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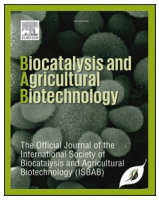

Growth performance, hematological and biochemical effects of iron oxide nanoparticles in *Labeo rohita*

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ABSTRACT

In aquaculture farming, iron deficiency in fish causes anemia and also causes improper growth. Biologically synthesized nanoparticles are widely used in micro-nutrient delivery in aquaculture as they are eco-friendly, and non-toxic to the environment. Iron oxide nanoparticles can be the alternative supplement to overcome iron deficiency. Therefore, the primary aim of this study was to determine the effect of *Amaranthus tricolor* mediated iron nanoparticles in the physiological, hematological, biochemical parameters of the Indian major carp *Labeo rohita.* In the present study characterization of the iron nanoparticles were done. Iron oxide nanoparticles were added to the experimental basal diets at various concentrations. Positive and negative controls were also taken. Feeding experiment showed that iron oxide nanoparticles incorporated diet showed positive result towards the survival, growth and biochemical compositions in *Labeo rohita.* The values of hemoglobin and erythrocyte count show a significant increase among the different concentration of diet. Fish fed with iron oxide nanoparticle supplemented diet produced a better performance in the biochemical compositions. The results from the study prove that the iron nanoparticles supplemented diet can improve the physiological, biochemical and hematological activities in *Labeo rohita.* Thus it is inferred that the iron oxide nanoparticles possess the capabilities of being the alternative source to rectify the iron deficiency and retarded growth related problems in *Labeo rohita.*

1. Introduction

As the world population is increasing, people are demanding for the resources which supply protein requirements. Animal protein plays a major role in terms of nutrition. Aquatic animals act as the resources to supply the proteins (Erkan et al., 2011). In Aquaculture, commercial and artificial fish feed does not fulfill the protein requirement in the development of the fish and cause many diseases to the fish. This condition affects aquaculture farming and fish consumption by the people. According to FAO 2006, continuous depletion of natural fish resources creates demand for the fish and other aquatic animals.

The aquaculture industry performs new scientific aspects and technology for the improvement of the qualified feed. The aquatic environment may act as a sink for the entry of the nanoparticles (Farre et al., 2009) that are easily taken by aquatic organisms such as mollusks, crustaceans and fish (Ward and Kach, 2009). To support high productivity and variety in fish farming, use of technology and adaptation of good fishery management practices are of primary importance (Lucas et al., 2012).

Iron is an essential nutrient for the growth of the fish and the improvement of their physiological and immunological parameters. It is an important micronutrient involved in oxygen transport and cellular respiration through its oxidation-reduction activity and electron transfer (Roeder and Roeder, 1968). Physiological abnormalities such as susceptibility to diseases, changes in hematological parameters, microcytic anemia, poor feed conversion, growth depression, and immune suppression in animals have been associated with iron deficiency (Andersen et al., 1996). The efficiency of the supplemented feed is not only to improve their nutritive profile but also on the animal's inherent ability to consume, digest, absorb nutrient utilization in aquatic animals depends on their activity of the digestive enzymes. Iron supplement for fish is essential because dietary requirements are not equal to natural iron sources due to low solubility and low bioavailability (Hilty et al., 2011). The important reason for using *L. rohita* as an experimental animal is because of its economic importance. *Labeo rohita* is one of the commercially remarkable cultured species in India. Recent studies have shown that the nano based feed additives promotes the positive impact in aquaculture (Onegbhu et al., 2018). However detailed studies on the

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role of *Amaranthus tricolor* mediated iron oxide nanoparticles as *Labeo rohita* has not been made. According to FAO/WHO limit (1989), the level of permissible limit of iron is 30 mg/g. In the study, the iron concentration used is below the permissible limit. The aim of this study is to investigate the physiological, biochemical, hematological, and enzymatic assay of *L. rohita* fed with iron oxide nanoparticles supplemented diets.

2. Materials and methods

2.1. Collection and identification of plant

The entire plant of *Amaranthus tricolor* belonging to *Amaranthaceae* family (red spinach) was collected from Kuniyamuthur, Coimbatore district, Tamilnadu, India. The collected plant material was identified and authenticated with the help of Dr. C. Murugan, Scientist 'E' & Head of Office, Botanical Survey of India (BSI), Southern Regional Centre, Tamilnadu Agricultural University (TNAU) Campus, Coimbatore, Tamilnadu, India, where the voucher plant was preserved.

2.2. Preparation of plant extract

The collected leaves of *Amaranthus tricolor* were washed thoroughly with tap water followed by distilled water to remove dust and they are allowed to air dry at room temperature for 4–5 days to remove moisture. Then, the dried leaves were crushed using mixer grinder, made into powder and stored for further use. About 2 g of leaf powder was added into 100 mL double distilled water and heated up to 60 °C. It was filtered using Whatman No.1. filter paper to get the pure aqueous leaf extract and stored in the air tight container for further experimental usage (Gottimukkala et al., 2017).

2.3. Green synthesis of iron oxide nanoparticles using Amaranthus tricolor

The FeONPs were synthesized using Phyto-reduction method. To 50 ml of 0.1 M Ferric nitrate solution, 40 mL of aqueous plant extract was added under constant stirring up to 30 min. The appearance of instantaneous black color indicated the formation of iron oxide nanoparticles. The formed black solution was centrifuged at 5000 rpm for 10 min. The supernatant was removed. The product was washed thrice with ethanol and allowed to dry overnight at room temperature and stored for further characterization (Bhavika et al., 2018).

2.4. Phytochemical analysis of plant extract

Freshly prepared leaf extracts were subjected to standard phytochemical analyses using the standard procedure (Trease and Evans, 1989) in order to find out the presence of various phytoconstituents such as alkaloids, terpenoids, flavonoids, tannins, steroids, anthraquinones, saponins, resins, glycosides and phenols.

2.5. Characterization of green iron oxide nanoparticles

The reduction of pure iron oxide nanoparticles was monitored by measuring the UV–Vis spectrum by a sampling of aliquots (0.3 mL) of iron oxide nanoparticle solution diluting the sample in 3 mL distilled water. It was done by using UV–Vis spectrophotometer Systronics 118 at the range of 200–800 nm. Fourier transform infrared spectroscopy measurements were carried out to explore the mode of interactions between *Amaranthus tricolor* leaf extract and the nanoparticle surface. The sample was mixed with KBr to obtain KBr pellets consisting of 1.5% (w/w) of the nanoparticles. The resulting mixture was pressed into disks (0.5 mm in thickness). FTIR spectra of the leaf extract and nanoparticles were achieved in a Shimadzu FT-IR spectrophotometer and registering amplitude waves ranging from 550 to 4000 cm^{-1}.The morphological characteristics of the nanoparticles were obtained through scanning electron microscopy (ZEISS MODEL) operated at 4 KV, magnification 41.17 KX. SEM is a quick and easy method to obtain details about the morphology of nanoparticles. Thin film of the sample was prepared on a carbon coated copper grid by just dropping the suspension of nanoparticles in water on the grid, extra solution was removed using blotting paper and then the film on the SEM grid were allowed dry by putting it under a mercury lamp for 5 min. Elemental analysis of nanoparticles was carried out using EDS instrument (ZEISS MODEL) in a resolution of 60 A°, operated at 4 kV with a magnification of 5 K.

2.6. Collection and acclimatization of fish

The Fingerlings of L. *rohita,* with an average size of 6 cm were purchased from Palar Fish Seed farm, Palani, Tamilnadu. They were safely transported in oxygenated plastic bags half filled with hatchery water to the laboratory. The purchased fingerlings were maintained in the plastic dubs of 30 L capacity containing siruvani water (25 L) with an automatic aerator (Aqra Air Pump). During acclimatization period of one week, fingerlings were fed with the mixture of basal ingredients (rice bran, groundnut oil cake and soy meal) of 2 g alternatively two times morning and evening (8.30 a.m. & 3 p.m.) per day and 80% of aquarium water was renewed daily at 3 p.m.

2.7. Diet formulation

Iron oxide nanoparticles were added to the experimental basal diets at the rates of 2.5, 5.0, 7.5 and 10.0 mg/kg dry feed weight. Basal diets were prepared with locally available feed ingredients. Soymeal (165 g/kg) was used as protein sources; rice bran (555 g/kg) was used as carbohydrate sources; Groundnut oil cake (275 g/kg) was used as a protein and lipid sources; gelatin was used as a binding agent.

Active healthy fishes were chosen from the acclimatized tank and made to starve for 48 h prior to the commencement of the experiment. In the study five groups (Treatment 1, 2, 3, 4, & 5) of *Labeo rohita* with 25 individuals were assigned for this experiment for 45 days. Group 1 was considered as positive control and was fed with *Amaranthus tricolor* supplemented diet. Treatment 2 was considered as negative control and was fed with commercial feed. Treatment 3, 4, 5 and 6 were fed with 2.5, 5.0, 7.5 and 10.0 mg/kg iron oxide nanoparticles supplemented diets respectively. During the feeding trial, the consumed feed and feces were removed on a daily basis by renewing the water (Asaikkutti et al., 2016).

2.8. Water quality analysis

The water sample was collected from the fish tub and the parameters were checked. The physico-chemical parameters such as pH, total dissolved solids, turbidity, chloride, alkalinity, calcium, sulphate, nitrite, aluminium and magnesium were checked periodically using American Society for Testing and Materials.

2.9. Growth parameters

All the fishes in different experimental groups were estimated for growth and food indices by the formulae, (Nasrin et al., 2018).

$$\text{Survival rate } (\%) = \frac{\text{Final fish number}}{\text{Initial fish number}} \times 100$$

Length gain (cm) = Final length (cm)-Initial length (cm)

Weight gain (g) = Final weight (g)- Initial Weight(g)

$$\text{Specific growth rate } (\text{SGR}) = \frac{\text{Log Final weight} - \text{logInitial weight}}{\text{Experiment period}} \times 100$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{Feed given (dry weight)}}{\text{Total wet weight gain}}$$

2.10. Biochemical profile

2.10.1. Estimation of carbohydrate

Carbohydrate level in the tissue of fish was estimated by the Anthrone method. 100 mg of muscle, liver and gills were homogenized in 2 mL tri-chloro acetic acid, and centrifuged at 5000 rpm for 10 min. The supernatant was collected in a test tube and 4 ml of anthrone was added. The mixture got evaporated by keeping the test tube in a boiling water bath for exactly 15 min. Later the mixture was cooled in running tap water. The absorbance of the reddish-brown colored mixture was read at 630 nm using photoelectric colorimeter and glucose acts as the standard for estimating Carbohydrates (Asaikkutti et al., 2016). Carbohydrate level in muscle, liver and gills of fish was calculated using the formula,

$$\text{Amount of carbohydrate present} = \frac{\text{Optical density of the sample}}{\text{Optical density of the standard}} \times \text{conc. of std.} \times 100\text{mg/g}.$$

2.10.2. Estimation of total protein

Estimation of the protein content of fish muscle, liver and gills were estimated by using the commercial kit (AUTOSPAN Liquid Gold Total Protein, Modified Biuret, End Point Assay). The absorbance was read at 578 nm using photoelectric colorimeter (Behera et al., 2014). The total protein level in muscle, liver and gills of fish is calculated using the formula,

$$\text{Total protein concentration (g/dL)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times 6.5$$

2.10.3. Estimation of lipid

Lipids are soluble in some organic solvents, which are utilized for extracting lipids from tissues. In biological materials, the lipids are generally bound to proteins and they are therefore extracted either with a mixture of ethanol and diethyl ether or a mixture of chloroform and methanol. The tissue homogenate mixture was prepared in a cold chloroform -methanol mixture (2:1). The homogenate was filtered through filter paper soaked in chloroform-methanol mixture. The filtrate was collected in a test tube and evaporated in desiccation. After the complete removal of the solvent, the tubes were taken out and 8.0 ml of 2% potassium dichromate was added. The tubes were kept in the boiling water bath for 15 min and then cooled. 45 ml of distilled water was added and the tubes were cooled again in running tap water. The intensity of color was measured at 590 nm using the formula given below (Barnes and Black Stock, 1973).

$$\text{Lipid present in the sample} = \frac{\text{OD of the sample}}{\text{OD of the satandard}} \times \text{Concetration of the standard} \times 100$$

2.11. Hematological studies

2.11.1. Blood sampling

Blood was drawn from all fish under control and experiment group by cardiac puncture using sterile disposable plastic syringe with a 22-gauge needle. The syringe and needle were moisturized with heparin sodium (1%) was used as an anticoagulant. Blood was transferred into small-sized vials, which was already rinsed with heparin and kept in the ice box. The blood sample was used for the estimation of hemoglobin, erythrocytes, and leucocytes count. Later the blood sample was centrifuged to separate the plasma for the estimation of biochemical parameters.

2.11.2. Erythrocytes count

The Erythrocytes were counted using hemocytometer. Red Blood Cells diluting fluid was used for determining total erythrocytes count. It was done by mixing 20 µl of blood with 3980 µl of the diluting fluid in a clean test tube. Cell counts were performed by using a Neubauer's counting chamber (Behera et al., 2014).

The total number of erythrocytes was calculated by,

$$\text{Number of RBC} = \frac{\text{Number of erythrocyte} \times \text{dilution counted}}{\text{Area counted} \times \text{depth of fluid}} \text{ (millions/cu.mm of blood)}$$

$$\text{Dilution} = 200$$

$$\text{Area counted} = 5 \times 0.04 = 0.2 \text{ square mm.}$$

$$\text{Depth of fluid} = 0.1 \text{ mm.}$$

2.11.3. Estimation of Hemoglobin

The hemoglobin level of blood was estimated by the cyanomethymoglobin method using Drabkins Fluid (Drabkin, 1950). The absorbance was measured using a spectrophotometer at 540 nm and the final concentration was calculated by comparing with the standard cyanomethymoglobin.

$$\text{Hemoglobin (g/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of standard}} \times \text{concentration of standard}$$

2.11.4. Leucocyte count

Leucocytes were counted by using the hemocytometer. White blood cells diluting fluid was used for determining the total leucocyte counts. It was done by mixing 20 µl of blood with 3980 µl of the diluting fluid in a clean test tube. Cell counts were performed by using a Neubauer's counting chamber (Behera et al., 2014).

The total number of leucocytes was calculated by the formula,

$$\text{Number of WBC} = \frac{\text{Number of leucocyte} \times \text{dilution counted}}{\text{Area counted} \times \text{depth of fluid}} \text{ (1000/cu.mm of blood).}$$

$$\text{Dilution} = 20$$

$$\text{Area counted} = 4 \times 1 = 4 \text{ square mm}$$

$$\text{Depth of fluid} = 0.1 \text{ mm.}$$

2.12. Assay of digestive enzymes

Activities of digestive enzymes (protease, amylase, and lipase) were assayed on the initial and final days of the feeding experiment. The whole digestive tract and hepatopancreas were homogenized in ice-cold double distilled water and centrifuged at 9300 g under 4 °C for 20 min. The supernatant was used as a crude enzyme source. Total protease activity was determined by the casein-hydrolysis method (Furne et al., 2005) where 1 unit of enzyme activity represents the number of enzymes required to liberate 1 µg of tyrosine per minute under assay conditions. Amylase activity was determined by the starch-hydrolysis method (Bernfeld, 1955). The specific activity of amylase was calculated as milligrams of maltose liberated per gram of protein per hour. Lipase activity was analyzed by the method of Furne. One unit of lipase activity was defined as the amount of free fatty acid released from triacylglycerol per unit of time estimated by the amount of NaOH required to maintain pH constant and represented as mille equivalents of alkali consumed (Annamali Asaikutti et al., 2016).

2.13. Statistical analysis

SPSS 20 version was used for the determination of DMRT, ANOVA and Mean ± Standard deviation.

3. Results

3.1. Phytochemical analysis of plant extract

The presence of phytochemicals was analyzed in the *Amaranthus tricolor* plant extract. They were represented in Table .1.

3.2. Characterization of iron oxide nanoparticles

The appearance of black color in the reaction mixture is the indication of the formation of iron nanoparticles. The formation of black color in the reaction solution is due to the surface plasmon excitation of the iron oxide nanoparticles. The UV–Vis spectra of the colloidal solution of green mediated iron oxide nanoparticles showed strong peaks at 250 nm (Fig. 1).FTIR spectra of *Amaranthus tricolor* plant extract and *Amaranthus tricolor* mediated iron nanoparticles were recorded in order to identify the functional groups involved in the formation of nanoparticles. FTIR spectrum of plant extract and iron nanoparticles are shown in (Fig. 2a and b).The aromatic C═C stretching frequency which appeared at 1647.21 and 1550.77 cm^{-1} were observed. The C–O stretching frequency was noticed at 1249.87 cm^{-1} and 1269.16 cm^{-1}. The FeO peaks were noted at 432.05 cm^{-1} and 540.07 cm^{-1}. The N–H stretching observed at 3425.58 cm^{-1} in the *Amaranthus tricolor* plant extract was shifted to 3402.43 cm^{-1} in *Amaranthus tricolor* mediated iron oxide nanoparticles. In addition to this, the C–H stretch appeared at 2924.09 cm^{-1} was also shifted to 2927.94 cm^{-1} in nanoparticles. The shift in the peaks confirmed that plant extract reduced the ferric ions for the formation of nanoparticles. Scanning Electron Microscopy of synthesized iron oxide nanoparticles revealed that the particles were spherical in nature (Fig. 3). The synthesized FeONPs showed individual particles as well as agglomerated particles. The agglomeration is due to the intermolecular interaction such as Vander walls force. Energy dispersive spectrum of iron oxide nanoparticles showed a strong signal at 6.612 keV in the iron oxide region and this confirmed the presence of iron oxide nanoparticles (Fig. 4). Additionally, the oxygen peak is also observed. The elemental analysis revealed that the iron oxide was a major constituent.

3.3. Water quality parameters

The water quality parameters such as temperature, pH, total dissolved solids (TDS), turbidity, alkalinity, chloride, sulphate, nitrite, aluminium, magnesium and calcium were monitored and listed out in Table 2.

Table. 1
Qualitative phytochemical analysis of *Amaranthustricolor* leaf extract.

S.no.	Name of thetest	RESULTS
1.	Alkaloids	+
2.	Steroids and sterols	_
3.	Flavanoids	_
4.	Triterpernoids	_
5.	Tannins	+
6.	Phenols	_
7.	Cardiac glycosides	+
8.	Volatile oil	_
9.	Saponins	_
10.	Fatty acids	_
11.	Glycosides	+
12.	Terpenoids	+
13.	Proteins	+
14.	Carbohydrates	+
15.	Quinone	+

'+' Present '_' Absent.

3.4. Analysis of survival, growth and food indices

The physiological parameters like survival rate, growth (length and weight) and food parameters like Food conversion ratio were calculated (Table 3). At the end of the experiment, the maximum survival rates of fingerlings at about 96% were seen in 10.0 (mg/kg) concentrations. The minimum survival rates of fish at about 89% were seen in a negative control. Likewise, the maximum length gain and weight gain attained at about 2.51 ± 0.04 cm and 1.89 ± 0.09 g in the 10. (mg/kg) concentrations followed by 7.5, 5.0, 2.5 (mg/kg) concentrations. The minimum weight gain and length gain at about 0.94 ± 0.08 g and 1.26 ± 0.05 cm were observed in positive control. The maximum SGR was obtained at about 0.19 ± 0.01 in 10.0 mg/kg concentration. The FCR ratio is lower in 10.0 mg/kg concentration (1.71 ± 0.04 g). Fish group which has low FCR was considered to be efficient. The DMRT statistical analysis of data represented in Table 4 shows that among all the groups the 10.0 (mg/kg) concentration is highly significant.

3.5. Biochemical analysis

3.5.1. Estimation of carbohydrates

Carbohydrate content in muscle, liver, gills of fish were estimated (Fig. 5). The carbohydrate level gets gradually increased in the 10.0 mg/kg concentration of iron oxide nanoparticles supplemented diet. The maximum amount of carbohydrate was seen in muscle, liver and gills about 16.34 ± 1.66, 14.67 ± 1.54, 15.89 ± 1.08 mg/g respectively. The minimum amount of carbohydrate in muscle, liver and gills was seen about 10.73 ± 1.32, 9.05 ± 1.11, 9.85 ± 1.11 mg/g respectively in negative control. The one way ANOVA analysis of iron oxide dietary supplement in fish revealed that it was significantly increased at different concentrations ($F = 0.137$; $P < 0.05$).

3.5.2. Estimation of total protein

This result shows that the increase in protein level of fish in muscle, liver and gills (16.45 ± 0.03, 13.68 ± 1.79, 15.42 ± 1.52 g/dl) in 10.0 mg/kg concentration of iron oxide nanoparticles supplemented diet when compared to other groups. Protein content in muscle, liver and gills of fish is represented in Fig. 6. The protein content gets gradually increased among the iron oxide supplemented diet. The minimum amount of protein was seen in muscle, liver and gills about 12.16 ± 0.09, 10.21 ± 1.06, 11.86 ± 1.06 g/dl in negative control group. The one way ANOVA analysis showed the significant increase in dietary supplement iron oxide nanoparticles supplemented feed in various concentrations ($F = 0.136$; $P = <0.05$).

3.5.3. Estimation of lipids

Lipid content in muscle, liver and gills of fish were estimated (Fig. 7). Comparing to the lipid content of control groups there were a gradual increase in the lipid content in the iron oxide nanoparticles supplemented groups. The maximum amount of lipid was observed in the fish supplemented with iron oxide nanoparticles in 10.0 mg/kg concentrations (T6 group). The recorded values of lipid content in the muscles, liver and gills were 14.99 ± 0.03, 13.83 ± 0.02, 12.96 ± 1.03 g/dl respectively. The minimum amount of lipid content was recorded in T2 group. The data were significantly increased in various concentration of iron oxide nanoparticle supplemented diet ($F = 0.328$; $P < 0.05$).

3.6. Haematological studies

Blood samples were collected from fish of T1, T2, T3, T4, T5, and T6 groups and checked for the Erythrocytes count, Hemoglobin and Leucocyte count. Table .5 represents the haematological parameters for all the fish of T1, T2, T3, T4, T5, and T6 groups.

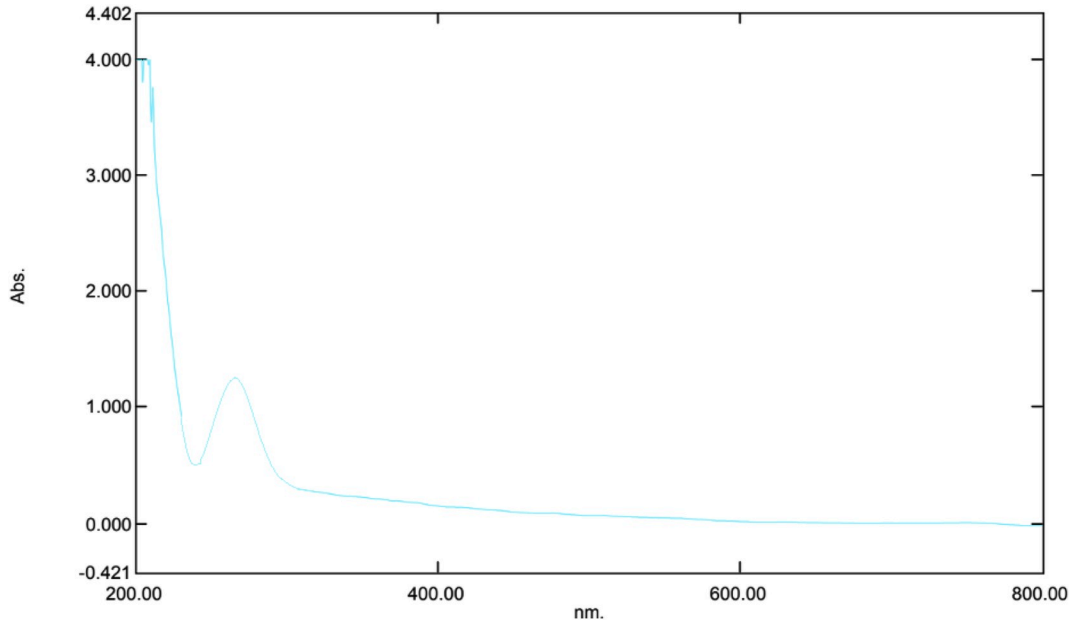

Fig. 1. UV spectrum of iron oxide nanoparticles.

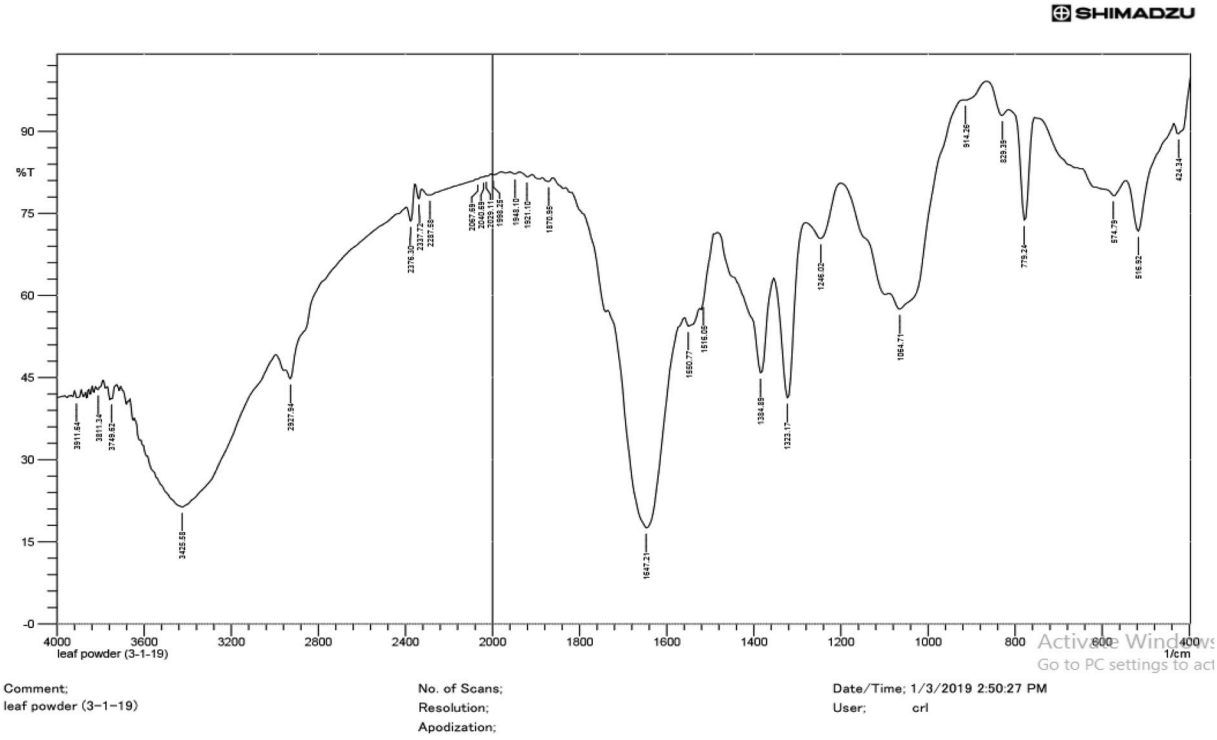

Fig. 2a. FTIR spectrum of *Amaranthus tricolor*.

3.6.1. Erythrocytes count

Erythrocyte counts in serum of fishes were estimated. The result showed that there was a gradual increase in Erythrocytes count based on the iron oxide nanoparticle supplemented diet concentration. The maximum count of Erythrocyte was observed about 2.4 ± 0.07 g/dl in the fish groups supplemented with iron oxide nanoparticles in 10.0 mg/kg concentrations. The minimum count of Erythrocyte was observed about 0.95 ± 0.03 g/dl in negative control. From this result, it is observed that RBC content in the iron oxide nanoparticle supplemented groups showed the improvement when compared to the control groups.

3.6.2. Estimation of hemoglobin

Hemoglobin level in fishes was gradually increased when exposed to iron oxide nanoparticle supplemented basal diet when compared to the control groups. The result obtained with maximum amount of hemoglobin at 8.9 ± 0.04 g/dl was seen in *Labeo rohita* fed with 10.0 mg/kg concentration of iron oxide nanoparticle supplemented diet followed by 7.5, 5.0, 2.5 mg/kg concentrations respectively. The minimum count of Hemoglobin was seen at about 6.2 ± 1.23 in negative control. From this data, the increased level of Hemoglobin was seen in the iron oxide nanoparticle supplemented diets.

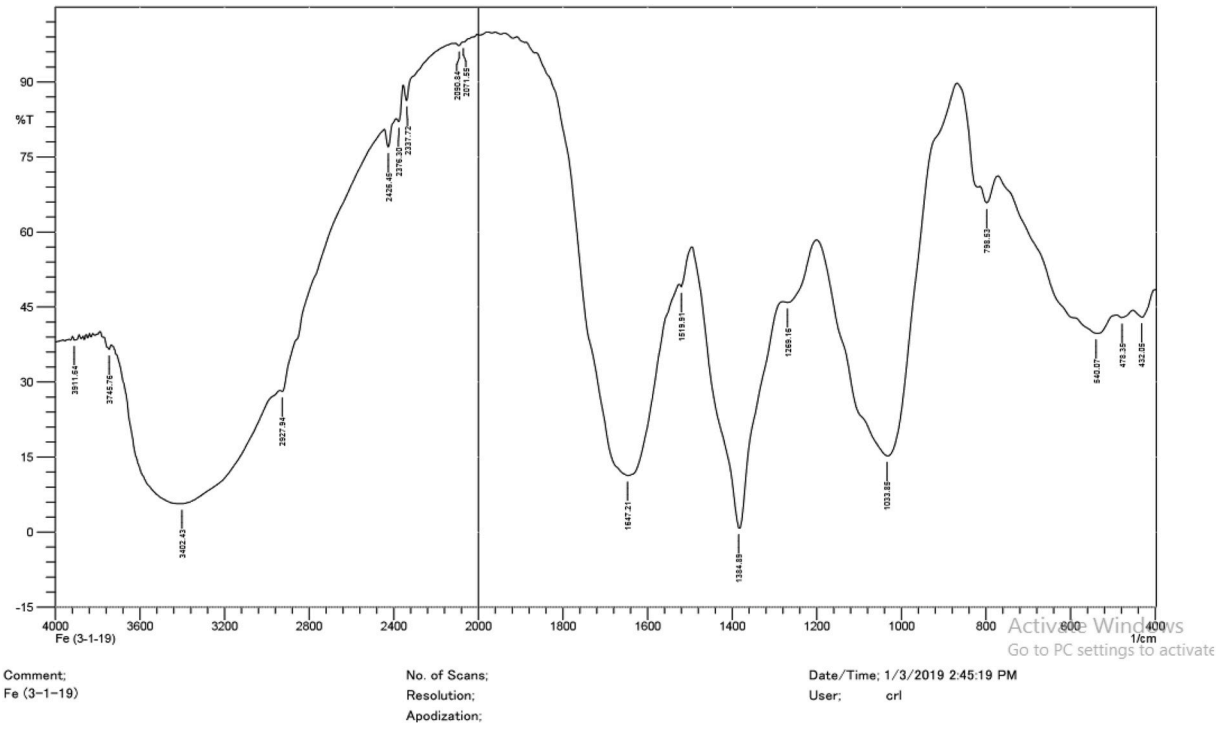

Fig. 2b. FTIR spectrum of iron oxide nanoparticles.

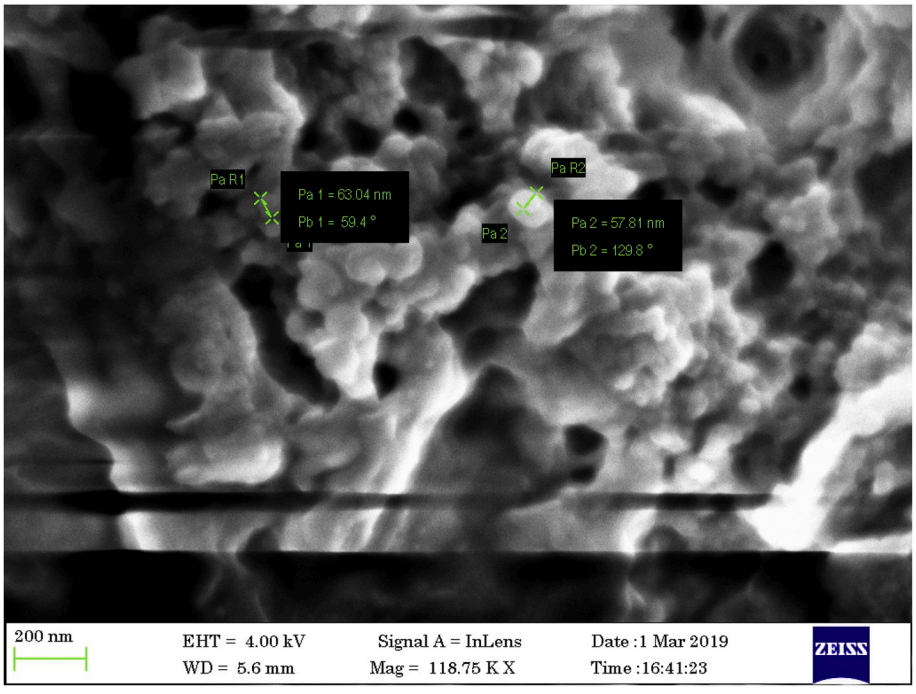

Fig. 3. SEM of iron oxide nanoparticle.

3.6.3. Leucocyte count

The Leucocyte count in the serum of fish was observed to be higher in control groups (93.12 1000 cells/cu.mm) when compared to those of respective control groups. The minimum amount of leucocyte content at about 65.68 ± 1.98 1000 cells/cu.mm was seen in the fish group fed with 10.0 mg/kg concentration. The data conveys that there was a gradual decrease in the WBC count in the iron oxide nanoparticle supplemented groups.

3.7. Assay of digestive enzymes

The activity of digestive enzymes such as Protease, Amylase and Lipase in L. *rohita* was significantly increased in FeONPs supplemented diets. The one way Analysis of Variance (ANOVA) revealed that the enzyme activities were improved ($F = 2.412$; $P < 0.05$) in fish fed with various concentrations of FeONPs supplemented diet. The increased activities of protease, amylase and lipase enzymes of fish supplemented with 10 mg/kg FeONPs are 1.21 ± 0.97, 1.29 ± 1.6 and 1.25 ± 1.05 U/

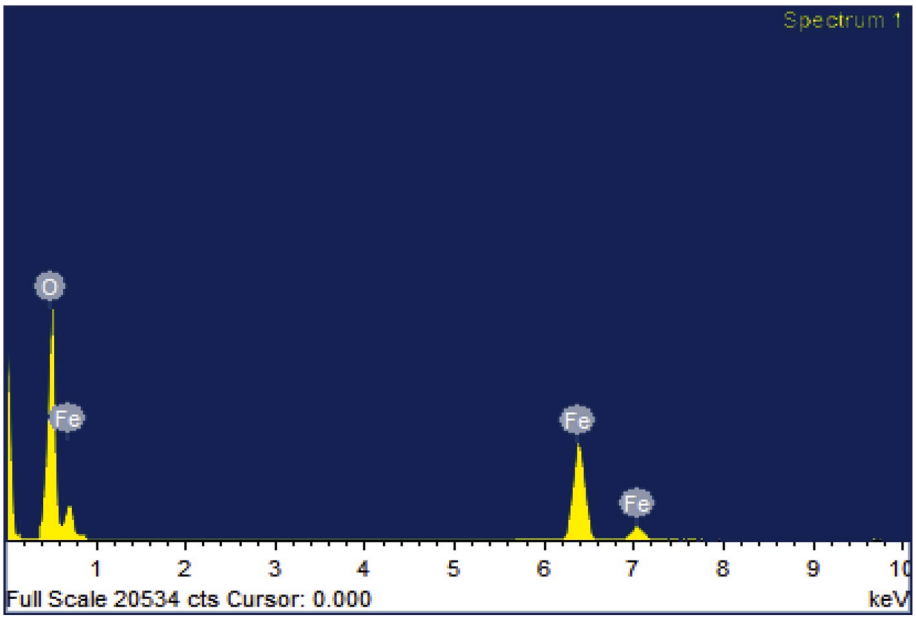

Fig. 4. EDS of iron oxide nanoparticle.

Table 2
Water quality parameters.

S.no	PARAMETERS	RESULTS
1	Temperature	26±2 °C
2	pH	7–8
3	Total dissolved solids	110 mg/l
4	Turbidity	0.1 N
5	Chloride	19.9 µg/l
6	Alkalinity	74 mg/l
7	Sulphate	1.8 mg/l
8	Nitrate	1.6 mg/l
9	Total hardness	26 mg/l
10	Magnesium	2.45 mg/l
11	Aluminium	Below the detection limit
11	Calcium	6.401 mg/l

mg protein respectively. The minimum enzymatic activities were recorded in negative control where the values of protease, amylase and lipase were 0.47 ± 0.2, 0.55 ± 0.9 and 0.52 ± 0.5 U/mg protein respectively (Fig. 8).

4. Discussion

In Aquaculture, the quality of feed is the major problem that is expressed at the farm level as poor yield performance and higher cost of production. As there is a restriction in the use of antibiotics as growth promoters in fish feed, feed additives in nano forms are reviewed to have various effects like enhancing the growth and immunity. Finding alternative sources to improve the fish feed is important if the growth of the aquaculture is to be sustained. Recent advancement in nanotechnology helps in revolutionizing different aspects of science and technology and also in aquaculture.

Table 3
Growth, survival and food indices of *LABEO ROHITA* fed with FeONPs supplemented basal diets.

Parameters	DAYS	+ve Control	-ve Control	CONCENTRATIONS OF FeONPs (mg/kg)			
				2.5	5.0	7.5	10.0
Survival rate (%)	**1**	100	100	100	100	100	100
	15	96	98	98	96	96	97
	30	94	92	96	96	95	96
	45	94	89	93	91	93	96
Length gain (cm)	**1**	00	00	00	00	00	00
	15	0.11 ± 0.02	0.09 ± 0.01	0.16 ± 0.04	0.14 ± 0.06	0.19 ± 0.03	0.14 ± 0.02
	30	0.96 ± 0.07	0.73 ± 0.04	1.31 ± 0.03	1.49 ± 0.03	1.75 ± 0.06	1.86 ± 0.05
	45	1.39 ± 0.07	1.26 ± 0.05	1.73 ± 0.02	2.02 ± 0.05	2.49 ± 0.02	2.51 ± 0.044
Weight gain (g)	**1**	00	00	00	00	00	00
	15	0.09 ± 0.01	0.06 ± 0.01	0.12 ± 0.02	0.09 ± 0.02	0.19 ± 0.03	0.18 ± 0.03
	30	0.24 ± 0.06	0.20 ± 0.05	0.32 ± 0.02	029 ± 0.05	0.34 ± 0.05	0.36 ± 0.04
	45	1.09 ± 0.02	0.94 ± 0.08	1.19 ± 0.12	1.18 ± 0.06	1.25 ± 0.06	1.89 ± 0.09
SGR (%)	**1**	0.00	0.00	0.00	0.00	0.00	0.00
	15	0.06 ± 0.00	0.04 ± 0.00	0.08 ± 0.01	0.07 ± 0.00	0.10 ± 0.01	0.09 ± 0.01
	30	0.10 ± 0.01	0.07 ± 0.02	0.011 ± 0.01	0.09 ± 0.01	0.12 ± 0.01	0.14 ± 0.02
	45	0.16 ± 0.02	0.09 ± 0.01	0.18 ± 0.02	0.15 ± 0.02	0.16 ± 0.00	0.19 ± 0.01
FCR (g)	**1**	00	00	00	00	00	00
	15	1.66 ± 0.01	1.44 ± 0.03	1.18 ± 0.01	1.20 ± 0.03	1.17 ± 0.02	1.09 ± 0.04
	30	2.08 ± 0.00	1.95 ± 0.06	1.42 ± 0.05	1.59 ± 0.01	1.29 ± 0.03	1.20 ± 0.05
	45	2.54 ± 0.05	2.21 ± 0.04	1.88 ± 0.03	1.96 ± 0.06	1.80 ± 0.01	1.71 ± 0.04

Table 4
DMRT Table for growth, survival and food indices.

PARAMETERS	POSITIVE CONTROL	NEGATIVE CONTROL	CONCENTRATIONS OF FeONPs (mg/kg)			
			2.5	0.5	7.5	1.0
Survival	94.75^{A}	92.03^{A}	95.75^{C}	95.42^{C}	96.85^{D}	97.25^{E}
Length (cm)	0.61^{B}	0.52^{A}	0.80^{C}	0.91^{C}	1.10^{D}	1.12^{D}
Weight (g)	0.35^{A}	0.30^{A}	0.40^{B}	0.39^{B}	0.44^{C}	0.60^{F}
SGR (%)	0.04^{A}	0.03^{B}	0.06^{C}	0.11^{D}	0.14^{E}	0.16^{F}
FCR (g)	1.99^{E}	1.96^{D}	1.62^{C}	1.70^{C}	1.23^{B}	1.17^{A}

*Capital letter represents comparison of row means. Mean values taken for the various days of similar concentration. The highest significance is observed in the group in which the FeONPs supplemented.

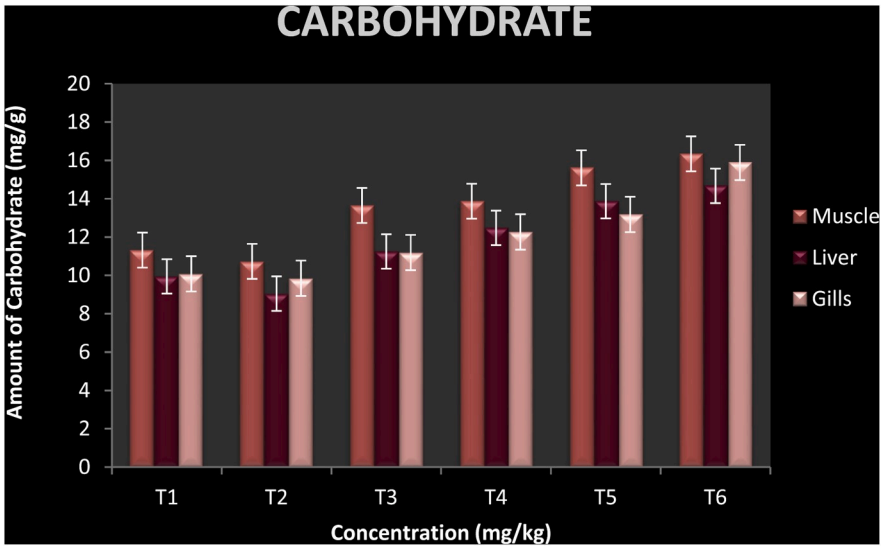

Fig. 5. Carbohydrate levels in muscle, liver and gills of *Labeo rohita*.

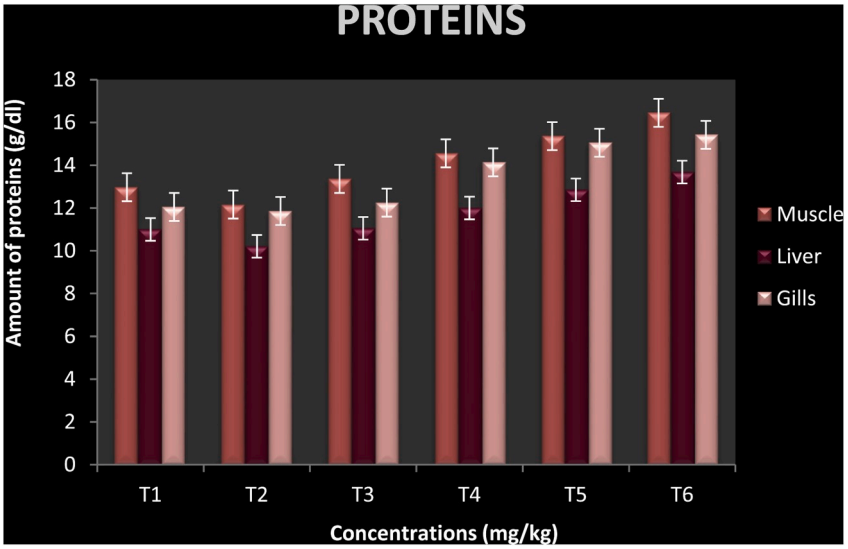

Fig. 6. Total Protein levels in muscle, liver and gills of *Labeorohit*

Green synthesis provides advancement over chemical and physical method and it is cost-effective and environment-friendly. The green synthesis of iron oxide nanoparticles can be achieved by vigorous and stirring technique (Karkuzhali and Yogamoorthi, 2015). The reduction of iron oxide metal nanoparticles was primarily observed by the color change of the starting material from yellow to black, by the addition of Ferric nitrate as the precursor to the plant extract which acts as the reducing agent. UV–Vis spectra shows strong absorption peak at 250 nm. The formation of color in the reaction solution arises from the excitation of surface plasmon vibration in the metal nanoparticles (Shahverdi et al., 2007). Monalisa and Nayak (2013) reported that UV–Vis spectra in the range of 200–600 nm and they observed peak at 216–265 nm range. Sable and Saswade (2017) studied the phytochemical components of ethanol extracts of *Amaranthus tricolor* which revealed the

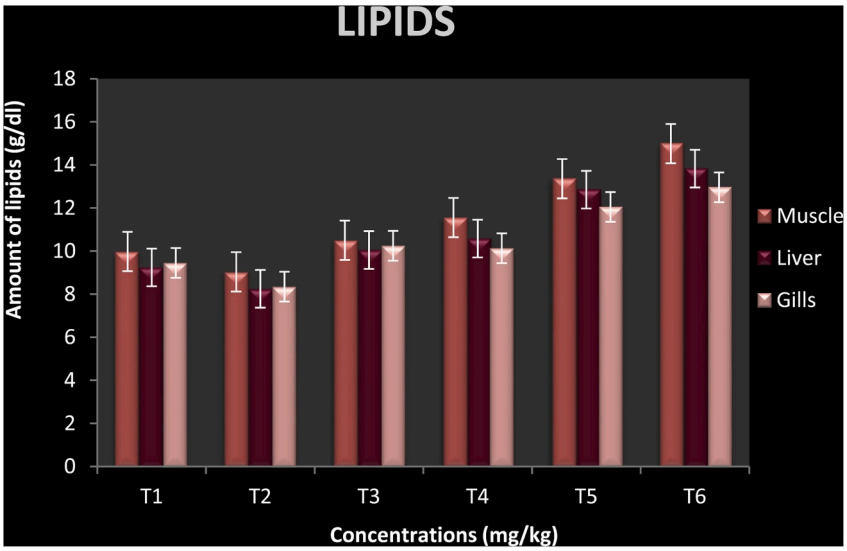

Fig. 7. Lipid content in muscle, liver and gills of *Labeo rohita*.

Table 5
Hematological parameters of *L. ROHITA* fed with feonps supplemented basal diets.

Parameters	+ve Control	-ve Control	Concentrations of FeONPs (mg/kg)			
			2.5	5.0	7.5	10.0
RBCs (million/ µl)	1.6 ± 0.06	0.95 ± 0.03	1.7 ± 0.03	1.7 ± 1.08	2.0 ± 1.02	2.4 ± 0.07
Haemoglobin (g/dl)	6.5 ± 0.03	6.2 ± 1.23	6.8 ± 0.01	7.5 ± 2.22	8.2 ± 0.67	8.9 ± 0.04
WBCs (1000 cells/cu) mm)	89.0 ± 0.42	93.12 ± 0.04	76.08 ± 1.28	73.0 ± 0.03	69.32 ± 0.42	65.68 ± 1.98

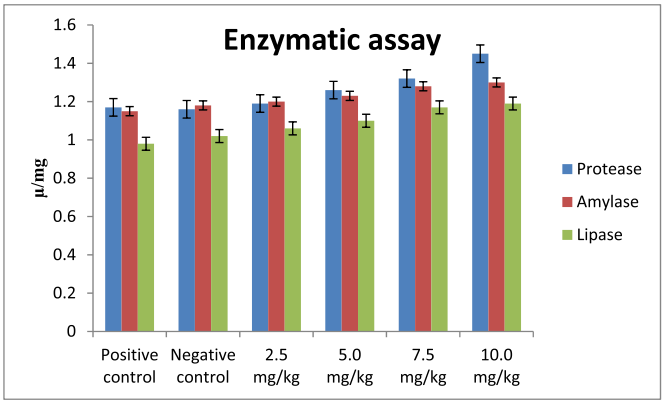

Fig. 8. Activities of digestive enzymes (Protease, Amylase and Lipase) in *Labeo rohita*.

presence of Alkaloids, Carbohydrates, Cardiac glycosides, Phenol, Tannins, Terpenoids and Quinones. The above results are similar to the data obtained in the present study. The SEM result revealed that the particles were spherical in nature. Similar kind of particles was reported by Karkuzhali and Yogamoorthi (2015). Maheswari and Sreenivasula, 2016 revealed that the nanoparticles containing iron and oxygen atoms and confirmed the purity of iron oxide nanoparticles. The present findings coincide with the earlier findings.

During the present study, iron oxide nanoparticles supplementation feeding at different concentration in the experimental diets resulted in higher growth performance as compared to the control diet. Increase in growth is due to the nutrient ability of iron oxide nanoparticles and also due to the enhancement of feed utilization. Behera et al. (2014) also observed that iron supplementation in diet improved the growth performance of *L. rohita.* The enhanced growth performances and feed utilization obtained in the present study may be due to the nanosized particles. In the present study, iron oxide nanoparticles supplementation feeding at different concentration in the experimental diets resulted in higher growth performance as compared to the control diet. Behera et al. (2014) also observed that iron supplementation in diet improved the growth performance of *Labeo rohita*. They stated that the final weight, weight gain, weight gain percentage, SGR and CF were significantly higher for the fish group fed with iron oxide nanoparticles. In the present study FCR decreased with increase in dietary iron oxide supplemented feed. This indicates the effective quality of this dosage when compared to control group and other doses. Similar study by Nasrin et al., 2018) confirmed that the FCR gets decreased with increase in dietary iron nanoparticles up to certain level of 40 (mg/kg) concentrations feed.

Evaluation of biochemical composition in fish is of immense importance in monitoring the health status and also for the evaluation of effect of diet supplements. The biochemical compositions like carbohydrate, proteins and lipids gets increased based on the concentrations. These components were significantly higher for the fish group fed with 10.0 (mg/kg) of iron oxide nanoparticles. This condition shows that dietary supplementation of iron oxide nanoparticles has influence on nutrient absorption and initiates the additional synthesis and storage of proteins and lipids in the fish *Labeo rohita*. Earlier study by Nasrin et al. (2018) showed that the biochemical composition like protein, cholesterol, triglycerides and lipid gets increased in *Clarias batrachus* fed with 40 mg/kg concentration of iron supplemented feed. Concentration based increase in the muscle biochemical compositions, such as protein and lipid compared to control group suggests that dietary Fe-NPs has influence on nutrient absorption and enhances the synthesis and storage of protein and lipid.

Blood parameters are one of the important indicators for monitoring the health condition of fish in terms of physiological as well as the pathological point of view (Mohseni et al., 2014; Dawood et al., 2015). Erythrocytes count and Hemoglobin show a significant increase in the iron oxide supplemented feed. Whereas, White Blood Cells (WBCs) counts were higher in the control group and gradually decreased throughout the iron oxide supplemented diet groups. This condition shows that there was an improved defense mechanism in the iron oxide nanoparticle supplemented diet group when compared with the control group. The blood parameters such as RBC, WBC and hemoglobin would

serve as baseline data for assessment of the health status of fish as well as reference point for future comparative surveys (Satheeshkumar et al., 2011).

The serum enzyme activity could provide further insight into the possible impacts of diets on fish growth performances and utilization (Dawood et al., 2015). Results of the presents study showed enhanced serum enzyme activity (Amylase, Protease, lipase) values in fish fed with the highest concentration of iron oxide nanoparticles. Swain et al. (2018) revealed that the diet supplemented with zinc oxide and selenium nanoparticles significantly increased the enzymatic activity when compared to control diet in the *L. rohita.*

5. Conclusion

Aquaculture plays a major role in providing animal protein to the human diet. In the Aquaculture industry, iron deficiency represents a critical problem for the health of the fish. Iron deficiency is one of the major cause of Anemia, poor physiological development, poor biochemical status and very low immunological status and increased mortality in fish. Iron is one of the most essential elements to maintain fish health. Nanotechnology is used as an alternative source to compete with the nutritional quality of the fish feed by means of metallic iron oxide nanoparticles. Results on the feeding trial of *L. rohita* with iron oxide nanoparticle supplemented diet showed a gradual increase in the physiological, biochemical, hematological and enzymatic activities. In conclusion, the present findings clearly indicate that *Amaranthus tricolor* mediated iron oxide nanoparticles supplemented basal diet possess the capabilities of being the alternative source to rectify the iron deficiency in *L. rohita.*

Ethics approval and consent to participate

Not applicable.

Availability of data and materials

All the data and materials presented in the manuscript are the original work of the authors.

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CRediT authorship contribution statement

S. Thangapandiyan: Visualization, Supervision, Data curation, Validation. **A.S. Alif Alisha:** Conceptualization, Investigation, Methodology, Writing - original draft. **K. Anidha:** Software, Formal analysis, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2020.101582.

Abbreviations

ANOVA	Analysis of Variance
DMRT	Duncan's Multiple Range Test
EDS	Energy Dispersive Spectroscopy
FCR	Food Conversion Ratio
Fl	femtoliter
FT-IR	Fourier Transform Infra-red Spectroscopy
G	Gram
SEM	Scanning Electron Microscopy
EDS	Energy Dispersive Spectroscopy

References

Andersen, F., Maage, A., Julshamn, K., 1996. An estimation of dietary iron requirement of Atlantic salmon, *Salmo salar* L., parr. Aquacult. Nutr. 2, 41–47.

Asaikkutti, Annamalai, Bhavan, P.S., Vimala, K., Karthik, M., Cheruparambath, P., 2016. Dietary supplementation of green synthesized manganese oxide nanoparticles and its effect on growth performance, muscle composition and digestive enzyme activities of the giant freshwater prawn *Macrobrachium rosenbergii.* J. Trace Elem. Med. Biol. 35, 7–17.

Barnes, H., Black Stock, J., 1973. Estimation of Lipids in marine animals and tissues detailed investigation of the Sulpho phosphor vanillin method for total lipids. J. Exp. Mar. Biol. Ecol. 12, 103–118.

Behera, T., Swain, P., Rangacharulu, P.V., Samanta, M., 2014. Nano-Fe as feed additive improves the hematological and immunological parameters of fish, *Labeo rohita* H. Appl. Nanosci. 4, 687–694.

Bernfeld, P., 1955. Amylases. In: Colowick, S.P., Kalpan, N.O. (Eds.), Methods in Enzymology. Academic Press, New York, pp. 149–158.

Bhavika, T., Paras, T., Sejal, S., 2018. Green synthesis of zero valent iron nanoparticles from *spinaciaoleracea* (spinach) and it's application in waste water treatment. IAETSD J. Adv. Res. Appl. Sci. 5, 2394–8442.

Dawood, M.A.O., Koshio, S., Ishikawa, M., Yokoyama, S., 2015. Interaction effects of dietary supplementation of heat-killed Lactobacillus *plantarum* and β-glucan on growth performance , digestibility and immune response of juvenile red sea bream, Pagrus major. Fish Shellfish Immunol. 45, 33–42.

Drabkin, D.L., 1950. The distribution of the chromoproteins, hemoglobin, myoglobin and cytochrome C, in the tissues of different species, and the relationship of the total content of each chromoprotein to body mass. J. Biol. Chem. 182, 317–333.

Erkan, C., Kizak, Volkan, Murathan, Kayim, SafakSeyhaneyilditz, C., Kutlu, Banu, Mehmet, A., Mehmet, K., Demirtas, Nida, 2011. Nanotechnological applications in aquaculture-seafood industries and adverse effects of nanoparticles on environment. J. Mater. Sci. Eng. 5, 605–609.

FAO, 2006. State of World Fisheries and Aquaculture (SOFIA) SOFIA. Food and Agriculture Organization of the United Nations, Rome, Italy, p. 162.

FAO/WHO, 1989. Evaluation of Certain Food Additives and the Contaminants Mercury, Lead and Cadmium. WHO Technical Report, Series No. 505.

Farre, E., Gajda-Schrantz, K., Kantiani, L., Barcelo, D., 2009. Ecotoxicity and analysis of nanomaterials in the aquatic environment. Anal. Bioanal. Chem. 393, 81–95.

Furne, M., Hidalgo, M.C., Lopez, A., Garcia-Gallego, M., Morales, A.E., Domenzain, A., Domenzain, J., Sanz, A., 2005. Digestive enzymes activities in adriatic sturgeon acipensernaccarii and rainbow trout Oncorhynchus mykiss. Comparitive Stud. Aquac. 250, 391–398.

Gottimukkala, K.S.V., Harika Reddy, P., Deeveka, Zamare, 2017. Green synthesis of iron nanoparticles using green tea leaves extract. J. Nanomed. Biother. Discov. 7 https://doi.org/10.4172/2155-983X.1000151.

Hilty, Knijnenburg, J.T.N., Teleki, K.F., Hurrell, R.F., Pratsinis, S.E., Zimmbermann, M. B., 2011. J. Food Sci. 76, 1–10.

Karkuzhali, Yogamoorthi, A., 2015. Biosynthesis of iron oxide nanoparticles using aqueous extract of *jatropha gossipifolia* as source of reducing agent. Inter. J. Nano Sci. Nanotech. 6, 47–55.

Lucas, M., Robinson, H., Oberle, F., 2012. Evaluation of corn gluten feed and cottonseed meal as partial replacements for soybean meal and corn in diets for pond-raised hybrid catfish, *Ictalurus punctatus I. furcatus.* J. World Aquacult. Soc. 43, 107–113.

Maheswari, K.C., Sreenivasula, R., 2016. Green synthesis of magnetite nanoparticles through leaf extract of *azadirachtaindica.* J. Nanosci. Tech.. 2, 189–191.

Mohseni, M., Pourkazemi, M., Bai, S.C., 2014. Effects of dietary inorganic copper on growth performance and immune response of juvenile beluga, Husohuso. Aquacult. Nutr. 20, 547–556.

Monalisa, Paattanayak, Nayak, P.L., 2013. Green synthesis and characterization of *zero valent* iron nanoparticles from the leaf extract of *azhadirachtaindica (neem).* World J. Nano Sci. Technol. 2, 06-09.

Nasrin, Akter, Alam, Jahangir, Jewel, Abu Sayed, Haque, Ayenuddin, Khatun, Samsad, Sumaiya, Akter, 2018. Evaluation of dietary metallic iron nanoparticles as feed additive for growth and physiology of Bagride catfish *Clariasbatrachus*(Linnaeus, 1758). Int. J. Fish. Aqua. Stud. 6, 371–377.

Onegbhu, C.U., Agarwal, A., Singh, N.B., 2018. ZnO nanoparticles as feed supplement on growth performance of cultured African Catfish Fingerlings. J. Sci. Ind. Res. 77 (4), 213–218.

Roeder, M., Roeder, R.H., 1968. Effect of iron on the growth rate of fishes. J. Nutr. 90 (1), 86–90.

Sable, K.V, Saswade, R.R, 2017. Preliminary phytochemical analysis of different solvent extracts from leaf of *Amaranthus tricolor*. Int. J. Recent Sci. Res. 8 (8), 19356–19358.
Satheeshkumar, P., Ananthan, G., Senthil Kumar, D., Jagadeesan, L., 2011. Haematological and biochemical parameters of different feeding behavior of teleost fishes from Vellar estuary, India. Comp. Clin. Pathol. 21, 1187–1191.
Shahverdi, A.R., Fakhimi, A., Shahverdi, H.R., Minaian, S., 2007. Synthesis and effect of silver nanoparticles on the antibacterial activity of different antibiotics against Staphylococcus aureus and Escherichia coli. Nanomed. Nanotechnol. Biol. Med. 3, 168–171.
Swain, P, et al., 2018. Effects of dietary zinc oxide and selenium nanoparticles on growth performance, immune responses and enzyme activity in rohu, *Labeo rohita* (Hamilton). Aquacult. Nutr. 27 (3), 1–9. In this issue.
Trease, G.E., Evans, W.C., 1989. Pharmacognosy. W.B. Scandars Company Ltd, London, pp. 269–300.
Ward, J.E., Kach, D.J., 2009. Marine aggregates facilitate ingestion of nanoparticles by suspension-feeding bivalves. US Nat. Libr. Med. Nat. Inst. Health 68, 137–142.