IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

Impact Of Aluminium Oxide Nanoparticles On Zebrafish *Danio rerio*

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Abstract : Aluminium oxide nanoparticles were synthesized using the co-precipitation method and characterized using UV-Vis, SEM, EDAX, FT-IR and XRD. Before the experiment, the physical-chemical parameters of water such as pH, temperature, oxygen, carbon dioxide, hardness, alkalinity and chloride were estimated. Behavioural studies like circular swimming, jerk movement, aggressive movement and surface respiration of Zebrafish *Danio rerio* were observed when exposed to aluminium oxide nanoparticles in water. The different concentrations of 0.380,0760 and 3.80 mg/l of aluminium oxide nanoparticles were used for the sublethal analysis on zebrafish. Biochemical composition such as protein, carbohydrate and lipid in muscle and gill of zebrafish, and haematological parameters such as red blood cells, white blood cells, hemoglobin and hematocrit were measured in the whole blood of *Danio rerio.* UV-Vis spectrum of aluminium oxide nanoparticles was observed at 230nm. SEM image showed crystalline structure. EDAX spectrum was recorded confirming the presence of Al and O elements. FT-IR spectra were analysed in the range of 500-4000 cm-1.The chemical composition of aluminium oxide nanoparticles was examined by using XRD and the diffraction peaks are indexed as 14.1291°, 17.8971°, 22.414°, 28.185° and 30.4804° are observed corresponding to 84.7,135,140,127 and 141. Biochemical parameters and haematological parameters decreased with an increased quantity of aluminium oxide nanoparticles exposed to zebrafish.

Keywords: Impact, Aluminium oxide, Nanoparticles, Zebrafish

INTRODUCTION

Nanoparticles hold a leading stage in the rapidly developing field of nanotechnology. The unique size-dependent properties make these materials superior and indispensable in many areas of human activity. It can be used as a surface for molecular assembly and may be composed of inorganic or polymeric materials[1]. The nanoparticles can be synthesized by biological systems such as plants and microorganisms. The biosynthesis of metal nanoparticles by plants is currently under development. Green nanotechnology eliminates toxic substances makes environmentally friendly and non-toxic is used to offer numerous benefits. During the synthesis of nanoparticles bioreduction of metal ions takes place[2].

In recent decades, various environmental challenges have been mitigated due to a boom in nanotechnologies and nanomaterials development. Nanoparticles have been also widely used in environmental applications and have soon promising performance in pollutant removal or toxicity mitigation. Several studies have also shown the application of nanoparticles on heavy metals removal from contaminated water. In addition to great removal performance, research has demonstrated the feasibility of reusing and regaining the removal capacity in successive treatment cycles. Additionally, with extensive usage of nanoparticles for a wide spectrum of applications such as energy, electronics and personal care products, nanoparticles have been released into the environment, heightening concern about the safety and toxicity of nanoparticles[3].

Among various nanoparticles, aluminium has vast applications in industries where it is used as an additive of fuel to increase fuel efficiency and also in Military applications. Aluminium nanoparticles bind to the gills of many fishes and cause toxicity[4]. The release of nanoparticles in aquatic systems is expected to increase in the future given in the continuous growth of nanotechnology applications. Aluminium oxide nanoparticles are usually considered as the nano toxic material. Acute toxicity bioassays (lethal and sub-lethal concentrations) are used to assess the toxicity of heavy metals to the potentials of various fish species. The toxic effects on fishes are examined by the effects on physiology, growth, biology and behaviour of various animals including fish species. In the green synthesis of aluminium oxide nanoparticles, fruit extract is used to offer numerous benefits.

The zebrafish *Danio rerio* is an important modal organism for nanotoxicology studies due to its small size, ease of maintenance, rapid development and available genomic information. The female zebrafish are able to spawn year-round every 2 to 3 days. And the fecundity rate is 200 eggs. This feature makes as a suitable test animal. Also, zebrafish are easily obtainable, maintainable and inexpensive.

Aquatic ecotoxicity studies on manmade nanoparticle effects grew rapidly and it affects more freshwater organisms than the terrestrial or saltwater species. The nanoparticles dispersed into the environment during the production processes, potentially entering the marine compartment and thus posing potential risks for biota [5]. The increase in large quantities of engineered aluminium oxide nanoparticles released to aquatic environments for pollution remediation has raised concerns about environmental safety [6].

Haematological studies are used to assess the physiological changes in fish and fish blood is used to detect the alterations in the fish. The most haematological variables measured include RBC and WBC count, haemoglobin content and haematocrit value [7]. The toxic nanoparticles can cause variations in many haematological parameters, either by increasing the number or concentration by boosting their biosynthetic activities or by decreasing its number or concentration by quelling its biosynthetic sites [8]. Being a novel attempt, this work is to evaluate the acute toxicity of aluminium oxide nanoparticles on Zebrafish (Danio rerio).

MATERIALS AND METHODS

Zebrafish is one of the most popular pets in the world and it is characterized by red and golden strip coloration. The zebrafish is an important vertebrate model organism used in scientific research. Due to its small size, ease of maintenance and rapid development it is used for nanotoxicity studies.

Plant Material (Fruit) Collection

For the experimental study, Aluminium oxide nanoparticles (Al_2O_3) were synthesized by using the fruit of Pumpkin (*Cucurbita pepo)*. Fresh fruit was collected from Dindigul main market, Tamil Nadu, India and it was cleaned with running water to remove debris and other contaminated organic contents.

Preparation of Fruit Extract

10g of *Cucurbita pepo* were weighed, washed and cleaned with distilled water and air dried. Then it was crushed with the help of a mortar and pestle.100 ml of distilled water was added to it and boiled for 20 minutes. Filtered by using Whatman No.1 filter paper. After filtration, an extract was obtained for further use.

Synthesis of Aluminium oxide Nanoparticles

The co-precipitation method was used for the synthesis of aluminium oxide nanoparticles. 0.1 M Aluminium nitrate and 1M Sodium hydroxide were dissolved separately in distilled water. 25 ml of prepared extract was added dropwise to the 100 ml of 0.1 M aluminium nitrate solution and kept for 2 hours under constant stirring conditions like pH, and temperature. A milky white-coloured precipitate occurred by adding NaOH to fix the pH. Then it was centrifuged at 2500 rpm for 20 minutes. The centrifuging process continued with distilled water and ethanol in trace volume. The resultant supernatant was discarded and the pellets were dried in a hot air oven at 100^0 C for 3 hours to obtain aluminium oxide nanoparticles.

Characterization of Aluminium oxide Nanoparticles

UV-Vis Spectroscopy

The UV-Vis Spectroscopy was used to analyse of aluminium oxide nanoparticles and the absorbance peak was 230 nm and the maximum absorbance at 150-500 nm of synthesized aluminium oxide nanoparticles.

Scanning Electron Microscope

The morphology of the aluminium oxide nanoparticles was examined using a scanning electron microscope (SEM) (LEO 1455 VP). SEM analysis is a powerful tool which uses a focused beam of electrons to produce complex, high-magnification images of a sample's surface topography.

Energy Dispersive X-Ray Spectroscopy

The EDAX spectroscopy (HORIBA 8121-H) is used to analyse the elemental composition of Al_2O_3 nanoparticles. It shows Al and O as the major components of aluminium oxide nanoparticles.

X-RAY DIFFRACTION

The structure and crystalline size of aluminium oxide nanoparticles were determined by XRD using X' pert powder X-ray diffractometer with nickel-filter CuKα radiation in the 20 range (=1.5418 Aº) from an X-ray tube run at 40 KV and 30ma.

Fourier Transform Infrared Spectroscopy

The interactions between the *Cucurbita pepo* extract and the nanoparticle surface were measured by the FT-IR. FTIR spectrometer using the KBr pellet method.

Collection and Acclimatization of Fish

For toxicity studies, Zebrafish (*Danio rerio*) (1.0 ± 0.5g) were collected from Live Tropics fish farm, Kadachanendhal, Madurai, Tamil Nadu, India and transported to the laboratory in polythene bags filled with oxygenated water. Fishes were acclimated for 15 days at 28 ± 2^0 C in round plastic troughs. During acclimation, fish were fed with control feed containing fish meal, ground nut oil cake, wheat flour and rice bran in the form of dry pellets.

Acute Toxicity Tests (LC_{50} Determination)

Acute tests were conducted in the study following the international standard guidelines (ASTM, 1993: [9] 96 h static-renewal acute aluminium oxide nanoparticle toxicity was conducted at room temperature. Glass tanks were filled with 15 litres of water. In each glass tank, different concentrations (0,150mg,300mg,450mg,600mg,750mg/15l) of aluminium oxide nanoparticles were taken. There were five treatments of test chemicals plus a control and three replications of each treatment. In each glass tank, 5 fishes were introduced. A control was maintained without aluminium oxide nanoparticles. 5 fish with an average length of 2cm and an average weight of 1g were selected and introduced into each glass tank. The behaviour and survival of each fish were observed in each concentration for 4 days. 0,10,20,30,40,50/l of nominal concentration of aluminium oxide nanoparticles were used in the study. The mortality rate of fish was assessed and counted in different aluminium oxide concentrations and also in the control group at 24,48,72,96 hours. This test is used to determine the 96h median lethal concentration (LC_{50}) value by the use of Finney's probit analysis.

Survival Studies

Different concentrations of (0,10,20,30,40,50 mg/l) aluminium oxide nanoparticles were taken and observed for the survival studies for a period of 96 hrs median lethal concentration (LC_{50}) value was calculated by the use of SPSS Software probit analysis.

Sub Lethal Concentration

Sub-lethal concentrations of aluminium oxide nanoparticles such as 0,0.380,0.760,3.80mg/l were confirmed from the survival studies. The behaviour of zebrafish was observed in each concentration for 14 days.

Biochemical Composition

Total protein [10], carbohydrate [11], and lipid [12] was estimated.

Hematological Parameters

The haematological parameters including haemoglobin (Hb), haematocrit (Hct), red blood cells (RBC) and white blood cells (WBC) were measured in the blood of Zebrafish (*Danio rerio*). Mean cell haemoglobin (MCH), Mean cell volume (MCV) and Mean cell haemoglobin concentration (MCHC) were calculated using standard formula.

RESULTS AND DISCUSSION

UV-Vis spectrum is the basic and important technique for the identification and characterization of nanoparticles. UV-Vis spectroscopy was used for the characterization of aluminium oxide nanoparticles[13]. The absorbance spectra of aluminium oxide nanoparticles were measured in wavelength within the range of 150 to 500. It exhibits a strong absorption band at 130nm. The maximum peak was observed between 150 to 250nm (Figure 1).SEM images of Al_2O_3 NP_s were taken for the analysis of the size and shape of Al_2O_3 NP_s. SEM results reveal that the aluminium oxide nanoparticles are crystalline structures (Figure 2). Gupta et al., (2018)[14] reported the crystalline structure Al_2O_3 NP_s using SEM. The presence of oxide (O_3) and Aluminium (Al_2) was revealed in the synthesized nanoparticles by EDAX spectral analysis. The recorded EDAX results show that aluminium oxide nanoparticles peak located between 0.10 Kev and 10 Kev. Peaks indicating the purity of aluminium oxide nanoparticles on the spectrum at 1.50 Kev and another peak of 0 element was located at 0.35 Kev (Figure 3). Dhawale et al., (2018)[15] reported that the quantitative analysis of Al_2O_3 nanoparticles was carried out by using EDAX spectroscopy and it shows Al and O as are the major components of aluminium oxide nanoparticles. The peak of Al and O is present between the 0Kev and 2.0Kev. Bhoi et al., (2020)[16] reported that the characterization of aluminium oxide nanoparticles by EDAX spectroscopy was used to analyze the presence of Al_2O_3 components.FT-IR spectroscopy is a useful technique for the analysis of structure and components and to identify the functional group present in the samples. The FT-IR spectra of the Synthesized Al_2O_3 powders in the range of 500- 4000 cm^{-1} and bands observed at 3362, 1638, 3431, 1063, 733 and 571 cm^{-1} which are associated with O-H stretch, C=C stretch, O-H stretch, S=O stretch, C-H stretch and C-Br stretch(Figure 4). Dhawale et al., (2018)[16] revealed that Al_2O_3 NP_s FT-IR spectra are at 410.84, 420.48, 445.56, 491.85, 501.49, 588.29, 636.51, 709.80. (O-Al-O) functional groups.

The crystalline structure and the phase composition of aluminium oxide nanoparticles were determined by using the XRD technique. The XRD analysis can be used to analyse the morphology of Al_2O_3 NP_s. Several Bragg's reflections with 2ø values of 14.1291^0, 17.8179^0, 22.414^0, 28.185^0 and 30.4804^0 are observed corresponding to 84.7, 135, 140, 127 and 141 planes(Figure 5). Dhawale et al., (2018)[16] reported that the peaks in the XRD pattern of aluminum oxide nanoparticles from the JCPDS file (71-1683) having rhombohedral structure at 2ø angles nine reflections were observed and are 25º(012), 35º(104), 43º(113), 52º(024), 57º(116), 61º(122), 66º(214), 68º(300), and 70º(119). Gazanfari et al.,(2014)[17] reported that XRD peaks were due to the factors of size and stretch. The resultant aluminium particles were pure face-centered cubic (fcc). Bhoi (2020) reported that the different peaks of Al corresponding to 39.7702^0, 46.0267º, 66.3436º, 79.4211º, and 83.5915ºwere observed.

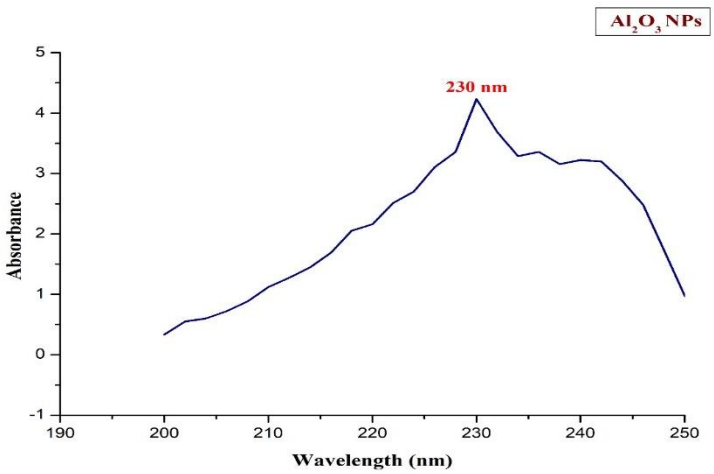

Figure 1. UV- Visible Spectroscopy of Aluminium oxide nanoparticles

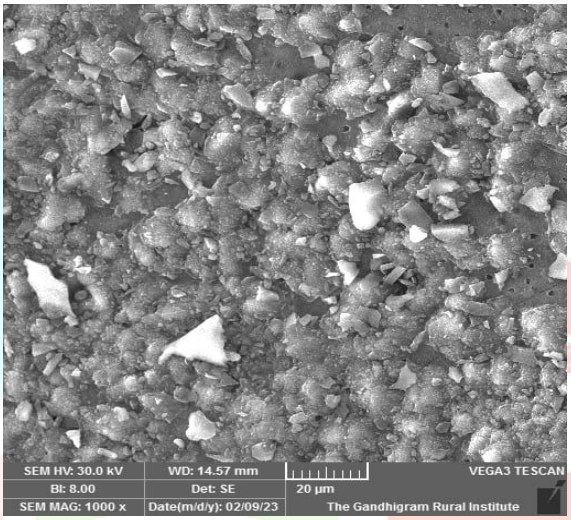

Figure 2. SEM image of Aluminium oxide nanoparticles

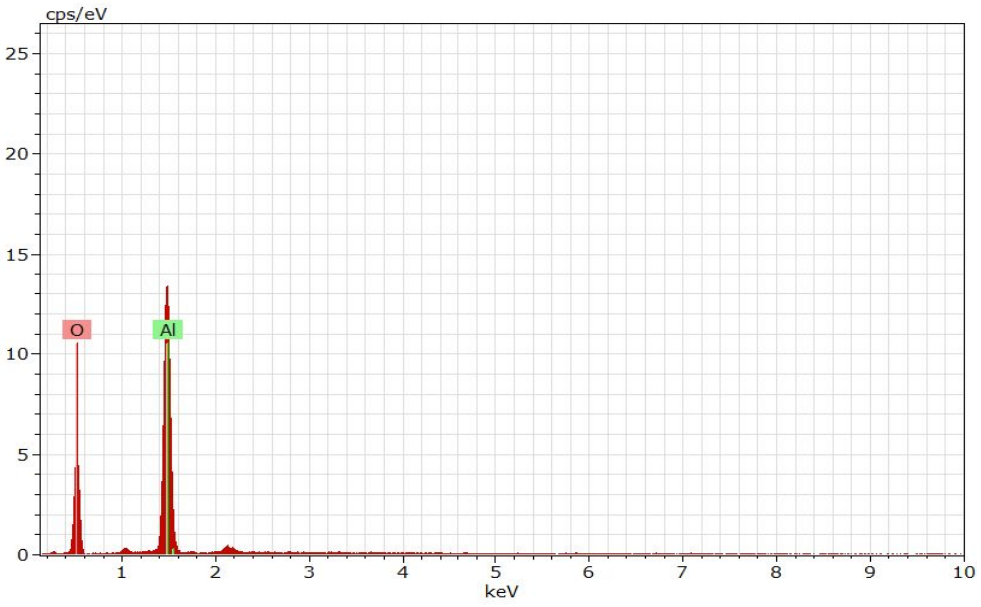

Figure 3. EDAX Image of Aluminium oxide nanoparticles

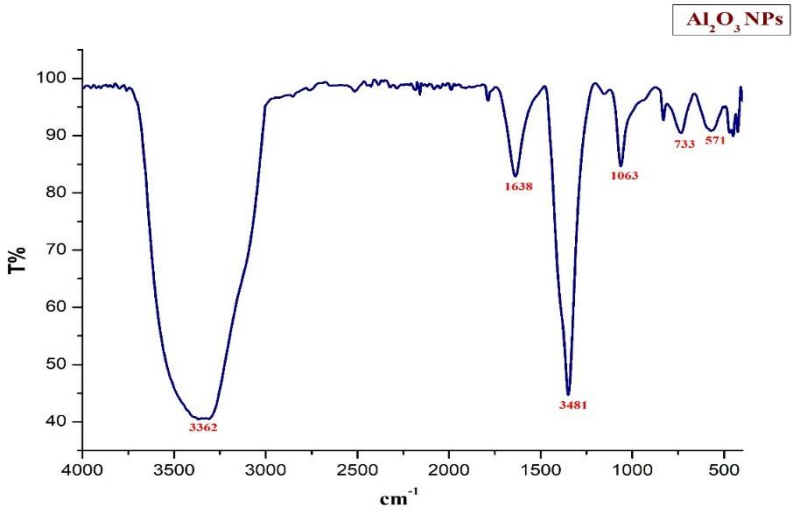

Figure 4.FT- IR image of Aluminium oxide nanoparticles

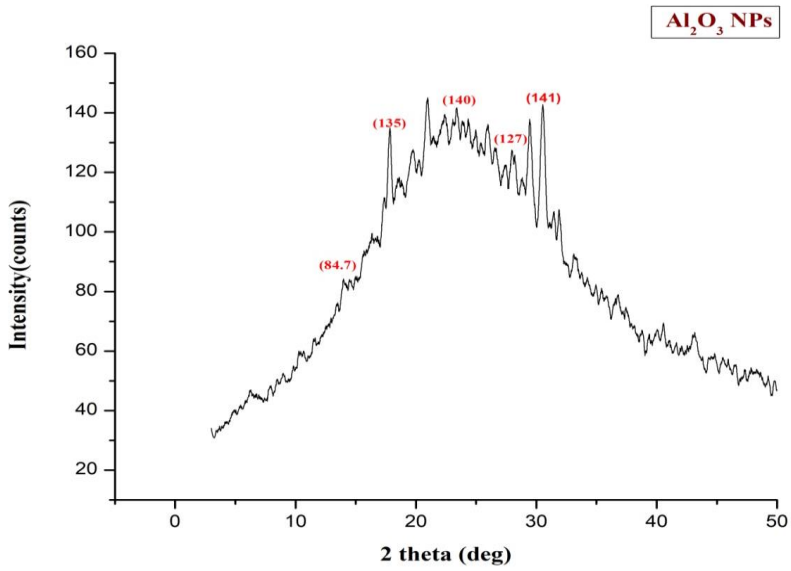

Figure 5. X-ray diffraction of Aluminium oxide nanoparticles

In this study Mortality/survival of zebrafish in control and aluminium oxide treated tanks was recorded after 96 hours and the median lethal concentration (LC_{50}) was identified from the survival studies the sublethal concentrations 1/10, 1/50, 1/100 of aluminium oxide nanoparticles were taken respectively(Table 1 & 2). Vidya (2017) [18] reported that the 96hrs exposure of Al_2O_3 nanoparticles at different concentrations such as 10,20,30,40,50,60 and 70mg/l show acute toxicity in fishes and eventually affect the health status of aquatic ecosystems.

Table 1. Survival studies of zebrafish exposed to aluminium oxide nanoparticles

Probability	95% Confidence Limits for Concentration(ppm)			95% Confidence Limits for Log(concentration ppm)		
	Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
LC_1	23.640	3.196	30.139	1.374	0.505	1.479
LC_{10}	29.259	9.346	34.715	1.466	0.971	1.541
LC_{15}	30.760	11.952	36.095	1.488	1.077	1.557
LC_{20}	32.008	14.478	37.371	1.505	1.161	1.573
LC_{25}	33.119	16.995	38.662	1.520	1.230	1.587
LC_{30}	34.149	19.526	40.064	1.533	1.291	1.603
LC_{35}	35.132	22.064	41.676	1.546	1.344	1.620
LC_{40}	36.091	24.573	43.624	1.557	1.390	1.640
LC_{45}	37.044	26.991	46.071	1.569	1.431	1.663
LC_{50}	**38.007**	26.242	49.212	1.580	1.466	1.693

Table 2. Survival studies of Lc_{50} concentration of Aluminium oxide nanoparticles

S.No	Concentration (mg/l)	Exposure Duration (Hrs)			
		24	48	72	96
1	150 mg/15 L	NM	NM	NM	NM
2	300 mg/15 L	NM	NM	NM	NM
3	450 mg/15 L	NM	NM	NM	NM
4	500 mg/15 L	NM	NM	NM	1
5	750 mg/15 L	NM	NM	2	3

NM – No Mortality

Biochemical parameters such as total protein, carbohydrate and lipid content (mg/g) in muscle and gill of zebrafish are higher in control(T_0) and lower in treatments 1 to 3(Fig.6). Vidya (2017) reported decreased protein levels in *Oreochromis mossambicus* fish exposed to Aluminium oxide nanoparticles. Canli and Canli (2019)[19] reported that the aluminium oxide nanoparticles were deposited in the liver of *Oreochromis niloticus* fish when exposed to aluminium oxide nanoparticles for 14 days. It shows that Al_2O_3 nanoparticles decrease the ATPase activities in the osmoregulatory organs (liver and kidney) of *O.niloticus* Thangapandiyan and Monika (2020)[20] reported that the level of carbohydrates present in fish tissues (muscle, liver, and gills) was gradually increased with increase in the ZnO NPs concentration. Ashouri et al., (2015)[21] reported that the selenium nanoparticles in the feed increased the protein, carbohydrates and lipid content of muscle, gill and liver on crucian carp. Sarkar et al., (2015)[22] also reported that the silver nanoparticles in the feed increased the protein, carbohydrate and lipid content of muscle, gill and liver of *Oreochromis mossambicus*. Keerthika et al., (2017)[23] reported that the iron oxide nanoparticles altered the biochemical parameters of *Labeo rohita*.

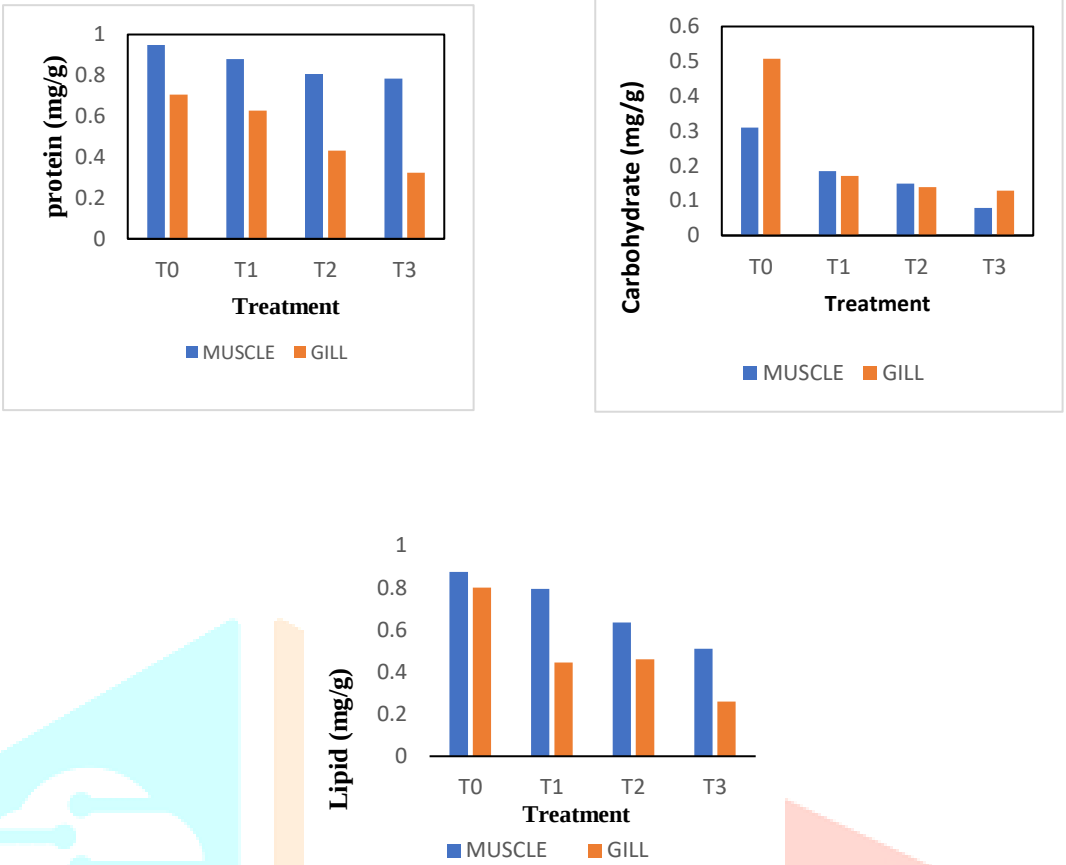

Figure 6 Protein(a), carbohydrate (b) and lipid(c) in muscle and gill of Zebrafish

Haematological parameters of zebrafish exposed to aluminium oxide nanoparticles were presented in Table 3. Haematological parameters are very helpful in the judgement of the health condition of fish species. The RBC and WBC in zebrafish were decreased. Such decreased RBC was reported on the differential quantities of zinc oxide and copper oxide nanoparticles incorporated feed on haematological characteristics of Zebrafish (*Danio rerio*) [24,25] The RBC and WBC count of Koi carp gradually increased as the quantity of ZnO NPs increased from feed I to feed VI[26]. The decreased HCT value is may be due to less oxygen content in the blood or shrinkage of the cell due to toxicant stress on erythropoietic tissue. In contrast to the above findings, CuO nanoparticles did not cause any major disturbance in the haematology of rainbow trout[8]. Haematological parameters such as RBC, WBC, blood glucose and haemoglobin of *Oreochromis mossambicus* exposure to nanoparticles showed ionoregulatory interference, but also compensatory responses to allow fish to ensure and showed that a change significantly affected the haematology of *Oreochromis mossambicus* [27]. Dawood et al.,(2020)[28] reported the exposure of selenium nanoparticles to *Oreochromis niloticus* in relation to haematological parameters such, as RBC, WBC, Hb, PCV, MCH and MCH. The Hb is slightly increased compared with control fish. reported that haematological parameters such as RBC and WBC, blood glucose and haemoglobin. After exposure, the parameters such as RBC and Hb were decreased. The observed decrease in Hct value may be due to the less oxygen content in the blood of fish. Moreover. erythropoietic tissue lower Hct values also indicate shrinkage of cells due to toxicant stress and changes in

haematological parameters such as leucocytes and erythrocyte counts, haemoglobin and haematocrit were apparent in the fish exposed to trivalent chromium

TABLE 3. Haematological Parameters of Zebrafish

S.No	Parameters	T_0	T_1	T_2	T_3
1	WBC(Cells/cumm)	20,100	7,300	6,800	5,900
2	RBC (Millions/cumm)	0.19	0.19	0.15	0.09
3	Hb (gm/dl)	0.8	0.5	0.3	0.3
4	HCT (%)	1.6	1.0	0.6	0.5
5	Platelets count (lakhs /cumm)	8.6	8.4	5.9	5.3
6	Polymorph (%)	67	36	80	90
7	Lymphocytes	32	19	37	21
8	Eosinophils (%)	01	03	01	01

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