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Toxic effects of Zinc Oxide Nanoparticles to *Scenedesmus quadricauda* Microalgae

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ABSTRACT
The increase utilization of Zinc Oxide nanoparticles in industry during current years has resulted in their prevalence in aquatic environment. In light of this, the aim of present study was to explore the toxicity of chemically synthesized zinc oxide nanoparticles at various concentrations on Scenedesmus quadricauda using an algal growth inhibition test. In this work, Zinc Oxide nanoparticles were chemically synthesized using zinc nitrate and potassium hydroxide. Chemically synthesized nanoparticles were analyzed by various techniques. The average particle size of zinc oxide nanoparticles was approximately 29.97 nm, calculated by x-ray diffraction (XRD), and confirmed by transmission electron microscopy (TEM). Scenedesmus quadricauda was grown in BG-11 broth media in an optimum growth condition. After achieving the substantial growth, the microalgae cells were exposed to varying concentrations starting from 20 mg/L to 100 mg/L at the interval of 20 of zinc oxide nanoparticles for 24, 72, 120 hours. Growth inhibition test of algal cells was determined using spectrophotometer and cell number counting by Neubauer hemocytometry. The results show that the growth of Scenedesmus quadricauda microalgae was significantly impacted by the short-term exposure of 100 mg/L zinc oxide nanoparticles concentration treated cells after 120 hours.
Keywords: *Nanoparticles, Ecotoxicity, Microalgae, Scenedesmus quadricauda, Cell Viability.*

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INTRODUCTION

Nanoparticles are the tiny particles with the size ranging in at least one dimension from 1 to 100 nanometres. Nanoparticles are synthesized to attain unique physicochemical property, generally superior from their corresponding bulk counterparts and can be customized due to presence of various electrons on their surface [1-3]. At nanoscale size, the ratio of volume to surface area is more than in the same bulk counterpart [4, 5]. Metal oxide nanoparticles like Zinc Oxide (ZnO), Copper Oxide (CuO), Titanium Dioxide (TiO_2), etc. as well as metallic nanoparticles are among the most used nanomaterials in various areas like pesticides, alternative energy, medicines, consumer products and have received significant attentions over their potential ecological effects [6]. Extreme use and excessive release of nano form in the environment create unfavorable effects on water and living organisms [7]. Various types of nanoparticles have been reported to be toxic to living organisms, as they are non-biodegradable and soluble in the aquatic environments. When they enter in food chain, their high concentrations may internalize in human body and get accumulated in various organs, causing serious health ailments. A ZnO nanoparticles most utilized nanoparticles in different areas especially in cosmetics industries. Thus, over the years, zinc oxide has been produced in higher quantities as compared to other nanomaterials [8]. These probably enter to water bodies through the sewages of the industries [9, 10]. Because of the high demand of ZnO nanoparticles, its toxic effects on different levels of food chain specifically phytoplankton as producers at basic level of aquatic food sound essential. Toxic effects of ZnO nanoparticles on microalgae are important concern for the aquatic ecosystem [11]. Algal cells are suitable candidates to study due to ease of culturing, small size (10 micron), etc. Therefore, alga is most popular model aquatic organism for the toxicity assessment of nanoparticles.

Scenedesmus quadricauda is a common freshwater microalga. *Scenedesmus* sp. is frequently used for toxicity tests as model microalgae [12]. Different metal oxide nanoparticles have been examined on *Scenedesmus* sp. including TiO_2, Al_2O_3, SiC, CeO_2, & ZnO etc. [12-15]. Short term toxicity studies have

revealed that at lower concentrations, metal oxide nanoparticles show negative effect on microalgae growth [12].
In the present study, we aimed to explore the toxic effects of ZnO NPs on freshwater *Scenedesmus* microalgae through assays of growth parameters.

MATERIALS AND METHODS

Nanoparticle synthesis

Zinc Oxide nanoparticles (ZnO NPs) were chemically synthesized by direct precipitation technique using 0.2 M Zinc Nitrate (Zn $(NO_3)_2.6H_2O$) and 0.4 M of potassium hydroxide (KOH) as precursor. KOH solution was mixed dropwise into the salt solution of Zinc nitrate under continuous stirring for 3 hours. This led to the formation of white precipitate product. The white precipitate was washed 4-5 times with ethanol for removal of impurities. The white precipitate was left to dry at about 80^{o}C for 24 h.

Characterization of synthesized ZnO Nanoparticles

UV-vis spectroscopy technique was used to record the spectra of ZnO NPs. The presence of functional groups of the zinc oxide nanoparticles was determined by Fourier transform infrared spectroscopy (FTIR) (Perkin Elmer, Spectrum Two). X ray diffraction (XRD) was used for crystal structure characterization, size and phase identification. The size and morphology of ZnO NPs were investigated by the transmission electron microscopy (TEM) (JEOL, JEM-1230) at the output voltage potential of 120 KV.

Isolation of *Scenedesmus quadricauda*

Algal sample was collected from Jal Vihar, Italian garden, Gwalior, Madhya Pradesh, India. Collected algal sample was transferred to sterilized fresh BG-11 broth media (7.4 pH) for enrichment [16]. Various isolation & purification methods were used to obtain the pure culture of *Scenedesmus quadricauda*, [17]. After several sets of purification experiments pure cultures were confirmed by observation under light microscope at 40 X magnification. Isolated individual colonies were maintained in BG-11 broth media as well as on agar plates at ambient temperature in fluorescence light with 16:8 hour light-dark cycle [18]. Every month pure culture was transferred to fresh BG-11 broth to make them in exponential growth phase condition.

Determination of algal cell viability

Cell biomass and measurements were performed until the cultures achieved their death phase. Two different methods were used: (1) spectrophotometric absorbance, by determining the optical density of cell suspension using UV-vis spectrophotometer (Medox, MX-1287-011) at ג=680 nm. BG-11without algal cells were used as blank and ZnO NPs suspension without algal cells was used as positive control [19]. (2) cell number counting by Neubauer hemocytometry (figure 1) [20]. The hemocytometer is consisting of 9 large squares of equal size. The central square is further divided into small squares with each having volume of 4×10^{-6} ml. *Scenedesmus quadricauda* were counted in small squares since they are less than 10 micron in size. The cell number per unit volume was calculated as:

Cell number per unit mL = Cell number per small square / 4×10^{-6} mL

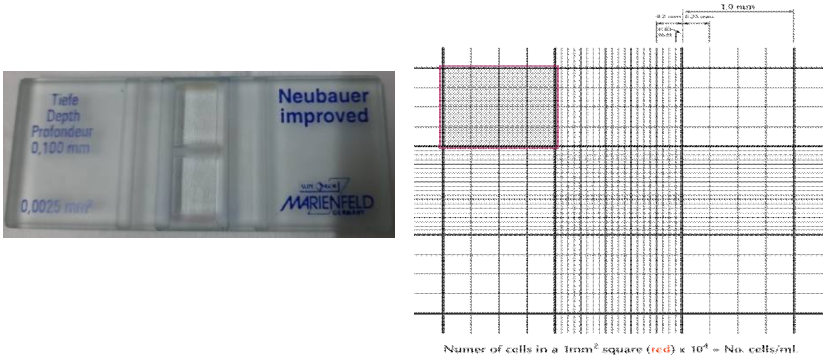

Figure 1. Hemocytometer image at normal (left) and at 40 X magnification (right)

ZnO nanoparticles exposure to *Scenedesmus quadricauda*

Toxicity experiments were performed in 250 ml flasks by introducing initial cell density of $1x10^{-6}$ cells/mL [21] containing 50 ml of BG11 broth having various ZnO NPs concentrations starting from 20 mg/L to 100 mg/L at the interval of 20 in fluorescence light with 16:8 h light-dark cycle for 24, 72 and 120 hrs. These conditions were maintained constantly during the 120 hours of the experiment. Negative

control consisted of cells cultures in BG-11 without ZnO NPs. All exposures tests were performed in triplicates.

RESULTS

UV-visible spectra analysis

The UV-visible absorbance spectra of prepared ZnO NPs are depicted in figure 2. An absorption peak was observed at 352 nm confirmed the successful synthesis of ZnO NPs.

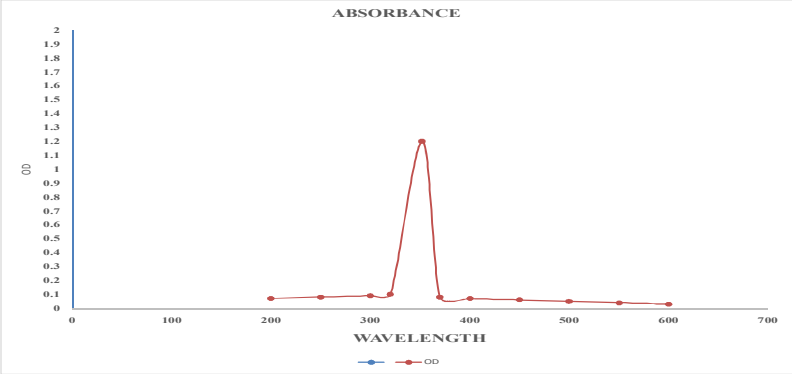

Figure 2. UV-vis spectra of ZnO nanoparticles solution

FTIR

The FTIR spectrum of sample has been shown in the range of 4000-450 cm^{-1} in Figure 3. The absorption bands of ZnO shows at 3574, 3471, 1637, 1368, 1014, 885, 838 and 463 cm^{-1} in table 1. The sharp peak positioned at 463 cm^{-1} is attributed to the presence of Zn-O stretching bonds [23, 24].

Table no 1: Peak area values and possible assignments of FTIR spectra for ZnO NP

Sr. No.	Frequency (cm^{-1}) ZnO NPs	Bond type	Bond Origin	Functional group
1.	3574.68	Sharp	O-H Stretching	Alcohol (free)
2.	3471.14	Strong, Medium	N-H Stretching	Alcohol (Intermolecular), Primary amine
3.	1637.25 885.38 838.36	Medium, Strong	C=C Stretching, N-H Bending, C=C Stretching, C-H Bending	Conjugated alkane, Amine, Alkene, 1,2,4-trisubstituted
4.	1368.65	Medium, Strong	C-H Bending, O-H Bending, O-H Bending, S=O Stretching	Alkene, Alcohol, Phenol, Sulfonate, Sulfonamide
5.	1014.04	Strong, Medium	C-F Stretching, C-O Stretching, C-N Bending	Fluoro compound, Alkyl aryl ether, Amine, Vinylether
6.	463.04	Strong	Zn-O Stretching	

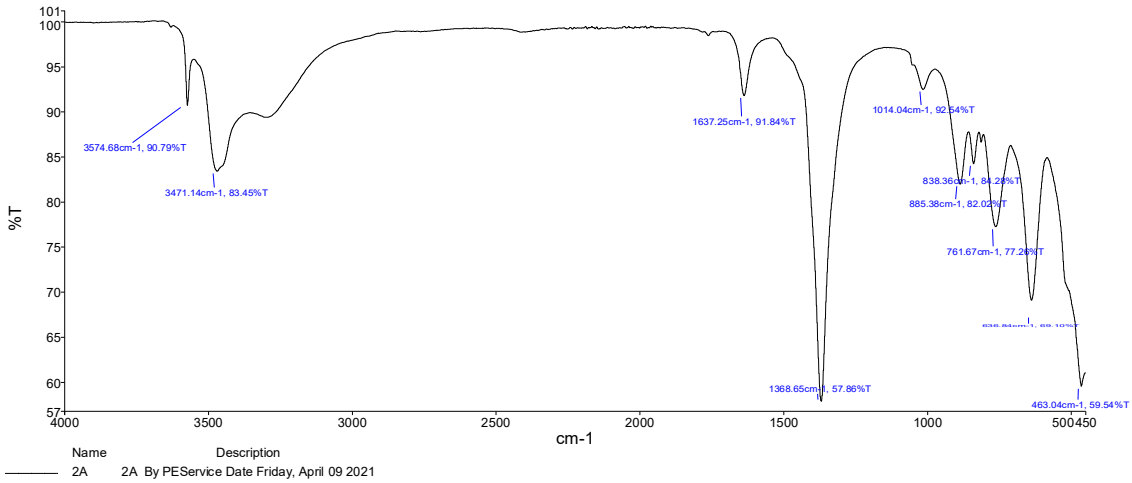

Figure 3. FTIR spectrum of chemically synthesized ZnO NPs

X-Ray Powder Diffraction

The powder X-ray diffraction (XRD) pattern of zinc oxide nanoparticles is depicted in figure 4. Various diffraction peaks are recognized, corresponding to characteristic peak of ZnO NPs. It has acquired hexagonal wurtzite structure as compared with the JCPDSICDD card: 75-0592 data. The diffraction peaks with 2 theta values of 31.674, 34.320, 36.110, 47.400, 56.412, 62.749, 67.797, and 76.82 correspond to the hkl (crystal plains) 100, 002, 101, 102, 110, 103, 112, and 202 mentioned in the JCPDS file No. 75-0592 [22]. The average size of ZnO NPs was evaluated using the "Debye-Scherrer's equation" [25].

$$d = k\lambda\,(\beta * cos\theta)$$

Where, 'K' is the Scherrer's constant (0.9), 'λ' is the x-ray wavelength (0.15406), 'θ' is the peak position, 'β' is peak width at half maximum, 'd' is the particle size of the crystal.
Using the above equation, the mean size of the sample was calculated as 29.97 nm as shown in Table 2.

Table no 2: The observed and calculated 2 values of XRD of Zinc Oxide nanoparticles

2 position	d-spacing	Hkl	()FWHM	Average particle size
31.674	2.8226	100	0.231	
34.320	2.6108	002	0.230	
36.110	2.4854	101	0.260	
47.400	1.9164	102	0.304	29.97 nm
56.412	1.62975	110	0.331	
62.749	1.4796	103	0.383	
67.797	1.3811	112	0.358	
76.82	1.2398	202	0.44	

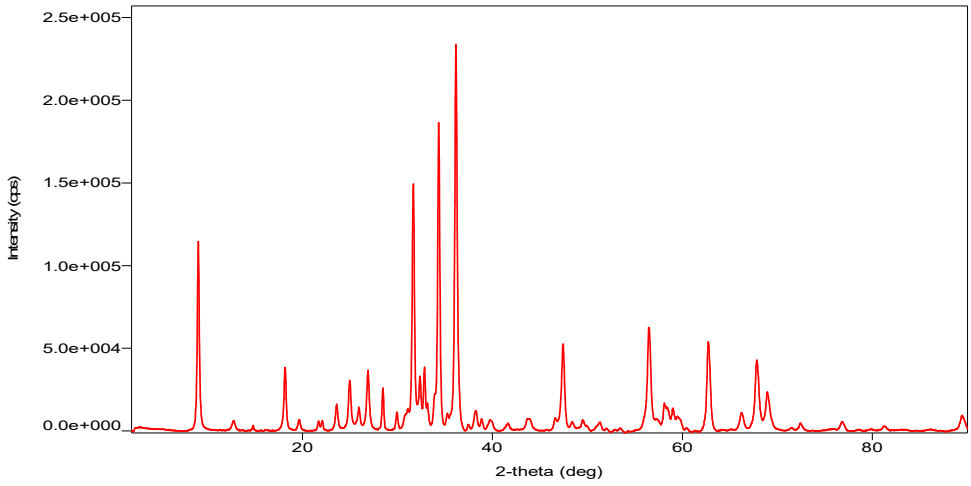

Figure 4: X-ray diffraction pattern of synthesized ZnO nanoparticles

TEM analysis

TEM was carried out to find the morphology and size of chemically synthesized ZnO nanoparticles. As depicted in Figure 5 chemically synthesized ZnO nanoparticles were in the size range of 30 nm.

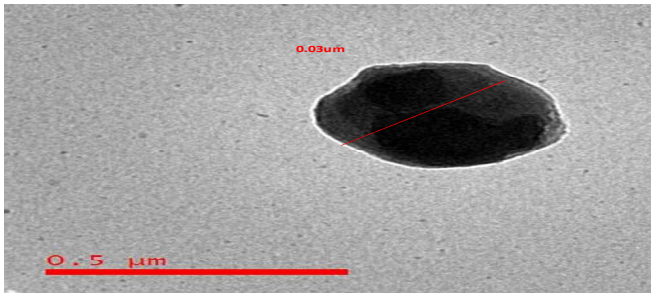

Figure 5. TEM Micrograph of ZnO nanoparticle

Determination of Growth Inhibition

ZnO nanoparticles with varying concentrations viz, 20, 40, 60, 80, and 100 mg/L) were prepared by suspending in BG11 broth. Growth inhibitory effect on microalgae was studied according to OECD201 guidelines [21]. Toxicity experiments were carried out by introducing initial cells (~ 10^4 cells/mL) in flask containing 50 ml of BG11 broth having ZnO NPs in different concentrations. The flasks were continuously agitated at 150 rpm in a rotatory shaker incubator at room temperature for 24, 72, and 120 hours. After the desired time interval, some solution was removed from flask using a pipette and growth inhibition test of algal cells was monitored by recording the absorbance at 680 nm using UV-vis spectrophotometer according to Lu, et al and cells were counted on Neubauer chamber under an optical microscope (EVOS, core cell imaging system transmitted light microscope) with 40X lens (figure 8). After counting the average number of cells in the top and bottom, and squares were observed for algal growth under different ZnO nanoparticles concentrations. The results showed that increasing concentrations of ZnO nanoparticles reduced growth of *Scenedesmus quadricauda* after 24 hours (figure 7).

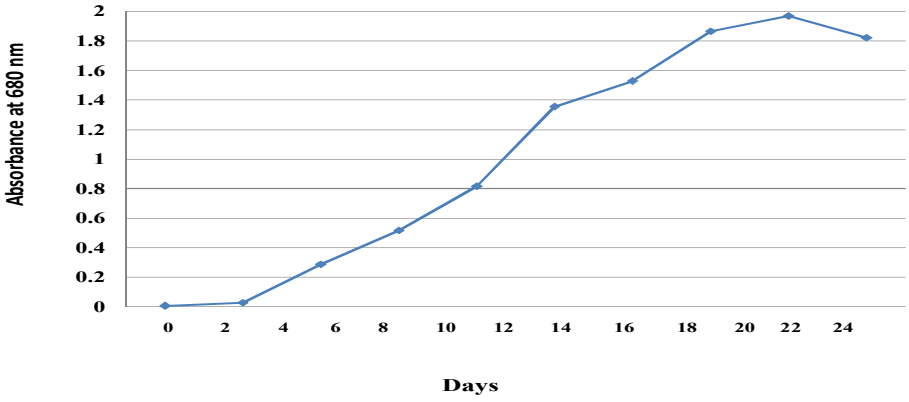

Figure 6. A. Growth curves of *Scenedesmus quadricauda* microalgae at 25±2℃ with 16:8 hours of light-dark cycle under Fluorescence light.

The number of cells in the culture medium reduced with rising concentration of nanoparticles; the decrease in cell numbers was significant in samples exposed to ZnO NPs of 100 mg/L concentration. This decline is significant in comparison to the control (figure 6B).

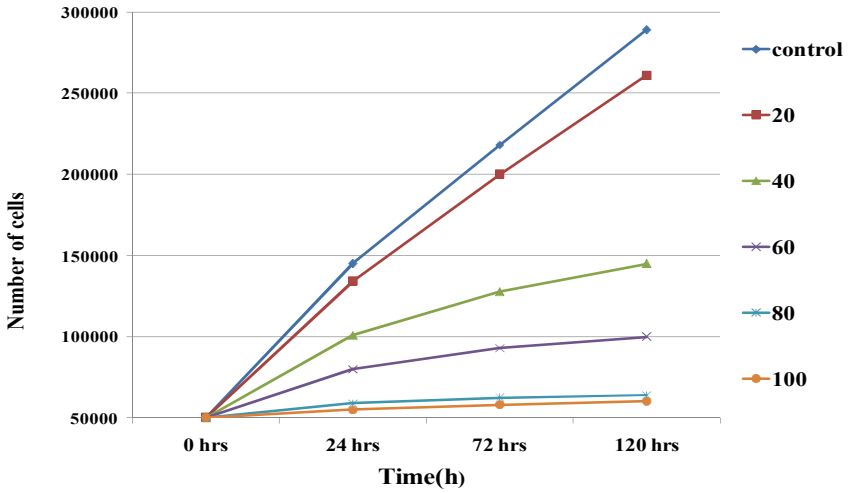

Figure 6.B. Average of *Scenedesmus* cells number at different time stage and different concentrations of ZnO nanoparticles (mg/L).

This reduction in cell density during 120 hrs indicates the severe toxicity of ZnO NPs and its effect on algal growth. The results confirmed that growth inhibitory effects and reduction in cell density appear after 24 hrs (figure 6 B) In contrast, the growth rate constantly increased in control samples throughout the experiment.

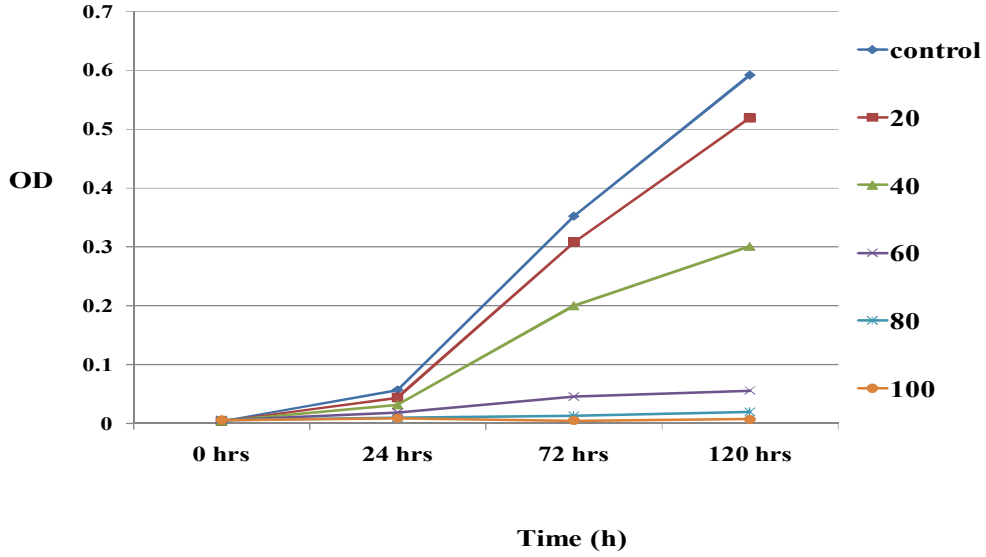

Figure 7: The number of *Scenedesmus quadricauda* cells against time at different concentration of ZnO nanoparticles

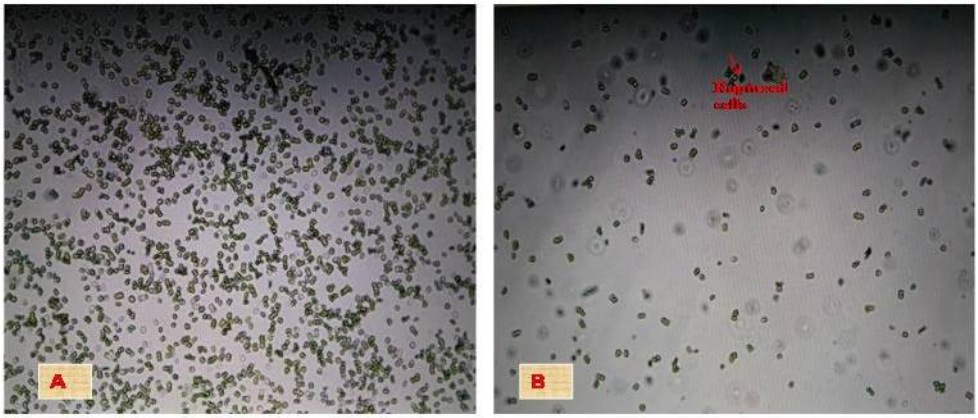

Figure 8. Microscopic image (magnification 40X) of *Scenedesmus quadricauda* cells (a) control cells (b) ZnO NP (40mg/L) treated cells after 48 hours.

DISCUSSION

The increasing exploitation of nanoparticles in everyday results into their release in the environment and aquatic ecosystem. Nanoparticles have been found in aquatic environment, still their quantification data is a meager [26]. In our study, ZnO NPs at lower concentrations (≤ 10 mg/L) did not reduce the microalgal growth, as compared to higher levels (≥ 40 mg/L). Cell number counting was used to estimate the biomass concentration (fig. 6B).

Many studies have concluded that the particle size has direct relation with toxicity [27, 28]. In general, nanoparticles with smaller size possess high surface area and attributed to more toxicity [29]. ZnO nanoparticles below 30 nm size can easily internalize into the cell and even in nucleus through the nuclear pore [30]. Andriana et al has investigated the long-term impacts of ZnO NPs on *Scenedesmus rubescens* at low concentration for a period of 28 days [31]. In our work, we have attempted to study the short-term toxicity of ZnO NPs to the *Scenedesmus quadricauda* at > 10 mg/L concentration.

ZnO nanoparticles with 30 nm size were successfully prepared by using zinc nitrate and KOH via chemical synthesis process. Synthesized ZnO NPs were characterized by various techniques like TEM, UV-vis, FTIR and XRD.

UV-vis spectroscopy revealed the formation of ZnO nanoparticles. The absorption maximum was observed at 352 nm, that aspect to the intrinsic bandgap of Zn-O absorption [32].

FTIR was done to study the functional groups on ZnO NPs (figure 3). The sharp peak positioned at 463 cm^{-1} is due to presence of Zn-O stretching bonds [24]. Therefore, FTIR results has indicated the high purity of chemically synthesized ZnO Nanoparticles.

XRD pattern revealed the hexagonal wurtzite crystalline structure, and the crystalline size was determined to be 29.97 nm as the diffraction peak seen to be narrower and intense. This result was also being compared with the given standard XRD pattern of ZnO (JCPDSICDD card: 75-0592) for confirmation purpose.

The morphological feature of synthesized ZnO NPs was characterized using transmission electron microscope (TEM) as shown in figure 5. Size of the chemically synthesized ZnO nanoparticles was approximately 30 nm.

Suspension of ZnO nanoparticles consist of ZnO particles and Zn^{2+} ions. Both these species contribute the toxicity. Accessible data on ZnO NPs toxicity propose several modes of action or mechanisms on microalgal cells. In most of the studies, it has been reported that upon release, Zn^{2+} becomes potent toxic for algae [33-35]. Previous reports have mentioned the toxic effects of ZnO Nanoparticles on oceanic as well as diverse fresh water algal species. Franklin et al has studied that ZnO NPs showed higher toxicity on algae at EC50 of 0.049 and 0.044 mg/L with 72 h exposure for total (both dissolved Zn^{2+}ions and ZnO particles in suspension), and ionic effects (dissolved Zn^{2+} from ZnO nanoparticles) as compared to bulk [36]. Another report by Ji et al. described the toxicity order of Zn^{2+} > ZnO NPs > bulk ZnO towards microalgae *Chlorella* sp. at ≤ 50 mg/L concentration. Once the nanoparticle concentration is above 50 mg/L, the ZnO NPs possess higher toxic effects than Zn^{2+}ions [37]. Wong and group reported that ZnO nanoparticles possess more toxicity than bulk ZnO particles at concentrations 2.36, and 2.97 mg/L on *S. costatum*. Some studies have reported the cell membrane damage causing growth reduction of algal cells are linear to the ZnO nanoparticles concentration and used dissolved Zn^{2+}ions [33, 38]. Released Zn^{2+} ions can easily enter algal cells by micropores present in their cell walls. Entered Zn^{2+} could damage

lysosomal, and mitochondria resulted in necrosis of microalgal cells [39]. The internalization and aggregate formation of ZnO NPs negotiated the morphology, growth, and the cell membrane integrity of microalgae *Chlorella* sp. [40]. They observed that the dissolved ZnO NPs is the probable cause for the cell damage mechanics.

In our study, *Scenedesmus quadricauda* culture was exposed to different concentrations starting from 20 mg/L to 100 mg/L at the interval of 20 of zinc oxide nanoparticles for 24, 72, 120 hours. The results revealed that the growth of *Scenedesmus quadricauda* microalgae was significantly impacted by the short-term exposure of 100 mg/L ZnO nanoparticles concentration treated cells after 24 hours. Growth inhibition test of algal cells was determined using the spectroscopic method and cell number counting was done by Neubauer hemocytometry.

CONCLUSION

It is now well understood that worldwide interest to exploit nanomaterials in industries will pose the environment and ecosystem to high threat. Results of this research show that the growth rate of *Scenedesmus quadricauda* cells inhibited with higher concentrations of ZnO NPs. The toxic effects of ZnO NPs compared with controls was observed after 120 hours at concentrations of 80 and 100 mg/L. In our study, we used cell viability and biomass as biomarkers to characterize the toxicity of ZnO nanoparticles on *Scenedesmus quadricauda*. Reduction in cell viability & algal biomass was strong evidenced the source of cellular toxicity and proven to be sensitive biomarkers for ZnO nanoparticles toxicity on *Scenedesmus quadricauda.* This study showed the potential of *Scenedesmus quadricauda* as the prospective bioindicators for ZnO nanoparticles toxicity with viable cell count and algal biomass as the biomarkers for toxicity testing.

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