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Toxicity assessment of iron oxide nanoparticles in *Labeo rohita*

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Abstract
The present study deals with the toxicity of Iron oxide nanoparticles in *Labeo rohita*. The iron oxide nanoparticles were synthesized by chemical precipitation method and characterized using Scanning Electron Microscopy, Energy Dispersive X-Ray Spectroscopy and Fourier Transform Infrared spectroscopy. SEM image of iron oxide nanoparticles shows spherical shape. EDAX spectrum showed three peaks located between 2KeV and 10KeV.The FTIR spectrum of iron oxide nanoparticles was observed and spectral bands confirm the formation of Fe–O bonds. Preliminary toxicity tests were conducted for the determination of 96 h median lethal concentration (LC_{50}) of Fe_3O_4 to *L. rohita* in the concentration of 100ppm, 1500ppm and 3000ppm for four days and mortality observed at high concentration. The concentration equivalent to 1/10 and $1/100^{th}$ of LC_{50} of Fe_3O_4 nanoparticles was selected for sublethal concentration of toxicological for about seven days and no mortality observed during this period. Haematological parameters and biochemical parameters such as protein, carbohydrate and lipid were estimated for control and treatments (300ppm and 30ppm) when *L. rohita* was exposed to Fe_3O_4 nanoparticles for a period of 7 days. The haematological parameters such as RBC, Hb and Hct were decreased and WBC increased in Fe_3O_4 nanoparticles treated fish when compared to control. Total protein, carbohydrate and lipid were consequently decreased in Fe_3O_4 nanoparticles treated fish when compared with control.

Keywords: Toxicity, iron oxide, nanoparticles, *Labeo rohita*

1. Introduction
Nanotechnology is the art and science of manipulating matter at the atomic or molecular scale and holds the promise of providing significant improvements in technologies for protecting the environment. Nanotechnology is likely to have a profound impact on economy and society in the early 21^{st} century, comparable to that of semiconductor technology, information technology, cellular and molecular biology [1]. Science and technology research in nanotechnology promises breakthroughs in areas such as materials and manufacturing, nanoelectronics, medicine and healthcare, energy biotechnology, national security, and industrial revolution [2]. Growing exploration of nanotechnology has resulted in the identification of many properties of nanomaterials such as enhanced magnetic, catalytic, optical, and mechanical properties when compared to conventional formulations of the same materials [3]. These materials are increasingly being used for commercial purpose such as fillers, catalysts, water filtrations, semiconductor, cosmetics, microelectronics, etc., leading to direct and indirect exposure in humans [4]. Among different nanoparticles, iron oxide nanoparticles have attracted much interest recently because of their interesting magnetic and electric properties which are not observed in bulk phases. Study of iron oxide nanoparticles were substantially increased because of their technological applications especially in biomedical science. Owing to their unique physical and chemical properties, magnetic iron oxide nanoparticles have become a powerful platform in many diverse aspects of biomedicine. However, the biomedical applications of magnetic iron oxide nanoparticles arouse serious concerns about their pharmacokinetics, metabolism, and toxicity. The aquatic environment may act as a sink for the entry of the metal, metal oxide, and magnetic nanoparticles that are easily taken by aquatic organism. Among the various metal oxide nanoparticles, iron oxide nanoparticles are widely used in environmental and medical applications [5]. Fish species have been extensively used for assessing the effects of NPs in aquatic ecosystem. In this current study, *Labeo rohita* is used because it is a traditional nutritious food fish for people in India. Further, it is available in abundance and withstands a wide range of environmental conditions.

Hence the present study is related to the synthesis, characterization, and toxicological evaluation of iron oxide nanoparticles (Fe_3O_4) in fish *Labeo rohita.*

2. Materials and Methods

The Precipitation method was adopted for synthesis of iron oxide nanoparticles. The morphology and composition of Fe_3O_4 nanoparticles were examined by Scanning Electron Microscopy (SEM) using a LEO 1455 VP equipped with energy dispersive. Fourier transform infrared spectroscopy (FTIR) is used to measure the vibration modes of functional groups of molecules and is sensitive to molecular structure, conformation, and environment. Therefore, in the current study it is possible to directly relate the intensities of the absorption bands to the concentration of the corresponding functional groups. FTIR spectroscopy was analyzed in the range of 4000 – 400 cm^{-1}. The FTIR spectrum of synthesized iron oxide nanoparticle was analyzed for knowing the possible functional groups. The measurement was carried out by JASCO (FTIR-6200) spectrum. An energy dispersive X-ray detection instrument (EDAX) (HORIBA 8121-H) was used to examine the elemental composition of the sample.

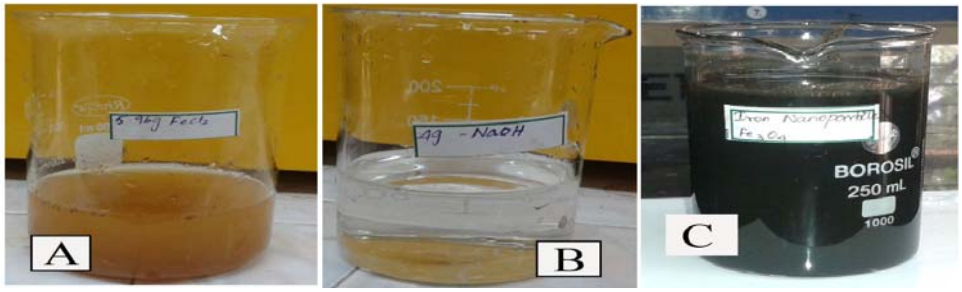
Healthy fingerlings of *Labeo rohita* were procured from SGC fish farm, E. Pudthupatti, Theni,Tamil Nadu, India and acclimatized to laboratory condition for about 20 days before the commencement of the experiment. During acclimatization, fish were fed with rice bran and ground nut oil cake once in a day. Feeding was given at least one hour prior to replacement of water. Water (one-third) was changed frequently to remove the excretory wastes. Feeding was withheld for 24 h before the commencement of the experiment to keep the experimental animals more or less in the same metabolic state. During acclimatization, the fish stock was maintained at natural photoperiod and ambient temperature. This ensures sufficient oxygen for the fish and the environment is devoid of any accumulated metabolic wastes. The initial body weight and length of the fish were measured as g and cm respectively.

For assessment of Fe_3O_4 NP toxicity, plastic trough with 10 L of water was taken. In each plastic troughs, different concentrations of the Fe_3O_4 nanoparticles (i.e. 100, 1500, 3000 ppm) were added. A control plastic trough with 10 L of water was also taken. Ten healthy fish, with an average length of 15 ±0.6 cm and average weight of 10.6 ±2 g were selected and introduced into each trough. The manifestation and survival time of fish was observed in each concentration for four days.

To assess the sublethal toxicity Fe_3O_4 NPs, 30 healthy fish were selected from the stock and divided into three groups (one control and two experiments) and then introduced into three separate plastic troughs (10 fish in each tank). 1/10th and 1/100th of LC_{50} value of Fe_3O_4 NP (300ppm and 30 ppm) was added directly into two experimental plastic troughs after removal of the same volume of water. Experiment was conducted for a period of 7 days. At the end of 7th day of exposed fish were randomly collected from control and experiment troughs for the study of haematological and biochemical assay.

3. Results and Discussion

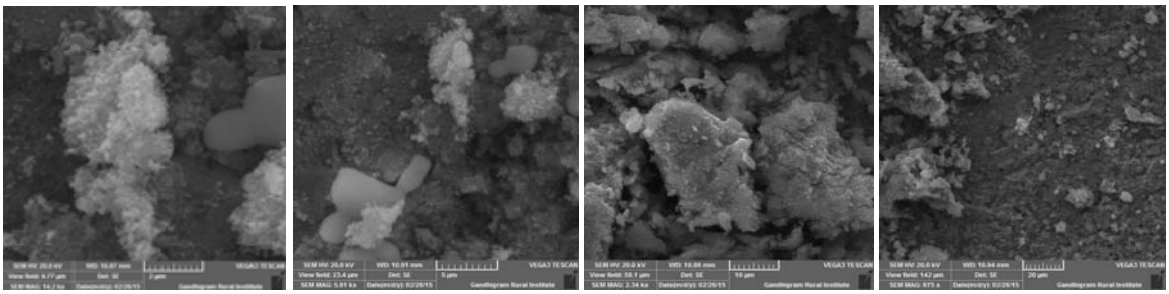
As NaOH was added to $FeCl_2$, it is found to change colour from brown to black colour as shown in (Figure1) and this colour change indicates the synthesis of iron oxide nanoparticles (Fe_3O_4)**.** Precipitation was observed by increasing the pH to 12.



(A) $FeCl_2$ (B) NaOH (C) Fe_3O_4 NPs

Fig 1: Synthesis of Iron oxide Nanoparticles (Fe_3O_4 NPs)

The SEM image (Figure 2) showing the high density chemical synthesized Fe_3O_4 further confirmed the development of iron oxide nanostructures. Chemically Synthesize iron oxide nanoparticles showed that hexagonal and spherical in nature. The microscopic image shows that the Fe_3O_4 nanoparticles did not appear as discrete particles but form much larger dendritic flocks whose size could reached micron scale size range about 10.87 mm (scale bar 2µm), 10.91mm (scale bar 5µm), 10.86mm (scale bar 10µm), 10.94mm (scale bar 20µm) for figure 2 a, b, c and d respectively. Nano particle are represented intermediated state between bulk and molecular material, and exhibit toxicity more than bulk because their greater surface reactivity and small size have ability to penetrate and accumulate with in cells [6]. Obtained nanoparticles are hexagonal and spherical in nature. SEM image of Fe_3O_4, in the picture appears that Fe_3O_4 particles composed of small particle [7]. Analysis of the SEM image of synthesized iron oxide nanoparticles, showed a clear image of highly dense iron oxide nanoparticles which are almost spherical in size. The size of most of the nanoparticles ranges from 30 nm to 110 nm. The average percentage of nanoparticles present in the synthesized sample is 66 nm. From the image, it is confirmed that the sample contains various sizes of nanoparticles which are indeed agreement with the result obtained from DLS particle analyses. Similar results on SEM analysis of iron oxide nanoparticles had been also reported [8]. Similar observation was taken in this study showed micron scale size range about 10.87 mm (scale bar 2µm), 10.91mm (scale bar 5µm), 10.86mm (scale bar 10µm), 10.94mm (scale bar 20µm).



(a) (b) (c) (d)

Fig 2: SEM Image of Iron oxide Nanoparticles

EDAX spectrum recorded on the iron oxide nanoparticles is shown as three peaks located between 2 KeV and 10 KeV (Figure 3). Those maxima are directly related to the iron characterized lines K. The maximum peak located on the spectrum at 6.4 KeV clearly coming from iron. The second maximum peak located on the spectrum at 0.3 KeV clearly indicates comes from oxygen. Third peak located at 2.6 KeV are connected with the chloride characteristics line.

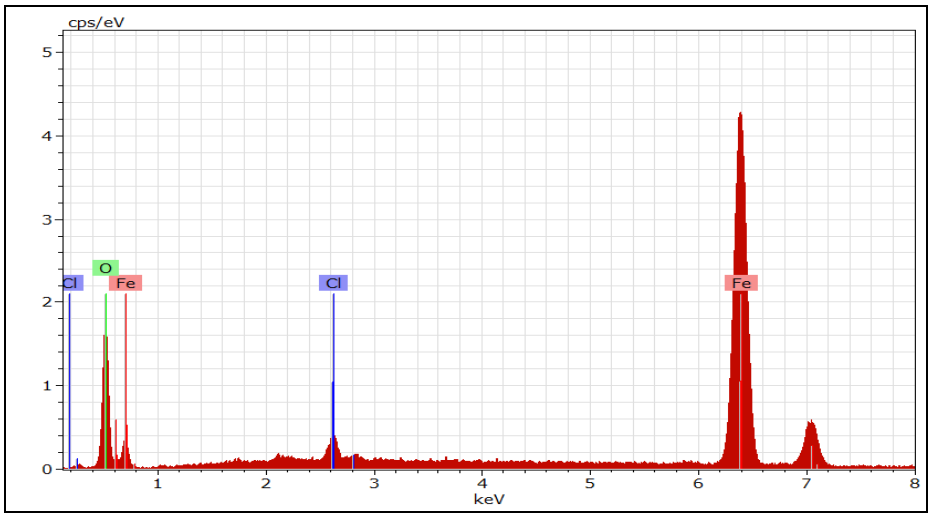


Fig 3: Energy Dispersive X-Ray Spectroscopy (EDAX) image of Iron oxide Nanoparticles

Fourier Transform Infrared spectroscopy measurements were carried out to identify the possible functional groups responsible for the reduction of the Fe ions in chemically synthesized iron oxide nanoparticles. The FTIR spectrum of iron nanoparticles was analyzed in the range 4000-400cm^{-1} (Figure 4) and bands observed at 2959.95, 1081.95, 1402.62, 1154, 897.07 and 576.67 which are associated with amide B: N–H stretching of proteins, PO_2^- symmetric stretch: mainly nucleic acids, COO^- symmetric stretch: fatty acids and amino acids, C–O asymmetric stretching of glycogen Carbonate Ion, aliphatic Iodo Compounds (Table1).

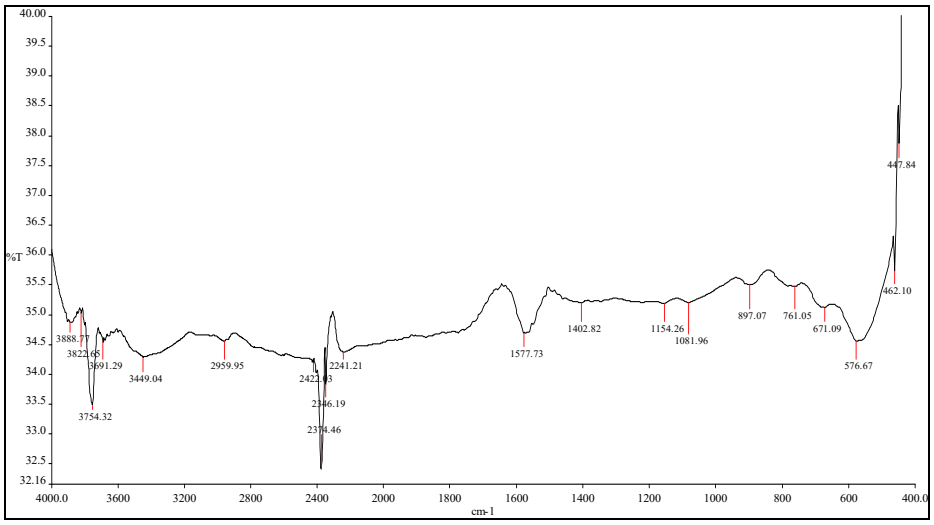


Fig 4: FTIR Spectra of 400-4000CM^{-1} region of chemically synthesized Iron oxide Nanoparticles

Table 1: FTIR Functional Group Representation OF Fe_3O_4 NPs

S. No	Wave Number (cm^{-1}) Fe_3O_4 NPs	Definition of the spectral assignments
1	2959.95	Amide B: N–H stretching of proteins
2	1081.96	PO_2^- symmetric stretch: mainly nucleic acids
3	1402.62	COO^- symmetric stretch: fatty acids and amino acids
4	1154	C–O asymmetric stretching of glycogen
5	897.07	Carbonate Ion
6	576.67	Aliphatic Iodo Compounds

Preliminary toxicity tests were conducted for the determination of 96 h median lethal concentration of Fe_3O_4 to *L. rohita*. The mortality/survival of fish was recorded at the end of 96 h and results are shown in Table 2. Among the various techniques to assess the toxicity of environmental contaminants on living organisms, static bioassay has considerable attraction in ecotoxicological studies. Similarly, Anand Sadanandan Remya *et al.,* (2014) [9] studied and observed mortality of fish in higher concentrations of Fe_2O_3 NP treated groups might have resulted from the excessive accumulation of Fe_2O_3 NPs in the body of fish. Similar observations are also done in this work in Mortality observation Fe_3O_4 nanoparticles exposed fish. In this study, the 1/10 and 1/100 96 h LC_{50} value of Na2SeO3 to the fish *L. rohita* was found to be 300 and 30ppm. In previous reports, the 96 h LC_{50} value of Na_2SeO_3 was found to be 85.8 mg L^{-1} in *Morone saxatilis* [10], 1–35 mg L^{-1} in *Danio rerio* [11], 39.0mg L^{-1} in *Oncorhynchus kisutch* [12] 11.7 mg L^{-1} *Juvenile walleye* [13] and 70.0 mg L^{-1} in *Pagrus major* [14].

Table 2: Mortality rate of *L. rohita* exposed to Fe_3O_4Nanoparticles

S. No	Concentration (ppm)	No. of mortality (in Hours)			
		24	48	72	96
1.	100	0	0	0	0
2.	1500	0	0	0	0
3.	3000	1	0	8	1

Haematological parameters of *L. rohita* exposed to Fe_3O_4 nanoparticles for a period of 7 days are represented in Table 3. During the above treatment period, both Hb and Hct contents were decreased in Fe_3O_4 nanoparticles treated fish and then increased from that of the respective control group. RBC count was decreased in Fe_3O_4 nanoparticles throughout the exposed period and WBC count was increased significantly in Fe_3O_4 nanoparticles treated fish when compared with respective control. Similarly, Heath, (1995) [15] reported that hematological parameters are potential biomarkers of exposure to agrochemicals due to their sensitivity to certain toxic agents. Clinical hematological parameters have been widely used as a potent bioindicators in aquatic toxicology [16]. Rajan *et al* (2016) [17] reported that when *Oreochromis mossambicus* exposed to sublethal concentration of Zinc oxide nanoparticles the Hb, Hct and RBC levels were decreased indicates a generalized immune response and a protective response to the toxicant. In the present study exposure of fish *L. rohita* to Fe_2O_3 NPs showed significant alterations in the hematological parameters such as RBC, Hb and Hct contents were decreased in Fe_3O_4 nanoparticles treated fish. Similar to present findings, Smith *et al.*, (2007) [18] reported a significant decrease in the hematocrit and blood hemoglobin in rainbow trout exposed to single wall carbon nanotube. In contrast to above findings, TiO_2 NPs did not cause any major disturbances in hematology of rainbow trout [19]. In the hemopoietic organism the inhibition of erythropoiesis resulted due to the decrease in these parameters indicates the anemic condition of the fish [20]. Release of oxygen radical brought about by sodium selenite may be another possible reason for the observed decrease in Hb content. Generally, the Hct value depends on the oxygen carrying capacity of blood. Moreover, lower Hct values also indicate shrinkage of cell due to toxicant stress on erythropoietic tissue [21]. Failure of erythrocyte production, internal hemorrhages or impaired osmoregulation during stress condition may leads to a reduction in RBC count [22-24].

Table 3: Haematological parameters of *L.rohita* exposed to Fe3O4 NPS for 7 days. Each valve is the average of five individual observations

Haematological parameter	Control	Low concentration(30ppm)	High concentration(300ppm)
RBC (cells/cumm)	1,48,000	1,16,000	95,000
WBC (cell/cumm)	6,450	17,800	28,000
Lymphocytes (%)	52	30	23
Hematopoiesis (%)	38	57	58
Erythrocytes (%)	4	6	7
Monocytes (%)	2	2	2
Basophils (%)	1	1	1
Neutrophils (%)	3	4	9
Hb (gms%)	1.0	0.9	0.7
Hct (%)	1.6	1.4	1.2
Platelets (cells/cmm)	1,00,000	48,000	12,000

Protein content in gill, liver and muscle of *L. rohita* in control and in exposed Fe_3O_4 nanoparticles (30ppm and 300ppm) is presented in Figure 5. Among the different tissues the protein content is higher in muscle and lower in gill and when compared to control the protein content decreased with increasing concentration of iron oxide nanoparticles. Mehjbeen Javed and Nazura Usmani, (2015) [25] reported an increase in total protein, albumin and globulin in liver and muscle. In the muscle of Oreochromis niloticus the increase in total protein was reported [26].

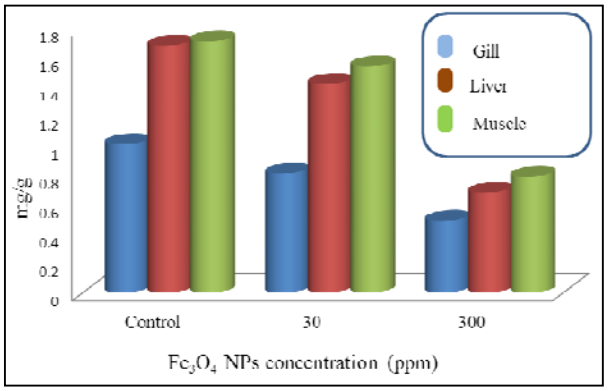


Fig 5: Protein content in gill, liver and muscle of *l. rohita*

Carbohydrate content in gill, liver and muscle *L. rohita* in control and exposed Fe_3O_4 nanoparticles (30ppm and 300ppm) is presented in Figure 6. Among the different tissues the carbohydrate content is higher in muscle and lower in gill and when compared to control the carbohydrate content decreased with increasing concentration of iron oxide nanoparticles. The serum glucose first increased and then decreased upon chronic exposure until depleted [27, 28].

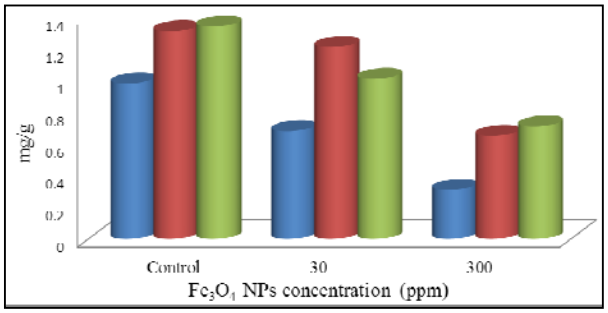


Fig 6: Carbohydrate content in gill, liver and muscle of *L. rohita*

Total lipid content in gill, liver and muscle *L. rohita* in control and exposed Fe_3O_4 nanoparticles (30ppm and 300ppm) is presented in Figure 7. Among the different tissues the lipid content is higher in muscle and lower in gill and when compared to control the lipid content decreased with increasing concentration of iron oxide nanoparticles. Similar observation was done, were significant decreased in the total lipid levels when compared to reference. Other workers also recorded significant elevations in these parameters [29, 28, 30, 31]. Elevation or depletion in lipid profile is either due to disturbance in the metabolism of lipids or may be due to impaired clearance from plasma which favours liver dysfunction.

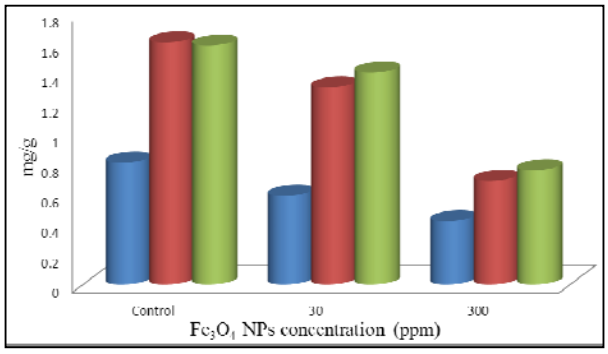


Fig 7: Lipid content in gill, liver and muscle of *L. rohita*

4. Conclusion

From this study, it is concluded that Fe_3O_4NPs altered the haematological and biochemical parameters of *Labeo rohita*.

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