(RESEARCH ARTICLE)

Differential quantities of zinc oxide nanoparticles incorporated feed on growth and haematological characteristics of Zebrafish (*Danio rerio*)

Muthuswami Ruby Rajan * and Gnanavel Roopashree

Department of Biology the Gandhigram Rural Institute- Deemed to be University Gandhigram -624 302, Tamil Nadu, India.

Abstract

Zinc oxide nanoparticles were synthesized and characterized using by UV-Visible Spectroscopy (UV-Vis); Scanning Electron Microscope (SEM); Energy Dispersive X–Ray Spectroscopy (EDAX); X-Ray Diffraction (XRD) and Fourier Transform Infrared Spectroscopy (FT-IR). Six feeds were prepared by using the differential quantities of Zinc oxide nanoparticles such as 0;20; 40; 60; 80 and 100 mg along with a fish meal; groundnut oil cake; wheat flour and tapioca flour. Feed utilization and haematological parameters of zebrafish were estimated after 21 days. The UV-visible absorption spectra demonstrate that Zinc oxide Nanoparticles were measured in wavelengths within 200 to 300 nm. SEM image shows that the Zinc oxide nanoparticles were observed at the wavelength range from 8.40 mm. EDAX spectrum recorded two peaks located between t 0.2KeV and 8.6 KeV. XRD image shows that the diffraction peaks of Zinc oxide nanoparticles are indexed as 041;142; 123; 281; 163; and 100;200. FT-IR spectrum was observed at the wavelength range from 3512 - 1500 cm-1. The condition factor of Zebrafish was decreased in the final when compared to the initial. The feed consumption and feed conversion efficiency of Zebrafish were higher in feed V. The feed conversion ratio of Zebrafish was best in feed III. Growth and the percentage growth of Zebrafish were higher in feed VI. Assimilation and metabolism of Zebrafish were higher in feed V. Gross and Net growth efficiency were higher in feed III. All the haematological parameters increased with increased quantities of zinc oxide nanoparticles.

Keywords: Differential; Growth; Haematological; Zinc; Nanoparticles; Zebrafish

1. Introduction

Among various nanoparticles, Zinc is the second most abundant trace element in the animal body. It can't be stored in the body [1]. Zinc is extensively used in various consumer products such as sunscreens and cosmetics with a high potential of being released into the aquatic environment. Zinc is a trace element known to have antibacterial, health-promoting, and anti-sterility effects in organisms. The most common and effective Zn-based nanoparticles are Zinc Oxide nanoparticles (ZnO NPs) supplemented in a basal diet to improve the quality and health of fish [2]. ZnO NPs, on the other hand, has previously been shown to be capable of removing lead (Pb) and cadmium (Cd) from aqueous solutions in different conditions, including time, absorbent dosages, pH, and temperature [3]. Fish, occupying high trophic levels in the aquatic ecosystem and being an important food source, are regarded as indicators of ZnO contamination in the aquatic environment [4]. Zebrafish *Danio rerio* has been considered one of the most potent model organisms for toxicity assessment of nanoparticles in the last few years. Moreover, it is easy to maintain in the laboratory with cost-effectiveness as compared to other fishes. The zebrafish has also emerged as a valuable model organism for to study of craniofacial cartilage development and pigment pattern formation [5-7]. In zebrafish, the pharyngeal skeleton, jaw and branchial arches arise from the cranial neural crest cells that develop from dorsal and lateral regions of the neural ectoderm and then migrate to pre-determined locations. The presence of zinc oxide in fish feed as nano form improved feed palatability thereby causing fish to take more feed. It would cause higher stimulation of the synthesis of DNA, RNA and protein leading increase in the body cells of fish. Supplementation of different

* Corresponding author: Muthuswami Ruby Rajan

concentrations of zinc oxide nanoparticles in basal feed greatly improves the weight and biochemical composition of fish [8]. Fish feed is an essential component for growth, immunity and health-promoting factors to achieve the farm net gain. Hence the work related to the differential quantity of ZnO nanoparticles incorporated feed on growth, haematological studies on Zebrafish Danio rerio is wanting. Hence the present study was carried out.

2. Material and methods

2.1. Materials

Zinc acetate (Zn $(CH_3COO)_2.2H_2O$), sodium hydroxide (NaOH) and zinc oxide (ZnO) were collected from the Department of Biology, The Gandhigram Rural Institute - Deemed to be University, Gandhigram, India. All the reagents used for the synthesis of zinc oxide nanoparticles were analytical grade and used without further purification. All the glass wares were washed thrice with deionized water and dried before use.

Synthesis of zinc oxide nanoparticles was carried out by a simple precipitation method for this study, 0.5M of zinc acetate (Zn $(CH_3COO)_2.2H_2O$) was dissolved in 100ML of distilled water and 1M of sodium hydroxide were also dissolved in 100 ML of distilled water precipitation was done by mixing of 1M NaOH which is to be added in dropwise to 0.5M of zinc acetate solution under vigorous stirring. The process continued until the appearance of a milky white precipitate. During this precipitation process, P^H was increased from 7 to 14. Following the precipitation, the solution was centrifuged at 3000 rpm for 10 min and washed several times with distilled water and ethanol to remove the by-products. The supernatant was then removed and the pellet was dried in an oven at 100^0 C for 3 hours. Finally, Nano ZnO was ground with mortar to be shaped into powder.

2.2. Characterization of ZnO NPs

The physical and primary characterization of chemically synthesized zinc oxide nanoparticles was analyzed by using UV-VIS spectroscopy using spectrophotometer UV-VIS Double Beam DUV 3500. The morphology and composition of ZnO nanoparticles were examined by Scanning electron microscopy. An energy-dispersive X-ray detection instrument was used to examine the elemental composition of the sample. X-ray powder diffraction (XRD) is a rapid analytical technique primarily used for phase identification of a given material and can provide information on unit cell dimensions. The analyzed materials are finely ground and homogenized and the average bulk composition is determined. The vibration modes of the functional group of nanoparticles were analyzed by Fourier Transform Infrared spectroscopy analysis using JASCO (FTIR-6200) spectra.

2.3. Growth Studies

For growth studies Zebrafish fingerlings (1.340 ± 0. 540 g) were collected from Aqua Garden Fish Farm, Kadachanenthal, Madurai, Tamil Nadu, India and transported to the laboratory in polythene bags filled with oxygenated water. Fishes were acclimated in glass aquaria (60 X 45 X 45 cm) for a period of 15 days at 28 ± 2^0C. During acclimation, a feed containing fish meal, groundnut oil cake, wheat flour and rice bran were used in the form of dry pellets. The raw materials are selected based on their ability to supply nutrients to the fish. After knowing the protein content by the Micro-Kjeldhal method, the feed was prepared in (Table 1). Fish meal and Ground nut oil cake, tapioca powder, and wheat flour is the protein source used as the components used for feed preparation was dried, powdered and sieved through a 425-micron sieve. The ingredients were weighed and mixed thoroughly with 130-150 ml of distilled water. The mixed feedstuff was put in an autoclave for 30 min at 100^0C and cooled. After cooling, fish oil, sunflower oil, supplevite mix, sodium chloride, sodium benzoate and differential quantity of Zinc oxide nanoparticles such as 0,20, 40, 60, 80 and 100 mg were mixed with the feed and it was extruded with the help of a pelletizer, the formulated feed was kept in an airtight container at -20^0C until used to prevent contamination. (Table 2).

Table 1 Protein level of major ingredients

S.No.	Ingredients	Protein (%)
1	Fish meal	58
2	Groundnut oil	44
3	Wheat flour	11
4	Tapioca	03

**Table 2** Composition of different ingredients in the experimental feed (g\100 gm) of zebrafish

Ingredients	Feed I	Feed II	Feed III	Feed IV	Feed IV	Feed IV
Fish meal	33.75	33.75	33.75	33.75	33.75	33.75
GNOC	33.75	33.75	33.75	33.75	33.75	33.75
Wheat flour	11.2	11.2	11.2	11.2	11.2	11.2
Tapioca	11.2	11.2	11.2	11.2	11.2	11.2
Fish oil	2	2	2	2	2	2
Sunflower oil	2	2	2	2	2	2
Supplevitemix	2	2	2	2	2	2
Sodium chloride	2	2	2	2	2	2
Sodium benzoate	2	2	2	2	2	2
Zinc Oxide Nanoparticles	-	20mg	40 mg	60 mg	80 mg	100 mg

GNOC – Ground Nut Oil Cake

For the present study uniform size of zebrafish *Danio ratio* (1.34±0.54g) was selected and then the fishes were introduced in a rectangular glass tank (45cm L X 22cm B X 22cm H) having a capacity of 18 litres. Five fish were introduced to each tank. For each treatment triplicates were maintained. During rearing, the fish were fed on an ad-Libitum diet of the prepared feed twice a day for 1 hour each from 9-10 am and 4-5 pm. The unfed were collected after one hour of feeding without disturbing the fish and dried to constant weight. The faecal matter was collected daily before changing the water with the least disturbance to the fish and dried at 95^0C. Approximately 70% of the water in the tank was replaced with tap water. The experiment was continued for 21 days. On the 21st day, the length and weight of the fish were measured in live conditions. Blood samples were collected from the gill of the fish for estimating red blood corpuscles, white blood corpuscles, haemoglobin, Hematocrit, and Platelets.

3. Results

The UV visible absorption spectra are a technique used to characterize zinc oxide nanoparticles. The absorbance spectra of ZnO nanoparticles were measured in wavelength within the range from 300nm to 400 nm. The sharp bands observed close to 260nm throughout the reaction indicate the formation of ions (Figure 1). Scanning electron microscopy shows that the nanoparticles formed the clustered form because of the adhesive nature of spherical shaped appearance as shown in Figure 2. EDAX Spectrum recorded on the ZnO nanoparticles is shown as three peaks located between 0.2 KeV and 8 KeV (Figure 3).
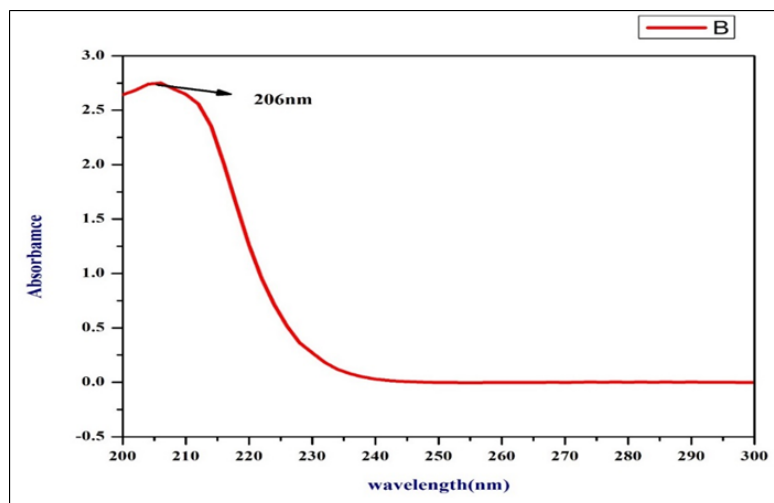
Figure 1 UV-Vis Image of Zinc oxide nanoparticles

Those maxima are directly related to the zinc-characterized line K. The maximum peak on the spectrum at 1 KeV comes from zinc. The maximum peak on the spectrum at 0.2 KeV comes from oxygen. The third peak located at 8.6 KeV is connected with the Sulphur characteristics line. The XRD diffraction peaks of ZnO nanoparticles are indexed as 4228, 4523, 6155, 1197, 1931and 1363 which is represented in Figure 4. The clear and sharp diffraction peaks confirmed that the prepared compounds are pure with a high degree of crystallinity. The FTIR spectrum of zinc oxide nanoparticles were analyzed in the range of 4000-500 cm^{-1}. The FT-IR analysis was carried out for identifying the functional groups of active components based on the peak value in the region of infrared radiation. Zinc oxide formation was confirmed with bands 3512, 1600, 1408, 1344, 1017, 861,675 and 577, which were associated with N-H Aliphatic primary amine=C Conjugated alkene, C-F Fluro compound, S=O Sulfonic acid-O Sulfoxide=C Alkene=C Alkene-I Halo compounds are respectively. (Figure 5).
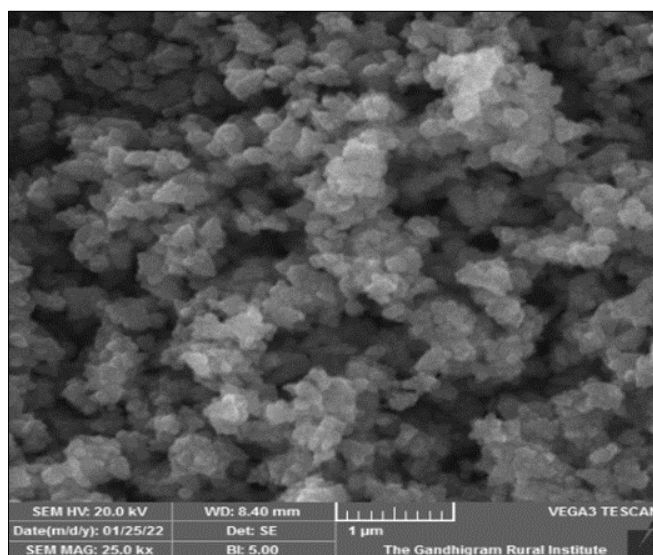

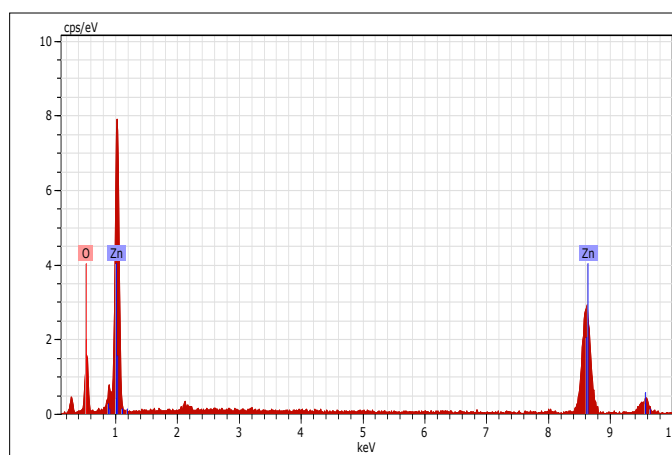**Figure 2** SEM Image of Zinc oxide nanoparticles

Figure 3 EDAX Spectrum of Zinc oxide nanoparticles

The condition factor of Zebrafish reared in different feeds were presented in Table 3. The final condition factor is decreased in all feeds. The different feed utilization and growth parameters are presented in Table 4. ANOVA (Analysis of Variance) of growth parameters (Feed consumption, Gross Growth Efficiency, Net Growth Efficiency) were presented in Table 5. The feed consumption was higher in feed V (0.68) and feed IV (0.663) containing 60 mg of ZnO nanoparticles and lower in feed VI (8.3) containing 20 mg of zinc oxide nanoparticles (Table 4) and significantly varied (Table 5). The feed conversion efficiency of zebrafish reared in feed I is 1.6. The feed conversion ratio is best in feed VI (3.443). The growth was higher in feed VI. Like growth, the percentage growth rate of zebrafish reared in feed VI is higher (21.72). The assimilation of Zebrafish reared in feed I, II, III, IV, V and VI are 2.90, 3.059, 2.863, 3.192, 3.346 and 3.276

respectively. The metabolism of Zebrafish reared in feed I, II, III, IV, V and VI are 2.85, 2.99, 2.70, 3.09, 3.21 and 3.10 respectively. The gross growth efficiency of Zebrafish reared in feed I, II, III, IV, V and VI are 13.03, 19.64, 32.91, 16.33, 19.75 and 29.29 respectively. The analytical variance (ANOVA) shows that the gross growth efficiency is significant. The net growth efficiency of Zebrafish reared in feed I, II, III, IV, V and VI are 1.93, 2.25, 7.06, 3.35, 4.04 and 4.30 respectively.
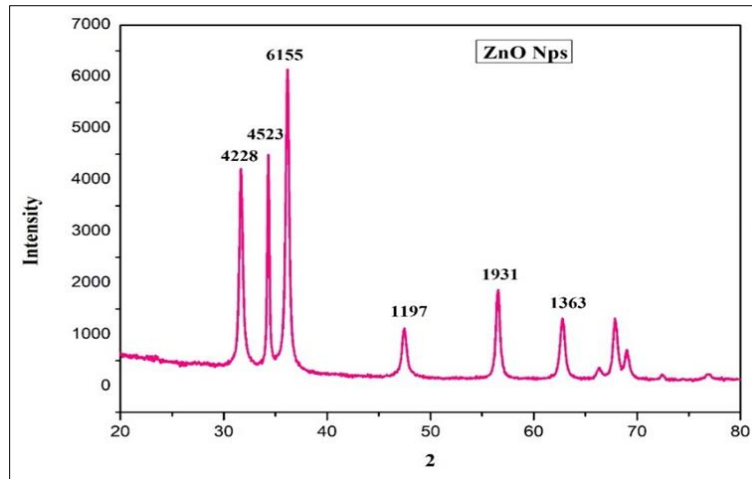

Figure 4 XRD Spectrum of Zinc oxide nanoparticles
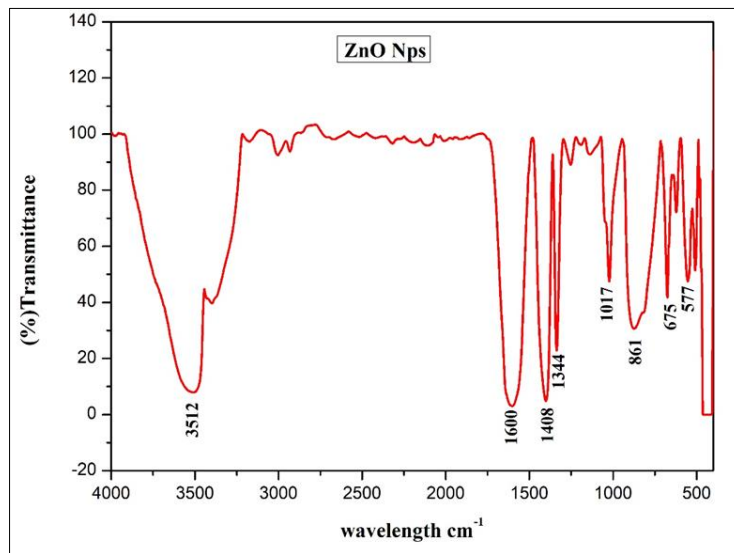
Figure 5 FTIR Spectrum of Zinc oxide nanoparticles

Table 3 Condition factor of Zebrafish

Feeds	Initial	Final
I (Control)	4.046±1.92	2..396±0.96
II	1.816±0.755	1.20±0.42
III	1.29±0.277	1.02±0.12
IV	2.183±0.472	1.04±0.17
V	1.82±0.331	1.18±0.27
VI	1.23±0.181	0.78±0.15

Table 4 Feed utilization and Growth parameters of zebrafish in relation to the different quantities of zinc oxide nanoparticles. Each value is the average (± SD) performance of five individuals in triplicates reared for 21 days

Parameters	Experimental Feeds					
	Feed I (Control)	Feed II (20 mg)	Feed III (40 mg)	Feed IV (60 mg)	Feed V (80 mg)	Feed VI (100 mg)
Feed consumption (g/g live wt./21 days)	0.45±0.19	0.32±0.09	0.52±0.10	0.66±0.19	0.68±0.07	0.58±0.09
Feed conversion Efficiency	0.39±0.17	0.45±0.34	0.75±0.50	0.68±0.30	0.82±0.22	1.07±0.07
Feed conversion Ratio	9.29±4.31	6.22±2.94	4.60±3.08	7.24±3.90	5.32±1.49	3.44±0.38
Growth	0.05±0.01	0.06±0.02	0.16±0.12	0.1±0.03	0.13±0.04	0.17±0.04
Percentage Growth	9.80±4.33	9.86±0.98	19.22±12.21	17.23±7.32	21.72±5.93	26.74±1.68
Relative Growth Rate	0.02±0.05	0.03±0.01	0.08±0.06	0.05±0.15	0.06±0.02	0.08±0.02
Assimilation (g/g live wt./21 days)	2.90±1.06	3.05±0.97	2.86±1.80	3.19±0.91	3.34±0.41	3.27±0.61
Metabolism (g/g live wt./21 days)	2.85±1.06	2.99±0.99	2.70±1.72	3.09±0.92	3.21±0.38	3.10±0.57
Gross Growth Efficiency (%)	13.03±7.54	19.64±11.64	32.91±21.19	16.33±7.17	19.75±5.35	29.29±3.51
Net Growth Efficiency (%)	1.93±0.93	2.25±1.65	7.06±4.79	3.35±1.47	4.04±4.01	4.30±1.77

Table 5 ANOVA (Analysis of Variance) of Growth parameters (Feed consumption, growth, gross growth efficiency, net growth efficiency) of Zebrafish

Parameters	Source	Sum of Squares	df	Mean Square	F	Sig.
Feed consumption	Between Groups	0.268	5	0.054	2.859	0.063
	Within Groups	0.225	12	0.019		NS
	Total	0.492	17			
Growth	Between Groups	0.035	5	0.007	16.695	0.000
	Within Groups	0.005	12	0.000		S
	Total	0.040	17			
Gross growth efficiency	Between Groups	428.028	5	85.606	7.775	0.002
	Within Groups	132.125	12	11.010		S
	Total	560.153	17			
Net growth efficiency	Between Groups	31.529	5	6.306	6.955	0.003
	Within Groups	10.879	12	0.907		S
	Total	42.208	17			

The haematological parameters of Zebrafish is presented in Table 6. The RBC, haemoglobin, haematocrit and platelets of zebrafish is gradually increased from feed I to feed VI. WBC decreased with the increasing quantity of Zinc oxide nanoparticles in the feed.

Table 6 Haematological parameters of zebrafish

Blood Parameters	Feed I	Feed II	Feed III	Feed IV	Feed V	Feed VI
RBC (millions/cumm)	0.03	0.02	0.02	0.02	0.02	0.03
Haemoglobin (gm/dl)	0.1	0.1	0.2	0.3	0.4	0.5
Haematocrit (Hct) (%)	0.2	0.1	0.1	0.0	0.1	0.2
WBC (Cells/ cumm)	1600	1200	900	800	600	700
Platelets (Lakhs/cumm)	24000	32000	34000	38000	46000	51000

RBC - Red Blood Corpuscles WBC - White Blood Corpuscles Hct - Hematocrit

4. Discussion

The primary characterization and synthesis of zinc oxide nanoparticles were analyzed by using a UV-Vis spectrophotometer and peak absorbance was observed at wavelength 206nm. Similarly, a peak at 320nm for zinc oxide nanoparticles [9, 10]. The UV-Vis spectra of ZnO NPs synthesized using zinc nitrate and zinc acetate were observed at 380nm. Also reported that a sharp peak of absorbance at 345 nm, which indicates an almost uniform size of ZnO nanoparticles [11]. The morphology of zinc oxide was rod-shaped, whereas chemically synthesized zinc oxide was clustered form of a spherical shape with uniform distribution, which was observed by SEM. The SEM image of ZnO NPs synthesized by zinc nitrate was spherical in shape [12] similar to the above finding whereas the SEM image of ZnO was rod-shaped synthesized by zinc chloride [13, 14]. The EDAX spectrum of chemically synthesized ZnO was recorded which has only Zn and O compound peaks and it confirms the purity of particles. EDAX spectrum of zinc oxide nanoparticles are shown as two peaks located between 1.0 KeV and 8.6 KeV. The maximum peak on the spectrum at 1.0 KeV comes from zinc. The second peak located on the spectrum of 0.6 KeV clearly indicates oxygen. It confirms that the chemically synthesized zinc oxide NPs have strong peaks of Zn and O. Farag et al., (2010) [15] reported that EDAX spectrums have only zinc and oxygen indicating the purity of the synthesized ZnO. Quantitatively the oxygen-to-zinc weight ratio was found to be approximately 1 to 4, indicating the stoichiometric formation of ZnO. Also reported that the EDAX spectrum zinc oxide nanoparticles shown as two peaks located between 2KeV and 10 KeV maximum related to the zinc characterized lines K[16]. The XRD technique is used for all compounds' structure and phase analysis. The XRD diffraction peaks of ZnO nanoparticles are indexed as 4228, 4523, 6155,1197,1931,1363. Kajbafvala et al., (2009) [17] reported that the XRD diffraction peak of sharp intensity between 30^0 and 40^0 theta scale can be indexed to the hexagonal wurtzite of ZnO (JCPDS card 36-1451) with a high crystalline structure. Elen et al., (2011) [18] also attributed each peak gained with X-ray diffraction of calcinated zinc oxalate obtained from the microemulsion method to the hexagonal wurtzite structure of ZnO NPs. Also reported that in order to evaluate and identified the reaction product XRD viewed the crystal size analysis and XRD viewed the crystal size analysis and XRD diffraction 2θ value[19]. The functional group of nano zinc oxide was analysed by Fourier transform infrared spectroscopy (FTIR) and peaks observed are 3512,1600,1408,1344,1017,861,675,571 associated to N-H Aliphatic primary amine, C=C Conjugated alkene, C-F Fluro compound, S=O Sulfonic acid, C-O Sulfoxide, C=C Alkene, C=C Alkene, C-I Halo compounds are respectively. Farag et al., (2010) observed peaks at 3441 cm^{-1} as a result of O-H stretching vibration, 1636 cm^{-1} is due to C=O stretching of carboxyl anion absorbed on the surface of ZnO particles and 435 cm-1 is ascribed to Zn-O stretching vibrations. Thilagavathi Thirugnanam (2013) [20] reported that the FTIR spectral peak at 417.52 cm^{-1} can be attributed to the characterized absorption of hydroxyl confirming the pure ZnO nanoparticles.

The condition factor (K) of zebrafish was estimated to assess the feed for comparative purposes. The final condition factor of zebrafish was decreased in all the feeds supplemented with ZnO nanoparticles. An increase in the condition factor of *Macrobachiium rosenbergii* post-larvae fed with 40g / kg^{-1} of iron oxide nanoparticles in the feed [21]. The final condition factor of koi carp was increased when fed with zinc oxide nanoparticles [22].

Feed consumption of zebrafish was higher in feed V containing 80 mg of ZnO NPs in the feed. The feed consumption of Mrigal increased when the concentration of Zinc Oxide nanoparticles increased in the feed. The feed consumption of Mrigal was higher in feed IV containing 15 mg/g^{-1} of zinc oxide nanoparticles [23]. The feed conversion efficiency of zebrafish was higher in feed III containing 40 mg of ZnO NPs in feed. Growth and the percentage growth of zebrafish were higher in feed V containing 80 mg of ZnO NPs in the feed. The growth of *Macrobrachium rosenbergii* was higher in copper-supplemented feed [24]. The assimilation and metabolism of zebrafish were higher in feed V and feed IV respectively. Sangeetha and Rajan (2021) [25] reported that the assimilation of Koi carp was higher in feed IV containing 30 mg of iron oxide nanoparticles in the feed. The gross growth efficiency of zebrafish was higher in feed III containing

40 mg of ZnO NPs in feed. The net growth efficiency was higher in feed III containing 40 mg of ZnO NPs in feed. The gross growth efficiency was higher in *Cirrhinus mrigala* fed with feed IV containing 15mg of ZnO NPs in the feed [23].

Haematological parameters are very helpful in the judgment of the health condition of fish species The WBC count of zebrafish is gradually decreased as the quantity of ZnO NPs increased from feed I to feed VI. The platelets are increased with the increasing quantity of Zinc oxide nanoparticles in the feed. The haematological parameters such as haematocrit, Hb, RBC and WBC are used to assess the functional status of the oxygen-carrying capacity of the bloodstream and have been used as indicators of metal pollution in the aquatic environment [26]. In the present study, the haematological analysis such as RBC and Haemoglobin of *Labeo rohita* exposed zinc oxide nanoparticles were significantly increased ($p<0.05$) with concentration increases. An increase in blood parameters with a high concentration of selenium nanoparticles supplemented in the feed of African catfish, *Clarias gariepinus* [27]. The haematological parameters were gradually increased with different doses of iron oxide nanoparticles fed on Indian major carp [28]. Also reported that haematological characteristics of grass carp fed with ZnO supplemented diet showed a significant decrease in WBCs, Hb, HCT, MCV, and MCH values but an increase in RBC and MCHC values [29].

5. Conclusion

The results conclude that feed III containing 40 mg of zinc oxide nanoparticles was suitable for the growth and feed VI containing 100 mg of zinc oxide nanoparticles enhanced the haematological parameters of Zebrafish.

Compliance with ethical standards

Acknowledgments

Disclosure of conflict of interest

The authors declare no conflict of interest.

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Author's Short Biography

	Dr. Muthuswami Ruby Rajan: Qualified M.Sc., M.Phil., M.Ed., PhD in Zoology from Madurai Kamaraj University, Madurai, Tamil Nadu, India. Presently working as Professor & Head, Dept. of Biology, The Gandhigram Rural Institute-Deemed to be University, Gandhigram, India. 30yrs teaching and 35 yrs of research experience. Areas of research are aquaculture, bionanotechnology, environment & probiotics. Completed 2 major and 7 minor research projects. Produced 17 Ph. Ds and published 194 research papers in National and International Journals. Editorial Board Member and reviewer of the Journal of Natural Resources and Conservation, USA and reviewer in several Journals such as the International Journal of Nano Dimension, Biological Trace Element Research, Journal of Environmental Biology, Uttar Pradesh Journal of Zoology, Journal of Ecology and Environmental Sciences and so on.