ONE POT SYNTHESIS OF COPPER OXIDE NANOPARTICLES FOR ANTIBACTERIAL AND ANTICANCER ACTIVITIES

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Abstract

Green synthesis of copper oxide NPs was done using an environment friendly and low cost method with aqueous extract of stem of *Cuscuta reflexa* which acted as an efficient reducing agent in the formation of CuONPs. The color of the colloidal solution changed from blue to brown indicating the synthesis of NPs. TEM and SEM study showed that the particles were mostly spherical in size with average particle size of 35 nm. DLS characterization revealed that the hydrodynamic radius of CuONPs was 103 nm with -14 mV as zeta potential. FTIR confirmed various phytochemical responsible for stabilization of CuONPs. Disc diffusion method was used to determine the antibacterial activity of CuONPs which showed significant inhibitory activity against pathogenic bacteria *S.aureus* and *E.coli*. The NPs also revealed anticancer activity against MCF-7 cell line.

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DOI: - 10.48047/ecb/2023.12.si5a.0278

INTRODUCTION

Over the last few years nanotechnology has gained enormous interest due to its wide range of application in the field of pharmaceutical and medicine. Specially metal and metal oxide NPs has slowly attracted the attention of the researchers as these NPs have been used widely in various fields such as electronics, photonics, catalysis, waste water treatment, medical, diagnostic, delivery, biosensor, bio- imaging and pharmaceuticals. NPs of silver, gold, copper oxide and zinc oxide are of prime important because of their unique physical, chemical, electrical and biological properties. These NPs have small size, shape and large surface to volume ratio. Two important approaches are used to synthesize these NPs: top down and bottom up approaches. Three different methods are included in these two approaches: Physical, Chemical and Biological.**[1-4]** Top down approach is the physical method and the other two lies under bottom up approach. Physical and chemical methods are not eco-friendly and leads to the production of hazardous byproducts due to the use of toxic chemicals which poses risk to environment and human health**.[5,6]** The use of biological components such as plants, bacteria, biomolecules, algae, fungi is the only alternative which is eco-friendly, cost effective and easily scalable.The use of plants for the synthesis of MNPs has advantage over other biological methods as it is exempted from the complicated step like sampling of microorganism, culturing, isolation, and storage of microorganism. The basic mechanism for the synthesis of metal NPs using plant extract is the reduction of metal ion to zerovalent metal which is nanosized by the biomolecules present in the plant extract, nucleation, then the NPs grows and lastly the nanoparticles are further stabilised by the phytoconstituents. CuONPs being less expensive, eco-friendly, low toxicity, simplicity, sustainability has gained significant attention recently. Copper oxide NPs have been utilised to prevent microbial infection and as an anticancer, antioxidant, anti-inflammatory agent**.[7-10]**

The inappropriate use of antibiotics has led to the development of resistant strains of bacteria which needs multiple drug therapy for the treatment. These medications are quite costly and have side effects**.[11,12]** Cancer is the leading cause of death worldwide. Cancer is rising due to less efficient diagnosis and helpful therapies. The current treatment for cancer therapy are radiation surgery, therapy, chemotherapy but the side effects related to chemotherapy is severe. Technological and scientific research are rapidly increasing to develop new formulations herbal nanobased medicines to challenge the threat related to infectious disease and cancer**.[13, 14]**

Various studies where researchers have used extracts of *Azadirachta indica, Broccoli, Ficus religiosa, Camellia sinensis ,Adhatoda vasica, Cynodon dactylon, Solanum nigrum, Ocimum tenuiflorum*for antibacterial and anticancer activities have been reported**.[15-22]**

With this intention we are trying to synthesise Copper oxide NPs from *Cuscuta reflexa* stem extract. *Cuscuta reflexa* belonging to family Convolvulaceae is a perennial, parasitic herb of commonly known as Akash Bel. Various phytochemicals such as cuscutin, quercetin, kaempferol, amarbelin, coumarin, stigmasterol, carbohydrates, proteins, tannins are present in *C.reflexa* extract which possess antibacterial,, anticancer, antihypertensive, antioxidant, ,antiviral, antifungal, and anticonvulsant properties**.[23]** Silver and zinc oxide NPs using *C.reflexa* have already been reported in previous studies but till now there is no report on copper oxide NPs using *C.reflexa* has been published**.[24,25]** So this study was aimed to biosynthesize copper oxide NPs from aqueous extract of fresh stem of *C.reflexa* and evaluating their antibacterial and anticancer activities.

Material and Methods

Material

Fresh stems were collected from Dehradun, in the month of October 2022. The taxonomy of the plant was identified by Dr Sunita Garg at (NISCAIR), National Institute of Science Communication and Information Resources (Ref. No NISCAIR /RHMD/CONSULT/2020/3766-67-3). All the reagents used were purchased from CDH (Central drug House) Fine Chemical, Delhi. Mueller-Hilton Agar (MHA) was obtained from Hi Media laboratories Pvt. Ltd, Mumbai.

Preparation of extract

Stems were washed with distilled water and then byethanol to avoid microbial contamination. The rinsed stems were shade dried at room temperature for 1-2 hrs. 5 g of the stem were chopped into fine pieces and was added into 100 ml of distilled water in conical flask and heated at 60˚-70˚C for1 h on temperature controlled magnetic stirrrer. Thereafter the cooled extract was filtered with the help of Whatman no 1 (11µm, Sigma Aldrich) filter paper. The filtrate was stored at 4°C and was used later for phytochemical screening and synthesis of copper oxide NPs.

Biosynthesis of Copper oxide NPs

50 mL of 10 mM aqueous copper sulphate solution was taken in the flask and placed on temperature controlled magnetic stirrer. To this 25mL of 50 mg/mL *C.reflexa* stem extract was added slowing continuous stirring at 500 rpm at 70°-80°C for 60 min when the color of the solution turns blue to brown which indicates the formation of CuONPs. Centrifugation of the colloidal solution was done at 8000 rpm and the NPs were collected, washed with distilled water followed with ethanol and dried in oven at 100°C to get brown colour powder which was stored for further use.

Characterization of nanoparticles

Synthesized CuONPs were further characterized by UV-Visible spectroscopy Shimadzu UV-1800, Perkin Elmer Frontier FTIR-FIR Spectrophotometer (ATR) was used. Shape, size ,morphology and crystallinity of the synthesised NPs was determined be Dynamic light scattering (DLS) and Zeta potential by Zetasizer nano series –Nano ZS90 (Malvern Panalytical), Transmission electron microscope (TEM) JEOL 2010 LaB6, Scanning electron microscope (SEM) -JEOL-SM-610LA, X-ray diffractometer (Bruker, D2-Phaser).

Antibacterial activity of CuONPs nanoparticles by disc diffusion method

Disc diffusion method (Kirby-Bauer method) was used to evaluate the antibacterial activity of CuONPs of C.reflexa extract using Gram positive bacteria *Bacillus subtilis* (MTCC 1133), and gram-negative bacteria *Escherichia coli* (MTCC 40). Mueller-Hilton Agar (MHA)was used as nutrient media. Bacterial strain conatining 106 CFU $mL^{-1}$of microorganisms was spread uniformly on the petri plates conatining agar media .20 µL of different concentration of CuONPs (1000µg/mL and 2000µg/mL) and 2000µg/mLof Cuscuta reflexa extract was added to filter paper disc and placed in the petri dish. Distilled water was used as negative control and Gentamycin 250 µg/mL was taken as positive control. After 24 h of incubation period at 37° C the inhibition zones was measured and recorded. Antibacterial investigation was carried in triplicate analysis and the zone of inhibition was indicated as as mean ± standard deviation (SD**).[26]**

MTT assay for cytotoxic potential

The cell lines MCF-7 were purchased from National Centre for Cell Science, Pune, India.MTT assay was used to evaluate the cytotoxic actiity on MCF-7 cells line.The (8000 cells/ well) were cultured in 96 well plates for 24h in DMEM medium supplemented with 10% FBS and 1% antibiotic solution at 37° C with 5% CO_2. Next day cells were treated from different dossage (10 µg/mL-1000µg/mL) of samples.Doxorubicin was taken as standard and untreated cells were taken as negative control.After 48 h the medium was removed and cells were incubated with 10µL of MTT for 4 h at 37° C. Formazan was formed which was solubilised in DMSO.The optical density was noted using a microplate spectrophotometer at a wavelength of 570 nm.**[27]** Percentage cell viability was calculated using the equation shown below:

Cell viability (%) = Absorbance of test/Absorbance of control *100

Result and Discussion

Visual and UV analysis of copper oxide NPs

Cuscuta reflexa stem extract **(Figure 1[A])** has been used to synthesise copper oxide NPs **(Figure 1B [E]).** The first indication for the synthesis of CuONPs was the color change from blue to green **(Figure 1B [C]).** Further confirmation of the synthesis of NPs was done using UV-Vis spectroscopy. SPR was further observed using UV-Vis spectroscopy for the biosynthesied NPs and it was found to be λ 271 nm which is similar to previous reports**(Figure 2).** Absorption of electromagnetic waves by the collected oscillation of electrons at the surface causes effect which is called Surface Plasmon Resonance (SPR**).[28]**

$$CuSO_4\ 5H_2O + \text{plant extract} \rightarrow Cu(OH)_2 \rightarrow Cu_2O \rightarrow CuO$$

Figure 1.A) *Cuscuta Reflexa* stem B) E.*Cuscuta Reflexa* stem extract, C. CuONPs in solution.

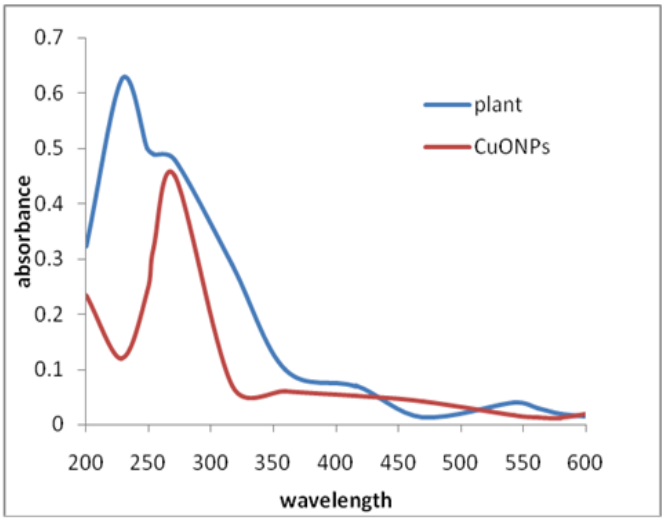

Figure 2. The UV-Visible spectrum of plant extract and CuONPs solution

FTIR analysis of CuONPs

The biomolecules present in the extract that reduced and capped CuONPs were identified through FTIR. FTIR spectra of both the stem extract and the synthesized copper oxide NPs displayed similar peaks with little shift in the spectra.

IR spectra of *C.Reflexa* stem extract and CuONPs(**Figure 3 [A]& [B])** show a strong peak at 3491 cm^{-1} and 3439 cm^{-1} which can be attributed to the stretching vibration of (O-H) of the phenolic group or amine functional group (N-H). The other band at 1620 cm^{-1} and 1690cm^{-1}corrosponds to the amide I band and -C=C- stretching vibration band. This amide I band could be due to the presence of proteins in the stem extract. Stretching vibration of ether group (C-O-C) was observed at 1090 cm^{-1}in plant extract and 1120 $cm^{-1}$is due to CuO vibration in nanoparticles respectively. From FTIR spectral study it can be concluded that the Phytoconstituents that could be involved in the stabilization and capping of copper oxide NPs could be proteins, tannins, phenolics or flavonoids present in the *C.reflexa* stem extract**.[29]**

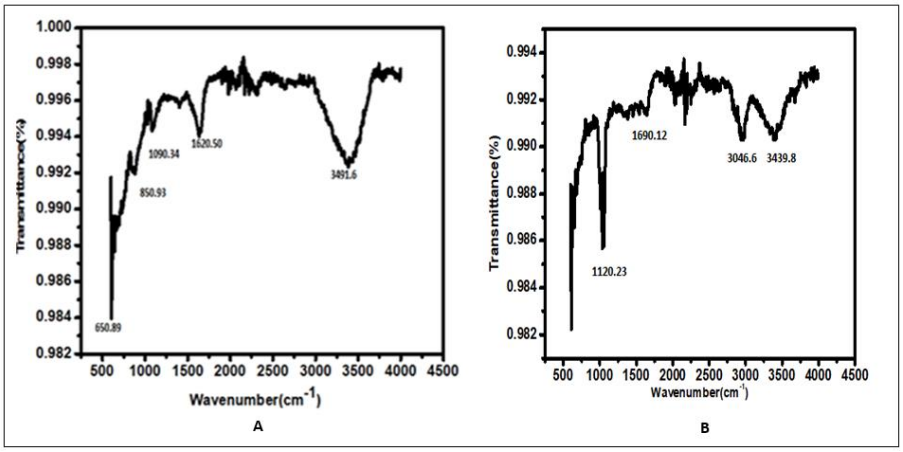

Figure 3. (A) FTIR spectrum of C.Reflexa stem extract, (B) FTIR spectrum of CuONPs

Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) analysis

SEM and TEM analysis were carried out to study the morphology, shape and size of the synthesized NPs. Results of SEM and TEM images investigation of *C.Reflexa* extract mediated CuONPs revealed that the NPs were mostly spherical in shape and polydispersed **(Figure 4. [A] & [B]).** The particle size of the NPs ranges from 25-60nm with the average particle size of 35nm. By comparing the SEM and TEM results, it is observed that the CuONPs forms aggregates by absorbing moisture and ultrasonication is required to disaggregate them

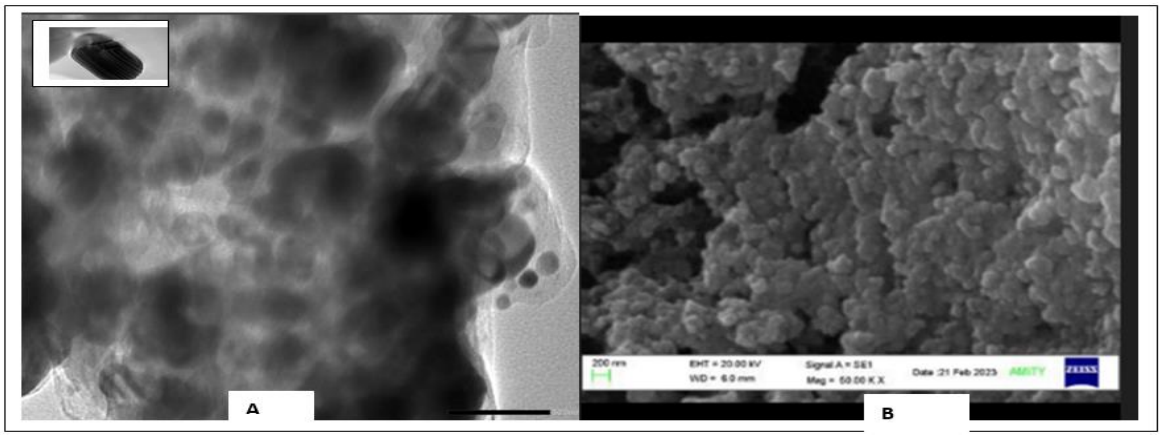

Figure 4. (A) TEM image of CuONPs, (B) SEM image of CuONPs

XRD diffraction study

XRD graph of CuONPs shows diffraction peaks at 2θ values 31.8, 35.8, 38.4, 48.7, 53.2, 58.5, 61.6, 66.1, 68.1 which corresponds to (110), (111), (200), (-202), (020), (202), (-113), (020) and (220) respectively **Figure 5.** The results obtained were similar to work done before. This result shows monoclinical structure of nanoparticles. The result was further assessed by comparing the data with (ICDD) file no. 80-1916**.[30]**

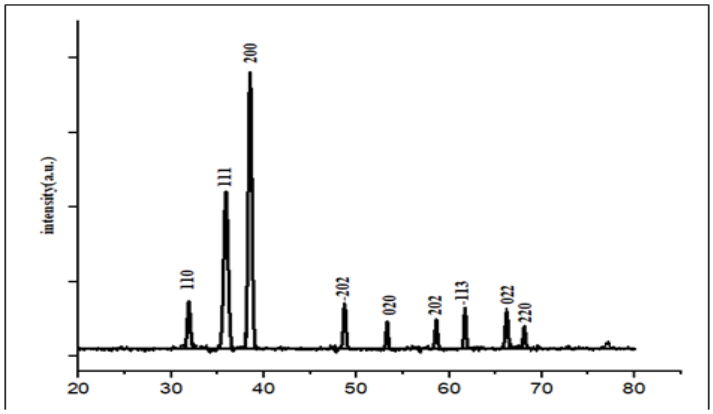

Figure 5. XRD spectrum of CuONPs

Dynamic light scattering and Zeta Potential Analysis

DLS is used to determine both the particle size distribution and Zeta potential of the biosynthesised CuONPs. The results are presented in **(Figure. 6 [A] & [B]).** The size measurement by DLS depends on the hydrodynamic size.The size obtained by DLS is greater than that determined by TEM.As seen in **Figure 6[A]**single peak is seen at 160 nm with the mean particle size distribution of CuONPs is 103 nm. The polydispersity index is 0.424.The zeta potential of CuONPs was -14 mV shown in **Figure 6[B]**. Zeta potential is an important to determine the stability of the colloid, higher negative value confirms high stability, and high dispersibility of CuONPs .

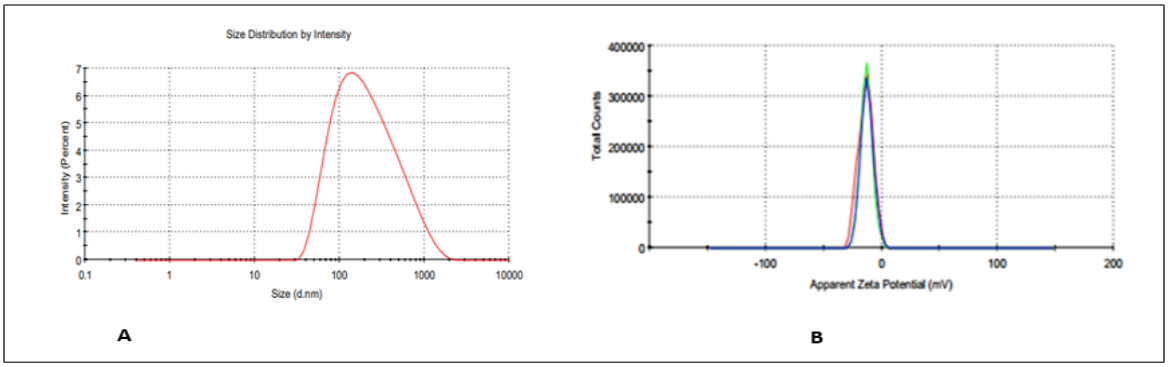

Figure 6. (A) DLS of CuONPs , (B) Zeta potential of CuONPs

Antibacterial Activity

The antibacterial activity of C. reflexa extract and CuONPs by disc diffusion method tested against Gram-positive bacteria *Bacillus subtilis* and gram-negative bacteria *Escherichia coli* are given in**Table 1** and **Figure 7**. The zone of inhibition (mm) was measured and calculated after the CuONPs with different doses were added and then incubated at room temperature for 24 h. Zone of inhibition was 14.5 mm and 13 mm for *E. coli* and *S. aureus*. Plant extract showed very less ZOI against the bacterial strains. The mechanism behind the antibacterial activity of CuONPs is that the nanoparticles binds to the outer membrane of the bacteria and then enters the cell by penetrating through the membrane and eventually binds to the carboxyl of sulphur group of amino acids and inactivates important enzymes and proteins of the bacteria thereby destroying the DNA.**[31]** The other theory is that these NPs generate reactive oxygen species (ROS) specifically superoxides thereby destroys DNA and various proteins and causes death of bacteria.

Compounds (µg/mL)	**Zone of inhibition (mm) Average ±SD**	
	E.coli	*S.aureus*
Distilled water (0)	*0*	*0*
Plant extract 2000	7 ± 0.14	6 ± 0.14
CuONPs 1000	14 ± 0.20	9 ± 0.07
CuONPs 2000	14.5 ± 0.03	13 ± .12
+ve control 250	22 ±0.04	20± 0.11

Table 1. Zone of inhibition (mm) for blank discs , plant extract, CuONPs and positive control against tested bacteria. Data represent mean ± SD (n=3, p< 0.05).

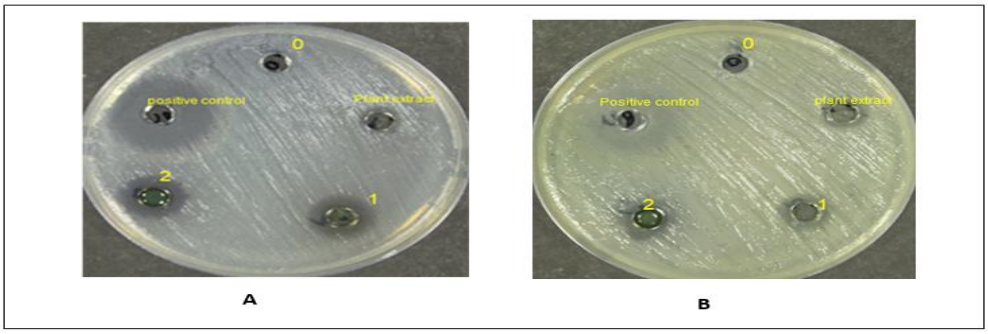

Figure 7. Zone of inhibition of CuONPs against bacterial strains A. E.coli and B. S.aureus, 0 : Distilled water, Plant extract , 1: 1000 µg/mL, 2: 2000 µg/mL, Positive control (Gentamycin).

In-Vitro cytotoxicity

In-Vitro cytotoxicity of CuONPs were assessed in MCF-7 cell line through MTT assay after 24 h of exposing the cells to different concentration (10-1000 µg/mL) of CuONPs **.(Table 2)**

The CuONPs treated MCF-7 cells were analysed through MTT assay after 24 h of adding various concentrations of 10-1000 µg/mL for CuONPs.IC_{50} value for CuONPs was calculated as 250 µg/mL.The anticancer activity depends on the shape size and the morphology of the NPs and also on the biomolecules which are adsorbed on the surface of the particles. The activity is in dose dependent manner, with the increase in concentration of CuONPs the viability of the cell decreases. CuONPs exerts its cytotoxic effect by permeating inside the cells through endocytosis and release of Cu+ ions increasing reactive oxygen species and Nitric oxide formation leading to apoptosis and cell death.(**Figure 8. [A]&[B])** shows the anticancer activity of CuONPs biosynthesised from fresh stem of *C.reflexa* against MCF-7 cells**.[32]**

A

Concentration of CuONPs (µg/mL)	**Absorbance @ 570nm (mean ± SD)**	**Percentage viability (Mean ± SD)**	**IC_{50}**
0	0.23±0.21	100±0.21	250 µg/mL
10	0.23±0.07	97.90±0.01	
20	0.21±0.012	88.87±0.23	
50	0.17±0.13	72.72±0.54	
100	0.14±0.21	60.44±1.2	
250	0.12±0.06	50.46±0.78	
500	0.095±0.02	40.16±0.34	
1000	0.056±0.01	23.53±0.16	

B

Concentration of Doxorubicin (µg/mL)	Absorbance @ 570nm (mean± SD)	Percentage inhibition(Mean ± SD)	IC_{50}
0	0.758±0.02	0.00	21.19 µg/mL
1.560	0.710±0.06	6.03±0.02	
3.125	0.629±0.12	17.84±0.06	
6.25	0.576±1.2	24.35±0.05	
12.5	0.417±0.34	45.15±0.06	
25	0.302±0.51	60.65±0.12	
50	0.181±0.07	76.54±0.23	
100	0.040±0.02	94.99±0.54	

Table 2 : Cytotoxic activity of (A) biogenic CuONPs , (B) Doxorubicin against MCF-7 cell line

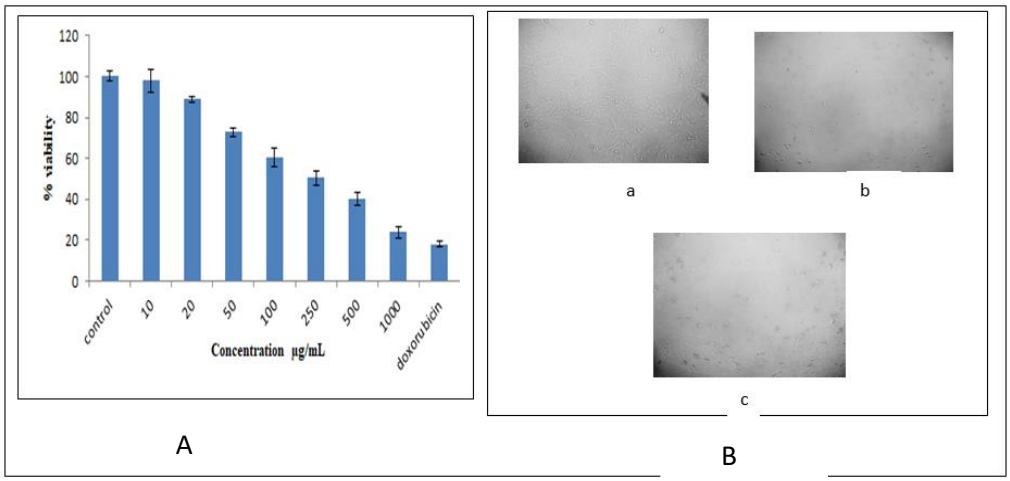

Figure 8. A. Anticancer activity of CuONPs against MCF-7 cell line. B. Images of MCF-7 cell line treated with (a) control (b) Doxorubicin (c) phytogenic CuONPs. Data represent mean ± SD (n=3, $p< 0.05$).

Conclusion

The plant extract mediated synthesis of CuONPs is simple, eco-friendly, safe and economical method for the formation of NPs . The study discloses that the nano particles were crystalline, spherical and within 100nm in size. The antibacterial activity of NPs against the pathogenic bacteria was quite significant but was less than the standard drug . The cytotoxic activity of the NPs against MCF-7 cells increased with increasing dose and IC_{50} value was found to be 250 µg/mL. Further in-vivo studies can be carried out to explore the detailed mechanism of cytotoxic activity and the production can be taken to large scale.

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