	PO_2	PO_3	F value	P value
SOD (µmol/min/mg protein)	43.5±0.01[a]	42.6±0.2[b]	40.7±0.03[c]	39.4±0.2[d]	58.02	0.000
CAT(µmol/min/mg protein)	124.6±0.09[a]	122.6±0.05[b]	121.6±0.005[c]	120.5±0.02[d]	70.5	0.000
POD(µmol/min/mg protein)	79.6±0.04[a]	77.9±0.07[b]	76.8±0.04[c]	74.6±0.05[d]	99.1	0.000
LPO(µmol/min/mg protein)	8.62±0.005[a]	8.27±0.005[b]	6.23±0.007[d]	7.32±0.004[c]	2576	0.000

Data is presented as Mean ± SE. (n=3) One-way ANOVA followed by LSD post hoc test shows a comparison between antioxidant activity of parsley oil supplemented fish at different dosage levels. Superscript letters show a comparison between groups. Means sharing different letters are significantly different from each other ($P < 0.05$).

Control = 35% CP diet without parsley supplementation, **PO_1** = 1ml/kg parsley oil in diet, **PO_2** = 2ml/kg parsley oil in diet and **PO_3** = 3ml/kg parsley oil in diet.

SOD = Superoxide Dismutase, **CAT** =Catalase, **POD** = Peroxidase, **LPO** = Lipid Peroxidation.

Table 14: Activity of intestinal digestive enzymes of *Labeo rohita* fingerlings after 75 days feeding of basal & experimental diets supplemented with graded levels of Parsley (*Petroselinum crispum*) leave powder

Digestive enzymes	Control	PL_5	PL_{10}	PL_{15}	F value	P value
Amylase (U/mg)	0.16 ± 0.01^{d}	0.23 ± 0.01^{c}	0.25 ± 0.01^{b}	0.27 ± 0.01^{a}	122	0.001
Protease(U/mg)	0.46 ± 0.01^{d}	0.53 ± 0.01^{c}	0.57 ± 0.01^{b}	0.64 ± 0.01^{a}	184	0.001
Lipase (U/mg)	2.39 ± 0.01^{a}	1.82 ± 0.01^{b}	1.10 ± 0.01^{c}	0.76 ± 0.01^{d}	264	0.001

Data is presented as Mean ± SE (n=9) One-way ANOVA followed by LSD post hoc test shows a comparison between dietary levels of parsley at four graded levels. Superscript letters show a comparison between groups. Means sharing different letters are significantly different from each other ($P < 0.05$).
Control = 35% CP diet without parsley supplementation, **PL_5** = 5g/kg parsley leave powder in diet, **PL_{10}** = 10g/kg parsley leave powder in diet, **PL_{15}** = 15g/kg parsley leave powder in diet.

Table 15: Activity of intestinal digestive enzymes of *Labeo rohita* fingerlings after 75 days feeding of basal & experimental diets supplemented with graded levels of Parsley (*Petroselinum crispum*) oil

Digestive enzymes	Control	PO_1	PO_2	PO_3	F value	P value
Amylase(U/mg)	0.16 ± 0.01^{a}	0.15 ± 0.01^{a}	0.14 ± 0.01^{a}	0.13 ± 0.01^{a}	1.62	0.26
Protease(U/mg)	0.46 ± 0.01^{d}	0.55 ± 0.01^{c}	0.67 ± 0.01^{b}	0.73 ± 0.01^{a}	348	0.001
Lipase (U/mg)	2.39 ± 0.01^{c}	2.72 ± 0.01^{b}	2.73 ± 0.01^{b}	2.96 ± 0.01^{a}	335	0.001

Data is presented as Mean ± SD. (n=9) One-way ANOVA followed by LSD post hoc test shows a comparison between dietary levels of parsley at four graded levels. Superscript letters show a comparison between groups. Means sharing different letters are significantly different from each other ($P < 0.05$).
Control = 35% CP diet without parsley supplementation, **PO_1** = 1ml/kg parsley oil in diet, **PO_2** = 2ml/kg parsley oil in diet and **PO_3** = 3ml/kg parsley oil in diet.

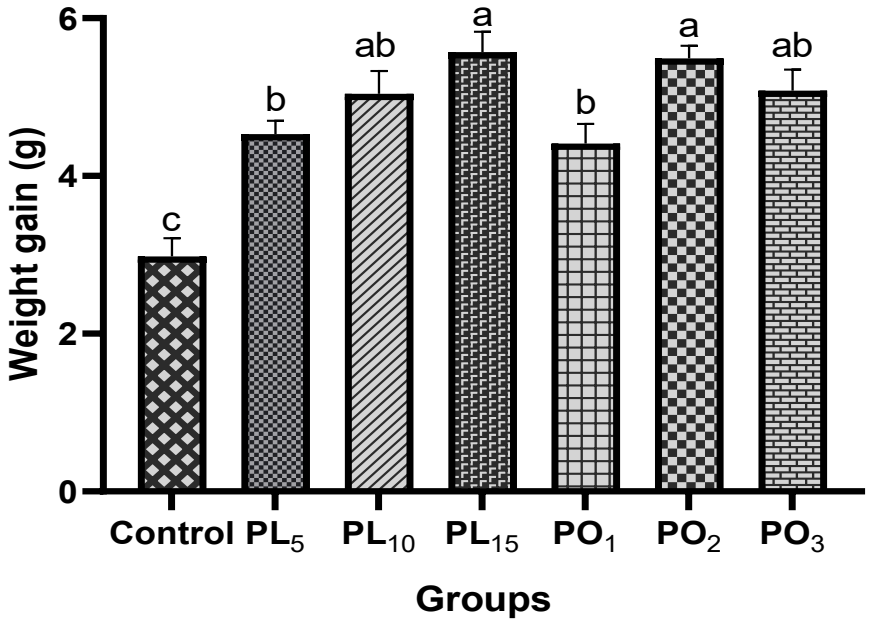

Figure 1: Comparative effect of Parsley (*Petroselinum crispum*) leaves and oil supplemented diet on weight gain of *L. rohita* fingerlings. **Control** = 35% CP diet without parsley supplementation, **PL_5** = 5g/kg parsley leave powder in diet, **PL_{10}**= 10g/kg parsley leave powder in diet, **PL_{15}** = 15g/kg parsley leave powder in diet, **PO_1** = 1ml/kg parsley oil in diet, **PO_2** = 2ml/kg parsley oil in diet and **PO_3** = 3ml/kg parsley oil in diet.

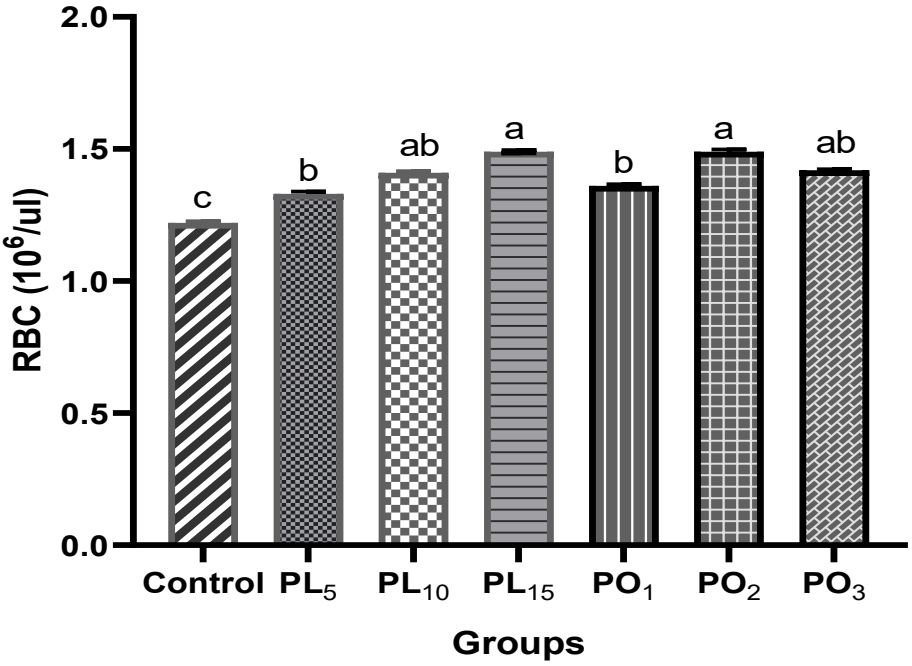

Figure 2: Comparative effect of Parsley (*Petroselinum crispum*) leaves and oil supplemented diet on RBC count of *L. rohita* fingerlings. **Control** = 35% CP diet without parsley supplementation, **PL_5** = 5g/kg parsley leave powder in diet, **PL_{10}**= 10g/kg parsley leave powder in diet, **PL_{15}** = 15g/kg parsley leave powder in diet, **PO_1** = 1ml/kg parsley oil in diet, **PO_2** = 2ml/kg parsley oil in diet and **PO_3** = 3ml/kg parsley oil in diet.

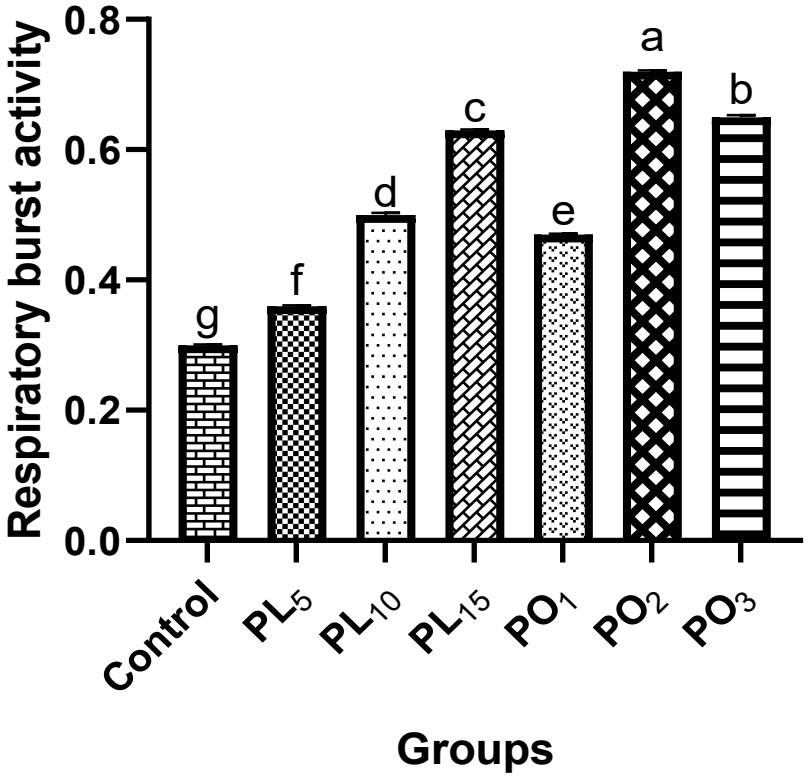

Figure 3: Comparative effect of Parsley (*Petroselinum crispum*) leaves and oil supplemented diet on Respiratory burst activity of *L. rohita* fingerlings. **Control** = 35% CP diet without parsley supplementation, **PL_5** = 5g/kg parsley leave powder in diet, **PL_{10}**= 10g/kg parsley leave powder in diet, **PL_{15}** = 15g/kg parsley leave powder in diet, **PO_1** = 1ml/kg parsley oil in diet, **PO_2** = 2ml/kg parsley oil in diet and **PO_3** = 3ml/kg parsley oil in diet.

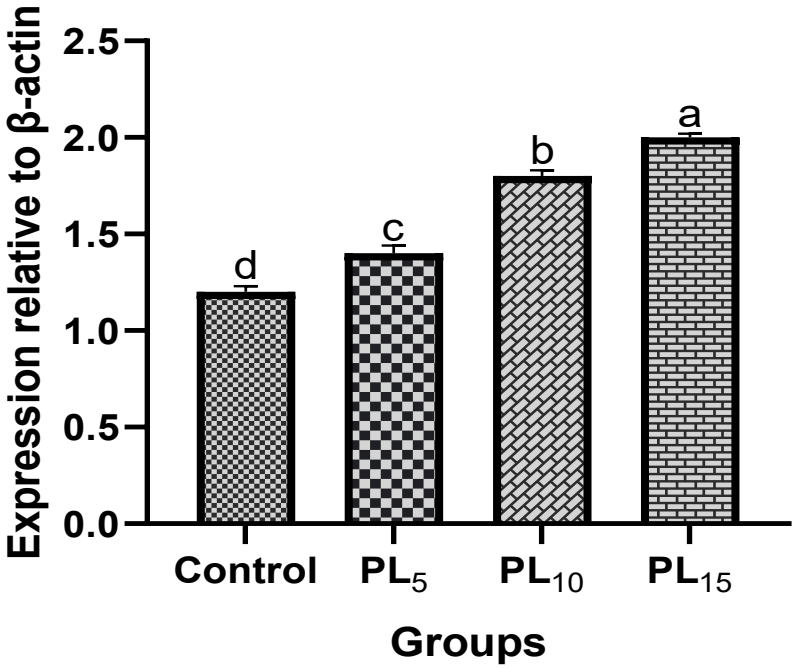

Figure 4: Analysis of gene expression of Myogenin in *L rohita* fingerlings after 75 days feeding of experimental diets supplemented with graded levels of Parsley (*Petroselinum crispum*) leave powder. **Control** = 35% CP diet without parsley supplementation, **PL_5** = 5g/kg parsley leave powder in diet, **PL_{10}**= 10g/kg parsley leave powder in diet, **PL_{15}** = 15g/kg parsley leave powder in diet, **PO_1** = 1ml/kg parsley oil in diet, **PO_2** = 2ml/kg parsley oil in diet and **PO_3** = 3ml/kg parsley oil in diet.

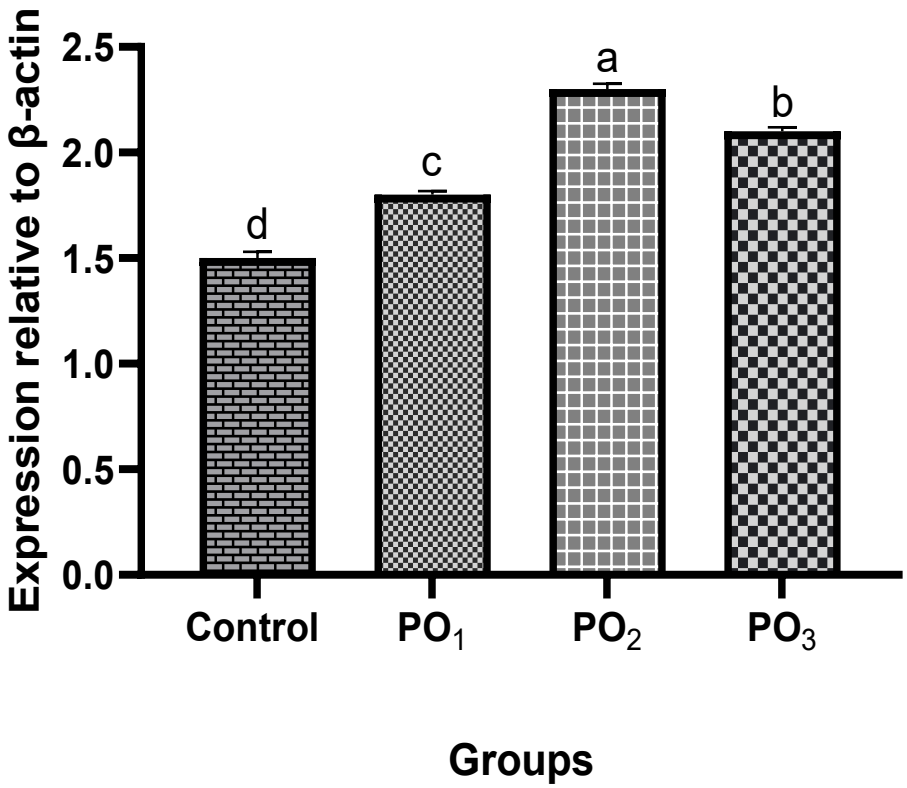

Figure 5: Analysis of gene expression of Myogenin in *L rohita* fingerlings after 75 days feeding of experimental diets supplemented with graded levels of Parsley (*Petroselinum crispum*) oil. **Control** = 35% CP diet without parsley supplementation, **PL_5** = 5g/kg parsley leave powder in diet, **PL_{10}**= 10g/kg parsley leave powder in diet, **PL_{15}** = 15g/kg parsley leave powder in diet, **PO_1** = 1ml/kg parsley oil in diet, **PO_2** = 2ml/kg parsley oil in diet and **PO_3** = 3ml/kg parsley oil in diet.

DISCUSSION

Aquaculture is the fastest-growing food industry, responsible for provision of food and nutritional reliability. With increase in human population, the demand for increased and high-quality fish feeds for human consumption aroused to reduce wild stock overexploitation and keep the ecosystem from declining (Hassan *et al*., 2021). Because of the high cost and fluctuating quality of fish meal and soybean meal, as well as their uncertain availability, alternative protein sources for fish feeds have become necessary (Parisi *et al*., 2020). Furthermore, substituting cheaper and locally available plant sources for expensive fish meals would reduce production costs and thus increase profit (Mallik et al., 2019).

A significant emphasis has been deposited on the use of traditional plant protein sources. However, their scarcity and competition from other sectors for such conventional crops for livestock and human consumption as well as industrial use help boost their costs, putting them out of reach for fish farmers or aqua feed producers (Fasakin *et al*., 1999). As a result, in order to achieve a more economically, environmentally friendly, and viable production, research interest has been directed towards the evaluation and use of unconventional protein sources, specifically from plant products such as seeds, leaves, roots and extracts of plants (Magouz *et al*., 2008).

In fish culture, health management is critical for the sustainable growth. During intensive culturing of aquatic organisms, a variety of microbial agents can cause diseases. Bacterial infections are responsible for most of the mortality in cultured fishes throughout the world (Austin *et al*., 2007). To avoid any damage to fish health, use of antibiotics and immunostimulants is common. Due to limited effectiveness and high-cost, the use of immunostimulants is not possible when large number of fishes are infected. To overcome these circumstances, traditional medicinal plants and herbs are used to control the diseases (Dugenci *et al*., 2003). Herbs can be used as functional feed additives as well as natural immunostimulants to boost culture productivity by enhancing the animal health and lowering the likelihood of output loss. (Hoseinifar *et al*.,2020)

Dietary supplementation of natural or artificial ingredients can have beneficial effects on welfare, growth as well as immune responses (Van Doan *et al*., 2020). Leafy vegetables are particularly important in many people's diets because they serve crucial roles in alleviation of hunger and ensure the food security. They are important sources of nutrition because they

significantly increase the amount of proteins, minerals, vitamins, fibers, and other nutrients that are typically deficient in everyday meals (Solanke & Awonorin, 2002).

Parsley (*Petroselinum crispum*), a culinary herb native to the Mediterranean region, is a member of the Umbelliferae family has been reported to have a lot of medicinal attributes including, antimicrobial, anticoagulant, antioxidant properties (Nielsen *et al*., 1999). Parsley has high iron content, and its high vitamin C content helps with iron absorption (Duke *et al*., 2009). Additionally, parsley is known to improve digestion, promote protein and fat absorption, and enhance digestive activity and elimination, thanks to its high enzyme content, as mentioned in a study by Bahnas *et al*. (2009).

In the present study, *Petroselinum crispum* was used as feed additive in *L rohita* diet in order to evaluate its effects on growth performance, haematological indices, immunity parameters, antioxidants and digestive enzymes. About 210, *L rohita* fingerlings were distributed equally in 21 glass aquaria and randomly divided into seven groups. Fishes that were fed with diets supplemented with parsley showed better growth, FCR and survival rate compared to control when challenged with Staphylococcus aureus after 75 days of feeding trial. The inclusion of parsley was done in two dietary forms i.e., leaves and oil. Parsley leaves were added in three different concentrations 5g, 10g and 15g, while parsley oil was added in amount 1ml, 2ml and 3ml in fish diet.

Fish growth and its associated qualities depend upon quality and quantity of feed. In the current study, the results showed that supplementation of parsley leaves in the feed at PL_{15} (15g/kg) caused a positive influence on the growth indices of L. rohita fingerlings. During the 75 days of feeding trial, the weight gain in the group fed parsley leaves at 15 g/kg of diet (i.e., PL_{15} group) increased compared to other parsley leave supplemented groups and control group fed the basal diet. Thus, resulted FCR was significantly lower for the PL_{15} fed group in comparison to the other groups. The fresh leaves of parsley contain minerals, manganese, chlorophylls, calcium and vitamins. The presence of essential minerals in leaves might cause an improved growth of fish.

Parallel to this, parsley oil supplemented groups revealed enhanced growth performance in PO_2 (2ml/kg) oil fed group. Parsley essential oil has immunomodulatory properties because of presence of flavonoids, myristicin, oleic acid, apiol and carotenoids. With the highest dose level supplementation, fish showed growth at some extent. But prolonged exposure of highest dose

showed reduced growth and other related parameters and highest FCR. The observed growth-promoting effects of the parsley-supplemented diet may be explained by the improved nutrient digestibility of experimental diets with parsley incorporation. It could be possible that fat was used as energy source and protein was utilized for growth of fish.

According to Abbas (2010), dietary parsley fed to chicken resulted in significantly improved body weight, feed efficiency and feed intake in broiler chicken. Similarly, in an experiment conducted on broiler chicken with parsley as feed additive to assess the effects of parsley on chick's performance. In this study, chicks were divided into five groups T1, T2, T3, T4 and T5 with supplementation of parsley at 0,3,6,9 and 12g/kg respectively. The group fed with 9g/kg parsley had significant effects on body weight and weight gain. The feed intake and feed conversion ratio also improved compared to control (Majeed et al., 2021). In line with present study, improved growth performance was demonstrated in juvenile *Cyprinus carpio*, which were fed parsley-supplemented diets. In this study, parsley was administered at 0.1,0.25,0.5 and 1% in diet. The results revealed that in treatment group given with 0.5% parsley significantly highest growth performance was observed (Moraki *et al*., 2014).

The growth and development of fish are greatly influenced by an organism's state of health. Hematological parameters are an essential physiological indication in the prognosis of health-related problems in fish. Fish morphology and physiological state can be determined by haematological characteristics (Fadeifared *et al*., 2018). In the present study, the results revealed a significant improvement in the hematological parameters like RBC, WBC, Hb, and Hct in the fingerlings fed with a diet supplemented with 10 g or 15 g of parsley as compared to the control.

The larger RBCs may have contributed to the rise in haemoglobin levels, which in turn may have improved oxygen delivery. The improvement is likely due to the rich iron, beta carotene, and vitamin C content found in parsley, which are important for overall health (Ragab *et al*., 2010). The consumption of fresh parsley leaves by geese has been shown to significantly enhance various haematological traits. Increased WBCs were reported in geese fed with parsley leaves (Al-Daraji et al., 2012). Almost similar resulted were demonstrated by inclusion of 15g of dietary ginger powder in feed of *L rohita* fingerlings (Rawat et al., 2022).

Likewise, the supplementation of parsley oil at inclusion level 2ml/kg lead to increase in RBC count, WBCs, hemoglobin, haematocrit and other indices. The use of parsley extract in diet of *O. niloticus* with aflatoxicosis abolished Hb reduction (El-Barbary & Mehrim, 2009). The Hct index is the volumetric percentage of erythrocytes in systemic circulation and is affected by erythrocyte number and size. Increased Haematocrit indicates that the blood has the ability to transport oxygen, indicating that the fish is in good health (Birchard, 1997). The antioxidant effect of PO, which protects the Hb molecule and erythrocyte membrane from oxidative damage in addition to restoring erythropoiesis, may be the cause of the improvement in Hb concentration and RBC count. The improvement in leukocytic and lymphocytic counts could be due to the maintenance of leukocyte redox state as well as the immunostimulant effect of PEO, which boosts leukocyte proliferation (Farag *et al*., 2021).

Proteins are the most important compounds in the serum with albumin and globulin being the major serum proteins. Albumin is synthesized in the liver and during oxidative stress induced by free radicals, it acts as an antioxidant. Another protein, globulins is absolutely essential for maintaining a healthy immune system (Kumar *et al*., 2005). The inclusion of parsley in diet of L rohita fingerlings showed elevated IgM, total protein, albumin and globulin levels, as well as enhanced respiratory burst activity. As we see the serum protein profile, elevation in levels of total protein, albumin might be resulted due to enhanced synthetic function of liver and the renal glomerular filtration, or increased in antibody production by leukocytes. In congruity, the studies on chicken illustrate that the inclusion of Parsley leaves in diet can lead to improve in some blood serum biochemical traits of broiler (Ali *et al*., 2016).

The elevated levels of immunoglobulins attribute the B-lymphocyte function and help in fighting against disease when fish is infected with bacteria. Thus, showing that parsley has immunity enhancing properties. In vertebrate phagocytic cells, an activity known as respiratory burst provides an indication of an oxygen-dependent defence mechanism in which reactive oxygen intermediates with potent microbicidal activity are produced. In the present study the respiratory burst activity of phagocytes was measured by reduction of nitroblue tetrazolium (NBT) by intracellular superoxide radicals produced by leucocytes. The results indicated that parsley fed fish had enhanced respiratory burst activity might be due to presence of higher flavonoid contents in parsley which scavenge the free radicals.

Superoxide dismutase (SOD) is a vital antioxidant that is present in the majority of living cells to counter oxidative stress. Superoxide dismutase protect cells from reactive oxygen species by catalyzing the disproportionation of superoxide anion radicals into molecular oxygen and hydrogen peroxide. It facilitates the conversion of superoxide into oxygen and hydrogen peroxide. Catalase is a key enzyme which uses hydrogen peroxide, a nonradical ROS, as its substrate. This enzyme is responsible for neutralization through decomposition of hydrogen peroxide, thereby maintaining an optimum level of the molecule in the cell which is also essential for cellular signaling processes. In the fish under study, the activity of CAT was decreased in dose-dependent manner in both leave and oil fed groups compared to control. Peroxidases are the ubiquitous enzyme and reported to be present in all living genera. They catalyze reduction of peroxide and generate reactive oxygen species. In the present study, the activity of antioxidant was decreased in dose-dependent manner in both leave and oil fed groups compared to control.

Lipid peroxidation is the process in which oxidants such as free radicals attack lipids containing carbon-carbon double bond(s), especially polyunsaturated fatty acids (PUFAs, that involve hydrogen abstraction from a carbon, with oxygen insertion resulting in lipid peroxyl radicals and hydroperoxides. Lipid peroxidation or reaction of oxygen with unsaturated lipids produces a wide variety of oxidation products. The main primary products of lipid peroxidation are lipid hydroperoxides. The LPO activity observed in control group was higher compared to both parsley leave and oil fed groups. As parsley contains myristicin, it caused detoxification of enzyme glutathione S-transferase (GST) in the liver and small intestinal mucosa of mice (Zheng *et al*., 1992). Similarly, it was reported by Nielsen *et al*. (1999) that human treated with parsley oil showed increase in levels of superoxide dismutase, glutathione reductase and total antioxidant activity when in stress.

Along with antimicrobial properties, herbs and their extracts can also increase appetite and digestion in fish. Parsley is rich in bioactive compounds like, polyphenols, flavonoids, carotenoids, cumarines and vitamin C. These compounds serve as catalysts for digestive system and enhance the elasticity of intestine by increasing the secretion of digestive enzymes. During this process, microbiota in gastrointestinal region also play significant role (El-Houseiny *et al*., 2022). The levels of digestive enzymes in fish may be influenced by type of feed given, biochemical composition of food and onset of sexual maturity. It is also known that age and stage of development significantly

influence the digestive enzyme activities in different fish species (Peres *et al*., 1998). In the present study, the positive effect of parsley on the growth efficiency may be related to its inducing effects on digestive enzymes, as digestive enzymes were significantly increased in Parsley-supplemented fish compared to the control group.

Amylase is one of the main enzymes that breaks down glycosidic connections between sugar residues in big carbohydrate molecules. Starch is particularly broken down into glucose molecules by amylase. Amylase is secreted by the entire intestine in the Indian major carps, *Catla catla, Labeo rohita and Cirrhinus mrigala*, and its activity is high towards the proximal end (Dhage *et al*.,1968). In the present study, *L rohita* fingerlings consumed parsley-supplemented diet showed significant increase in amylase activity compared to control when fed with 10g/kg parsley and decrease in activity observed in 15g/kg supplemented dietary group. Previous studies supported higher amylase activity in the intestine of herbivorous carp as compared to carnivorous fish (Fernandez *et al*., 2001). *O. niloticus* depicted significantly higher amylase activity in in response to dietary *Astragalus* polysaccharides (Zahran *et al*., 2014).

Proteases are digestive enzymes that break down the links between neighboring amino acids in proteins to form peptides. According to Kumar *et al*. (2006) evaluations on the functional efficacy of digestive proteases from catla (*Catla catla*), rohu (*Labeo rohita*), and silver carp (*Hypophthalmichthys molitrix*), the intestine protease activity was higher than that of the liver. Rohu had the highest total protease activity, followed by silver carp and catla. The ester linkages between fatty acids and glycerol are hydrolyzed by lipase. In the current study, the protease activity was elevated in parsley fed groups compared to control but the lipase activity in PL fed diets is low compared to the control one. Whereas, the lipase activity in PEO diets increased in dose dependent manner compared to control.

Myogenesis is the generation of muscular tissue during embryonic development from stem cells, by fusion of myoblasts into multinucleated fibers (myotubes) (Johnston, 2006). This process is highly conserved in all vertebrates and requires the synchronized participation of four myogenic regulatory factors (MRFs): MyoD, Myf5, myogenin and MRF4 (Massari & Murre, 2000). In the present study, the mRNA expression of myogenin gene is upregulated as compared to housekeeping B-actin genes in parsley supplemented groups.

CONCLUSION

From the present study, it is concluded that parsley has significant effect on growth performance, haemato-immunological parameters, antioxidants and digestive enzymes. Parsley leave powder and essential oil are beneficial for fish health in many aspects, as they have immune-modulatory, anti-microbial and anti-fungal properties. They are affordable, environmental-friendly and can be administered in diet at commercial level to prevent the fish from certain microbial and bacterial infections.

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