Original Article

Toxic Effects of Copper Oxide Nanoparticles on *Chlorella vulgaris*

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Abstract

Aim: Exploitation of nano-based materials has increased the disposal of nanoparticles into the environment. Toxic nanomaterials pose their adverse effects and severely impact the human and environmental health. The aim of this study was to explore the toxic effects of chemically-synthesized copper oxide nanoparticles (CuO-NPs) on *Chlorella vulgaris* using an algal growth inhibition assay. **Materials and Methods:** Nanoparticles were synthesized using cupric sulfate and sodium hydroxide. Synthesized nanoparticles were characterized by Fourier transform infrared spectroscopy (FTIR), ultraviolet-visible (UV-vis) spectroscopy, X-ray diffraction (XRD), and transmission electron microscopy. *C. vulgaris* culture was exposed to various concentrations of CuO-NPs at intervals of 3, 6, 12, and 24 h. Growth inhibition assay of algal culture was estimated via the spectroscopic method and cell enumeration was done by Neubauer hemocytometry. **Results:** The average diameter of CuO-NPs was ~35.85 nm. UV-Vis spectroscopy was used to confirm the configuration of CuO-NPs. Therefore, FTIR results has indicated high purity of synthesized CuO-NPs. Size of the CuO-NPs was determined by XRD. Results showed that growth of *C. vulgaris* microalgae was notably affected by the exposure of 2 mg/L CuO-NPs concentration exposure for 6 h. **Conclusion:** The current work showed that CuO-NPs have significant toxic to *Chlorella* sp. Data analysis showed NPs have broad effects on growth of *C. vulgaris* and there is a correlation between nanoparticles concentration as well as their toxicity on the microalgae.

Keywords: Cell growth, *Chlorella vulgaris*, copper oxide nanoparticles toxicity, ecotoxicity, microalgae, nanoparticles

Introduction

Nanomaterials have received immense attention due to their versatile properties such as high reactivity and huge surface to volume ratio.[1,2] Nanoparticles, discharged from industrial, household, and medical products are categorized as novel biohazard for the environment.[3] Copper oxide nanoparticles (CuO-NPs) are among the most recurrently used NPs, largely employed in gas sensors, antifouling agents, glass, ceramic and superconducting materials.[1,4-6] CuO-NPs have exclusive antifouling and antimicrobial properties, making them broadly used in soaps and antifouling paints on the hulls of boats and other underwater surfaces.

Enormous applications of CuO-NPs have increased their discharge into the environment, causing unwanted effects on aquatic organisms, including animals, bacteria, and microalgae. Previous studies have reported that *in vitro* toxicity of CuO-NPs is more as compared to other metal oxide NPs.[1] Toxicity posed by CuO-NPs is mainly due to intracellular penetration and Solubilization.[7] Solubilization of CuO-NPs produce free Cu ions, which at high concentrations promote the fabrication of excessive reactive oxygen species that can oxidize biomolecules such as lipids, proteins, and nucleic acids, thereby causing oxidative stress. Penetration of NPs into cells causes membrane break and leaking of cellular contents.[8-11] Considering the alarm regarding toxicity of CuO-NPs on different organisms, various studies have been done to explore their destructive effects on diverse aquatic organisms.[12,13]

Algae are one of the major components of all marine organisms and have ability to accumulate various NPs and heavy metals.[3] Aruoja *et al*. have studied the toxic effects of CuO, ZnO and

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TiO_2 nanoparticles on *Pseudokirchneriella subcapitata* and confirmed the toxic effects of CuO-NPs in green alga *Chlamydomonas reinhardtii*. Moreover, it was revealed that CuO-NPs pose toxicity on *Chlorella vulgaris* and *Microcystis aeruginosa*.[6,14,15] The toxicity mechanism of nanoparticles in algae is less explored. Aruoja *et al*., have indicated that CuO-NPs pose more toxicity on algae than bulk counterparts because of release of Cu^{2+} ions.[6,16,17]

The green micro algal species, *C. vulgaris* (a nonmobile cell) is one of the most found freshwater microalgae. *Chlorella* species is a suitable for biological toxicity tests as preferred model organisms.[18] On the other hand, *C. vulgaris* is the dominant species of microalgae and the base of food chain in freshwater ecosystems which are frequently exposed to various contaminants.[19]

Previous studies have shown toxic effects of CuO-NPs on eukaryotic and prokaryotic algae for short term duration at relatively high concentrations. Wan *et al*. have reported the toxicity of CuO-NPs at higher concentration. Similarly, Zhang *et al*. 2013 have assessed the toxicity of CuO-NPs on *Chlorella pyrenoidosa* at higher concentration at longer duration. Another study from Zhao *et al*. have investigated the CuO-NPs toxicity on *Chlorella pyrenoidosa* with 72 h and exposure time.[9] CuO-NPs toxicity studies have also been conducted in the presence of organic matters. Fathi *et al*. have investigated the CuO-NPs toxicity along with humic acid on *Chlorella* with exposure duration up to 96 h.

Toxicity of CuO-NPs alone on microalgae *C. vulgaris* at lower concentration at short exposure duration have rarely been explored. Presence of organic matter affects the toxicity of CuO-NPs and accurate data cannot be generated. Apart from this, the establishment of microalgae as an indicator of aquatic toxicity, particularly nanotoxicity is a meager and very less data is available for the short-term toxicity.

In the current study, we aimed to investigate the short-term toxicity of CuO-NPs alone on freshwater *C. vulgaris* at lower concentration through assays of growth parameters.

Materials and Methods

Copper oxide nanoparticles synthesis

Copper Oxide NPs were synthesized by co-precipitation method with 0.1M of cupric sulfate and 2M of sodium hydroxide (NaOH) as precursor. The NaOH solution was added drop wise into cupric sulfate solution at room temperature under continuous stirring on a magnetic stirrer for 3 h [Figure 1]. This resulted in the formation of a white precipitate. The white precipitate was then washed 4–5 times with ethanol to remove the impurities. Finally, the precipitate was dried at 80°C for 24 h. All the chemicals were purchased from HiMedia Laboratories Pvt. Ltd., India.

Characterization of synthesized copper oxide nanoparticles

Ultraviolet Visible absorption spectrum of Copper Oxide NPs was recorded in the wavelength range of 200–600 nm

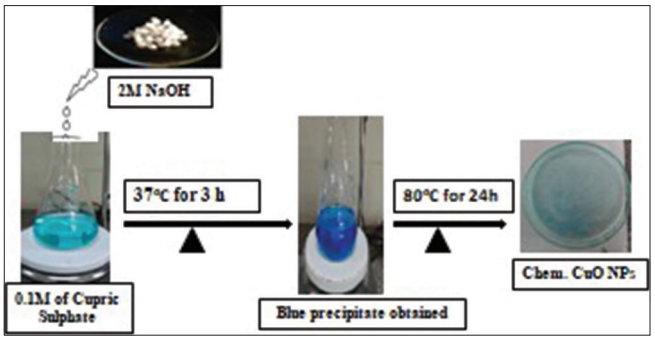

Figure 1: Schematic representation of chemically synthesized CuO nanoparticles. CuO: Copper oxide

to confirm the presence of nanoparticles. The occurrence of functional groups of the CuO-NPs was determined by Fourier transform infrared spectroscopy (FTIR) (Perkin Elmer, Spectrum Two) in the selection of 450–4000 cm^{-1}. X-ray diffraction (XRD) was used for crystal structure characterization, size and phase identification. The morphology and size of CuO-NPs were investigated by the transmission electron microscopy (TEM) (JEOL, JEM-1230) at an accelerating voltage potential of 120 KV.

Isolation and cultivation of *Chlorella vulgaris*

The algal sample was collected from Betwa river, Orchha, Madhya Pradesh, India (25.35°N, 78.64°E). Collected algal sample was transferred to sterilized fresh BG-11 broth media for enrichment.[16] The pH of the medium was adjusted to 7.4. Isolation and purification were done to obtain the pure strain of *Chlorella* sp. was used as the test organism in this study.[21] After several sets of purification experiments pure cultures were confirmed by observation under the light microscope at ×40 magnification. All pure cultures were maintained in BG-11 broth media ($NaNO_3$ 1.5 g/dm^3; K_2HPO_4 0.04 g/dm^3; $MgSO_4.7H_2O$ 75 mg/dm^3; $CaCl_2.2H_2O$ 36 mg/dm^3; $C_6H_8O_7.1H_2O$ 6 mg/dm^3; $C_{12}H_{22}FeN_3O_{14}$ 6 mg/dm^3; Na_2CO_3 20 mg/dm^3; Na-EDTA 1 mg/dm^3' H_3BO_3 2.86 mg/dm^3; $MnCl_2.4H_2O$ 1.81 mg/dm^3; $ZnSO_4.7H_2O$ 0.22 mg/dm^3; $Na_2MoO_4.2H_2O$ 0.39 mg/dm^3; $CuSO_4.5H_2O$ 79 ug/dm^3; $Co(NO_3)$ $2.6H_2O$ 49.4 ug/dm^3)[20] as well as on agar plates at 27.0 ± 2.0°C under Fluorescence light with a 16:8 h light-dark cycle.[22] After 15–20 days pure culture was transferred to fresh BG-11 broth to maintain their exponential growth phase condition. All the experiments were performed under the unchanged culture conditions in triplicates.

Determination of algal cell growth

After 3, 6, 12, and 24 h, cells were calculated using a Neubauer hemocytometer chamber and microscope lens ×40.[23,24] After counting the algae cells, the average number of cells was counted then the specific growth rate was calculated via the following equation:

Where μ is the specific growth rate, and N1 and N2 are the cell number at time 1 (t_1) and time 2 (t_2), respectively. Absorbances of cell biomass were recorded using a

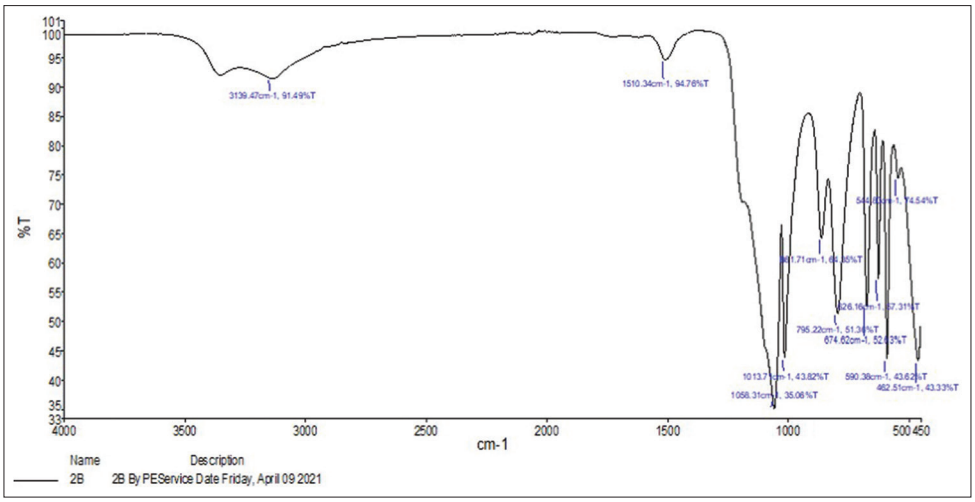

Figure 2: FTIR spectrum of chemically synthesized CuO NPs

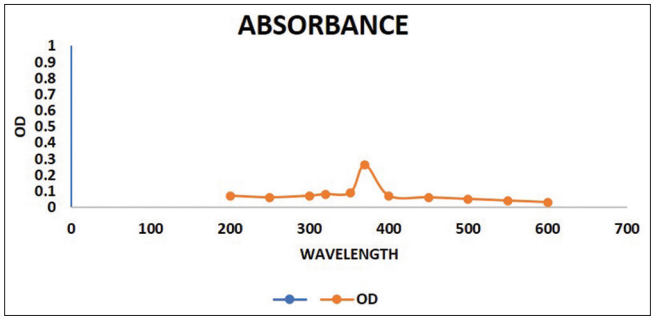

Figure 3: Optical absorption spectrum of CuO nanoparticles solution

ultraviolet-visible (UV-vis) spectrophotometer (Medox, MX-1287-011) at wavelength 720 nm. BG-11 culture media was used as blank and CuO-NPs suspension without algal cells was used as a control.[25]

Toxicity testing of copper oxide nanoparticles on *Chlorella vulgaris*

The strain of *C. vulgaris* was achieved from culture stock during the exponential phase of growth. Then the cultures were mixed homogeneously, and the cell digit was performed using a neubauer chamber.[24] For treatments, the BG-11 media containing ~4 $\times 10^4$ cell/ml of microalgae were incubated for 4 days and afterward the algal cultures were exposed to different concentrations of CuO-NPs (Viz.,1, 2, 4, 5, and 10 mg/L) for 3, 6, 12, and 24 h. The control and samples treated with CuO-NPs were aerated and stirred by a shaker. Each treatment and the control were exposed at room temperature under Fluorescence light with a 16:8 h light-dark cycle.[34,37] After the desired time interval, some solution was removed from flask using a pipette and growth inhibition test of algal cells was monitored by quantify the absorbance at 720 nm using spectrophotometer according to Lu *et al.*[42] (2017) and cells were counted on Neubauer chamber under an optical microscope (EVOS, core cell imaging system transmitted light microscope) with ×40 lens. After counting the average number of cells in the top and bottom, and squares were observed for algal growth under different CuO-NP concentrations. The microalgae toxicity analysis was performed for nanoparticles according to the modified guideline of Organization for Economic Cooperation and Development.[26,27] In toxicity testing, we used cell expansion and biomass as biomarkers to characterize the toxicity of CuO-NPs on *C. vulgaris* cells.

Statistical analysis

All the assays were performed in triplicates. All the data presented are the mean of generated data.

Ethical clearance

Ethical approval for this study (Ethical Committee, AIB, AUMP) was provided by the Ethical Committee of Amity Institute of Biotechnology, Amity University Madhya Pradesh, Gwalior on 13 January 2022.

Results

Fourier transform infrared spectroscopy studies

FTIR spectroscopy is valuable in measuring the absorption of IR radiations by a sample, and the outcome were shown by means of a wavelength. The assessment of the IR spectrum includes the relationship of the vibration bands (absorption bands) and the element compounds in the sample.[28] The FTIR band of the sample is confirmed using a direct method at room temperature in the range of 4000-450 cm^{-1} [Figure 2]. The functional groups responsible for identifying the possible stabilizing and reducing biomolecules using FTIR analysis are depicted. The absorption bands of CuO-NPs show at 3139.47, 1510.34, 1058.31, 1013.71, 861.71, 795.22, 674.62, 590.38, 544.83, and 462.51 cm^{-1} in Table 1. FTIR spectra at 3139.47 cm^{-1} shown a strong peak in the higher energy region due to O-H stretching, indicates the presence of alcohols and carboxylic group.[29] The N-O stretching shows the presence of nitro compound, and the bending vibration bond arises at 1510.34 cm^{-1}.[30] The FTIR spectra exhibited the strong, medium peak region at 1058.31 cm^{-1}, representing C-O stretching amine, vinyl ether, primary alcohol sulfoxide, and alkyl aryl ether compound. The C-F

Table 1: Assignments of Fourier transform infrared spectroscopy bands for copper oxide nanopowder

Frequency (cm^{-1}) CuO NPs	Bond type	Bond origin	Functional group
3139.47	Strong, weak	O-H stretching	Alcohol (intramolecular bonded), carboxylic acid (usually centered)
1510.34	Strong	N-O stretching	Nitro compound
1058.31	Strong, medium	C-C stretching, C-N stretching, S=O stretching	Alkyl aryl ether, amine, vinyl ether, primary alcohol, sulfoxide
1013.71	Strong, medium	C-F stretching, C-O stretching, C-N stretching	Fluoroc ompound, alkyl aryl ether, amine, vinyl ether
861.71	Strong	C-Cl stretching,	Halo compound
795.22	Medium	C=C bending	Alkene
674.62	Strong	C-Br stretching	Halo compound
590.38	Strong	Cu-O stretching	-
544.83	Strong	Cu-O stretching	-
462.51	Strong	Cu-O stretching	-

CuO NPs: Copper oxide nanoparticles

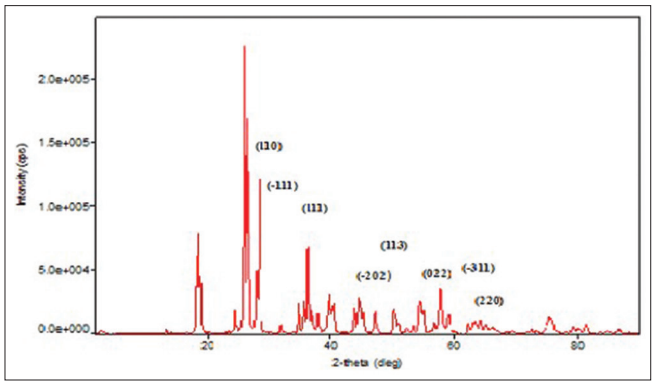

Figure 4: X-ray diffraction patterns of CuO nanoparticles

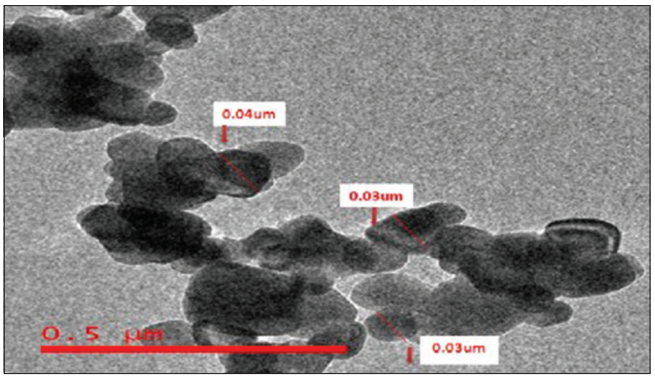

Figure 5: TEM Micrograph of CuO nanoparticles

stretching shows the presence of fluoro compound, the vibration bond arises at 1013.71 cm^{-1}. Strong stretching vibrational mode of halo compound was shown at 861.71 cm^{-1}. FTIR spectra exhibited the strong, medium peak region at 795.22 cm^{-1}, representing C = C stretching alkene. The C-Br stretching shows the presence of halo and Alkyl and Aryl Halides compound, bond arises at 674.62 cm^{-1}. CuO-NPs exhibited vibrational modes at 462.51, 544.83, and 590.38 cm^{-1}were assigned for Cu-O stretching vibration.[31]

Ultraviolet-visible spectra studies

The optical properties of synthesized copper oxide NPs have been studied by UV-Visible absorbance spectrum, which is shown in Figure 3. UV-Visible spectroscopy is commonly used technique to study the optical properties of the nanoparticles. The analysis was done within wavelength of 200–600 nm. A broad absorption peak was observed around 370 nm that confirmed the fabrication of CuO-NPs.[30,31]

X-ray diffraction studies

Power XRD was a fast analytical technique principally worn for phase identification of a crystallite material and can give information on unit cell dimensions. XRD pattern of CuO NPs are in monoclinic structre is shown in Figure 4. Several diffraction peaks are clearly recognized to the characteristic peak of CuO-NPs. It can be well indexed to BCC structure by assessment with the data from JCPDSICDD card: 01-089-5897. The diffraction peaks values of 31.973, 35.626, 37.963, 47.400, 53.440, 58.927, 62.269, and 66.24 correspond to the crystal plan values (miller indices) 110,-111, 111, 202, 113, 022,-311 and 220 reported in the JCPDSICDD card: 01-089-5897 and JCPDSICDD card: 05-0661.[32]

Particle size calculation

The average crystal size of CuO-NPs was determined using the "Debye-Scherrer's equation."[33]

$$d = k\lambda\ (\beta \times cos\theta)$$

Where,

K-is the Scherrer's constant (0.9)

λ- is the x-ray wavelength (0.15406)

θ-is the peak position

β-is the peak width at half maximum (FWHM)

d -is the particle size of the crystal.

The average particle size of CuO nanoparticles was found to be 35.85 nm, as calculated using above equation [Table 2].

Transmission electron microscopy studies

Transmission electron micrograph studies were carried out to locate the size and shape of the synthesized CuO-NPs. Size

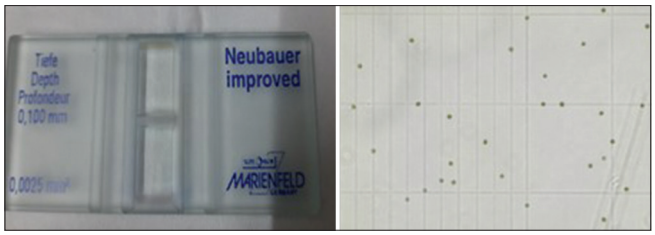

Figure 6: A hemocytometer (left) and the Chlorella vulgaris image at 40X microscopic magnification (right)

of the synthesize CuO-NPs was found to be approximately 30–40 nm. This further confirms the size determined using XRD pattern. Figure 5 shows the TEM image of the chemically synthesized CuO-NPs.

Algal growth inhibition and copper oxide nanoparticles treatment assays

Significant reduction in cell number was observed as compared to control under optical microscope (EVOS, core cell imaging system transmitted light microscope) with ×40 lens [Figure 6]. The cell growth in the culture broth was significantly decreased with a rising concentration of nanoparticles. In cytotoxicity assay, 2 mg/L of nanoparticles showed reduction in cell numbers after 6 h when compared to the control. Highest concentration (10 mg/L) of NPs observed nearly 95% toxicity after 6 h [Figure 7].

Growth of *Chlorella* sp. was inhibited at 2 mg/L CuO-NPs concentration. The reduction in cell number during 6 h exposure of CuO-NPs indicates the toxicity of nanoparticles [Figure 8a]. The results established that growth inhibitory effects and reduction in cell biomass appear after 6 h [Figure 8b]. In contrast, the growth rate constantly increased in control samples throughout the experiment. Similarly, reduction in the optical density of *C. vulgaris* with increasing concentrations of CuO-NPs indicate reduced growth of microalgae after 6 h. Reduction in cell expansion and algal biomass was significant, as evidenced by the source of cellular toxicity and proven to be sensitive biomarkers for CuO-NPs toxicity on *C. vulgaris*.

Discussion

CuO-NPs with 35.85 nm size were successfully prepared using cupric sulfate and NaOH via chemical synthesis process. Synthesized CuO-NPs were confirmed by various characterization techniques like TEM, UV-Vis, FTIR, and XRD.

UV-Vis spectroscopy was used to confirm the configuration of CuO-NPs. The absorption peak was observed at 370 nm, which characteristic to the intrinsic band-gap of Cu-O absorption. FTIR was carrying out to determine the functional groups of synthesized CuO-NPs. The sharp peak at 590.38, 544.83, and 462.51 cm − 1 are recognized to the presence of Cu-O stretching bonds.[31] So, FTIR results has indicated that synthesized CuO-NPs are high purified. XRD pattern of synthesized CuO-NPs is shown in Figure 5, based on

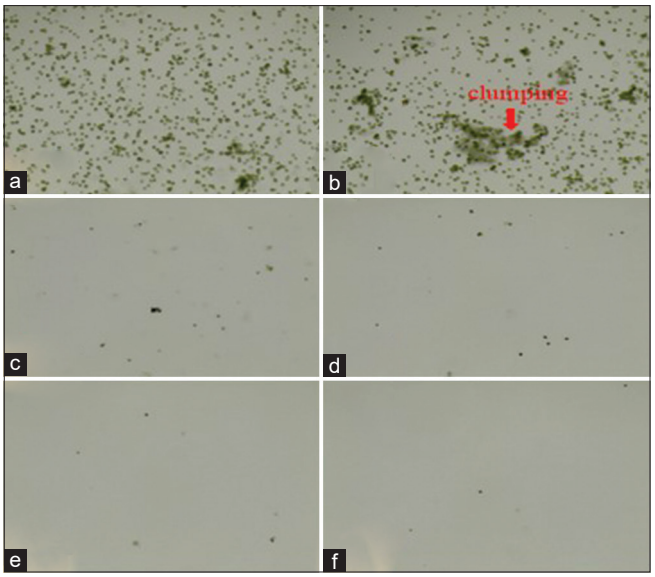

Figure 7: Morphological features (magnification 40X) of Chlorella vulgaris exposed to CuO-NPs (a) control cells (b) CuO-NP (1 mg/L), (c) CuO-NP (2 mg/L), (d) CuO-NP (4 mg/L), (e) CuO-NP (5 mg/L), (f) CuO-NP (10 mg/L), treated cells after 12 hours. CuO-NPs: Copper oxide nanoparticles

Table 2: The observed and calculated 2θ values of X-ray diffraction of copper oxide nanoparticles

2θ position	d-spacing	Hkl	β-FWHM	Average particle size (nm)
31.973	2.7969	110	0.213	35.85
35.626	2.5180	−111	0.232	
37.963	2.3682	111	0.20	
47.400	1.9241	202	0.230	
53.440	1.7132	113	0.233	
58.927	1.56605	022	0.41	
62.269	1.4898	−311	0.23	
66.24	1.4097	220	0.85	

FWHM: Full width at half maximum

the XRD blueprint, synthesized CuO-NPs has monoclinic structure, and the crystalline size was 35.85 nm. This result was also being compared with the given standard XRD pattern of CuO (JCPDSICDD card: 01-089-5897 and JCPDSICDD card: 05-0661) for confirmation.[32] The morphological feature of manufactured CuO-NPs was characterized using a transmission electron microscope. Size of the synthesized CuO-NPs was found to be approximately 30–40 nm.

In this study, CuO-NPs significantly reduced the cell growth [Figure 8b]. CuO-NPs at low concentrations (≥1 mg/L) did not influence the biomass of microalgae, in contrast to higher levels (≥2 mg/L) where microalgae biomass was declined. As a result of toxic effects of CuO-NPs, the lowest rate of growth compared with controls was observed after 6 h at concentration of 2 mg/L. Wang *et al*. found in their studies, 10 mg/L CuO-NPs with 15–50 nm particle size shows high suppressed cell density at 5 days.[35,36] The toxic effects of NPs have been related to the

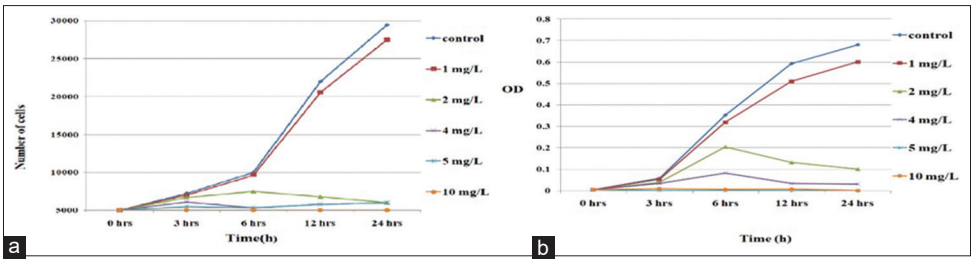

Figure 8: (a) Average of Chlorella cells number at different time stage and different concentrations of CuO nanoparticles (mg/L). CuO: Copper oxide. (b) The Biomass of Chlorella vulgaris cells against time at different concentration of CuO nanoparticles. CuO: Copper oxide

surface properties and size of NPs, the release of metal ions or the attachment of nanoparticles to the cell surface of the organisms[38,39] and ROS production, Oxidative stress plays the major part in the toxicity of various nanoparticles.[40,41] The toxicity of CuO-NPs in different organism is due to the high absorption rate of CuO-NPs and intracellular communications in various organisms.[8,42] In terms of morphology, nontreated Chlorella cells were spherical to ovoid in shape, size, and pigmentation, without clumping or agglomeration [Figure 7]. When cell cultures exposed to copper oxide NPs, clumping of (2 mg/L) nanoparticles with Chlorella cells were seen. Some researchers attribute the toxicity of NPs to the release of metal ions.[8] Aruoja et al. has reported that Cu^{2+} ion contributes to the toxicity of CuO NPs to Pseudokirchneriella subcapitata.[6] Another research indicates that toxicity in the fronds of Landoltia punctata was four times higher when exposed to Cu2 + ion of CuO-NPs as compared to same dose of dissolved copper.[41] Wenqiu *et al*.[41] explained the toxic effect of nano-CuO testes on two marine algae *Skeletonema costatum* and *Nitzschia closterium.* Results indicate that CuO-NPs toxicity on *S. costatum* and *N. costatum* were 0.663 and 2.455 mg/L respectively at 96 hour exposure.

As such, in our study, we attempt to investigate the toxicity of copper oxide NPs to the *C. vulgaris* at ≥1 mg/L concentration.

In toxicity assay, *C. vulgaris* culture was exposed to different concentrations (1, 2, 4, 5, and 10 mg/L) of CuO-NPs at intervals of 3, 6, 12, and 24 h. The results explained that the growth of *C. vulgaris* microalgae was drastically affected by the short-range exposure of 2 mg/L CuO-NPs concentration treated cells after 6 h. Growth inhibition study of *Chlorella* cells biomass was determined using the spectroscopic method and cell number counting was done by Neubauer hemocytometry.

Our study reports the toxic effects of CuO-NPs alone on microalgae *C. vulgaris* at relatively lower concentration with exposure duration up to 24 h. The study is not affected by the presence of background organic matter. Therefore, the accurate toxicity data of CuO-NPs have been generated. Apart from this, the present study opens a new avenue for the establishment of microalgae as an indicator of aquatic toxicity, particularly nanotoxicity. The present study has not been conducted with copper salts in bulk. Therefore, we are not comparing the results with bulk.

Conclusion

The escalating exploitation of nanoparticles in many industries will expose the environment to the high threat. The current study showed that CuO-NPs have significant toxic to Chlorella sp. Data analysis showed NPs have broad effects on growth of *C. vulgaris* and there is a direct association between the concentration of nanoparticles and their toxicity on the microalgae. As a result of toxic effects of CuO-NPs, the lowest rate of growth evaluate with controls was observed after 6 h at concentrations of 2, 4, 5, and 10 mg/L. This study showed the potential of *C. vulgaris* as the prospective bioindicators for CuO-NPs toxicity with live cell count and algal biomass as the biomarkers for toxicity assays. Hence, discharge of CuO-NPs in the environment reduces the growth and physiological characteristic of *C. vulgaris* cell and other aquatic organisms. Therefore, deterioration of growth and other parameters of *C. vulgaris* can be established as indication of CuO-NPs toxicity.

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Conflicts of interest

There are no conflicts of interest.

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