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Genotoxic Effect of Iron Oxide Nanoparticles Treated Tannery Effluent on Zebrafish *Danio rerio*

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ABSTRACT

The present study deals with the genotoxic effect of iron oxide nanoparticles treated tannery effluent on zebrafish *Danio rerio*. The chemical co-precipitation method was used for the synthesis of iron oxide nanoparticles which were characterized by SEM, EDAX, XRD, FTIR and VSM. Physico-chemical characteristics of tannery effluent were also estimated. Iron oxide nanoparticles were used as nano-adsorbents in reducing the toxic substances present in tannery effluent. Behavioural studies and genotoxic effect on zebrafish exposed to different concentrations of iron oxide nanoparticles treated tannery effluent and control (raw tannery effluent) were carried out. Biochemical composition such as protein, carbohydrate and lipid were estimated in the muscles and gills of zebrafish on 14^{th} day after exposure. SEM images of iron oxide nanoparticles were observed at 5 μm and 10 μm which were spherical. EDAX spectrum recorded on synthesized iron oxide nanoparticles was identified in 7 peaks. FT-IR spectrum of iron oxide nanoparticles was analysed in the range of 500-4000 cm^{-1} and spectral bands were observed. Physico-chemical parameters of treated tannery effluent were decreased as the different concentrations of iron oxide nanoparticles increased. 200, 225 and 250 ppm treated tannery effluent were selected for median lethal concentration. No mortality was found in both control and iron oxide nanoparticles treated tannery effluent. The number of micronuclei was increased with increasing concentration of iron oxide nanoparticles when compared to control. Biochemical characteristics such as protein, carbohydrate and lipid in muscle and gills of zebrafish were higher in T_2 (225ppm) than control and other concentrations. From this, it can be concluded that iron oxide nanoparticles can be used as nano-adsorbent in treating tannery effluent for effective removal of toxic substances.

INTRODUCTION

One of the most serious environmental problems is the existence of hazardous and toxic pollutants in industrial wastewater because most of these wastewaters end up into the environment. The main source of freshwater pollution can be attributed to the discharge of untreated waste, dumping of industrial effluent and run-off from agricultural fields. Nowadays, there is continuously increasing worldwide concern for the development of wastewater treatment technologies. Different treatment technologies are available for the removal of toxic substances. Adsorption is considered as one of the most effective, efficient and economical methods for the removal of pollutants from wastewater. Among the different treatment methods, the utilization of nanoparticles for the removal of pollutants from industrial effluents has arisen as an attractive research direction. This is because, compared to bulk materials, nanomaterials based adsorbents possess much larger surface area, which can provide a greater number of active sites for adsorption. Some kinds of nanomaterials such as carbon nanotubes, manganese oxides, zinc oxide, titanium oxide and iron oxide have been used as nano-adsorbents in many studies and showed excellent adsorption capacity for various heavy metals including chromium. Multi-wall carbon nanotubes have high dispersion and adsorption ability to remove heavy metals in wastewater treatment. Among these nano-adsorbents, the use of iron oxide nanoparticles has been received much attention due to their unique properties and effective in adsorption of toxic substances and heavy metals in tannery effluent (Lkhagvadulam et al. 2017).

Embryonic and larval *Danio rerio* (zebrafish) is increasingly used as a toxicological model to conduct rapid *in vivo* tests and developmental toxicity assays; the zebrafish features high genetic homology to mammals, robust, phenotypes, and high-throughput genetic and chemical screening have made it a powerful tool to evaluate *in vivo* toxicity. Recent studies employing zebrafish as an experimental model, comparing it with other *in vivo* and *in vitro* models, presenting zebrafish as a potent vertebrate tool to evaluate drug toxicity and efficacy to facilitate more extensive, easy and comprehensive knowledge of new generation drugs (Caballero & Candiracci 2018).

The application of genotoxicity biomarkers in organisms allows for the assessment of mutagenic hazards and the identification of the sources and fate of the contaminants. Micronucleus (MN) test is an index of accumulated genetic damage during the lifespan of the cells is one of the most suitable techniques to identify integrated response to the complex mixture of contaminants. The large majority of studies on the genotoxic effect of the polluted water environment have been carried out with the use of bivalves and fish. Haemocytes and gill cells are the target tissues most frequently considered for the MN determination in bivalves. The erythrocyte MN test in fishes was also widely and frequently applied for genotoxicity assessment of freshwater and marine environment in situ using native or caged animals following different periods of exposure (Bolognesi & Hayashi 2011). The study related to the genotoxic effect of iron oxide nanoparticles treated tannery effluent on zebrafish *Danio rerio* is very important and required, hence the present investigation was made.

MATERIALS AND METHODS

Materials

Ferrous chloride ($FeCl_2$), ferric chloride ($FeCl_3$) and sodium hydroxide were used for the synthesis of the iron oxide nanoparticles (Fe_3O_4) and purchased from Loba Chemicals, India. All the reagents used for the synthesis of Fe_3O_4 nanoparticles were of analytical grade and used without further purification. All the glassware were washed, rinsed with deionized water, dried and heat sterilized in a hot air oven.

Methods

Synthesis of Iron Oxide Nanoparticles

The co-precipitation method was used for the synthesis of iron oxide nanoparticles. The aqueous solution of $FeCl_2$ and $FeCl_3$ was prepared in 1:2 ratio and NaOH (0.1N) was added with constant stirring within 30 minutes, the solution gets brownish yellow colour. The pH of the solution was 1. The aqueous solution with a further constant stirring, within 30 minutes a visible colour change was observed. The yellow colour aqueous solution turned into greenish-black precipitate and the pH was adjusted to 12. After the precipitation, it was centrifuged at 500 rpm within 3 minutes and added with ethanol in trace volume to collect the iron oxide nanoparticles which were allowed to air dry/dissolve in distilled water for 8 h sonication (50Hz frequency Vibronics -230V).

Characterization of Iron Oxide Nanoparticles

(a) **Scanning Electron Microscope (SEM):** SEM analysis is a powerful investigative tool which uses a focused beam of electrons to produce complex, high magnification images of a sample's surface topography. Morphology of the sample (Fe_3O_4NPs) was investigated using a scanning electron microscope (SEM) (LEO 1455 VP).

(b) **Energy Dispersive X-ray Spectroscopy (EDAX):** A minute drop of nanoparticles solution was cast on aluminium foil and subsequently dried in the air before transferring it to the microscope. An energy dispersive X-ray detection instrument (EDAX) (HORIBA 8121-H) was used to examine the elemental composition of the sample.

(c) **X-ray Diffraction (XRD):** Structure and crystalline size of Fe_3O_4 nanoparticles were determined by using XRD diffractometer with nickel-filter CuKα radiations in the 2θ range (λ = 1.5418Å) from an X-ray tube run at 40kV and 30ma.

(d) **Vibrating Sample Magnetometer (VSM):** The magnetic property of Fe_3O_4 nanoparticles was determined by vibrating sample magnetometer.

Collection and Acclimation of Fish

For toxicity studies, zebrafish fingerlings (3.5±1.5g) were collected from Aqua garden, Madurai and transported to the laboratory in polythene bags filled with oxygenated water. Fish were acclimated in round plastic troughs for 15 days at 28±2ºC. During acclimation, fish were fed with trainee feed containing fish meal, groundnut oil cake, wheat flour and rice bran in the form of dry pellets.

Sample Collection (Tannery Effluent)

Tannery effluent samples were collected in plastic containers from the discharged stream of tannery effluent situated in the central part of Dindigul town.

Physico-Chemical Characteristics of Tannery Effluent

The physico-chemical parameters such as colour, odour, pH, electrical conductivity, total solids, total dissolved solids, total suspended solids, hardness, sodium, potassium, calcium, magnesium, sulphate, chloride, dissolved oxygen, dissolved carbon dioxide, BOD, COD and copper were estimated by standard methods (APHA 2012).

Role of Iron Oxide Nanoparticles on Physico-Chemical Parameters of Tannery Effluent

The role of iron oxide nanoparticles on the physico-chemical characteristics of tannery effluent was examined. Iron oxide nanoparticles were used as nano-adsorbents in treating tannery effluent with different concentrations such as 50ppm, 100ppm, 150ppm, 200ppm and 250ppm of Fe_3O_4NPs.

Acute Toxicity Tests (LC_{50} Determination)

Acute toxicity tests were conducted in this study following International Standard Guidelines (ASTM 1993). This study was conducted for 96 hours at room temperature. The raw tannery effluent was taken as a control. Different concentrations of iron oxide nanoparticles treated tannery effluent such as 200ppm, 225ppm and 250ppm were used for further studies. Ten fish were maintained in triplicates.

Genotoxicity (Micronucleus Assay)

Blood samples were taken from the fish in control and each concentration of iron oxide nanoparticles treated tannery effluent, smeared on clean glass slides and dried for 10 minutes for removal of water content, and fixed in ethanol for 10 minutes. Then, the slides were stained with Geisma stain (3%). The slides were evaluated under a light microscope and erythrocytes were counted for each sample (Suganya et al. 2018).

Collection of Organ Samples

The organs such as muscle and gills were dissected from the fish in control and different concentrations of iron oxide nanoparticles treated tannery effluent on 1^{st} day, 7^{th} day and 14^{th} day and stored frozen for further investigation.

Biochemical Characteristics

Total protein was determined spectrophotometrically at 660nm based on Lowry's method (Lowry et al. 1951). Total carbohydrate was determined based on the Anthrone method (Carrol et al. 1956). Total lipid was estimated by the method of Barnes & Blackstock (1973).

RESULTS AND DISCUSSION

Fe_3O_4 nanoparticles were synthesized by chemical co-precipitation method. Iron oxide nanoparticles can be synthesized through co-precipitation of Fe^{2+} and Fe^{3+} by the addition of a base. The size, shape and composition of iron oxide nanoparticles synthesized through chemical methods depend on the type of salt used, Fe^{2+} and Fe^{3+} ratio, pH and ionic strength (Ali et al. 2016). Complete precipitation of Fe_3O_4 should be expected between pH 9 and 14 (Gupta & Wells 2004). The precipitated magnetite is black in colour. Fe_3O_4 produced are usually coated with organic or inorganic molecules during the precipitation process (Kim et al. 2001).

The morphology of the iron oxide nanoparticles was studied by scanning electron microscopy. It revealed the spherical and rectangular shapes of synthesized nanoparticles. It showed a clear image of highly dense iron oxide nanoparticles and micron-scale size range about 9.48mm (scale bar at 10μm) and 9.48mm (scale bar at 5μm) (Fig.1). Keerthika et al. (2017) reported that the iron oxide nanoparticles having micron-scale size range about 10.91mm (scale bar 5μm) and 10.86mm (scale bar 10μm). Hariani et al. (2013) reported that the SEM image of synthesized iron oxide nanoparticles have a clear image ranges from 30nm to 100nm. Similar results on SEM analysis of iron oxide nanoparticles had also been reported by Lida et al. (2007).

The presence of carbon (C), oxygen (O) and iron (Fe) was revealed in synthesized nanoparticles by EDAX spectral analysis. EDAX spectrum recorded on the iron oxide nanoparticles is shown at three peaks located between 0.5KeV and 6.5KeV. The two peaks of Fe element were located on the spectrum at 0.8 KeV and 6.5 KeV and another peak of O element was located on the at 0.5 KeV (Fig. 2). Keerthika et al. (2017) reported the EDAX spectrum of iron oxide nanoparticles which showed three peaks located between 2 KeV and 10 KeV maxima related to the iron characterized lines K.

The FTIR spectrum of iron oxide nanoparticles was analysed in the range of 400-4000cm^{-1}. Fourier Transform Infrared Spectroscopy revealed the functional groups of iron

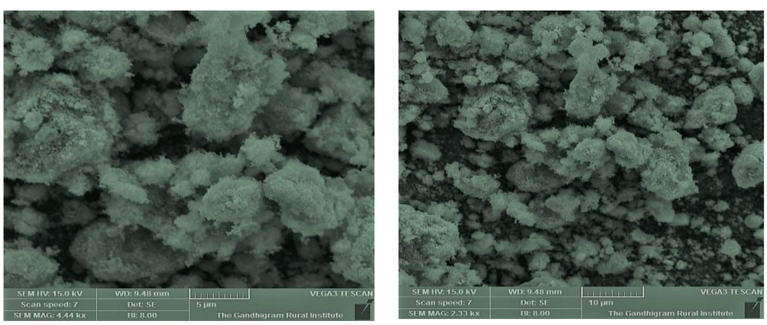

Fig. 1: SEM images of iron oxide nanoparticles.

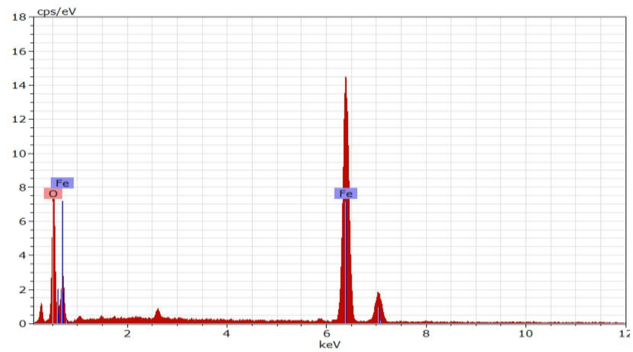

Fig. 2: EDAX image of iron oxide nanoparticles.

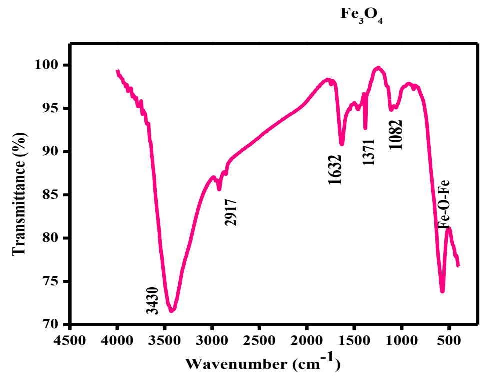

Fig. 3: FT-IR image o iron oxide nanoparticles.

oxide nanoparticles and viewed the functional groups of alcohol, phenol, alkanes, ketones, saturated aliphatic, alkyl halides and O-H, C-H, C-Br, C=O, C-I stretching of proteins (Fig. 3 and Table 1). Arokiyaraj et al. (2013) reported that the main functional groups of iron oxide nanoparticles are alcohol, phenols and primary amines. Similar results were obtained from the FT-IR spectrum of iron oxide nanoparticles confirmation peak at 511-535cm^{-1} (El-Kassas Hala et al. 2016).

The XRD diffraction peaks indexed with crystal planes are 19.5º (111), 30.02º (220), 35.22º (311), 54.08º (422), 64.55 º(440) and 78.36º (444). The samples were scanned between angles 0º to 90º to obtain the equatorial reflection. Structure and crystalline size of nanoparticles were determined by XRD with JCPDS Card No.89-2355 and 89-3854. The average crystalline size of chemically synthesized iron oxide nanoparticles was 15.58 and 21.34nm (Fig. 4). Suganya et al. (2016) reported that the synthesized iron oxide nanoparticles are crystal in nature and further confirmed by XRD and the nano-crystal average size is 10 to 16nm. Wu et al. (2011) reported that XRD patterns of synthesized particles with the standard diffraction spectrum of the synthesized product are crystalline iron oxide. The sharpness of XRD reflections indicates that the synthesized iron oxide is crystalline.

The magnetic properties of iron oxide nanoparticles were measured by Vibrating Sample Magnetometer (VSM) (Fig. 5). The synthesized iron oxide nanoparticles exhibit low saturation magnetization at 8.865 emu.g^{-1} than the bulk Fe_3O_4. Similarly, Mahdavi et al. (2013) reported that all the nanoparticles exhibit superparamagnetic behaviour and have low saturation magnetization (Ms) values than the bulk Fe_3O_4 (92 emu.g^{-1}). The saturation magnetization (Ms) of the Fe_3O_4 magnetic nanoparticles increase from 58.60 to 78.00 emu.g^{-1} with an increase of the nanoparticle sizes from 7.83 to 9.41nm. The result was due to the surface order or disorder interaction of the magnetic spin moment.

The physico-chemical characteristics of raw tannery effluent are presented in Table 2. All the parameters were higher than treated tannery effluent. Unpleasant odour of tannery effluent was due to microbial growth (Muthukkauppan & Parthiban 2018). Very high EC is due to the higher concentration of acid-base and salt in water (Kataria et al. 1995). Also increase in BOD level is a reflection of microbial oxygen demand, leading to depletion of dissolved oxygen (Poole et al. 1977). Similarly, Rajan &

Table 1: FT-IR functional group representation of iron oxide nanoparticles.

Bands	Functional groups	Type of vibration	Intensity
3439.42	Alcohol, phenol	O-H stretch	Strong
2924.03	Alkanes	C-H stretch	Medium
1631.00	Ketones, saturated aliphatic	C=O stretch	Strong
1109.35	Alkane	C-OH stretch	Strong
1026.90	Alcohol group	C-F stretch	Strong
582.87	Alkyl halides	C-Br stretch	Medium
479.70	Alkyl halides	C-I stretch	Strong

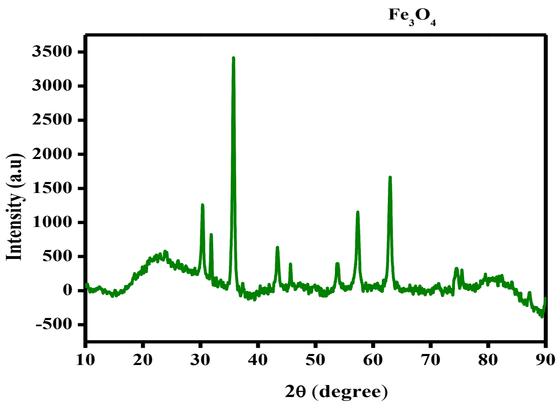

Fig. 4: XRD image of iro oxide nanoparticles.

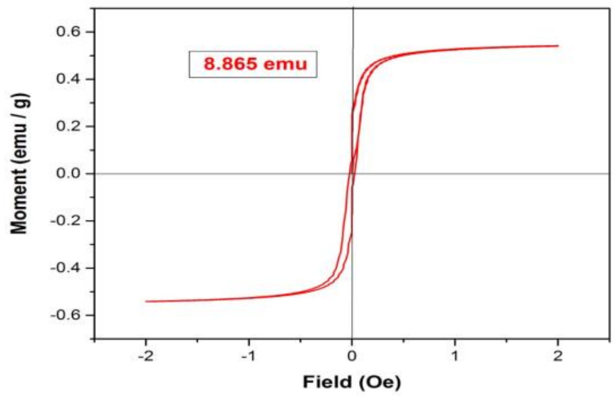

Fig. 5: VSM image of iron oxide nanoparticles.

Murali (2011) reported the physico-chemical characteristics of tannery effluent collected from a Common Tannery Effluent Treatment Plant located near Senkulam lake in Dindigul and treated with different leaves of plants for the removal of chloride. Lkhagvadulam et al. (2017) reported the use of maghemite nanoparticles for removal of Cr in tannery wastewater and maximum adsorption efficiency of total Cr was achieved 96.7% at an optimum condition and also observed the best efficiency in removing major physico-chemical parameters (TCr, SS, COD, sulphide and turbidity) in tannery wastewater.

Different concentrations of iron oxide nanoparticles (50ppm, 100ppm, 150ppm, 200ppm and 250 ppm) were used as nano-adsorbents in treating tannery effluent to reduce the physico-chemical contaminants (Table 3). The maximum adsorption efficiency of iron oxide nanoparticles

Table 2: Physico-chemical characteristics of tannery effluent.

S. No.	Parameters	Units	Values
1.	Colour	-	Dark brown
2.	Odour	-	Unpleasant smell
3.	pH	-	8.55
4.	Electrical conductivity	Ms/cm	251.24
5.	Total solids	mg/L	17444.4
6.	Total dissolved solids	,,	15190.4
7.	Total suspended solids	,,	2254
8.	Total hardness	,,	5844.8
9.	Sodium	,,	2760
10.	Potassium	,,	63.96
11.	Calcium	,,	1840
12.	Magnesium	,,	410.8
13.	Sulphate	,,	62.12
14.	Chloride	,,	4890.8
15.	Dissolved oxygen	,,	3.39
16.	Dissolved carbon dioxide	,,	25.2
17.	BOD*	,,	1570
18.	COD**	,,	21480
19.	Nitrogen	,,	155
20.	Copper	,,	0.016

*Biological Oxygen Demand, **Chemical Oxygen Demand.
All the values are an average of ten individual observations.

Table 3: Physico-chemical characteristics of Fe_3O_4 NPs treated tannery effluent.

S.No.	Parameters	Unit	Different concentration of iron oxide nanoparticles treated tannery effluent				
			50ppm	100ppm	150ppm	200ppm	250ppm
1.	Colour	-	Dark brown	Light brown	Light green	Light yellow	colourless
2.	Odour	-	-	-	-	-	-
3.	pH	-	8.30	8.22	7.92	7.54	7.11
4.	Electrical conductivity	Ms/cm	242.1	226.4	216.28	200,84	182.55
5.	Total solids	mg/L	3433	3233	2933	2850	2833
6.	Total dissolved solids	,,	2912	2780	2655	2624	2100
7.	Total suspended solids	,,	521	300	278	226	100
8.	Total hardness	,,	5200	4400	3200	2800	2000
9.	Dissolved oxygen	,,	4.04	6.464	6.48	6.52	6.56
10.	Dissolved carbon dioxide	,,	18	16	14	10	6
11.	Chloride	,,	3834	3550	3266	2840	2414
12.	Magnesium	,,	290	282	266	250	243
13.	Sulphate	,,	59	55.6	54.54	54.25	53
14.	BOD	,,	1433	734	406	118.2	80.8
15.	COD	,,	20000	12800	11200	9600	5600
16.	Nitrogen	,,	124	109	90	72	55
17.	Sodium	ppm	2752	2715.4	2533	2032	1667
18.	Potassium	,,	63.82	63.70	54.04	43.12	36.606
19.	calcium	,,	908	400	342	301	297.8
20.	Copper	,,	0.004	0.003	0.003	0.002	0.001

in treating tannery effluent was observed at low pH and high concentration of iron oxide nanoparticles (200 and 250 ppm). With the increase of adsorbent dose, the reduction of physico-chemical parameters of tannery effluent increase due to the increased available binding sites in the nano-composite for the complexation of metal ions (Saravanan et al. 2013). A faster initial removal rate was possible due to the availability of sufficient vacant adsorbing sites in the adsorbent (Sivakami et al. 2013).

No mortality was found in both control and different concentrations of iron oxide nanoparticles for LC_{50} determination. The behavioural response gives a direct response of the animals to the pollutant. Observation of basic behavioural responses of zebrafish was made which showed jerking movement and breathing in water surface (Table 4). Suganthi et al. (2015) reported the behaviour patterns such as jerky movements, continuous opercular movement, and reduction in dorsal and anal fin movements in *O.mossambicus* treated with ZnO NPs. Sivakumar et al. (2014) evaluated the behavioural responses of *Danio rerio* exposed to raw tannery effluent which showed rapid swim with random movements and coughing due to inconvenience in breathing.

Micronucleus assay was done on 1st, 7th and 14th day (Table 5). Micronuclei in erythrocytes of zebrafish increases with increasing concentrations of iron oxide nanoparticles treated tannery effluent when compared to the control group. The number of micronuclei present in different concentrations of iron oxide nanoparticles treated tannery effluent differ significantly from the control group at ($P < 0.001$) on 1st day, ($P < 0.01$) on 7th and 14th day based

Table 4: Basic observation of zebrafish exposed to control and iron oxide nanoparticles treated tannery effluent.

S.No.	Activity	Observation
1	Circular swimming	Yes
2	Jerk movement	Yes
3	Bottom resting	No
4	Surface respiration	Yes
5	Aggressive movement	Yes
6	Excess of mucous secretion	No
7	Mortality observation	Yes
8	Behaviour observation	Yes
9	Breathing movement	Surface

Table 5: Micronucleus Assay.

Treatments	Micronuclei 0^{th} day	Micronuclei 7^{th} day	Micronuclei 14^{th} day
T_0	3	13	18
T_1	7**	15*	25*
T_2	9**	18*	30*
T_3	10**	20*	31*

Significantly different from the control group at **P < 0.001 and *P < 0.01 based on *t*-test.

on *t*-test. Similarly, Suganya et al. (2018) reported the increased amounts of micronuclei with increasing of the concentration of iron oxide nanoparticles when compared to control group and less amount of micronuclei with a lower concentration of iron oxide nanoparticles. The micronucleus (MN) test in fish erythrocytes been has been successfully applied for the *in situ* detection of mutagenicity in a polluted environment and a significant increase in MN frequency in erythrocytes of fish collected in polluted marine or freshwater environments (Gustavino et al. 2001).

Biochemical characteristics such as protein, carbohydrate and lipid in zebrafish are higher in T_2 (225 ppm iron oxide nanoparticles treated tannery effluent) when compared to control (raw tannery effluent) and T_1 (200 ppm iron oxide nanoparticles treated tannery effluent) and T_2 (250 ppm iron oxide nanoparticles treated tannery effluent) (Figs. 6, 7 &

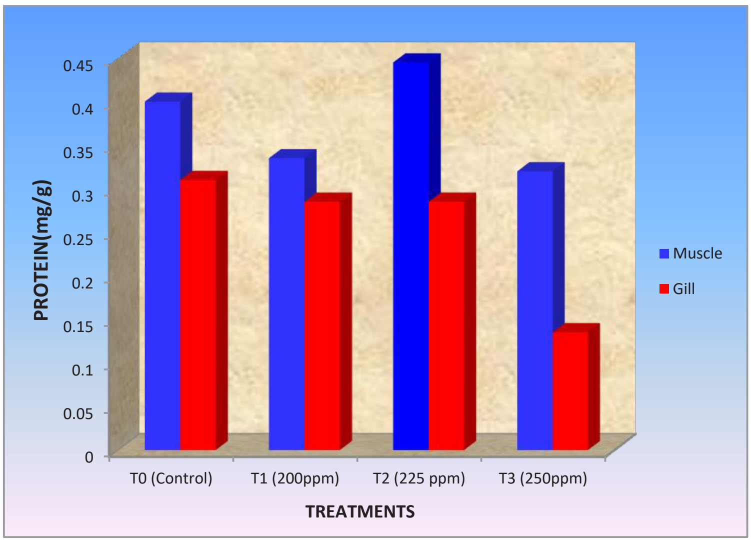

Fig. 6: Total protein level in muscle and gill of zebrafish on 14^{th} day.

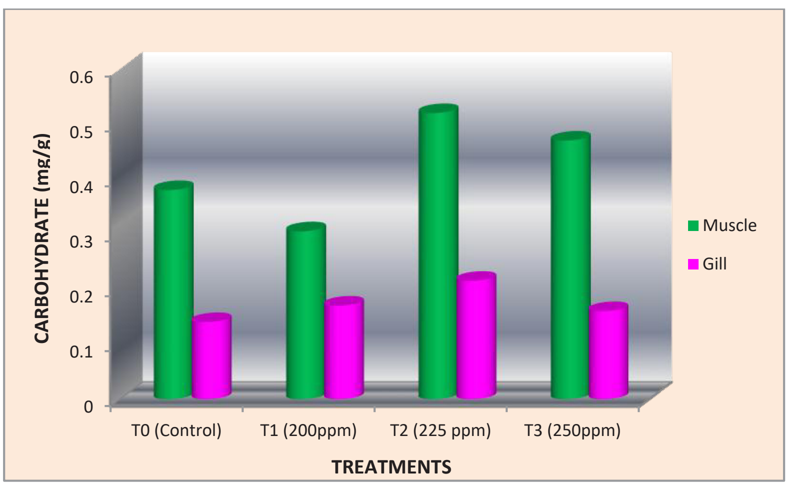

Fig. 7: Total carbohydrate level in muscle and gill in zebrafish on 14^{th} day.

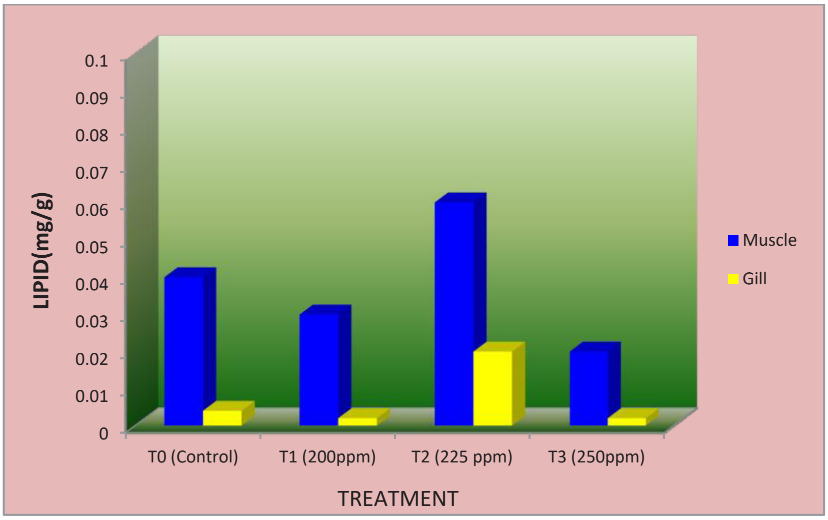

Fig. 8: Total lipid level in muscle and gill of zebrafish on 14th day.

8). The biochemical mechanisms in an organism play an important role during stress conditions due to the presence of toxicants in aquatic ecosystems. Thangam (2014) reported that pollutants in the aquatic media cause effects on fishes at a cellular or molecular level which results in significant changes in biochemical characteristics. Zhu et al. (2009) studied that the interaction of nanoparticles with chemical or biological systems may lead to biochemical disturbances or adaptive responses and these responses can be used to assess the health conditions of aquatic organisms. Keerthika et al. (2017) reported that the iron oxide nanoparticles altered the biochemical characteristics of *Labeo rohita*. Sivakumar et al. (2015) similarly reported that the biochemical parameters of muscle, gill and liver of zebrafish *Danio rerio* decrease significantly from control when exposed to raw tannery effluent. Palaniappan & Vijayasundaram (2008) reported that the arsenic intoxication induces significant alteration in the major biochemical composition of muscle proteins due to arsenic intoxication.

CONCLUSION

From this study, it is concluded that iron oxide nanoparticles can be used as nano-adsorbent in treating tannery effluent for effective removal of toxic substances.

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