Research Article


Biosynthesis of copper oxide nanoparticles and its therapeutic efficacy against colon cancer

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Abstract: In the present study, pumpkin seed extract was used to synthesize copper oxide nanoparticles (CuO NPs) along with evaluating its anticancer activity using different molecular biology tools in the human colorectal cancer cell line (HCT-116). Morphological and structural properties of the biogenically synthesized CuO NPs were characterized by UV-visible spectrophotometry (UV-vis), energy-dispersive X-ray spectroscopy (EDS), scanning electron microscopy (SEM), and transmission electron microscopy (TEM). For estimating the anticancer efficacy, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cytotoxicity, morphological alteration, reactive oxygen species (ROS) formation, and alterations in the mitochondrial membrane potential (MMP) were determined. SEM and TEM data revealed the formation of spherical nanoparticles possessing an average size of 20 nm. The CuO NPs showed 50% inhibitory concentration (IC50) at 25 µg/mL against the HCT-116 cell line. The treatment with IC50 concentration of CuO NPs showed significant shrinking, detachment, membrane blebbing, and shape distortion of cancer cells. Similarly, the IC50 dose of CuO NPs showed significantly early apoptosis in cancer cells compared to late apoptosis. The cancer cell line also showed a dose-dependent increase and decrease in ROS formation and MMP, respectively. The results obtained through various assays indicated significant anticancer efficacy of biogenically synthesized CuO NPs. Thus, further studies are recommended to validate our results using ex vivo and in vivo models.

Keywords: biogenic CuO NPs, cytotoxicity, electron microscopy, ROS

1 Introduction

Nanotechnology and nanostructured materials have contributed to the development of advanced materials with applications in the field of medicine, called nanomedicine [1]. It involves the catalytic synthesis of pharmaceutical drugs in nanoform that offers high surface area and small particle size [2,3]. Utilizing natural resources as the raw material for the synthesis of NPs leads to reduced waste production and enhances the E-factor and efficiency [4,5]. In the last two decades, the researchers have attempted to reduce waste, following a sustainable approach based on the 12 basic principles of green chemistry. Such an approach discourage the use of hazardous products and improve the current chemical synthesis procedures [6,7]. Hence, green nanoscience has paved a way to identify and develop inexpensive, eco-friendly, reliable, and safe nanomaterials, which offer more efficient processes, along with providing a range of applications for the welfare of human beings [8,9].

Copper is an essential micronutrient and plays an important role in several enzymatic activities in plants and animals [10,11]. Copper nanoparticles offer many applications such as in plant disease management,
2.1 Preparation of the extract

Pumpkin seeds are small and contain various nutrients. Pumpkin is generally utilized as a vegetable and comprises of 27 species. The most commonly grown pumpkin species include *Cucurbita maxima*, *Cucurbita pepo*, and *Cucurbita moschata* [26]. Pumpkin seeds were collected from a vegetable shop near the Jaipur National University, India. 2.5 g pumpkin seeds were washed, dried, and ground to a fine powder. The powdered seeds were refluxed for 45 min in deionized water in a round bottom flask and cooled at room temperature (RT). The resultant solution was filtered through a Whatman filter paper to obtain a purified crude extract. The filtrate was stored in a cool environment for further analysis.

2.2 Biosynthesis of CuO NPs from pumpkin seed

A 250 mL of 3 mM copper acetate solution was prepared in double-distilled water. 20 mL of pumpkin seed extract was then added in a dropwise manner in the prepared copper acetate solution kept under a magnetic stirrer to facilitate vigorous mixing for 2 h at RT. The reaction mixture was heated at 80°C for 3 h. Simultaneously, during the reaction, 0.2 g NaOH pellets dissolved in 5 mL of distilled water was added to the reaction mixture. The reaction mixture was heated again until the color changed to black, which indicated the formation of CuO NPs, and then centrifuged at 15,000 rpm for 10 min and washed multiple times with deionized water. The transparent solution was discarded, and a viscous layer consisting of the CuO NPs was collected and subsequently dried in an oven at 50°C.

2.3 Characterization of CuO NPs

The characterization of CuO NPs was done via various spectral techniques such as UV-Vis absorption spectroscopy, FTIR spectroscopy, X-ray diffraction (XRD) spectroscopy, EDX analysis, SEM, and TEM. The absorbance of CuO NPs was recorded within the range of 200–800 nm. The synthesized CuO NPs showed absorbance at 336 nm.
The FTIR analysis of CuO showed a peak at 481/cm, which indicates the formation of CuO NPs. A broad peak at 3,452/cm suggests the presence of stretching vibration of the aliphatic –OH of carboxylic acid. The images obtained through X-ray diffraction, SEM, and TEM analyses confirm the spherical shape of CuO NPs with an approximate size of 20 nm.

3 Biological evaluation of CuO NPs

Dulbecco’s modified eagle’s medium (DMEM), fetal bovine serum (FBS), streptomycin, penicillin, l-glutamine, phosphate-buffered saline (PBS), 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2’-dichlorofluorescein diacetate (DCFH-DA), trypsin-EDTA, acridine orange (AO), ethidium bromide (EO), rhodamine-123 (Rh-123), triton X-100, ethanol, dimethyl sulfoxide (DMSO), and bovine serum albumin (BSA) were purchased from Sigma Aldrich Chemicals Pvt. Ltd, India. All the other chemicals used were of analytical grade and were locally purchased.

3.1 Cell culture maintenance

The human colon cancer cell line (HCT-116) was procured from National Center for Cell Sciences, Pune, India. DMEM was supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 µg/mL) and used for maintaining the cell line in a humidified environment at 37°C with 5% CO₂.

3.2 MTT-based cytotoxicity assay

The cytotoxicity was estimated based on the reduction of MTT to purple formazan crystals [27]. Briefly, the viable cells were harvested and counted using a hemocytometer. A density of 1 x 10⁴ cells/mL was seeded/well in 96-well microplates. After 24 h, HCT-116 cells were treated with different concentrations (5–35 µg/mL) of the biogenically synthesized CuO NPs and incubated at 37°C in a 5% CO₂ incubator. Following the treatment, the HCT-116 cells were washed with a fresh culture medium, and 10 µL of MTT (stock solution 5 mg/mL of PBS) was added to each well, followed by incubating the HCT-116 cells again for 2–3 h at 37°C. The precipitated purple formazan crystals were dissolved in 100 µL of DMSO, and the absorbance was measured at 540 nm using a multiwell plate reader. The results were expressed as the percentage of treated cells with respect to the control cells.

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\text{Inhibition of cell proliferation (\%)} = \frac{\text{Mean absorbance of the control} - \text{Mean absorbance of the sample}}{\text{Mean absorbance of the control}} \times 100.
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The IC₅₀ value was determined for the CuO NPs from a dose–responsive curve. Based on the IC₅₀ value, the optimum concentrations of the CuO NPs were selected for further experiments.

3.3 Measuring the apoptotic induction of HCT-116 cells via AO/EB dual staining method

The apoptotic induction in HCT-116 cells were analyzed through the microscopic fluorescence according to the method described by Baskic et al. [28]. The cells were seeded in a 6-well plate (5 x 10⁶ cells/well) and then treated with different concentrations of CuO NPs (20 and 25 µg/mL) for 24 h. After the treatment, the cells were washed with cold PBS and stained for 5 min with 100 µg/mL AO/EB staining solution mixed in a ratio of 1:1 and examined immediately under a fluorescent microscope (40x magnification). The number of cells undergoing apoptosis was counted as a function of the total number of cells.

3.4 Measuring the extent of ROS formation

The intracellular level of the ROS in the HCT-116 cells was measured by the dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay [29,30]. The HCT-116 cells were seeded in 6-well plates (2 x 10⁶ cells/well) and then treated with varying concentrations of CuO NPs (20 and 25 µg/mL) for 24 h at 37°C. After the treatment, the cells were subjected to washing with PBS and then treated with 25 µM of DCFH-DA for 30 min at 37°C. Thereafter, the cells were washed with DMEM, and the fluorescence spectra were recorded every 5 min, up to 30 min (excitation 485 nm and emission 535 nm) using a spectrofluorometer (Shimadzu, Columbia, USA). The increase in ROS formation was calculated by a mean slope/min and normalized with respect to the unexposed control.
3.5 Measuring the mitochondrial membrane potential (MMP)

The MMP of the HCT-116 cells was measured by the method described by Bhosle et al. [31]. The cells were seeded into 6-well plates and then treated with varying concentrations of CuO NPs (20 and 25 µg/mL). Following the treatment, the cells were stained with Rh-123 dye and incubated for 15 min. Thereafter, the cells were washed twice with PBS and fixed. The fluorescence intensities were measured at 535 nm.

4 Results and discussion

4.1 Validating the biosynthesis of CuO NPs and their stability by UV-Vis spectroscopy

The synthesis of CuO NPs was confirmed by a characteristic peak in the 200–800 nm range. A continuous rise in the distinct peak with respect to the increase in the reaction time and concentration of the biological extract with salt ions showed a clear indication of CuO NPs synthesis. The distinct peak reflects a feature of the nano-sized particles with a surface plasmon resonance of 336 nm. Figure 1a and b shows UV-Vis spectra of the extract and biosynthesized nanoparticles (CuO NPs).

4.2 Spectroscopic analysis of CuO NPs by FTIR

FTIR analysis of the synthesized CuO NPs was recorded in the range of 4,000–400/cm of wavelength. The major Infrared (IR) vibration functional bands in the spectrum of CuO NPs were observed at 2,932, and 2,856/cm depicting the stretching vibration of the aliphatic −CH₂ groups, whereas a broad peak at 3,452/cm indicates an aliphatic −OH of carboxylic acid, and a weak peak at 1,733/cm is found to be similar to the keto group stretching vibrational mode. Several functional groups found in the pumpkin seed extract act as reducing and limiting agents for synthesizing stable nanoparticles. The IR, as mentioned above, could be aliphatic −OH carboxylic acid and its derivatives. A peak around 1,634/cm is attributed to the aromatic C=C bond stretching vibrations, whereas a peak at 481/cm indicates the formation of CuO NPs (Figure 2).

4.3 XRD analysis of the CuO NPs

The crystallinity of the biogenically synthesized CuO NPs was determined using the XRD analysis through the generation of various spectra (Figure 3). The observed diffraction sharp peaks at \(2\theta = 32.22, 35.33, 38.51, 48.44, 61.47, \) and 65.93 corresponded to miller indices planes of 110, 111, 202, 020, 113, and 311, which are consistent

![Figure 1: UV-Vis spectrum of (a) pumpkin seed extract and (b) biogenic CuO NPs.](image-url)
with JCPDS-01-080-0076 and ICSD-087122 of CuO NPs with a monoclinic phase and correspond to the face-centered cubic. Recently, other researchers have also reported the amorphous and less crystalline nature of CuO NPs using the XRD analysis [32,33].

4.4 SEM and EDX analysis of the CuO NPs

The morphology of the biosynthesized CuO NPs was studied by SEM analysis which showed the dissemination at the surfaces of the CuO NPs (Figure 4a). The SEM micrograph was also analyzed through the EDX spectroscopy to detect the presence of elements in the sample in a qualitative as well as quantitative manner. EDX spectroscopy showed strong peaks corresponding to copper (Cu) and oxygen (O), depicting the formation of nanoparticles. Gold (Au) is also found to be present in the EDX spectrum because the sample was coated with gold for the analysis. The recorded O and Cu values in CuO NPs were 89.42% and 20.11%, respectively (Figure 4b). Hence, the EDX analysis revealed the presence of a lesser amount of Cu and a higher amount of O.

4.5 TEM and selected area electron diffraction (SAED) analysis

TEM is a critical technique that provides direct information regarding the particle size distribution, average particle size, and size of the nanoparticles. TEM analysis was carried out to give more insight into the size, shape, and morphology of the biogenically synthesized CuO NPs (Figure 5a). TEM images revealed that the synthesized CuO NPs possess circular shapes with an average size of 20 nm. On the other hand, the SAED images (Figure 5b) determined the nature of CuO NPs revealing circular impression that can be indexed according to the reflection planes such as (110), (111), (202), (020), (113), and (311) planes, as marked by the XRD analysis.
4.6 Cytotoxicity assay and morphological alterations in the HCT-116 cells

A dose-dependent decrease in the cell viability by the CuO NPs was recorded in the colorectal cancer cells. The synthesized CuO NPs showed an IC$_{50}$ value at 25 µg/mL (Figure 6). The percentage of cell viability decreased up to 21% at 35 µg/mL. Gnanavel et al. [34] reported the IC$_{50}$ value of the synthesized CuO NPs to be 40 µg/mL in HCT-116 cell lines. Several other researchers have biosynthesized metal nanoparticles and demonstrated their anticancer potential at much higher concentrations [35,36]. This suggests better anticancer efficacy of our biogenically synthesized CuO NPs. The photomicrograph (10X)
represents morphological changes in the HCT-116 cancer cells in the form of shrinking, detachment, membrane blebbing, and distortion in the shape of the cells, which was induced by the treatment of CuO NPs (25 µg and 30 µg/mL). The control cells without any treatment showed normal and intact cellular morphology (Figure 7).

4.7 Evaluating the apoptotic induction in the HCT-116 cells by CuO NPs

To study the possible induction of apoptosis, HCT-116 cells were stained with the dual dye, AO/EB, and observed under the fluorescence microscope. Living cells displayed green fluorescence with intact nuclei, while early apoptotic cells showed fragmented nuclei with yellow fluorescence and condensed chromatin. On the other hand, late apoptotic cells displayed orange fluorescence along with the condensation or fragmentation of the chromatin (uniformly red/orange-stained cellular nuclei). Significant apoptotic induction was observed after treating the cells with 25 µg/mL CuO NPs (Figure 8). The results from the current study are in agreement with the earlier reports that observed apoptotic induction in cells treated with green synthesized nanoparticles causing damage to the DNA, and in turn leading to apoptosis/necrosis [37,38]. It is linked to the excessive production of ROS, causing oxidative stress, and eventually leading to the sub-G1 arrest of the cancer cells [37,39]. Similarly, another study also reported apoptotic induction via the upregulation of tumor suppressor genes (p53, Bax, caspase-3, and caspase-9) and the downregulation of oncogenes (Ras and Myc) in response to the green synthesized copper nanoparticles in MCF-7 cells [40].

4.8 Inducing the formation of ROS by CuO NPs

We observed a dose-dependent induction in the formation of ROS due to the treatment of HCT-116 cells with CuO NPs (Figure 9). The untreated control cells displayed a dull green fluorescence, while the cells treated with CuO NPs and stained with 2,7-dichlorodihydrofluorescein (DCF) displayed a bright green fluorescence. Our results agree with the previous studies, which reported an increase in

![Figure 7: Morphological alterations in HCT-116 cells treated with CuO NPs for 24 h.](image)

![Figure 8: Induction of apoptosis as a result of CuO NPs treatment in HCT-116 cells.](image)
the ROS production, following the main toxicity approach of the green synthesized CuO NPs [37,41].

4.9 Modulating the MMP

The results show a gradual decrease in the green fluorescence with increasing concentrations of CuO NPs. This indicates a dose-dependent decline in the MMP in HCT-116 cells (Figure 10). The fluorescent images show Rh accumulation in control cells, while it is absent in the treated cells. The green synthesized CuO NPs obtained from the black bean extract have also been reported to alter the mitochondrial structure, accompanied by the loss of membrane potential in HeLa cell lines [42]. Several mechanisms of action associated with green synthesized NPs have been reported in the scientific literature that includes apoptotic induction, increase in the production of ROS/NO, loss of MMP, etc. [43–45].

5 Conclusion

The current study involves the use of an eco-friendly and biogenic method to prepare CuO NPs from pumpkin seed extract. The results of our study show a significant anticancer potential of CuO NPs, which is mediated by the formation of ROS, induction of apoptosis and alteration in MMP in the cancer cells. Considering the benefits associated with plant-based NPs along with the significant anticancer efficacy of our biogenic CuO NPs, it is recommended to validate the results within ex vivo and in vivo models. Successful validation of our results in in vivo model could lead to beneficial applications of the biosynthesized CuO NPs in the pharmaceutical, medical, and biotechnological fields. However, the precise mechanism of action of the CuO NPs needs to be studied further to provide a better insight into their application in various fields.

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