



Green Synthesized Zinc Oxide Nanoparticles as Feed Additives to Improve Growth, Biochemical, and Hematological Parameters in Freshwater Fish *Labeo rohita*

S. Thangapandiyan¹ · S. Monika¹

Received: 4 July 2019 / Accepted: 18 August 2019
© Springer Science+Business Media, LLC, part of Springer Nature 2019

Abstract

Zinc deficiency in aquatic animals affects the biological processes and physiological functions. Thus, the supplement of ZnONPs can be used as an alternative method to overcome zinc deficiency. Nanoparticles have the potential to enhance the growth and health of the fish. The main aim of this study is to evaluate the growth efficacy of ZnONP-supplemented diet with fingerlings of *Labeo rohita*. The green synthesized ZnONPs were characterized by ultraviolet-visible (UV-Vis) spectroscopy, Fourier transformer infrared spectroscopy (FTIR), X-ray diffraction (XRD), scanning electron microscopy (SEM), and energy dispersive spectroscopy (EDS). Different concentrations of ZnONPs (5, 7.5, and 10 mg/kg) were administered in the basal diet of freshwater fish *Labeo rohita* for 45 days to observe the growth and metabolic functions of the body. However, the fish fed with 10 mg/kg ZnONP-supplemented diet shows that the growth performance was highly increased followed by 7.5 mg/kg and 5 mg/kg ZnONPs when compared with the control. The biochemical, hematological, and digestive enzyme activities were also significantly increased with different concentrations of ZnONPs. The effects of zinc oxide nanoparticles show the higher improvement of growth and metabolic functions in *Labeo rohita*. These results suggest that the nanotechnology could apply for feed formulation technology and pave the way for the dietary supplementation of zinc oxide nanoparticles as safe ingredients for aquatic animals to overcome the zinc deficiency.

Keywords Zinc oxide nanoparticles · *Labeo rohita* · X-ray diffraction (XRD) · Scanning electron microscopy (SEM) · Energy dispersive spectroscopy (EDS)

Introduction

Fish provides macronutrients and micronutrients that are essential to cognitive and physical development of human beings. Zinc is one of the essential micronutrient which is required for the maintenance of normal body functions of all living organisms. It regulates protein synthesis and acts as a regulator of energy, vitamin A, and lipid metabolism [1]. The availability of zinc from feed ingredients which have been historically the main protein source in feeds is typically low due to the presence of tricalcium phosphate which interferes with the absorption of zinc [2]. The zinc deficiency in fish leads to reduced growth and appetite, depressed bone Ca

and Zn content, high mortality, cataracts, dwarfism, and erosion of fins [3].

Nanotechnology provides major opportunity for the economy and sustainable development of aquatic resources in many countries. Although the application of nanotechnology is at a very early stage in aquaculture, it may have the potential to solve most of the problems in aquaculture and fisheries with better technical innovation at different levels. At present, Central Institute of Fisheries Education (India) has initiated research on the application of nanotechnology in aquaculture and fisheries [4]. Nanotechnology is termed as a result of the synthesis, characterization, exploration, and application of nanoparticles in the field of science and technology [5]. Nanoparticles are tiny materials having size ranging from 1 to 100 nm. These nanoparticles showed characteristic colors and properties with the variation of size and shape, which can be utilized in bioimaging applications [6].

Zinc is a natural component and a part of nature's inherent, which fulfills important metabolic functions in humans, animals, and plants. Zinc oxide is considered as a valuable

✉ S. Thangapandiyan
stp.nano@gmail.com

¹ Department of Zoology, PSG College of Arts and Science, Civil Aerodrome Post, Peelamedu, Coimbatore 641014, India

nutritional additive. Zinc oxide exists within the earth crust as a mineral zincite; while, most of it being used commercially is produced through synthetic methods [5]. Among the inorganic metal oxides, ZnONPs are non-toxic, biosafe, biocompatible, and heat-resistant and are being used as drug carriers, cosmetics, and fillings in medical materials. Another advantage of using ZnONPs as antibacterial agent is that they effectively inhibit activity of pathogenic microbes at small concentration [7]. Nanoparticles have the potential of controlling the pathogens, improving the immune and growth functions in aquaculture [8].

Increasing awareness towards green chemistry and other biological processes has led to the development of an eco-friendly and cost-effective approach for the synthesis of nanoparticles than conventional physical and chemical methods of synthesis [9]. The main reaction involved in the green synthesis of ZnO nanoparticles using plant leaf extract is reduction/oxidation reaction due to the presence of phytochemicals and enzymes in that biological materials takes part for the conversion of metal compounds to nanoparticles [10].

Spinach is a green leafy vegetable belonging to Amaranthaceae family. Normally, it is an annual plant (rarely biennial) growing up to 30-cm height. It is a rich source of vitamin A, vitamin C, vitamin K, magnesium, manganese, folate, and iron [11]. It also helps to fight diseases of the bone and skin disorders and helps to protect the health of infants and newborns [12].

Labeo rohita, commonly called as Rohu and one of the three Indian major carps, is the right choice for the present study because of its easy availability, of its soft nature, and of its adaptable nature to the laboratory condition. The proper amount of micronutrients is required for the improvement of fish growth and physiological health [13]. The major aim of the study is to investigate the growth, survival, biochemical, hematological, and enzymatic activities of fingerlings of *Labeo rohita* supplemented with different concentrations of zinc oxide nanoparticles.

Materials and Methods

Collection and Identification of Plant

The entire plant of *Spinacia oleracea* which belongs to Amaranthaceae family was collected from a nearby agricultural farm in Marudhampatti, Namakkal district, Tamil Nadu. The plant was identified and authenticated by Dr. C. Murugan, Scientist “E” & Head of Office, Botanical Survey of India (BSI), Southern Regional Centre, Tamil Nadu Agricultural University (TNAU) Campus, Coimbatore, Tamil Nadu.

Preparation of Leaf Extract

The collected fresh leaves were washed thoroughly with tap water followed by distilled water and they were air-dried at room temperature for 4–5 days. The dried leaves were powdered using mixture grinder. Five grams of dried leaf powder was added in 100 mL of 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 airtight container for further experimental usage [14].

Green Synthesis of Zinc Oxide Nanoparticle

To 50 mL of 2 M zinc acetate solution, 5 mL of aqueous plant extract and 2–3 mL of 2 M NaOH solution were added under constant stirring resulting in the formation of pale white precipitate which indicates the formation of zinc oxide nanoparticles. The precipitate was washed thrice with distilled water followed by ethanol to get free of impurities and allowed for air dry at room temperature overnight [10, 14].

Characterization

Phytochemical Analysis of Leaf Extract

Freshly prepared leaf extracts were subjected to standard phytochemical analysis using standard procedure [15] in order to find out the presence of various phytoconstituents such as alkaloids, terpenoids, flavonoids, tannins, steroids, anthraquinones, sponins, resins, glycosides, and phenols.

UV-Vis Spectroscopy

UV-Vis spectroscopy of zinc oxide nanoparticles was performed on a Shimadzu dual beam spectrophotometer (model UV-2000 Shimadzu) operated at a resolution of 1 nm.

Fourier Transformer Infrared Spectroscopy

Fourier transformer infrared spectroscopy (FTIR) measurements were carried out to explore the mode of interactions between spinach leaf extract and the nanoparticle surface. FTIR spectra of the spinach leaf extract and nanoparticles were achieved in a Shimadzu FTIR spectrophotometer and registering amplitude waves were ranging from 550 to 4000 cm^{-1} .

X-ray Diffraction

The crystallographic analysis of the samples was performed by powder X-ray diffraction. The X-ray diffraction patterns were recorded in the scanning mode on an X’pert PROPAN

analytical instrument operated at 40 kV and a current of 40 mA with Cu K α radiation ($\lambda = 1.54060 \text{ \AA}$). The different intensities were recorded from 35° to 79.93° , in 2θ angles. The diffraction intensities were compared with the standard JCPDS files. The software gave the information about the crystal structure of the particle.

The average size of the nanoparticles can be estimated using the Debye-Scherrer equation.

$$D = K\lambda/\beta \cos\theta,$$

where

- D thickness of the nanocrystal
- K constant
- λ wavelength of X-rays
- β width at half maxima of reflection at Bragg's angle
- $2\theta, \theta = \text{Bragg}$

Scanning Electron Microscopy

Morphology of the synthesized zinc oxide nanoparticles was investigated with the scanning electron microscope (ZEISS) operated at a resolution of 60 \AA at 4.00 kV magnification of 34.82 k.

Energy Disperse Spectroscopy

EDS analysis gives qualitative as well as quantitative status of elements that may be involved in the formation of nanoparticles. It was used for the determination of elemental composition and purity of the sample.

Collection and Acclimatization of Fish

The fingerlings of *Labeo rohita* with an average size of 6 cm were procured from the Palar Fish Farm, Palani, Tamil Nadu. The collected fingerlings were maintained in the plastic troughs with 30 L capacity containing Siruvani water (25 L) from college campus with automatic aerator (aqua air pump).

Water Quality Parameters

The water quality parameters such as temperature, pH, total dissolved solids (TDS), turbidity, alkalinity, determination of chloride, sulfate, nitrite, magnesium, and calcium were monitored frequently using standard methods [16].

Basal Diet Formulation

ZnONPs were used as the zinc source and added to the experimental basal diets at the rates of 0, 50, 75, and 100

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. The basal diets were prepared in the form of pellets [17]. Active healthy fishes were chosen from the acclimation tank and starved for 48 h prior to the commencement of the experiment [18]. In the study, five groups (treatments 1, 2, 3, 4, and 5) of *L. rohita* with 25 individuals were assigned for this experiment for 45 days. Treatment 1 was considered as positive control and was fed without ZnONPs. Treatment 2 was considered as negative control and was fed with commercial feed. Treatments 3, 4, and 5 were fed with 5.0, 7.5, and 10.0 mg/kg ZnONP-supplemented diets, respectively.

Bioassay

Analysis of Growth, Survival, and Nutritional Indices

At the end of the feeding trial, the survival rate, growth (length and weight), and other food parameters such as feed intake, specific growth rate, feed conversion ratio, and protein efficiency ratio were individually calculated by the following equations [1]:

$$\text{Survival (\%)} = \frac{\text{number of fish survived}}{\text{initial number of fish}} \times 100$$

$$\text{Length gain (cm)} = \text{Final length} - \text{Initial length}$$

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

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

$$\text{Food conversion ratio (FCR)} = \frac{\text{Total feed intake (g)}}{\text{Total wet weight gain (g)}}$$

Biochemical Analysis

Estimation of Carbohydrates

Carbohydrate level in the muscle, liver, and gills of fishes was estimated by anthrone method [19]. The absorbance of the reddish brown-colored mixture was read at 630 nm using a photoelectric colorimeter, and glucose acts as the standard for estimating the carbohydrates.

Carbohydrate levels in tissues of the fishes were calculated using the following formula:

$$\text{Amount of carbohydrate present} = \frac{\text{OD of the sample}}{\text{OD of the standard}} \times \text{concentration of standard} \times 100 \text{ mg/gm}$$

Estimation of Total Protein

Protein content of fish muscle, liver, and gills was estimated by using the commercial kit (Autospan – Liquid Gold Total Protein, Modified Biuret, end point assay). The absorbance was read at 578 nm using a photoelectric colorimeter.

The total protein concentrations were calculated using the following formula:

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

Estimation of Lipids

Lipids are soluble in some organic solvents, which are utilized for extracting lipids from tissues. Total lipid content was measured by Folch's method at 590 nm using the formula given below [20, 21].

$$\text{Lipids present in the sample} = \frac{\text{OD of the sample}}{\text{OD of the standard}} \times \text{concentration of standard} \times 100$$

Hematological Studies

Blood Sampling

Blood samples were collected from the fish by sacrificing them by cardiac puncture with the help of a disposable syringe fitted with a 26-gauge needle which was rinsed with heparin (Heparin Sodium I.P 5000 I.U. in 5 mL derived from porcine mucosa), an anticoagulant. The stored blood samples were used for hematological studies [22].

Erythrocytes Count

The erythrocytes were counted by using a hemocytometer. Cell counts were performed by using a Neubauer's counting chamber [23]. The total number of erythrocytes was then calculated using the following formula:

$$\text{No. of RBCs} = \frac{\text{No. of erythrocyte} \times \text{dilution counted}}{\text{Area counted} \times \text{depth of fluid}} \text{ (millions/cu mm of blood)}$$

$$\text{Dilution} = 200, \text{Area counted} = 5 \times 0.04 = 0.2 \text{ mm}^2, \text{ and Depth of fluid} = 0.1 \text{ mm}$$

Estimation of Hemoglobin

The hemoglobin level of blood was estimated by the cyanomethemoglobin method using Drabkin's fluid [24]. The hemoglobin concentration was then calculated using the following formula:

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

Hematocrit (Packed Cell Volume)

Hematocrit was determined by micro-hematocrit (capillary) technique described by Dacie and Lewis [25] using RM 12 °C micro-centrifuge and a micro-hematocrit reader.

Mean Cell Volume

The mean cell volume (MCV) is the average volume of red blood cells and is calculated from the hematocrit (HCT) and the red blood cell count (RBC) [26].

$$\text{MCV} (\mu\text{m}^3) = \frac{\text{HCT} (\%)}{\text{RBC count in millions /mm} \times 1000} \times 10$$

Mean Cell Hemoglobin

The mean cell hemoglobin (MCH) is the content (weight) of hemoglobin of the average red cell. It is calculated from the hemoglobin concentration and the red blood cell count [24].

$$\text{MCH} (\text{pg}) = \frac{\text{hb (g/dL)}}{\text{RBC count in millions /mm} \times 1000} \times 10$$

Mean Cell Hemoglobin Concentration

The mean cell hemoglobin concentration (MCHC) is the average concentration of hemoglobin in a given volume of packed red blood cells. It is calculated from the hemoglobin concentration and the hematocrit [26].

$$\text{MCHC (g/dL)} = \frac{\text{Hb} \left(\frac{\text{g}}{\text{dl}} \right)}{\text{HCT} (\%)} \times 100$$

Leucocyte Count

Leucocytes were counted by using the hemocytometer. Cell counts were performed by using a Neubauer's counting chamber [23]. The total number of leucocytes was then calculated using the following formula:

$$\text{No. of WBCs} = \frac{\text{Number of leucocyte} \times \text{dilution counted}}{\text{Area counted} \times \text{depth of fluid}}$$

(1000/cu mm of blood) Dilution = 20,
Area counted = $4 \times 1 = 4 \text{ mm}^2$,
and Depth of fluid = 0.1 mm

Assay of Digestive Enzymes

Activities of digestive enzymes such as protease, amylase, and lipase were assayed. The whole digestive tract and hepatopancreas were homogenized in ice cold double distilled water and centrifuged at 9300g 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 of Furne et al. [27]; amylase activity was determined by the starch-hydrolysis method of Bernfeld [28]; and lipase activity was analyzed by the method of Furne et al. [27].

Statistical Analysis

The data were analyzed by one-way analysis of variance (ANOVA) using SPSS 20 version for the estimation of biochemical parameters and digestive enzymes activities. The growth performance was analyzed by Duncan's Multiple Range test (DMRT) using SPSS 20 version. The data were represented as mean \pm standard deviation.

Results

Characterization

Phytochemical Analysis

The results of the phytochemical analysis of the aqueous leaf extract of *Spinacia oleracea* are shown in Table 1.

UV-Vis Spectroscopy

UV spectrum of colloidal solution of zinc oxide nanoparticles shows strong peak at 377 nm (Fig. 1). The zinc oxide nanoparticles exhibit an intense absorption peak due to the surface

Table 1 Phytochemical analysis of aqueous plant extract

S. no.	Phytoconstituents	Test adopted	Results
1	Alkaloids	Mayer s test	+
2	Steroids and sterols	Chloroform test	+
3	Flavonoids	Liermann-Burchard test	+
4	Triterpenoids	Ferric chloride test	–
5	Tannins	Knollar s test	+
6	Phenols	Ferric chloride test	+
7	Cardiac glycosides	Ferric chloride test	–
8	Volatile oil	Keller-Killiani s test	–
9	Saponins	Foam test	+
10	Fatty acids	Foam test	–
11	Glycosides	Chloroform test	+
12	Terpenoids	Keller-Killiani s test	+
13	Proteins	Biuret	+
14	Carbohydrates	Benedict's test	+
15	Quinone	Benedict s test	+

“+” = present; “–” = absent

plasmon around 377 nm which really indicates the formation of zinc oxide nanoparticles.

Fourier Transform Infrared Spectroscopy

FTIR spectra of *Spinacia oleracea* leaf extract and *Spinacia oleracea*-mediated zinc oxide nanoparticles were recorded in order to identify the functional groups involved in the formation of nanoparticles (Fig. 2a, b). The ZnO peak was noted at 447.49 cm^{-1} . The C=C stretching observed at 1647.21 in *Spinacia oleracea* was shifted to 1635.64 in *Spinacia oleracea*-mediated zinc oxide nanoparticles. In addition to this, the C=C=C stretch appeared at 1998.25 in leaf extract which was shifted to 1967.39 in nanoparticles. The N=O stretch appeared at 1550.77 in leaf extract which was shifted to 1539.20 in nanoparticles. The C-H stretch appeared at

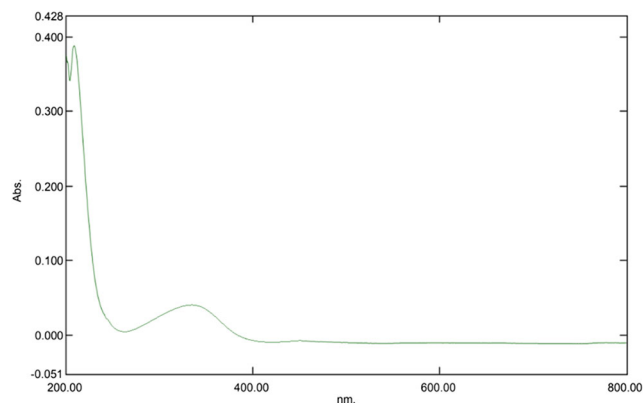
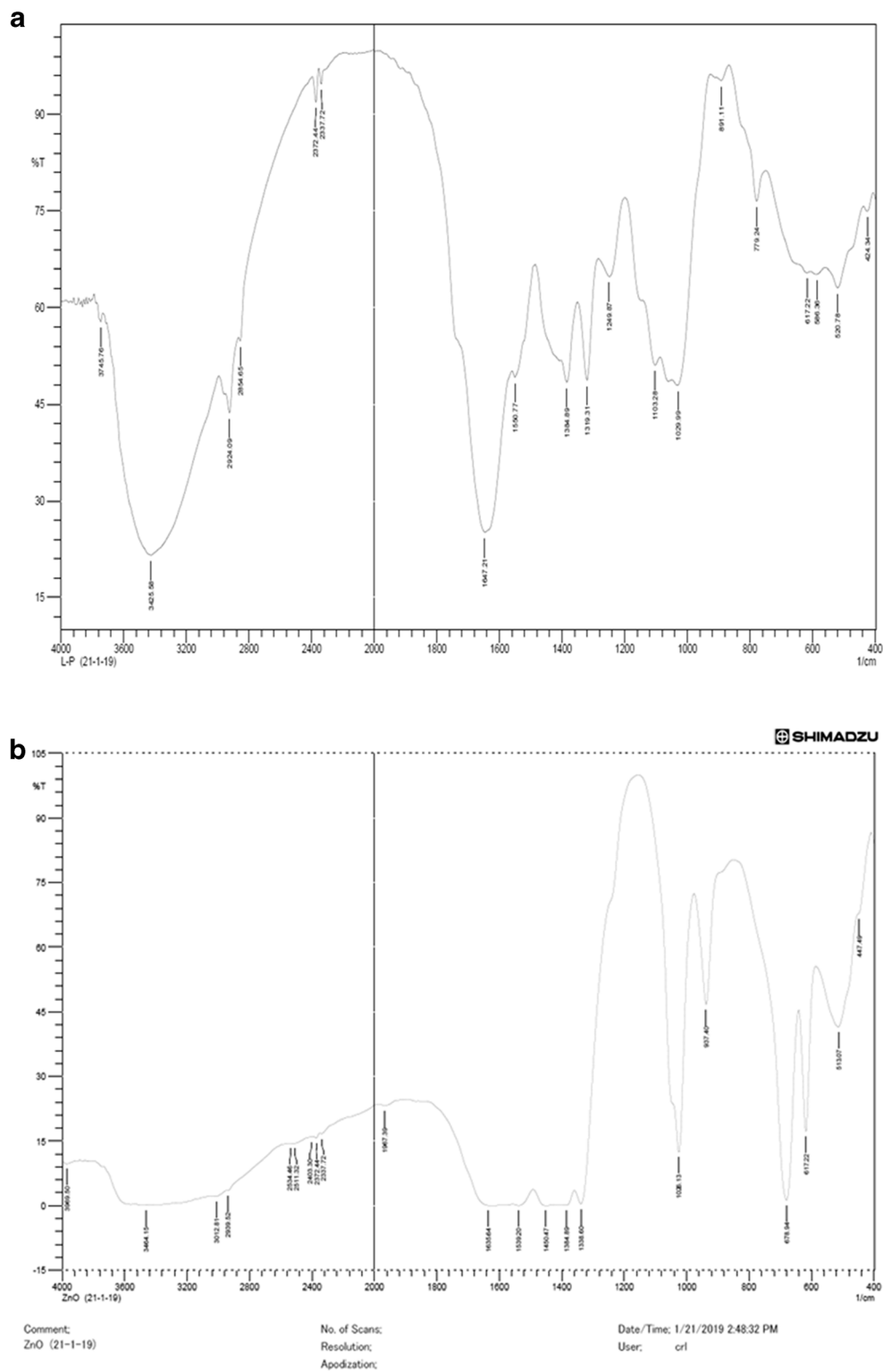


Fig. 1 UV spectrum of zinc oxide nanoparticles

Fig. 2 **a** FTIR spectrum of *Spinacia oleracea*. **b** FTIR spectrum of *Spinacia oleracea*-mediated zinc oxide nanoparticles

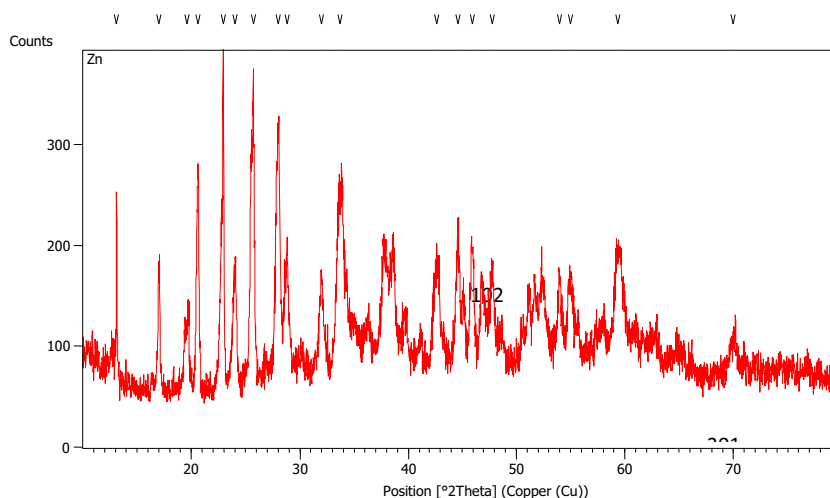


1384.89 in leaf extract which was shifted to exactly 1384.89 in nanoparticles. The C-O stretch appeared at 1064.71 in leaf extract which was shifted to 1026.13 in nanoparticles. The shift in the peaks confirmed that plant extract reduced zinc oxide for the formation of nanoparticles.

X-Ray Diffraction

The X-ray diffraction of zinc oxide nanoparticles is depicted in Fig. 3. The pattern shows the particle is crystalline in nature. The intensive diffraction peak at 2θ value of 47.76° from the

Fig. 3 X-ray diffraction pattern of zinc oxide nanoparticle



(102) lattice plane of face centered cubic (fcc) unequivocally indicates that the particles are made of pure zinc oxide. The additional peaks were observed at 69.98° which corresponds to the (201) planes of zinc oxide, respectively. The result showed that the particles were more or less spherical in nature. The size of the zinc oxide nanoparticles was found to be 22.61 nm.

Scanning Electron Microscopy

Scanning electron micrograph of synthesized zinc oxide nanoparticles revealed that the particles were more or less spherical in nature. It is depicted in Fig. 4.

Energy Dispersive Spectroscopy

Energy dispersive spectrum of zinc oxide nanoparticles showed a strong signal at 1 keV in the zinc region and thus

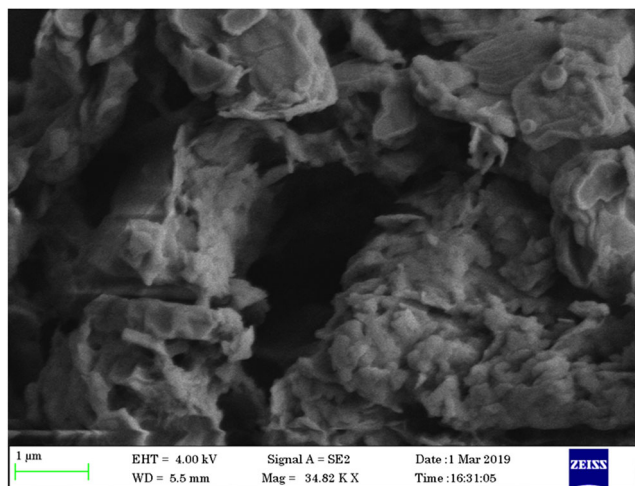


Fig. 4 Scanning electron microscopy of zinc oxide nanoparticles

confirmed the presence of zinc oxide nanoparticles in the prepared sample as depicted in Fig. 5.

Water Quality Parameters

The results of water quality parameters such as temperature, pH, total dissolved solids, turbidity, chloride, alkalinity, sulfate, nitrate, total hardness, magnesium, and calcium are given in Table 2.

Analysis of Growth, Survival, and Nutritional Indices

The results on growth performance, survival rate, and nutritional indices of *L. rohita* diets supplemented with different concentrations of ZnONPs are represented in Table 3. The fish supplemented with 10 mg/kg ZnONPs shows maximum growth performance such as weight gain (50 mg) and length gain (0.4 cm) when compared with control.

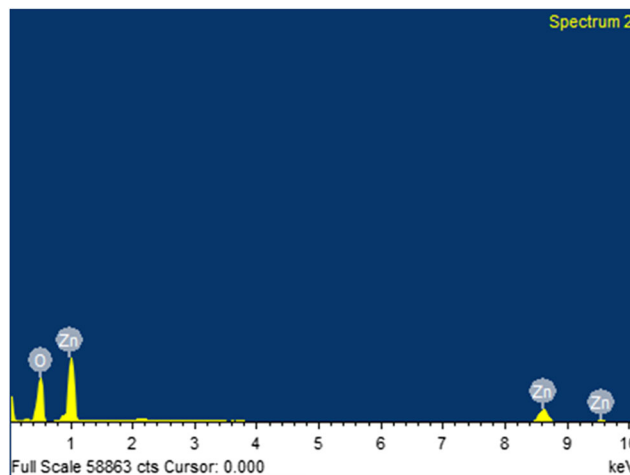


Fig. 5 Energy dispersive spectrum of zinc oxide nanoparticle

Table 2 Water quality parameters

Parameters	Results
Temperature	26 ± 2 °C
pH	7–8
Total dissolved solids	110 mg/L
Turbidity	0.1 N
Chloride	19.9 µg/L
Alkalinity	74 mg/L
Sulfate	1.8 mg/L
Nitrite	1.6 mg/L
Total hardness	26 mg/L
Magnesium	2.45 mg/L
Calcium	6.401 mg/L

Estimation of Carbohydrates

The level of carbohydrates present in fish tissues (muscle, liver, and gills) was gradually increased with increase in the ZnONP concentration (Fig. 6). The results indicate that the increased levels of carbohydrates present in the muscle, liver, and gills of fish supplemented with 10 mg/kg ZnONPs are 14.10 ± 1.42, 12.94 ± 1.09, and 11.89 ± 1.02 mg/g, respectively, when compared with the control. The analyzed data using one-way analysis of variance

(ANOVA) revealed that the levels of carbohydrates in fish were significantly increased at different concentrations of samples ($F = 4.307$; $P < 0.05$).

Estimation of Total Protein

The results of the present study indicate that the level of total protein present in fish tissues (muscle, liver, and gills) was significantly increased with increase in the ZnONP concentration (Fig. 7). There is an increased level of total protein present in the muscle, liver, and gills of fish supplemented with 10 mg/kg ZnONPs which is 17.03 ± 1.48, 16.93 ± 1.69, and 16.54 ± 1.38 g/dL, respectively, when compared with the control. The one-way analysis of variance (ANOVA) is used to analyze the data and it proved that the level of total protein was significantly increased at different concentrations of samples ($F = 3.563$; $P < 0.05$).

Estimation of Lipids

The estimated lipid levels in fish tissues (muscle, liver, and gills) were increased in the various concentrations of ZnONPs (Fig. 8). The level of lipids present in the muscle, liver, and gills of fish supplemented with 10 mg/kg ZnONPs is 14.94 ± 1.05, 14.87 ± 0.21, and 14.64 ± 1.09 mg/g followed by 7.5

Table 3 Survival, growth, and nutritional indices of *L. rohita* fed with ZnONP-supplemented basal diets

Parameters	Days	Positive control	Negative control	Concentrations of ZnONPs (mg/kg)		
				5.0	7.5	10.0
Survival rate (%)	1	100	100	100	100	100
	15	100	100	100	96	97
	30	96	92	95	95	96
	45	88	92	94	95	96
Length gain (cm)	1	6.93	7.03	6.7	5.3	7.1
	15	6.93	7.03	6.73	5.35	7.12
	30	7.02	7.07	6.79	5.4	7.15
	45	7.03	7.08	7.10	5.40	7.30
Weight gain (g)	1	2.52	2.16	2.03	2.27	2.05
	15	2.53	2.16	2.04	2.28	2.07
	30	2.55	2.17	2.06	2.3	2.07
	45	2.56	2.17	2.07	2.32	2.07
SGR (%)	1	0.02	0.02	0.02	0.05	0.05
	15	0.03	0.05	0.05	0.09	0.08
	30	0.05	0.06	0.09	0.1	0.09
	45	0.06	0.08	0.1	0.12	0.12
FCR (g)	1	1.86	1.78	1.12	0.91	0.75
	15	1.84	1.69	1.09	0.85	0.72
	30	1.74	1.25	1.0	0.82	0.65
	45	1.25	1.12	0.98	0.75	0.52

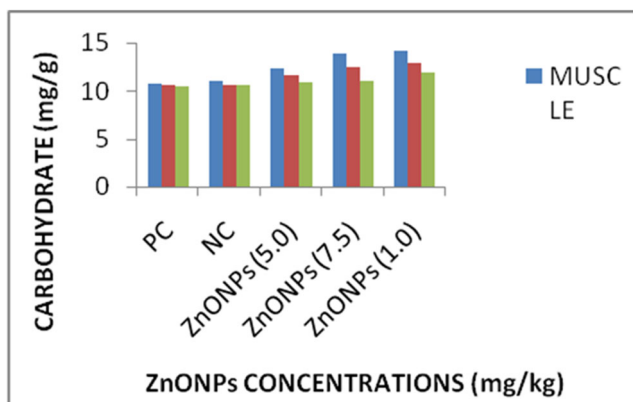


Fig. 6 Carbohydrate levels in muscle, liver, and gills of *Labeo rohita* fed with ZnONP-supplemented diet. PC = positive control; NC = negative control

mg/g and 5.0 mg/g of ZnONPs, respectively, when compared with the control. The analyzed data using one-way analysis of variance (ANOVA) revealed that the level of lipids was significantly increased at different concentrations of samples ($F = 22.146$; $P < 0.05$).

Blood Sampling

Erythrocytes Count

The erythrocytes (RBCs) count of *L. rohita* was gradually increased with increasing concentrations of ZnONPs. The fish fed with 10.0 mg/kg ZnONP-supplemented diet perform increased erythrocyte counts of 1.9 million/cu mm, respectively. The decreased erythrocyte counts (0.95 million/cu mm) were observed in control fish fed without ZnONPs (Table 4).

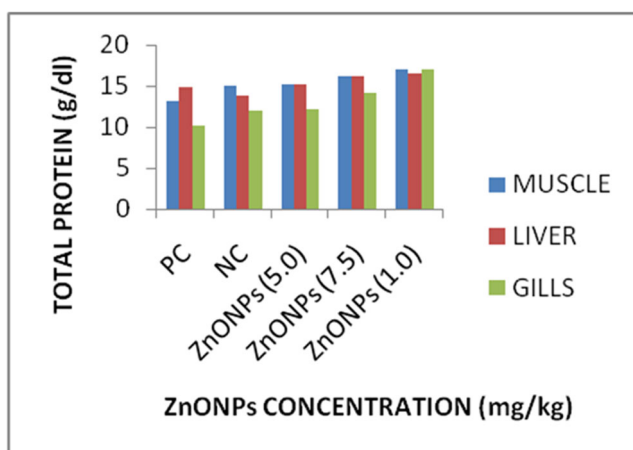


Fig. 7 Total protein levels in the muscle, liver, and gills of *Labeo rohita* fed with ZnONP-supplemented diet. PC = positive control; NC = negative control

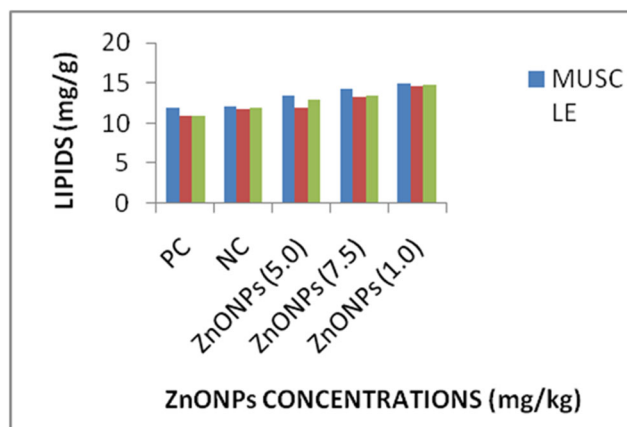


Fig. 8 Lipid content in the muscle, liver, and gills of *Labeo rohita* fed with ZnONP-supplemented diet. PC = positive control; NC = negative control

Estimation of Hemoglobin

The obtained results proved that the hemoglobin content in fish blood was significantly increased in various concentrations of ZnONPs. The fish fed with 10 mg/kg ZnONP-supplemented diet perform increased hemoglobin content of 8.5 g/dL followed by 7.5 g/dL in 7.5 mg/kg ZnONPs and 6.5 mg/kg ZnONPs which is shown in Table 4.

Hematocrit

The results of hematocrit are represented in Table 4. The hematocrit percentage of the fish fed with 10 mg/kg ZnONP-supplemented diet was 14.8% followed by 14% in 7.5 mg/kg and 6.5% in 5 mg/kg ZnONPs, respectively. The data revealed that the hematocrit content was increased in the fish fed with different concentrations of ZnONPs.

Mean Cell Volume

The increased mean cell volume (MCV) values of 87.0 fl was performed by the fish fed with 10 mg/kg ZnONP-supplemented diet. The MCV value observed in control fish fed without ZnONPs was 67.5 fl and is represented in Table 4.

Mean Cell Hemoglobin

The value of mean cell hemoglobin (MCH) in *L. rohita* was increased in various concentrations of ZnONPs. The fish fed with 10 mg/kg ZnONP-supplemented diet performs increased MCH values of 43.2 pg followed by 41.6 and 38.2 pg in 7.5 and 5 mg/kg ZnONPs, respectively. Thus, Table 4 shows that the MCH values were increased in the fish fed with different concentrations of ZnONPs when compared with control.

Table 4 Hematological parameters of *L. rohita* fed with ZnONP-supplemented basal diets

Parameters	Positive control	Negative control	Concentrations of ZnONPs (mg/kg)		
			5.0	7.5	10.0
RBCs (million/uL)	1.6 ± 0.1	0.95 ± 0.12	1.7 ± 0.11	1.8 ± 0.9	1.9 ± 0.88
Hemoglobin (g/dL)	5.2 ± 0.01	5.2 ± 0.63	6.5 ± 0.02	7.5 ± 0.05	8.5 ± 0.11
Hematocrit (%)	10.8 ± 1.25	8.3 ± 1.12	12.0 ± 1.23	14.0 ± 1.22	14.8 ± 1.21
MCV (fl)	67.5 ± 1.56	69.3 ± 1.89	70.5 ± 1.68	77.7 ± 1.35	87.0 ± 1.57
MCH (pg)	32.5 ± 1.26	34.9 ± 1.45	38.2 ± 1.49	41.6 ± 1.54	43.2 ± 1.64
MCHC (g/dL)	48.0 ± 1.23	50.4 ± 1.58	54.0 ± 1.56	55.5 ± 1.54	63.4 ± 1.24
WBCs (cells/cu mm)	32,000	22,400	30,000	27,000	32,000

Mean Cell Hemoglobin Concentration

There is an increase in mean cell hemoglobin concentration (MCHC) in *L. rohita* fed with various concentrations of ZnONPs. The fish fed with 10.0 mg/kg ZnONP-supplemented diet performs increased MCHC values of 63.4 g/dL, respectively (Table 4).

Leucocyte Count

The leucocyte counts in *L. rohita* does not undergo any changes with increase in ZnONP concentration. The leucocyte counts observed in fish fed with ZnONPs and fish fed without ZnONPs were maintained around 32,000 cells/cu mm. This results show that the fishes were free from any kind of diseases. The observed results are shown in Table 4.

Assay of Digestive Enzymes

The activity of digestive enzymes such as protease, amylase, and lipase in *L. rohita* was significantly increased in ZnONP-supplemented diets, as shown Fig. 9. The one-way analysis of

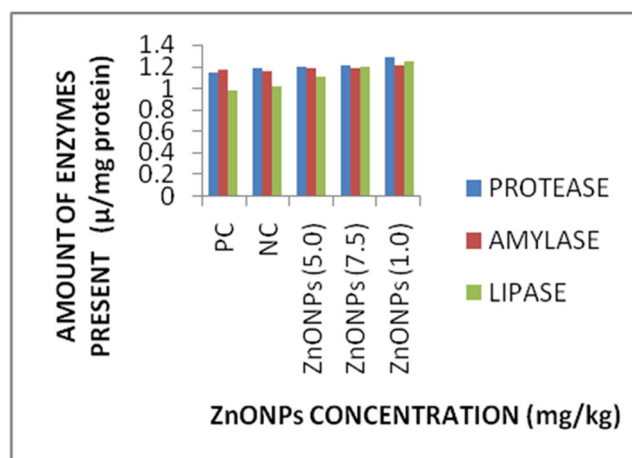


Fig. 9 Activities of digestive enzymes (protease, amylase, and lipase) in *Labeo rohita* fed with ZnONP-supplemented diet. PC = positive control; NC = negative control

variance (ANOVA) revealed that the enzyme activities were improved ($F = 2.412$; $P < 0.05$) in fish fed with various concentrations of ZnONP-supplemented diet. The increased activities of protease, amylase, and lipase enzymes of fish supplemented with 10 mg/kg ZnONPs are 1.21 ± 0.97 , 1.29 ± 1.6 , and 1.25 ± 1.05 U/mg proteins, respectively.

Discussion

Nanoparticles have enormous potential in several biological, physical, and chemical applications due to their smaller sizes and larger surface areas. They have enormous applications in biological field including aquaculture. The current trend in aquaculture development is to increase the intensification and commercialization of fish production. The improvement of immunity in fishes through dietary supplementation is always prioritized [29].

In the present study, zinc oxide nanoparticles were synthesized using phyto-reduction method. The green synthesis of zinc oxide nanoparticles was achieved by vigorous stirring method. The zinc acetate is used as a precursor and the *Spinacia oleracea* leaf extract and sodium hydroxide were used as reducing agents. Sagili et al. [14] synthesized the zinc oxide nanoparticles by phyto-reduction method using *Spinacia oleracea* as reducing agent. Olasupo et al. [11] reported that the aqueous spinach extract shows the abundant presence of saponins, flavonoids, steroids, terpenes, glycosides, tannins, and phenols. The results of the present experiment showed the similar presence of phytonutrients.

Biosynthesis of zinc oxide nanoparticles using *Eichhornia crassipes* showed strong absorbance peak at 378 nm [30]. The UV results of the present study coincide with earlier findings.

The FTIR results of the present study revealed the shift in the peaks confirmed that plant extract reduced zinc oxide for the formation of nanoparticles. The ZnO peak was noted at 447.49 cm^{-1} . Elumalai and Velmurugan [9] reported the similar FTIR results at 457 cm^{-1} by green synthesis of zinc oxide nanoparticles using the leaf extract of *Azadirachta indica*. The absorption peak at 437 cm^{-1} indicates the presence of ZnO nanoparticles [31].

Sachudanandam et al. [32] observed the characteristic main diffraction peak at $2\theta = 47.76^\circ$ confirming that the particle is made up of pure zinc oxide. The present result depicts the crystalline nature of zinc oxide nanoparticles. The calculated size of the particle was 22.61 nm. The morphology of green synthesis of zinc oxide nanoparticles was observed under a scanning electron microscope. The SEM result revealed that the particles were more or less spherical in nature. Similar kind of particles with increased size was reported by Sangeetha et al. [33]. The EDS spectrum revealed that the zinc is the major constituent in the sample and showed a strong signal at 1 keV in the zinc region. Along with zinc peak, oxygen peak is also observed. Gnanasangeetha and Sarala [34] reported the elemental analysis of the zinc oxide nanoparticles with Zn peaks. Similar kind of results was reported by Salam et al. [35].

Using nanotechnology in fish feeds, food packaging can provide health benefits to the fisheries and aquaculture [36]. Muralisankar et al. [1] reported the dietary supplementation of Zn nanoparticles to overcome Zn deficiency and improve the health in the freshwater prawn, *Macrobrachium rosenbergii*.

The present study deals with the growth performance, biochemical, hematological, and digestive enzyme activities and muscle composition of fingerlings of *Labeo rohita* fed with different concentrations of ZnONP-supplemented diet. The results of the growth performance revealed that the increased survival (96%), weight gain (2.54 g), length gain (7.12 cm), specific growth rate (0.13 %), and food conversion ratio (0.76 g) were observed in fish fed with 10.0 mg/kg zinc oxide nanoparticle-supplemented diet. Swain et al. [29] reported that 10 mg/kg zinc oxide nanoparticle-supplemented diet fed *Labeo rohita* performs increased growth rate.

The results of biochemical parameters such as carbohydrates, total protein, and lipids revealed that the increased level of carbohydrates, total protein, and lipids was observed in fish fed with 10.0 mg/kg zinc oxide nanoparticle-supplemented diet. Tawfik et al. [37] reported that the tilapia fish fed with various concentrations of zinc oxide nanoparticle-supplemented diet shows increased total protein concentrations. Annamalai et al. [19] reported that the muscle biochemical compositions of total protein, carbohydrate, and lipids were drastically increased in prawns fed on supplemented diet with 0–18 MnONPs/kg compared with the control.

The results indicate that the hematological parameters were increased in fish supplemented with zinc oxide nanoparticles diet while the WBC level does not undergo any variations among all the treated groups. This result indicates that the zinc oxide-supplemented diet fed fish are healthy and they are free from deficiencies. Behera et al. [22] demonstrated that iron nanoparticles could induce the hematological and immunological parameters of Indian major carp, *Labeo rohita*.

The digestive enzyme in fish plays an essential role in nutritional physiology and regulates the growth [13]. In this study, the important digestive enzymes such as protease,

amylase, and lipase activity shows that 10 mg/kg supplemented zinc oxide nanoparticles had improved digestive enzyme secretion in *Labeo rohita* compared with control. Similarly Zn, MnO, and Cu nanoparticles-supplemented feed produced significant improvements in digestive enzyme secretion in fishes and crustaceans [1, 19].

The obtained results clearly indicate that the zinc oxide nanoparticles can be used as the best alternative for the zinc deficiencies in fish. The nutrient deficiencies are being a major concern in aquaculture; the supplement of nanoparticles in the diet can provide a better solution to prevent the aquatic organisms from nutrient deficiencies.

Conclusion

Zinc deficiency in fish could affect the normal functioning of the body. Thus, nanotechnology can revolutionize aquaculture and ZnONP-supplemented diet can provide a solution for the zinc deficiency in fisheries. In the present study, the obtained results reveal that the zinc oxide nanoparticle-supplemented diet shows the higher improvement in growth of *Labeo rohita*. In conclusion, the green synthesized ZnONPs could apply for feed formulation technology and found the way for zinc oxide nanoparticles as safe ingredients for aquatic animals to overcome the zinc deficiency.

Acknowledgments The authors greatly acknowledge the Department of Zoology, PSG College of Arts and Science, for the facilities extended towards the research.

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

Authors' Contributions SM performed the experiment. SM has written the manuscript. ST was sourced as a research supervisor and designed the work. Both authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

Ethical Approval Not applicable.

Abbreviations ANOVA, analysis of variance; DMRT, Duncan's Multiple Range test; EDS, energy dispersive spectroscopy; FTIR, Fourier transformer infrared spectroscopy; SEM, scanning electron microscopy; XRD, X-ray diffraction pattern; ZnONPs, zinc oxide nanoparticles

References

1. Muralisankar T, Saravana Bhavan P, Radhakrishnan S, Seenivasan C, Srinivasan V, Santhanam P (2014) Effects of dietary zinc on the growth, digestive enzyme activities, muscle biochemical

- compositions and antioxidant status of the giant freshwater prawn *Macrobrachium rosenbergii*. *Biol Trace Elem Res* 160(1):56–66
2. Bharadvaj AS, Patnaik S, Browdy CL, Lawrence L (2016) Availability of dietary zinc sources and effects on performance of pacific white shrimp *Litopenaeus vannamei* (Boone). *Int J Recirculating Aquac* 13: 1–10. <https://doi.org/10.21061/ijra.v13i0.1514>
 3. Subcommittee on Fish nutrition, National Research Council (1993) Nutrient requirements of fish. Washington, DC: The National Academies Press, Page no. 20. <https://doi.org/10.17226/2115>
 4. Rather MA, Sharma R, Aklakur M, Ahmad S, Kumar N, Khan M, Ramya VL (2011) Nanotechnology, a novel tool for aquaculture and fisheries development a prospective mini-review. *Fish Aquac J* 16:1–6
 5. Hamed M, Darroudi M (2017) Zinc oxide nanoparticles: biological synthesis and biomedical applications. *Ceram Int* 43(1):907–914
 6. Khan I, Saeed K, Khan I (2017) Nanoparticles: properties, applications and toxicities. *Arab J Chem* <https://doi.org/10.1016/j.arabjc.2017.05.011>
 7. Krithika G, Saraswathy R, Muralidhar M, Thulasi D, Lalitha N, Kumararaja P, Nagavel A, Balaji A, Jayavel R (2017) Zinc oxide nanoparticles-synthesis, characterization and antibacterial activity. *J Nanosci Nanotechnol* 17(8):5209–5216
 8. Umalatha, Sridhar N, Kushwaha JP, Gangadhar B (2016) Digestive enzyme activities in different size groups and segments of the digestive tract in *Labeo rohita* (Day, 1878). *J Aquacult Mar Biol* 4(5):00098
 9. Elumalai K, Velmurugan S (2015) Green synthesis, characterization and antimicrobial activities of zinc oxide nanoparticles from the leaf extract of *Azadirachta indica* (L.). *Appl Surf Sci* 345(22):329–336
 10. Tamanna B, Kavita M, Manika K, Prasad R, Varma A (2015) Biosynthesis of zinc oxide nanoparticles from *Azadirachta indica* for antibacterial and photocatalytic applications. *Mater Sci Semicond Process* 32:55–61
 11. Olasupo AD, Aborisade AB, Olagoke OV (2018) Phytochemical analysis and antibacterial activities of Spinach leaf. *Am J Phytomed Clin Ther* 2(2):8. <https://doi.org/10.21767/2321-2748.100344>
 12. Soni and Sosa (2013) Phytochemical analysis and free radical scavenging potential of herbal and medicinal plant extracts. *J Pharmacogn Phytother* 2(4):22–29
 13. Debnath D, Pal AK, Sahu NP, Yengkokpam S, Baruah K, Choudhury D, Venkateshwarlu G (2007) Digestive enzymes and metabolic profile of *Labeo rohita* fingerlings fed diets with different crude protein levels. *Comp Biochem Physiol B Biochem Mol Biol* 146(1):107–114
 14. Lakshmi, Bai RS, Sharanagouda H, Ramachandra CT, Sushila N, Doddagoudar SR (2017) Biosynthesis and characterization of ZnO nanoparticles from Spinach (*Spinacia oleracea*) leaves and its effect on seed quality parameters of green gram (*Vigna radiata*). *Int J Curr Microbiol App Sci* 6(9):3376–3384
 15. Evans WC (1989) Trease and Evans pharmacognosy. *Balliere Tindall*, London, pp 216–217
 16. Uddin MN, Alam SN, Mobin MN, Miah MA (2014) An assessment of the river water quality parameters: a case of Jamuna River. *J Environ Sci Nat Res* 7(1):249–256
 17. Tapati D, Bipasha DG, Narayan DD (2016) Formulation of low-cost fish feed using locally available agro-based wastes and its efficacy in growth performance of common carp (*Cyprinus carpio* L.) – a case study from Apatani landscape of Arunachal Pradesh in Northeast India. *Int Res J Biol Sci* 5(3):61–67
 18. Onuegbu CU, Agarwal A, Singh NB (2018) ZnO nanoparticles as feed supplement on growth performance of cultured African catfish fingerlings. *J Sci Ind Res* 77(4):213–218
 19. Annamalai A, Bhavan PS, 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(3):7–17
 20. Folch J, Lees M, Bloane-Stanley GH (1957) A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* 266(1):497–509
 21. 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(1):103–118
 22. Remya AS, Ramesh M, Saravanan M, Poopal RK, Bharathi S, Natraj D (2014) Iron oxide nanoparticles to an Indian major carp, *Labeo rohita*: impacts on hematology, iono regulation and gill Na⁺/K⁺ ATPase activity. *J King Saud Univ- Sci* 27(2):151–160
 23. Behera T, Swain P, Rangacharulu PV, Samanta M (2014) Nano-Fe as feed additive improves the hematological and immunological parameters of fish, *Labeo rohita* H. *Appl Nanosci* 4(6):687–694
 24. Drabkin DL (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(1):317–333
 25. Dacie V, Lewis SM (1991) Practical hematology, 7th edn. *Churchill Livingstone*, London, p 556
 26. Stoskopf MK (1993) Fish medicine. *W B Saunders Company*, Philadelphia
 27. Fume M, Hidalgo MC, Lopez A, Garcia-Gallego M, Morales AE, Domenzain A, Domezain J, Sanz A (2005) Digestive enzyme activities in Adriatic sturgeon *Acipenser naccarii* and rainbow trout *Oncorhynchus mykiss*. A comparative study. *Aquaculture* 250(1–2):391–398
 28. Bernfeld P (1955) Amylases. In: Colowick SP, Kaplan NO (eds) *Methods in enzymology*. Academic Press, New York, pp 149–158
 29. Swain P, Das R, Das A, Padhi SK, Das KC, Mishra SS (2018) Effects of dietary zinc oxide and selenium nanoparticles on growth performance, immune responses and enzyme activity in rohu, *Labeo rohita* (Hamilton). *Aquac Nutr* 27(3):1–9
 30. Vanathi P, Rajiv P, Narendhran SS, Rajeshwari S, Rahman PKSM, Venckatesh R (2014) Biosynthesis and characterization of phytomediated zinc oxide nanoparticles: a green chemistry approach. *Mater Lett* 134(9):13–15
 31. Elizabeth V, Mary G (2015) Green synthesis of zinc oxide nanoparticles. *Int J Adv Res Sci Eng* 4(1):307–314
 32. Sachudanandam N, Shakila D, Geetha K, Dinesh Karthik A (2017) Novel green synthesis of zinc oxide nanoparticles & study of its in vitro antimicrobial activity. *IOSR J Pharm* 2:01–06
 33. Sangeetha G, Rajeshwari S, Venckatesh R (2011) Green synthesis of zinc oxide nanoparticles by *Aloe barbadensis miller* leaf extract: structure and optical properties. *Mater Res Bull* 46(12):2560–2566
 34. Gnanasangeetha D, Sarala TD (2013) One pot synthesis of zinc oxide nanoparticles via chemical and green method. *Res J Mater Sci* 1(7):1–8
 35. Salam HA, Rajeshwari S, Venckatesh R (2014) Green synthesis and characterization of zinc oxide nanoparticles from *Ocimum basilicum* L. var. *purpurascens* Benth-LAMIACEAE leaf extract. *Mater Lett* 131:16–18
 36. Handy RD (2012) FSBI Briefing paper: nanotechnology in fisheries and aquaculture. Fisheries Society of the British Isles, 1–29
 37. Tawfik MMM, Moustafa MM, Abumourad IMK, EI-Meliegy EM, Refai MK (2017) Evaluation of nano zinc oxide feed additive on tilapia growth and immunity. In 15th International Conference on Environmental Science and Technology, Rhodes, Greece, 1342:1