E-ISSN: 2347-5129
P-ISSN: 2394-0506
(ICV-Poland) Impact Value: 5.62
(GIF) Impact Factor: 0.549
IJFAS 2017; 5(1): 327-332
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www.fisheriesjournal.com
Received: 18-11-2016
Accepted: 19-12-2016

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Assessment of acute toxicity (LC_{50}-96 h) of aluminium oxide, silicon dioxide and titanium dioxide nanoparticles on the freshwater fish, *Oreochromis mossambicus* (Peters, 1852)

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Abstract
The acute toxicity of three nanoparticles, such as aluminium oxide (Al_2O_3-NPs), silicon dioxide (SiO_2-NPs) and titanium dioxide (TiO_2-NPs), were assessed on the freshwater fish, *Oreochromis mossambicus* for 96 h. Nanoparticles with different sizes as 16.7 nm of Al_2O_3, 1 nm of SiO_2 and 11.4 nm of TiO_2 were evaluated for the median lethal concentration by probit analysis. Different concentrations of nanoparticles of Al_2O_3-NPs at 10, 20, 30, 40, 50, 60 and 70 mg/ L; SiO_2-NPs at 5, 25, 50, 75, 100, 125, 150 and 175 mg/ L; and TiO_2-NPs at 25, 50, 75, 100, 125, 150, 175, 200 and 225 mg/ L were exposed to fishes for 96 h maintaining 10 animals per group. Mortality of fish was observed and recorded throughout the experiment. Median lethal concentrations that was determined by probit analysis is found to be 40 mg/L for Al_2O_3-NPs, 120 mg/L for SiO_2-NPs and 164 mg/L for TiO_2-NPs. The results proved that nanoparticles showed acute toxicity to fish when exposed for 96 h and this could reveal the fact that exposure of nanoparticles may result in adverse toxic effects to fish that eventually affects the health status of aquatic ecosystem.

Keywords: Nanoparticles, LC_{50}, aluminium oxide, silicon dioxide, titanium dioxide, *Oreochromis mossambicus*.

1. Introduction
Nanotoxicology, a new and evolving branch of toxicology, mainly deals with the adverse effects of nano-scale materials on the living system. The wide range of applications and the specific properties made nanoparticles more popular and as a result, the rate of production also increased in large scale. Advances in nanotechnology created the newly engineered nanoparticles of newly improved nano-scale particles yielding completely new products with different physical and chemical properties. However, the newly engineered nanoparticles are associated with different health impacts and its products have become a major concern in the emerging area of nanotoxicology. More recently, nanoparticles are considered as a potential environmental health hazard and its increased environmental persistence lead to harmful impact on both aquatic and terrestrial organisms [1].

In environmental toxicology, experiments often examine the response of toxic chemicals at different concentrations to the test organisms for specific period of time. The concentration-response relationship using the median lethal concentration (LC_{50}) determines the potency of the toxicant in test animals [2, 3]. Nanoparticles are comparatively new in toxicological sciences thus the toxicity data of nanomaterials remain curtailed and, therefore, prior to the conduct of any nanotoxicological investigation on experimental model, the standardized assessment of physicochemical characteristics including particle size, particle-type, dynamic light scattering or surface chemistry of nanoparticles is necessary [4]. Consistency in the results of nanoparticles on same test organisms is also questionable, as the same particles of different sizes, coatings and dispersions has been shown to provide with different results [5]. When the particle size is reduced, the proportion of atoms found at the surface is enhanced relative to the proportion inside its volume and result in more reactive and effective nano-scale particles that form several health implications and also modify the potential biological effects. Therefore, the present study determines the characterization of nanoparticles such as the particle size and purity, prior to the ecotoxicological testing.

The potential ecotoxicity effects of engineered nanoparticles on aquatic animals remain scanty. However, the rapid growth of nanotechnology sector in recent years targeted variety of consumer products as well as on pharmaceutical, gene therapy, cosmetics, electronics, and in industrial applications. It is a well-known fact that most of the industrial wastes and urban water sewage would find the way to end up in aquatic ecosystems, which ultimately results in the uptake of nanoparticles by aquatic organisms. Hence the ambitious use of nanoparticles raises concern to the public that it could pose a risk to both humans as well as on ecosystems.

Although there are several methods to evaluate the acute toxicity effects of such nanoparticles on test animals, the most convenient and statistically precise system to determine the ecotoxic effects of any toxicant is the conventional LC_{50} method. In the conventional LC_{50}-based acute toxicity testing, the exposure duration is set for 96 h as it estimates the lethal concentration at 50% as less variability than those at higher or lower centiles [2]. Several experiments on aquatic organisms have been shown to demonstrate that the presence of nanoparticles in a medium leads to decreased fertility, physiological changes, behavior abnormalities, and an increased mortality rate [6-8].

Metal oxide nanoparticles such as Aluminium oxide (Al_2O_3-NPs), silicon dioxide (SiO_2-NPs) and titanium dioxide (TiO_2-NPs) selected in the present study has wide range of applications ranging from personal care products to drug and gene delivery. Al_2O_3-NPs are widely used in pigments, paints, coatings, propellants, artillery as well as an explosive additive. SiO_2-NPs, one of the most widely used nanomaterials, commonly developed for a broad spectrum of biomedical and biotechnological applications such as biosensors for DNA, cancer therapy, gene delivery, drug delivery and in industrial manufacturing, packaging and ceramic synthesis. TiO_2-NPs is the most familiar nanoparticles used mainly in commercial products including personal care products, paints, and polishes, coatings and pigments. It is renowned fact that metal oxide nanoparticles are highly stable in the ecosystem [9] as they have higher bioaccumulation rate in the environment and are likely to enter into the food chain. Toxicity studies of nanoparticles in fish model remain scanty and thus the present investigation was aimed to assess the acute toxicity effects of selected nanoparticles for 96 h in the freshwater fish, *Oreochromis mossambicus*.

2. Materials and Methods

2.1 Preparation and characterization of test solutions

TiO_2-NPs (Cat. No: 634662; Titanium (IV) oxide, mix of anatase and rutile) were obtained from Sigma Aldrich, Germany. Al_2O_3-NPs (Cat. No: 0140408) and SiO_2-NPs (Cat. No: 1940323) were obtained from SISCO Research Laboratory (SRL), India. The purity and size of the nanoparticles are further confirmed by X-ray diffraction and Transmission Electron Microscopy. The nanodispersions were prepared just before exposure by ultra-sonication at 100 kHz for 30 min (except SiO_2 for 10 min) using double distilled water and maintained as stock.

2.2 Test animal

Oreochromis mossambicus weighing 6 ± 1.5 g and length 6.5 ±1cm were collected from local fish farm, Safa Aquarium, Kozhikode, Kerala (11º22'N, 75º85'E). Fishes were acclimatized to the laboratory conditions prior to experiments with exposure of constant supply of water and good lighting system. They were maintained in well-aerated glass tanks (40 L capacity), which was dechlorinated at regular intervals.

2.3 Preliminary Tests

The physico-chemical features of the tap water were estimated as per APHA guidelines [10]. Water temperature (28 ± 2 °C), oxygen saturation of water (70% and 100%), pH (7.4 to 7.6) were monitored using the standardized procedures and was maintained throughout the experiment.

2.4 Experimental procedure

To determine the acute toxicity effects of selected nanoparticles, the median lethal concentration or LC_{50} values for 96 h were determined by probit analysis, with a confident limit of 5% level [11]. The concentration of the nanoparticles at which 50 percent of the test animals dies during a specific period is referred to as median lethal concentration (LC_{50}) or median tolerance limit. In order to assess LC_{50} of the nanoparticles, the fishes were not fed a day prior to and during the test period to reduce faecal and excess food contaminating the test solution. Ten specimens were maintained in each treatment tanks and aerated using tubed motorized pumps. Control tank without toxicants were also maintained along with the treatment groups. The movement and the behaviour along with the mortality of fishes were continuously monitored throughout the study.

For determining LC_{50} concentration of nanoparticles, it is necessary to understand that how much concentration of a toxicant is required to cause 50% mortality. In the experiments, the concentrations were selected based on the purity of test materials and size of test animal, so that the concentrations of test chemicals were increased in geometric proportion series in each groups as follows:

Group I: Al_2O_3-NPs at seven different concentrations, ie., 10, 20, 30, 40, 50, 60 and 70 mg/ L for 96 h

Group II: SiO_2-NPs at eight different concentrations, ie., 5, 25, 50, 75, 100, 125, 150 and 175 mg/ L for 96 h

Group III: TiO_2-NPs at nine different concentrations ie., 25, 50, 75, 100, 125, 150, 175, 200 and 225 mg/ L for 96 h

All treatment groups at different concentrations of nanoparticles were tanked separately along with control animal. Fishes without any movement for long period were considered as dead and were removed from the tanks immediately to prevent contamination.

2.5 Statistical Analysis

All experiments were performed in triplicates for the accuracy of the results. Total number of animal used in the experiments, the exposure concentrations and the mortality rate in each experiment were fit to a probit model using $\log_{10}$ concentration transformation using the statistical package SPSS 17.0. The correlation between mortality on Y-axis and concentrations on X-axis and the best-fit line was obtained by plotting graph using MS Excel 2007.

3. Results

3.1 Characterization of nanoparticles

TEM images of nanoparticles indicated crystalline, irregular and roughly symmetrical structure of nanoparticles and the size ranged between 1 and 100 nm (Figure 1). The characterization of the selected three nanoparticles were confirmed using X-ray diffraction (Rigaku Miniflex) and it is found that the particles are pure and free from impurities

(Figure 2). The average particle size is calculated using Sherrer's formula and it was found as 16.7 nm of Al_2O_3, 1 nm of SiO_2 and 11.4 nm of TiO_2. The size and crystalline structure were then confirmed using High Resolution Transmission Electron Microscope (Jeol/JEM 2100) having resolution point 0.23 nm and lattice 0.14 nm and magnification 2000 X-1500000 X. TEM results confirmed that the nanoparticles used in the present study have the size similar to the manufacturer's details and the XRD data.

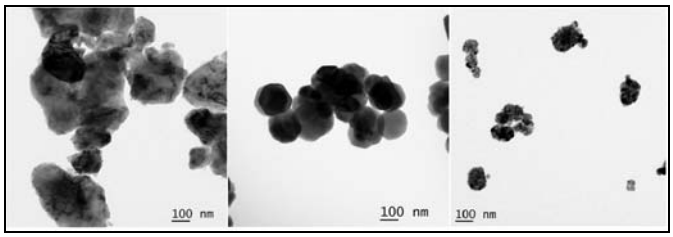

Fig 1: TEM images showing the morphology of Al_2O_3-NPs, SiO_2-NPs and TiO_2-NPs aggregates, respectively dissolved in double distilled water. Scale bar = 100 nm

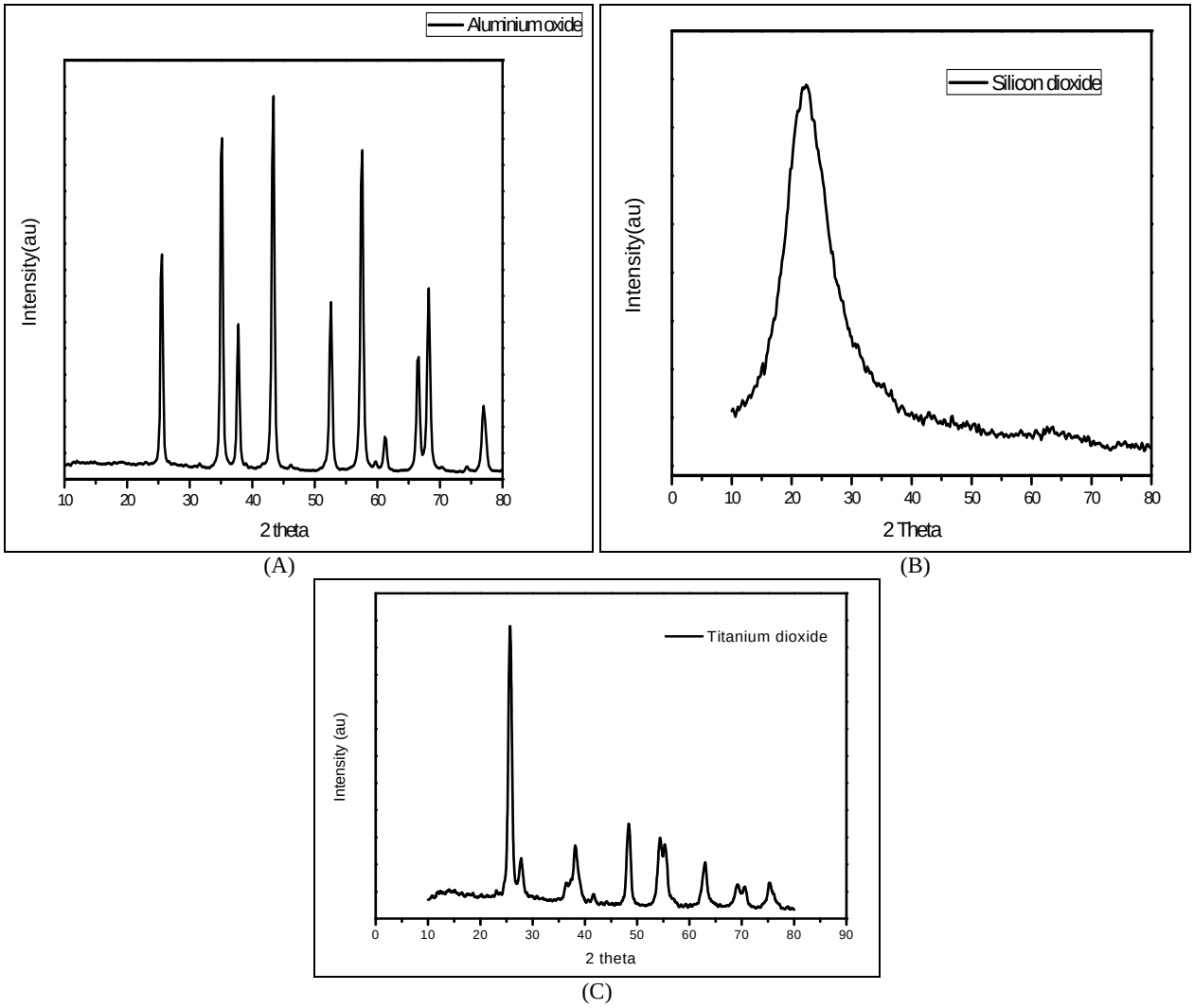

Fig 2: XRD (Rigaku Miniflex) images showing structural and crystalline nature of the powdered samples of nanoparticles. Fig. A denotes XRD peaks correspond to Al_2O_3-NPs with particle size of 16.7 nm range. Fig. B denotes XRD showing the characteristic peak of SiO_2-NPs with particle size of 1nm Fig. C shows the XRD peaks of TiO_2 denoting the peaks of both anatase and rutile at an angle of 27.6º and 28.9º with particle size of 11.4 nm, respectively.

3.2 Median lethal concentrations

In the present study mortality of the fishes in each group were continuously monitored throughout the experiment. It was observed that in Group I – Al_2O_3-NPs-exposed fishes showed no mortality at 10 and 20 mg/L concentrations for 96 h duration. At 30, 40 and 50 mg/L concentrations, fishes showed 20, 50 and 80% mortality, respectively. However, at 60 and 70 mg/ L concentrations showed total mortality of fish at 96 h and 24 h, respectively (Table 1). In Group II - SiO_2-NPs treated fishes, no mortality was observed at 5, 25, 50 and 75 mg/L concentrations up to 96 h. At 100 and 125 mg/L concentrations 40 and 50% mortality was observed after 96 h whereas at 150 and 175 mg/L concentrations showed 100% mortality at 96 h and 48 h, respectively (Table 3).

In TiO_2-NPs exposed fish (Group III) showed no mortality at 25, 50, 75 and 100 mg/L concentrations for 96 h. At 125, 150, 175 and 200 mg/L concentrations of TiO_2-NPs killed 20, 40, 50, and 80% of animals, respectively for 96 h of exposure. However, at 225 mg/L concentration, the total mortality was seen immediately within 24 h of treatment (Table 5). Computation of median lethal concentration by probit analysis is found to be 40 mg/L for Al_2O_3-NPs, 120 mg/L for SiO_2-NPs and 164 mg/L for TiO_2-NPs (Table 2,4 and 6; Figs. 3-5). The results of probit analysis indicated that the percentage of mortality is positively correlated (r = 0.97, 0.947 and 0.91) against the concentrations of Al_2O_3, SiO_2 and TiO_2-NPs, respectively which indicated high degree of positive correlation.

Table 1: Percentage of fish mortality exposed at different concentrations of Al_2O_3-NPs in *Oreochromis mossambicus* for 96 h

Concentrations	Total (No. of animals)	Mortality (%)	Hour of mortality
10.00	10.00	0	96 h
20.00	10.00	0	96 h
30.00	10.00	20	96 h
40.00	10.00	50	96 h
50.00	10.00	80	96 h
60.00	10.00	100	96 h
70.00	10.00	100	24 h

Table 2: Probit analysis of 95% confidence limits for effective concentrations of Al_2O_3-NPs in *Oreochromis mossambicus*

Prob	Concentration (mg)	95% Confidence Limits	
		Lower	Upper
.01	17.85793	0.34029	25.58665
.02	20.45254	4.15094	27.51176
.03	22.09874	6.98513	28.74855
.04	23.33712	9.10728	29.68883
.05	24.34444	10.82603	30.46114
.06	25.20182	12.28286	31.12458
.07	25.95359	13.55501	31.71150
.08	26.62670	14.68943	32.24165
.09	27.23887	15.71694	32.72801
.10	27.80237	16.65887	33.17959
.15	30.13542	20.51079	35.09719
.20	31.98966	23.50084	36.69257
.25	33.58043	25.99746	38.12984
.30	35.00899	28.16990	39.49015
.35	36.33276	30.11054	40.82314
.40	37.58889	31.87595	42.16408
.45	38.80422	33.50441	43.54106
.50	40.00027	35.02475	44.97850
.55	41.19632	36.46139	46.49963
.60	42.41165	37.83759	48.12886
.65	43.66778	39.17782	49.89498
.70	44.99155	40.51014	51.83628
.75	46.42011	41.86986	54.00932
.80	48.01088	43.30662	56.50646
.85	49.86512	44.90154	59.49697
.90	52.19817	46.81872	63.34930
.91	52.76167	47.27023	64.29131
.92	53.37384	47.75650	65.31890
.93	54.04696	48.28658	66.45340
.94	54.79872	48.87342	67.72562
.95	55.65611	49.53678	69.18254
.96	56.66343	50.30900	70.90137
.97	57.90180	51.24920	73.02361
.98	59.54800	52.48589	75.85789
.99	62.14262	54.41089	80.34924

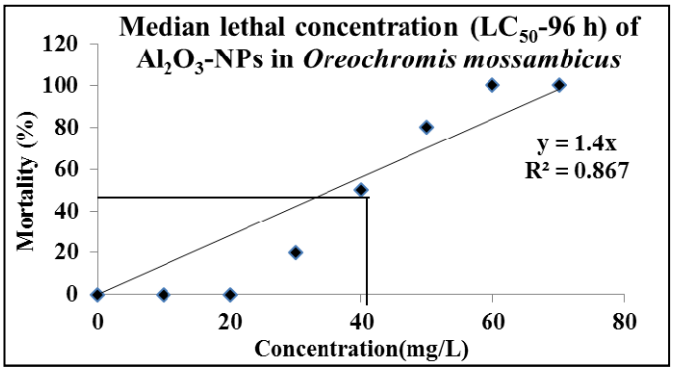

Fig 3

Table 3: Percentage of fish mortality exposed at different concentrations of SiO_2-NPs in *Oreochromis mossambicus* for 96 h

Concentrations	Total (No. of animals)	Mortality (%)	Hour of mortality
5.00	10.00	0	96 h
25.00	10.00	0	96 h
50.00	10.00	0	96 h
75.00	10.00	0	96 h
100.00	10.00	40	96 h
125.00	10.00	50	96 h
150.00	10.00	70	96 h
175.00	10.00	100	48 h

Table 4: Probit analysis of 95% confidence limits for effective concentrations of SiO_2-NPs in *Oreochromis mossambicus*

Prob	Concentration (mg)	95% Confidence Limits	
		Lower	Upper
.01	62.74245	32.99795	79.67651
.02	67.75591	38.16381	84.13493
.03	71.14254	41.83916	87.12121
.04	73.80127	44.82570	89.45680
.05	76.03706	47.40370	91.41789
.06	77.99336	49.70766	93.13367
.07	79.75001	51.81357	94.67580
.08	81.35643	53.76904	96.08849
.09	82.84548	55.60601	97.40110
.10	84.24024	57.34712	98.63424
.15	90.26922	65.07972	104.02869
.20	95.36712	71.83233	108.72215
.25	99.96951	78.03545	113.12803
.30	104.29158	83.88921	117.47702
.35	108.46326	89.49669	121.93910
.40	112.57596	94.91051	126.66699
.45	116.70341	100.15529	131.81541
.50	120.01315	105.24370	137.55034
.55	125.27474	110.19154	144.05462
.60	129.86778	115.03202	151.53713
.65	134.79210	119.82688	160.25490
.70	140.18380	124.67484	170.56019
.75	146.24449	129.72339	182.99327
.80	153.30221	135.19818	198.47538
.85	161.95987	141.48462	218.77992
.90	173.55114	149.38724	248.00626
.91	176.47298	151.30907	255.72014
.92	179.70294	153.40629	264.40444
.93	183.32274	155.72553	274.32992
.94	187.45174	158.33449	285.89723
.95	192.27452	161.33721	299.73505
.96	198.09943	164.90621	316.91156
.97	205.50279	169.36134	339.46452
.98	215.77438	175.41100	372.07509
.99	233.01591	185.27462	430.21187

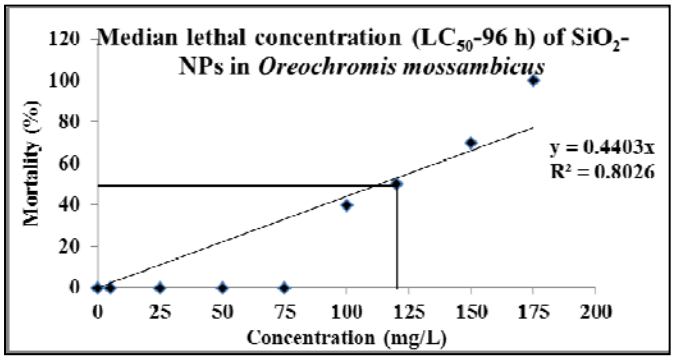

Fig 4

Table 5: Percentage of fish mortality exposed at different concentrations of TiO_2-NPs in *Oreochromis mossambicus* for 96 h

Concentrations	Total (No. of animals)	Mortality (%)	Hour of mortality
25.00	10.00	0	96 h
50.00	10.00	0	96 h
75.00	10.00	0	96 h
100.00	10.00	0	96 h
125.00	10.00	20	96 h
150.00	10.00	40	96 h
175.00	10.00	50	96 h
200.00	10.00	80	96 h
225.00	10.00	100	24 h

Table 6: Probit analysis of 95% confidence limits for effective concentrations of TiO_2-NPs in *Oreochromis mossambicus*

Prob.	Concentration (mg)	95% Confidence Limits	
		Lower	Upper
.01	79.36112	11.13292	106.02350
.02	89.30182	29.37677	113.22481
.03	95.60887	40.88573	117.86000
.04	100.35343	49.49879	121.39155
.05	104.21275	56.46989	124.29916
.06	107.49765	62.37387	126.80351
.07	110.37786	67.52439	129.02545
.08	112.95674	72.11224	131.03876
.09	115.30213	76.26246	132.89203
.10	117.46107	80.06164	134.61907
.15	126.39964	95.51704	142.04371
.20	133.50374	107.36139	148.38369
.25	139.59842	117.06739	154.27823
.30	145.07163	125.30259	160.05278
.35	150.14338	132.43471	165.90280
.40	154.95597	138.70534	171.95095
.45	159.61220	144.30158	178.27325
.50	164.19462	149.38457	184.91986
.55	168.77704	154.09873	191.93529
.60	173.43327	158.57529	199.37729
.65	178.24586	162.93704	207.33432
.70	183.31761	167.30727	215.94622
.75	188.79082	171.82537	225.43788
.80	194.88550	176.67621	236.18758
.85	201.98960	182.15581	248.89231
.90	210.92817	188.86256	265.06559
.91	213.08711	190.45871	268.99566
.92	215.43250	192.18412	273.27374
.93	218.01138	194.07191	277.98711
.94	220.89159	196.16980	283.26169
.95	224.17649	198.55038	289.28943
.96	228.03581	201.33276	296.38575
.97	232.78037	204.73472	305.12841
.98	239.08742	209.23020	316.77708
.99	249.02812	216.26592	335.18653

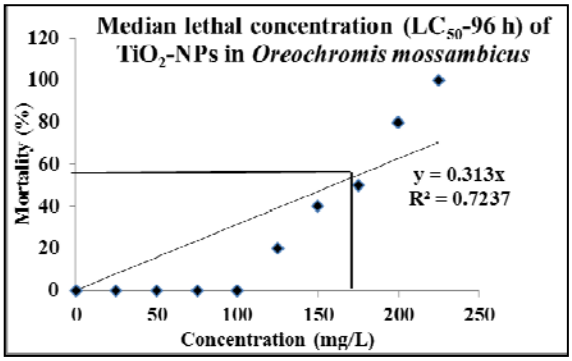

Fig 5

4. Discussion

Aquatic ecosystem is considered as the sink of several environmental contaminants consequently due to industrialization, agricultural productivity and also from urban wastewater discharges. The environmental contaminants can drift several kilometres from the point of application, and even at very little concentrations, lead to several adverse effects to most of the aquatic organisms. Nanoparticles have gained much attention in recent years owing to the production and manifold use in various products thereby gained the status of environmental toxicant within a short duration. The present study was aimed to assess the acute toxicity of different nanoparticles in the freshwater fish, *Oreochromis mossambicus*.

For ecotoxicity studies of nanoparticles it is crucial to demonstrate its characterization because the size, shape, structure, solubility, stability, surface area, surface coatings etc. contribute to the properties, fate and behaviour of nanoparticles in the biological system [12]. Characterization of nanoparticles, Al_2O_3, SiO_2 and TiO_2 showed the particle size as 16.7, 1 and 11.4 nm, respectively and it is found that the particles are pure and free from impurities. TEM images of nanoparticles indicated crystalline, irregular and roughly symmetrical in structure (Figures 1 and 2). Release of toxicants to aquatic environment has major impact on the production, spawning and in fecundity rate of fishes [13].

Toxicological assessment studies primarily focus to investigate the effects of toxicants based on the median lethal concentration, as it can directly measure the toxicity of compounds on the exposed organism. In this study the mortality of fish at different concentrations was observed for 96 h and also determined LC_{50} values of nanoparticles. The median lethal concentrations of Al_2O_3-NPs, SiO_2-NPs and TiO_2-NPs are 40, 120 and 165 mg/L, respectively, in the freshwater fish, *Oreochromis mossambicus*. Comparison of different nanoparticles based on the number of dead fishes at different concentrations indicates the toxicity of nanoparticles in the order of Al_2O_3, SiO_2 and TiO_2. Besides 100% mortality of fish was observed at 60 mg/ L concentration of Al_2O_3-NPs whereas in SiO_2-NPs and TiO_2-NPs exposure, 100% mortality rate was observed only at 175 and 225 mg/ L concentrations. Therefore, the nanoparticles Al_2O_3 is comparatively more toxic to the fish than SiO_2-NPs and TiO_2-NPs. However, mortality of the fish increased to the increase in the concentrations of nanoparticles. The disparity observed in the median lethal concentrations of nanoparticles might be due to its physicochemical properties rather than other factors as water salinity, pH, temperature or oxygen saturation, because all these factors are maintained to normal standard range throughout the experiment [14].

The toxicity of nanoparticles is consequently dependent on the particle size, composition and characteristics. This is the first comparative study on the acute toxicity of nanoparticles, as well as it is the first report stating the median lethal concentrations of the three nanoparticles Al_2O_3-NPs, SiO_2-NPs and TiO_2-NPs in the freshwater fish, *Oreochromis mossambicus*. The present study is the traditional acute toxicity tests to assess the LC_{50} values of nanoparticles, however, further studies are recommended to determine the toxic impacts of nanoparticles by the exposure to nanoparticles at sublethal concentrations in fish.

5. Conclusion

Assessment of acute toxicity, ie., median lethal concentrations

(LC_{50}-96 h) of nanoparticles, Al_2O_3, SiO_2 and TiO_2 by probit analysis was 40, 120 and 165 mg/L, respectively, in the freshwater fish, *Oreochromis mossambicus*. On comparison, an Al_2O_3 nanoparticle is comparatively more toxic to the fish than SiO_2 and TiO_2 nanoparticles when exposed for 96 h.

6. Acknowledgment
The authors acknowledge UGC-SAP/ BSR for the financial assistance during this study.

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