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A facile approach towards copper oxide nanoparticles synthesis using *Spirulina platensis* and assessment of its biological activities

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Abstract. There is a budding need to develop a method for environmentally benign metal nanoparticle synthesis, that do not use toxic chemicals in the synthesis protocols to avoid adverse effects in medical applications. The present investigation dealt with the synthesis of copper oxide (CuO) nanoparticles from blue green alga, *Spirulina platensis*. The algal extract consisting of phytochemicals was used as the reducing agent and copper sulphate as the substrate. Synthesised nanoparticles were characterized by UV-Vis spectrophotometry, FT-IR spectroscopy, XRD and SEM. Antibacterial and anticancer activities were assessed for the CuO nanoparticles. The results indicated that the formed CuO nanoparticles were observed to be nanosheets. FT-IR spectral analysis elucidated the occurrence of biomolecules required for the reduction of copper oxide ions. The synthesized nanoparticles were found to be effective at the concentration of 1 mg/mL against *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Serratia marcescens*. The cytotoxicity activity of CuO nanoparticle was evaluated by MTT Assay against colon cancer cell lines and confirmed that CuO nanoparticle at a concentration of 125 µg/mL had cytotoxic activity. In conclusion, the CuO nanoparticles were synthesized at a low energy supply, in an ecologically safe mode which could be utilized for pharmacological applications and various biotechnological studies.

Keywords: *Spirulina platensis*; Copper nanoparticles; Well diffusion; Colon cancer; MTT assay.

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Introduction

Nanoparticles are defined as particles having more than one dimension measuring 100 nm or less. The physical properties of nanoparticles differ significantly from their bulk materials because of their large surface atoms, large surface energy, spatial confinement and reduced imperfections (Rai and Posten, 2013). Among various transition metal oxides, copper oxide has attracted greater attention due to its fascinating properties such as electrocatalytic activity (Yang et al., 2010), antimicrobial activity (Azam et al. 2012), anticancer activity (Sankar et al., 2014). One of the first CuO-based products was put forth by a private company, Cupron. This firm incorporates micron-sized CuO particles into polymers which are further made into wound dressings and these dressings effectively prevent infection and increase the rate of wound healing, compared to standard treatments (Gabbay et al., 2006). Despite great potential of CuO nanoparticles only few studies have explored the nanoprepation of CuO.

Mode of synthesis is an essential part in the preparation of CuO nanoparticles, since it may control the size and morphology of nanoparticles which in turn affect the properties of nanoparticles. The conventional methods used for the synthesis of CuO nanoparticles are electrochemical, sonochemical, sol-gel, microemulsion, precipitation and microwave irradiation (Grigore et al., 2016). Though these methods help in creating nanocopper particles with defined size and shape, the chemicals used possess hazardous effect on environment. Therefore biological methods of nanoparticle synthesis using microorganisms, plant or plant extracts or enzymes have been suggested as possible eco friendly alternatives to chemical and physical methods.

Algae are a diverse group of plant kingdom that is being explored for application in nanotechnology. *Spirulina platensis* also known as *Arthrospira platensis* is a spiral unicellular prokaryote usually found in fresh water (Hetta et al, 2014). Over the past decade *Spirulina platensis* has been used for synthesis of silver nanoparticles and gold nanoparticles (Verma et al., 2014; Suganya et al., 2015). Various phytochemicals, peptides, polysaccharides and algal pigments in cyanobacteria and microalgae play an essential role in the preparation of nanoparticle (Patel et al., 2015).

Nanoparticles have a potential to be good antibacterial agents. Nanoparticles are able to attach to the membrane of bacteria by electrostatic interaction and disrupt the integrity of the bacterial membrane. Nanotoxicity is generally triggered by the induction of oxidative stress by the formation of reactive oxygen species following the administration of Nanoparticles. The mechanisms of different Nanoparticle toxicity depend on its own physical and chemical properties such as composition, surface modification, intrinsic properties, and the bacterial species (Hajipour et al., 2012).

Nanoparticles for anti-cancer drug delivery had reached the first clinical trial in the mid-1980s, and the first nanoparticles (e.g. liposomal with encapsulated doxorubicin) had entered the pharmaceutical market in 1995 (Nguyen, 2012). Other than targeted

drug delivery, nanoparticles itself has the potential to kill cancer cells. For example, Gold nanoparticles is known to induce apoptotic cell death in many tissues and has been shown to increase local control and overall survival in combination with radiotherapy and chemotherapy in randomised clinical trials by a process called hypothermia (Jain et al., 2012). Copper nanoparticles had also been utilised for the same purpose in various studies. CuO nanoparticles had been effective against human cervical carcinoma cells (Nagajyothi et al., 2017), human breast cancer cell line (Jeronsia et al., 2016), chronic myeloid leukemia (Shafagh et al., 2015).

In this study, an attempt was carried out to check the ability of *Spirulina platensis* to produce CuO nanoparticles and to examine the antibacterial and anticancer properties of biosynthesized nanoparticles. This is the first study describing the preparation of nanomaterial of copper oxide from Spirulina species.

Experimental methods

Culture collection and culturing

A volume of 250 g of *Spirulina platensis* culture was obtained from Oferr Nallayan Research Centre, Chennai, Tamilnadu, India, in a sterile flask, transferred to laboratory under aseptic conditions and stored at 4 °C. The culture was cultivated and maintained in CFTRI medium composed of, in g/L, $NaHCO_3$ 4.5; K_2HPO_4 0.05; $NaNO_3$ 1.5; K_2SO_4 1.0; NaCl 1.0; $MgSO_4$ 0.2.

$CaCl_2$ 0.04 and $FeSO_4.7H_2O$ 0.015 . The growth of alga was maintained at 28 °C with 4000 lux light intensity for 30 days. After 30 days of growth biomass was harvested, it was dried in hot air oven at 45 °C for 24 h and homogenized. The fine powder was stored for further studies at 18 °C.

Preparation of algal extract

Hot aqueous extract was prepared by taking 1 g of powdered biomass in 50 mL of water and heated for 1 h at 60 °C. The mixture was then centrifuged at 5,000 rpm for 10 min. The supernatant was collected and stored for the synthesis.

Phytochemical analyses

Phytochemical analyses were performed in order to identify the best solvent which could extract maximum of the phytochemicals that act as reducing and capping agents.

Test for alkaloids

Meyer's test: A volume of 0.5 mL of the extract was mixed with few drops of Meyer's reagent (mercuric chloride and potassium iodide in water) and checked for cream color precipitation Wagner's test: A volume of 0.5 mL of extract was taken and Wagner's reagent (iodine and potassium iodide in water) was added in drops and checked for reddish brown coloration/precipitation.

Test for proteins

Bradford's test: A volume of 0.5 mL extract was added with few drops of Bradford's reagent and checked for blue coloration

Test for carbohydrates

To a volume of 0.5 mL of the extract, few drops of Fehling's reagent was added and checked for its red color precipitate.

Test for saponins

Froth test: A volume of 0.5 mL of extract was diluted with twice the volume of distilled water and checked for froth.

Test for phenols

Ferric chloride test: 3-5 drops of 5% ferric chloride solution was added to 0.5 mL of extract and checked for red, blue, green or purple coloration

Test for cholesterol

To a volume of 1 mL of extract, 2 mL of acetic acid was added followed by few drops of conc. H_2SO_4 and checked for bluish green coloration.

Synthesis of Copper oxide nanoparticles

A volume of 5 mL aqueous extract of algal biomass was added to 5 mL of 7mM cupric sulphate solution. The colour change from blue to green indicated the formation of copper oxide nanoparticles which was observed as precipitate. The precipitate was centrifuged at 10,000 rpm for 10 min. The pellet was collected and dried in hot air oven at 60 °C overnight. The dried pellet was heated in muffle furnace at 700 °C for 7 h. The powdered nanoparticles were stored in capped vials for further analyses.

Characterization of nanoparticles

The preliminary detection of copper nanoparticle was observed by visual colour change. Copper oxide nanoparticles were characterized by UV-Vis spectroscopy, SEM, XRD and FT-IR. Preliminary confirmation was carried out using UV-Visible spectrophotometer (Model: G10S, Thermo Fisher Scientific Ltd., USA). The sample was scanned at a range of wavelengths between 200 nm and 700 nm for checking the maximum absorbance. Functional groups and chemical composition were analysed by Fourier Transform Infrared spectroscopy. The spectrum was obtained by recording the wavelength of 400-4000/cm. The shape and morphology of CuO nanoparticles was examined using Scanning Electron Microscopy. The particle size was determined by X Ray Diffraction studies using an X-Ray diffractometer (XPert Pro). The size was calculated from the spectrum using the Debye-Schrerr's equation:

$$D = (k\lambda) / (\beta\cos\theta)$$

where D is the particle size of the nanoparticle, k is the shape factor, λ is the wavelength, θ is the Bragg's angle and β is the line width (FWHM) in radians.

Antimicrobial activity of CuO nanoparticles

The antimicrobial activity of CuO nanoparticles were evaluated against laboratory isolates *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Serratia marcescens* by agar well diffusion method. Approximately 10^6 colony forming units (CFU) of the microorganisms were inoculated on Muller Hinton Agar plate, and then different concentration of CuO nanoparticles (200, 400, 600 and 1,000 µg/mL) were added to the well present in the Muller Hinton agar plate. Chloramphenicol (1 mg/mL) was used as control. All the plates were incubated at 3 °C overnight. After incubation, the plates were observed for the zone of inhibition and IC_{50} values were calculated for four organisms.

Anticancer activity of CuO nanoparticles

Anticancer activity of CuO nanoparticles were evaluated against Colon cancer cell line by MTT Assay. Cells at 90% confluence were exposed to nanoparticles of different concentration (25, 50, 75, 100, and 125 µg/mL) for 48 h and 72 h. After exposure, 20 µL of MTT solution (5 mg MTT in 1 mL PBS) was added to each well and incubated for 4 h under 5% CO2 atmosphere at 37 °C. Then the solution was removed, the microtitre plate was washed with PBS thrice and 200 µL of DMSO was added to each well. The plate was read for formazan product formation using a microtitre plate reader at 620 nm and the results were expressed as absorbance values of treated and untreated (control) cells.

Results and discussion

Spirulina platensis and CuO nanoparticle synthesis

Spirulina platensis was cultured in CFTRI medium and the biomass was harvested after 30 days. The biomass was collected from the medium by centrifugation and placed in hot air oven to obtain dried biomass (Figure 1A and 1B). The dried biomass is then homogenised to fine powder. Dried biomass yield was found to be 1 g/L. The phytochemicals identified in the extract of the culture were alkaloids, carbohydrates, glycosides, phenolics, proteins, saponins, cholesterol and flavonoids. Using hot aqueous extract of the biomass as reducing agent and copper sulphate as the substrate, copper oxide nanoparticles were produced and colour change from brown to green was observed visually with green precipitate (Figure 1C). Optimisation of concentration of copper sulphate showed that at 7 mM maximum yield of 0.069 g of nanoparticle/dry biomass was achieved (Figure 1D). The synthesis might be aided by the phytochemicals present in the extract which might apparently act as reducing agents and stabilizing agents. Extracellular polysaccharide and c-phycocyanin, a blue accessory pigment from the cyanobacterium, were tested for their ability to produce nanoparticles (Patel et al., 2014).

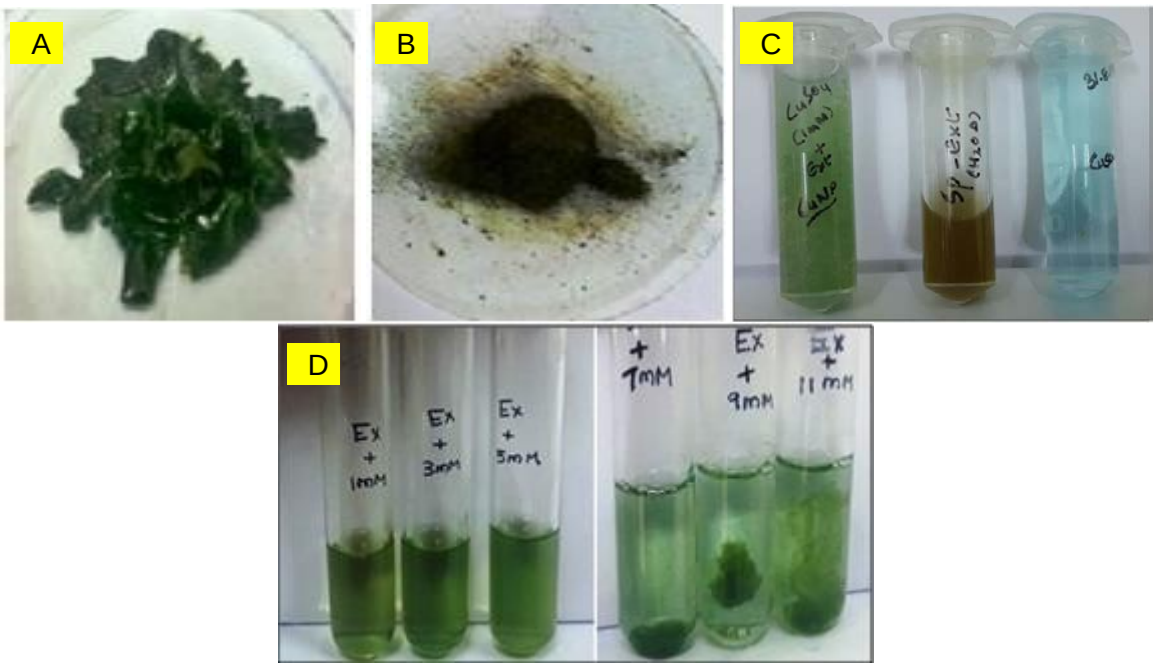

Figure 1. (A) Wet biomass of *S. platensis*, (B) Dried powder of the culture, (C) Green precipitation of CuO nanoparticle and (D) Maximum yield of nanoparticle at 7mM

Characterization studies

The visual observation was confirmed with the analysis by UV-Vis spectroscopy, which is the unique and simple protocol that analyses the sample with their optical properties. In the case of copper nanoparticles the peak absorbance between 580 nm and 590 nm

indicates formation of Cu nanoparticles (Cuevas et al., 2015). In the present study, maximum absorbance was observed at 641 nm which indicated that the copper oxide nanoparticles had been synthesized (Figure 2). This might apparently be due to the light absorption and transmittance which resulted in excitation generating high free electron density eventually leading to high plasmon frequency. Surface plasmon resonance (SPR) band may be due to electrons vibrating in resonance with the light waves (Dallas et al., 2011; Kanmani et al., 2011).

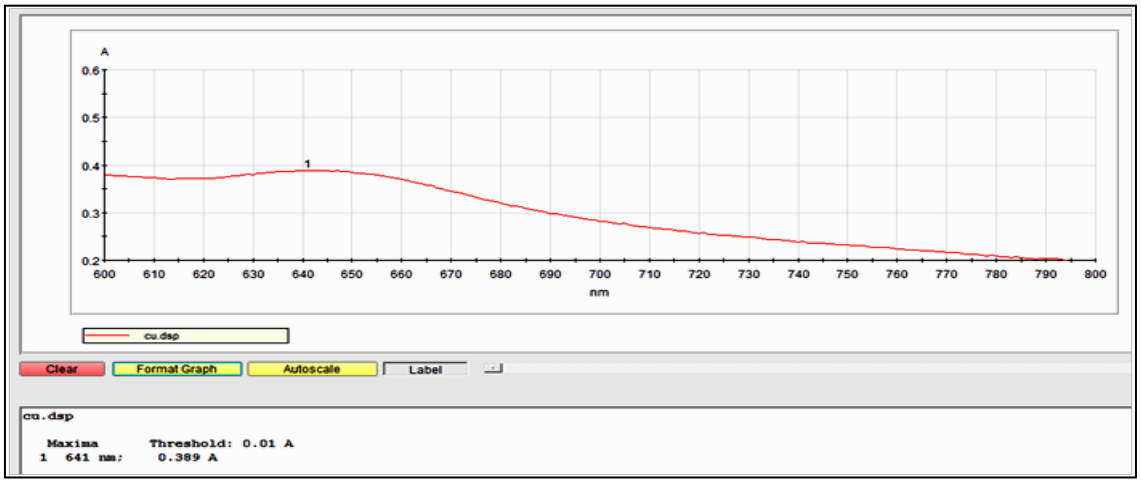

Figure 2. UV-Vis spectroscopic analysis of the synthesized CuO nanoparticle.

X-Ray diffractometer study was performed to identify the nature of the nanoparticle and its crystalline size. The particles were crystalline and the average crystalline size as calculated to be approximately and the thickness was calculated to be 5 nm (Figure 3A). The morphology of nanoparticles was determined using Scanning Electron Microscopy. Figure 3B illustrated that the synthesized nanomaterial was nanosheets. The individual sheets were seen to be overlapping and aggregrated to form a network kind of structure. Nanosheets demonstrating thin petal format illustrate a high degree of crystallinity that correlated with the observed XRD results. The agglomeration showed no deterioration in any activities against microbial and cancerous cells.

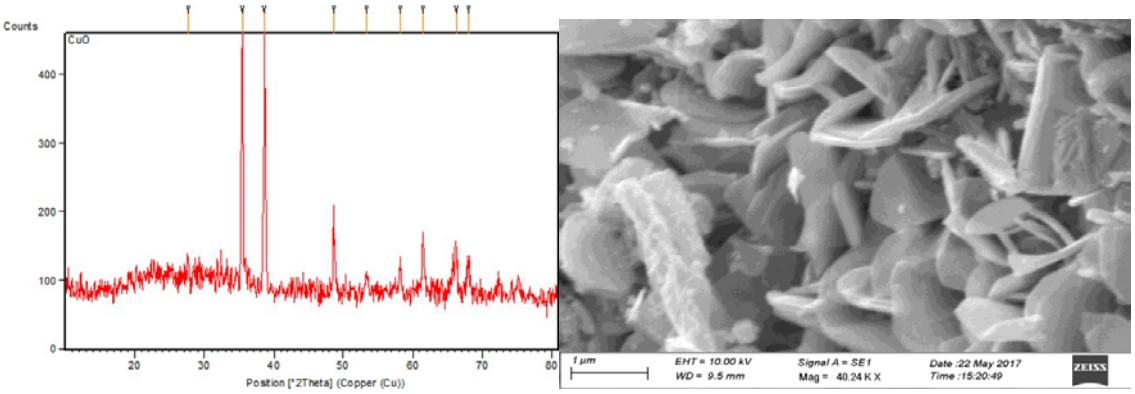

Figure 3. (A) XRD spectrum and (B) SEM image of synthesized CuO nanosheet.

FTIR spectroscopy was performed to determine the functional groups in the sample. The spectrum showed peaks ranging between 4,000-400 cm^{-1}. Figure 4 represents the FT-IR spectrum of copper oxide nanoparticle. The observed peak of CuO nanoparticles were 3406.12, 1713.13, 1363.44, 1145.90, 1025.17, 978.48, 916.57, 649.56, 613.15, 530.15, 447.26 and 481.12 cm^{-1} (Figure 7). The absorption peak at 3406.12 is due to stretching of primary amines. The peak at 1713.13 represented strong stretching of carboxylic acid. The peak at 1363.44 attributed to strong stretching of nitro compound and 1145.90 was due to strong stretching of sulphone groups. An absorption peak at 1025.17 represents strong stretching of vinyl ether and peak at 978.48 represents strong bending of alkene group. The peak at 649.56, 530.15 represented strong stretching of halo compound. The peak at 613.15 is due to Cu-O (Niraimathi et al., 2016). Hence FT IR spectrum confirmed the formation of CuO nanoparticles.

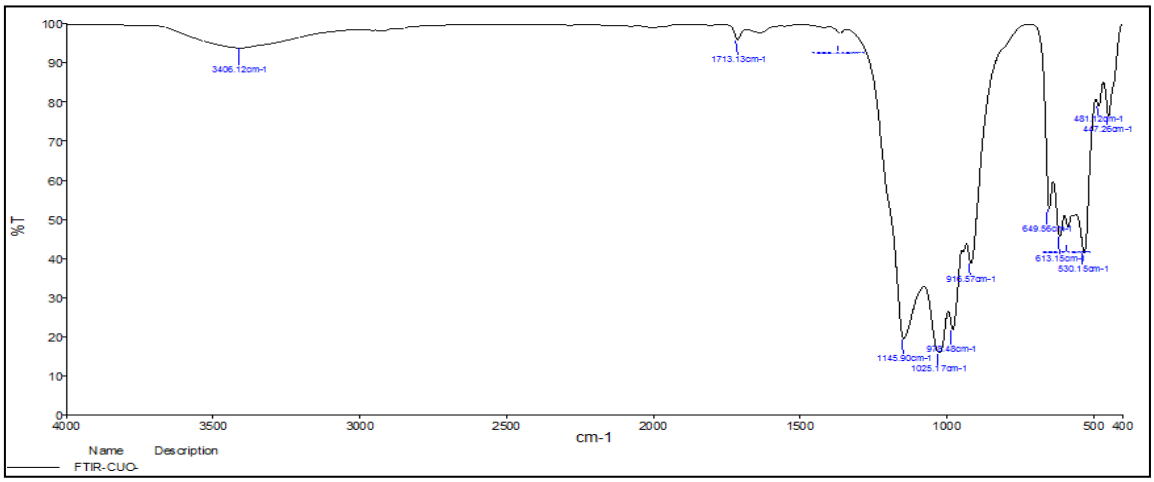

Figure 4. illustrates the FT-IR spectrum of CuO nanoparticle.

Antimicrobial activity of CuO nanoparticle

Antimicrobial activity was performed to check the efficiency of CuO nanoparticles to inhibit the growth of microorganisms. Antimicrobial activity of CuO nanoparticle was checked at the concentration ranging from 200 to 1,000 µg/mL (Figure 5). The result indicated that the zone of inhibition formed is higher at higher concentration of nanoparticles (1,000 µg/mL). The antimicrobial activity is mainly influenced by the nature (size and shape) of the nanoparticle. The antimicrobial activity of the nanoparticle is mainly because of the small size of approximately 5 nm (Padil and Černík, 2013). The IC_{50} values to inhibit the *E. coli*, *B. subtilis*, *P. aeruginosa* and *S. marcescens* using nanoparticles were calculated to be 3.66, 2.83, 3.03 and 0.217 mg, respectively.

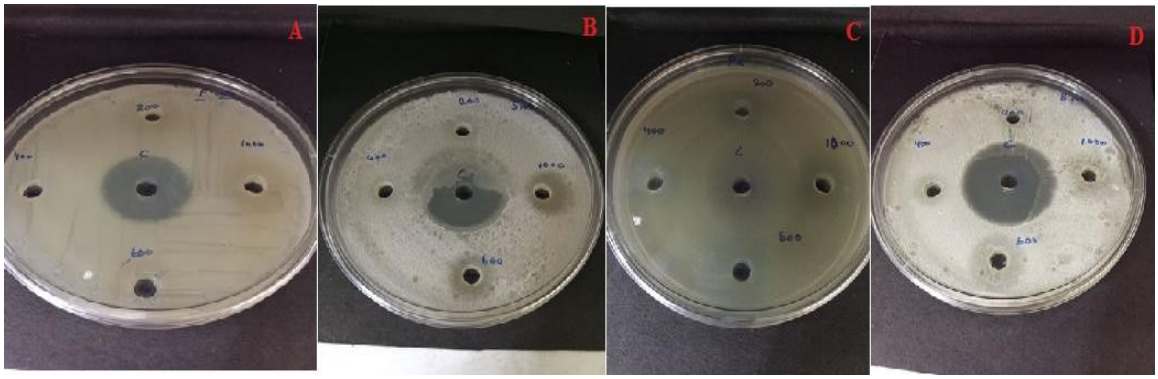

Figure 5. Antimicrobial activity of synthesized nanoparticle against (A) *E. coli*, (B) *B.subtilis*, (C) *P. aeruginosa* and (D) *S. marcescens.*

Anticancer activity of CuO nanoparticle

Anticancer activity of CuO nanoparticles was determined using MTT assay at different concentration of nanoparticles (25, 50, 75, 100, 125 µg/mL) against colon cancer cell lines for 48 and 72 h, respectively (Figure 6). There was decrease in absorbance at higher concentration of CuO nanoparticles (125 µg/mL), indicating the decrease in number of viable cells. Therefore, CuO nanoparticles are effective against colon cancer cell line. No reports showed anticancer studies using CuO nanoparticles and *S. platensis* extract on colon cancer. The suppression of cell viability as studied through MTT assay might apparently be due to cellular death mechanisms like apoptosis and necrosis. The prime focus of the nanomaterial is to induce the apoptosis of the cancer cells. Changes in morphological features like chromatin condensation, cell shrinkage, blebbing and nucleic acid fragmentation and biochemical processes via extrinsic and intrinsic pathways of the tumor cell would lead to apoptosis and thereby cell death occur leading to reduction in viability of cells.

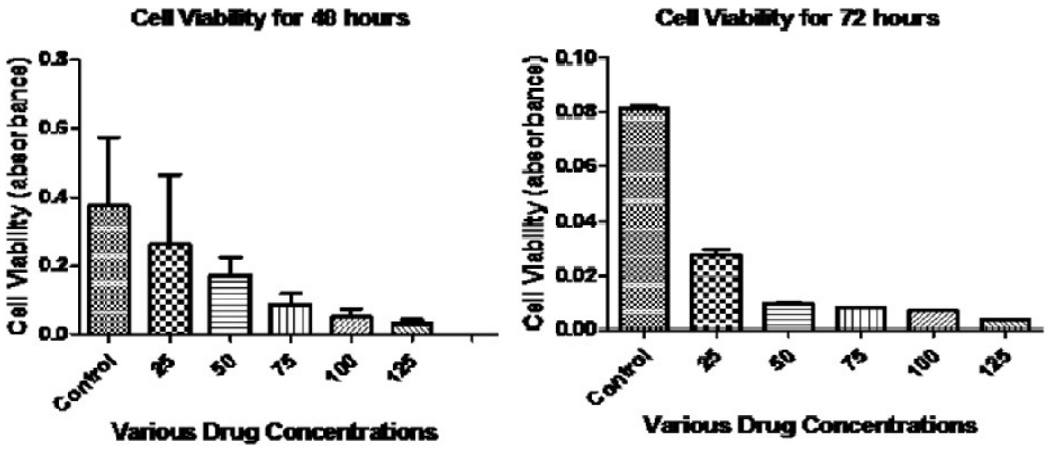

Figure 6. Cell viability at various drug (CuO nanoparticle) concentration for 48 h and 72 h.

Conclusion

In summary, CuO nanoparticles were attempted to be prepared using the hot aqueous extract of *S. platensis*. The maximum absorption was obtained at 641 nm in UV absorption spectroscopy. CuO nanoparticles have been proven to be good antibacterial and anticancer activities. At a maximum concentration of 1 mg/mL, the organisms turned susceptible to the synthesized nanoparticle. Also, from MTT assay it has been confirmed that CuO nanoparticles exhibited good cytotocix effect against colon cancer cell line at higher nanoparticle concentration (125 µg/mL). In conclusion, this study projected the utility of algal samples in the production of copper nanoparticles which revealed potent biological effects against microbial cells and cancerous cells. Thus the biosynthesized nanoparticles could be used up as an effective drug in the near future.

Conflicts of interest

The authors declare that have no conflict of interests.

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