Green synthesis of CuO NPs, characterization and their toxicity potential against HepG2 cells

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Abstract

In this study, copper oxide nanoparticles (CuO NPs) were green synthesized using the leaf extract of Momordica cochinchinensis (Lour.). Various characterization techniques such as Energy-dispersive x-ray spectroscopy (EDS), Ultraviolet Visible (UV–vis) Spectroscopy, x-ray diffraction (XRD), Transmission Electron Microscopy (TEM) and Fourier-transform Infrared (FTIR) Spectroscopy were utilized to study the formation of CuO NPs. LSA and TEM analysis revealed the formation of sphere-shaped CuO NPs with mean particle size of 56 nm. Additionally, the prepared CuO NPs were incubated with Hepatic (HepG2) cells to check their cell viability and evaluate the formation of reactive oxygen species (ROS). The results of the current work exhibited a concentration-dependent decline in the viability of HepG2 cells with half maximal inhibitory concentration (IC50) value of 75 µg ml−1. The cytotoxic effect of CuO NPs was responsible for the cell apoptosis as well as ROS induction in the HepG2 cells. Further, the prepared CuO NPs could act as possible chemotherapeutic agent for the treatment of liver hepatocellular carcinoma (HCC) in future.

1. Introduction

Cancer is the second most widespread cause of mortality and an important medical health issue of public all over the world [1]. More than 21 million cases of cancer and 13 million cases of mortality were estimated globally in accordance with the reports of World Health Organization (WHO) [2]. As per literature reports, Hepatocellular carcinoma (HCC) is the third major cause of mortality due to cancer [3, 4]. Even though, surgical resection is considered as standard medical therapy for HCC, most of the patients are not suitable because of the advanced extension of tumor at early diagnosis or/and inadequate hepatic functional reservoirs [5]. Additionally, chemotherapy regimes in appropriate candidates often have been restricted due to inefficacious medicines, poor prognosis and major organ injury. Long-term survival of patients with hepatocarcinoma is low and hence HCC is considered as one among the major causes of deaths related to cancer [6]. In addition, the occurrence of hepatocellular carcinoma (HCC) is expected to rise in the coming decades [7]. Hence, investigations for novel anticancer agents with higher efficacy regimes and less side effects are continued.

On the other hand, copper oxide nanoparticles (CuO NPs) can provide novel solution because of their good compatibility with biomolecules. They can be synthesized with unusual crystal morphologies with extremely high surface areas, which makes them useful in the diagnosis and therapy of cancer [8, 9]. Furthermore, CuO NPs are also reported to exhibit potential cytotoxic effects on different types of cancer cells such as MCF-7 breast cancer cells and A549 human lung cancer cells [10]. The interaction of nanoparticles with the living cells is enormously influenced due to their composition, size and shape [11]. CuO NPs have drawn significant attention by the researchers owing to the fact that they are ~ten fold cheaper than gold and silver in the market and hence the approaches involving CuO NPs will be very economical.
On the other hand, CuO NPs have attained great scientific attention because of their broad applications in nanoscience and nanotechnology such as superconductors, giant magneto resistance (GMR) materials, lithography, gas sensors, lubricants and nanocomposites [12–16]. CuO NPs are the nanomaterials of p-type semiconductor having 1.7 band gap. Moreover, the CuO NPs are used in fabrication of antibacterial products such as in paints, clothes and anti-fouling agents [17]. CuO NPs were also been used in pesticide production due to their extra-ordinary properties [18].

Many synthetic methods were developed for preparation of CuO NPs, including by electrochemical reduction [19], chemical [20] and microwave irradiations [21] etc. It is also noted that CuO NPs synthesized through chemical procedures suffer by adsorption of hazardous chemicals on their surface and hence can’t be used in biomedical applications. Hence, there is a need for the development of modern eco-friendly methods for CuO NPs synthesis through eco-friendly methods.

The biosynthesis of metal NPs like silver and gold utilizing microbes and polyphenols from plants have been already reported [22, 23]. For example, *Aloe vera* [24], *Albizia lebbeck* [25] and *gum karaya* [26] plant extracts were utilized for CuO NPs synthesis. Although, various synthetic reports were available for the biosynthesis of CuO NPs, the fabrication of CuO NPs using leaf extract of *M. cochinchinensis* is not yet reported.

In our current study, we used the leaf extract of *M. cochinchinensis* plant to synthesize CuO NPs. Various characterization techniques were used for investigating the morphology and size of the formed CuO NPs. Further, we evaluated the cell viability of HepG2 cells after incubation with CuO NPs and the formation of reactive oxygen species (ROS) to check their possible use for future treatment of liver hepatocellular carcinoma (HCC).

2. Experimental section

2.1. Materials
Copper sulfate (CuSO$_4$), Dimethyl sulfoxide (DMSO), MTT, other solvents and chemicals used in the experiment were obtained from Sigma Aldrich, Shanghai, China. Double distilled water was used throughout the experiment.

2.2. Preparation of *M. cochinchinensis* extract
About 1 g of dried leaf powder of *M. cochinchinensis* was added to 100 ml of deionized water in a round bottom flask (RBS) followed by heating on a water bath for about 1 h at 90 $^\circ$C. A clear extract was collected by passing the obtained plant extract mixture through cellulose nitrate filter paper (0.22 $\mu$m pore size). Later, the extract collected was kept in a refrigerator for future use for the preparation of CuO NPs.

2.3. CuO NPs preparation
Green synthesis of CuO NPs using leaf extract of *M. cochinchinensis* was carried out by slightly modifying the reported procedures of CuO NPs synthesized using *Cassia auriculata* and *Ericostemma axillare* leaf extract [27, 28]. About 50 ml of fresh leaf extract of *M. cochinchinensis* was added to 10 ml of 0.01 M aqueous CuSO$_4$ solution. Then, the reaction mixture was heated on a water bath for about 30 min at 90 $^\circ$C. Formation of CuO NPs was indicated by noticing the colour change of reaction mixture visually from dirty yellow to brown after 20 min. The nanoparticles obtained were centrifuged and washed several times with double distilled water and the obtained pellet containing NPs was dried at 60 $^\circ$C in an oven for overnight.

2.4. Characterization
High-resolution TEM instrument (HR-TEM, JEOL JEM 2100, Japan) was utilized for studying size and surface morphological features of the fabricated CuO NPs. The prepared NPs colloidal solution of 100 $\mu$l was added to 1 ml distilled water followed by placing in an ultrasonicator for 10 min to obtain a diluted colloidal dispersion with uniformly distributed NPs. One droplet of sonicated colloidal solution was placed onto the surface of copper grid, vacuum dried, and then was visualized under TEM instrument. TEM instrument was operated at 15 Kev acceleration voltage to record TEM images for the formed NPs. EDS analysis was conducted simultaneously using the same instrument. XRD pattern was recorded for prepared NPs using an Advanced diffractometer (Bruker D8, Germany) which was scanned at 4 $^\circ$/min with Cu K$\alpha$ radiation ($\lambda$ = 1.54 A$^\circ$) and 0.02$^\circ$ step size. Prior to this analysis, calibration of XRD was carried out utilizing Lanthanum hexaboride (LaB$_6$). In addition, average size and the surface charge of CuO NPs were examined with the help of Horiba Scientific SZ-100 NP Analyzer, Japan. CuO NPs dispersed in double distilled water was used for DLS analysis. Additionally, FTIR analysis was performed to know the capping groups that were existing on the surface of CuO NPs using JASCO-FT-IR spectroscopy instrument. Sample for FTIR analysis was prepared by mixing NPs powder with KBr and made a pellet using pellet maker and the obtained pellet was used for measurements under FTIR instrument.
XPS analysis was performed using the ESCA-3000 VG Scientific UK instrument operated at current of 150 W utilizing a non-monochromatic Al Kα radiation (1486.6 eV) with a 0.2 eV spectral resolution. An UHV chamber (< 5 × 10⁻⁸ Pa) was utilized at room temperature to out-gas the sample for overnight prior to the analysis. Deconvolution of Cls was performed using the XPS Peak 4.1 software. The sample powder was grounded in ceramic mortal followed by mixing with isopropyl alcohol in order to make a semisolid paste, which then drop casted on a stainless-steel substrate to obtain the final sample.

2.5. Cell culture

HepG2 cells were cultured in accordance with cell bank guide book. The RPMI medium was used to culture the cells by adding 10% concentrated fetal bovine serum (FBS), 100 μg ml⁻¹ of streptomycin and 100 units ml⁻¹ of penicillin followed by incubation at humidified atmosphere maintained with 5% CO₂ under 37 °C temperature.

2.6. Determination of cell proliferation

MTT assay was performed to study the effects of CuO NPs on HepG2 cell lines proliferation [29]. Cells were seeded in a 96-well microplate comprising of 200 μl RPMI growth medium with 1.2 × 10⁴ cells/well seeding density. Later, the cells were administrated with different concentrations of CuO NPs (0, 25, 75, 50 and 100 μg ml⁻¹) at various incubation periods (24, 48 and 72 h). After the treatment period, both untreated and treated control cells were subjected to incubation with 50 μl MTT solution (2 mg ml⁻¹ in PBS) and 150 μl fresh medium at a temperature of 37 °C for about 4 h. Then, the MTT medium was discarded and a solution mixture consisting of DMSO (200 μl) and Sorenson’s glycine buffer (25 μl) (0.1 M NaCl, 0.1 M glycine, pH 10.5) was supplemented to each well followed by incubation for about 30 min at a temperature of 37 °C. Biotek, ELx800 USA microplate reader was utilized to measure the optical absorbance of plates at wavelength of 570 nm.

2.7. Determination of ROS formation

2',7'dichlorofluorescin diacetate (DCFHDA), a non-fluorescent dye is oxidized by hydroperoxides to fluorescent dihydrofluorescein (DCF) was used for determination of relative levels of cellular ROS generation [30]. The cells at 3 × 10⁴ cells/well density was administrated with CuO NPs at various concentrations of 0 to 100 μg ml⁻¹ for a period of 48 h at a temperature of 37 °C. Furthermore, the formation of ROS in the cells treated with CuO NPs of 200 μM concentration was measured using a potential anti-oxidant in the presence of N-Acetyl cysteine (NAC). All the untreated/treated control group cells were removed by EDTA- trypsin solution which comprises of 0.02% EDTA and 0.25% trypsin, followed by washing with 0.1 M concentrated PBS (pH 7.2). Later, these cells were incubated in culture medium free from FBS and consisting of 50 μM dye for 30 min. The cells were washed again and the cell dispersions were subjected to centrifugation for 10 min. After removal of the supernatant layer, 1% triton X100 solution was used to dissolve the cell plates. Jasco RFP-750 spectrophuorometer (Jasco Corporation, Tokyo, Japan) was used to measure the changes in the fluorescence with the emission and excitation wavelengths of 530 nm and 485 nm respectively [31].

3. Results and discussion

3.1. Characterization

The current study showed the use of M. cochinchinensis plant leaf extract as reducing and stabilizing agent in the green fabrication of CuO NPs. Formation of CuO NPs was visually noticed by change in colour of reaction mixture from dirty yellow to brown colour after 20 min. Same experiment was carried out without the addition of M. cochinchinensis extract and the reaction solution exhibited no colour change even after 72 h, representing the role of M. cochinchinensis extract in fabrication of CuO NPs.

The crystal structure of biosynthesized CuO NPs was known by powder diffraction pattern, as showed in figure 1. The strong diffraction peaks found at 32.5°, 35.4°, 38.7°, 46.2°, 48.7°, 53.4°, 58.3°, 61.5°, 66.2 and 68.1° are corresponding to (110), (002), (200), (112), (202), (020), (202), (113), (022) and (220) planes, respectively, indicating the cubic structure of the synthesized CuO NPs.

Figure 2 showed the HRTEM images of CuO NPs which revealed their particle size, structural morphology. HRTEM Images confirmed that the particles existed in the size ranging from 40 to 80 nm, and the formed CuONPs were irregularly arranged with spherical shape. As shown in figures 2(A), (B), the HRTEM images confirmed the existence of bio-constituents of leaf extract of M. cochinchinensis onto the surface of NPs. These bio-constituents present on the NPs surface plays a major role in colloidal stability of CuO NPs [22, 23]. In the same way, figure 2(C) shows the SAED pattern of the green fabricated CuO NPs, which revealed crystalline and monoclinic CuO NPs formation.

Further, EDS analysis was used to study the elemental composition of the synthesized CuO NPs (as shown in figure 3). From EDS analysis, it is found the existence of strong signals corresponding to both oxygen and copper.
Figure 1. XRD pattern of prepared CuO NPs.

Figure 2. HRTEM images (A), (B) and SAED pattern (C) of prepared CuO NPs.

Figure 3. EDS spectrum of *M. cochinchinensis* extract mediated CuO NPs.
at approximately 0.2 keV and 1 keV respectively confirming the CuO NPs. Moreover, the purity of synthesized CuO NPs using the leaf extract of *M. cochinchinensis* was confirmed by the absence of extra peaks corresponding to impurities. However, the presence of additional peaks corresponding to chlorine and phosphorous may be attributed to the bioconstituents present on the surface of CuO NPs [32, 33]. Similar results were observed in previous reports where the synthesis of CuO NPs was performed using the sublimated precursors [34, 35].

DLS instrument was used for determining surface charge and average particle size of the formed CuO NPs. The particle size distribution profile of the fabricated CuO NPs was shown in figure 4(A). From figure 4(A), the mean size of the formed CuO NPs was found to be 56 nm and the obtained particle size distribution is in good accordance with the TEM results. Alternately, the surface electrokinetic zeta potential on NPs was found to be $-24.0 \text{ mV}$, indicating the capping of *M. cochinchinensis* extract polyphenolic molecules onto the surface of NPs (figure 4(B)). This negative surface charge value of CuO NPs was because of the adsorption of bio-constituents of *M. cochinchinensis* extract over the surface of CuO NPs. Moreover, the strong electrostatic repulsive forces in the CuO NPs were generated due to this negative charge present on surface, which leads to the stability of CuO NPs by preventing the aggregation [36].

The FTIR analysis was performed to know the probable functionalities present over the CuO NP’s surface. The presence of absorption bands at a wavenumber 630 cm$^{-1}$, 1076 cm$^{-1}$, 1620 cm$^{-1}$ and 3428 cm$^{-1}$ respectively were revealed by the FTIR spectroscopic results (figure 5). These absorption bands are owing to stretching vibrations of O-H bond (3428 cm$^{-1}$), tertiary alcohol group (1620 cm$^{-1}$) and stretching vibrations of carboxylic bond –C-O-C (1076 cm$^{-1}$) respectively, which are responsible for the colloidal stability of green fabricated CuO NPs using polyphenols of *M. cochinchinensis* extract. Moreover, the fabrication of CuO NPs was indicated by the existence of absorption band at a wavenumber 630 cm$^{-1}$ which corresponds to the stretching of Cu-O bond. On the other hand, the FTIR spectrum of plant extract exhibited the similar peaks as found in spectrum of CuO NPs, which further indicating the surface functionalization of CuO NPs with plant extract biomolecules. All these results showed that the biomolecules of *M. cochinchinensis* extract are accountable for the surface stabilization of CuO NPs.

**Figure 4.** (A) DLS size distribution and (B) Zeta potential of prepared CuO NPs.

**Figure 5.** FTIR spectrum of prepared CuO NPs (orange) and Plant extract (green).
The surface oxidation states of metal are measured with the help of XPS spectrum. The significant peaks observed in the broad XPS survey scans of CuO NPs were predominantly focused on O1s, C1s and Cu2p, confirming the presence of C, O and Cu in the sample (figure 6(A)). In addition, the high-resolution scans have determined the elemental composition of the sample (figure 6(B)). A strong core level spectrum of Cu2p was noticed, showing a characteristic peak with binding energy of 933.9 eV corresponding to the Cu2p3/2 [37]. The presence of two other peaks observed at 77.2 and 121.1 eV are corresponding to the Cu3p and Cu3s, respectively. Additionally, the high-resolution O1s scans were observed to be present with low binding energy peaks at 529.8 eV, representing the existence of oxygenated groups in CuO (as shown in figure 6(C)).

Typically, as represented in figure 6(D), the C1s peak with a 284.8 eV binding energy that is assigned to the functional groups C = O, C–O, and C–C, is due to the low amounts of amorphous carbon which is adsorbed onto the surface of the NPs [38]. The above findings confirmed the formation of nanocrystalline CuO NPs with functional groups, which is further noticed to be in accordance with the results of an earlier report [37].

Figure 6. (A) XPS spectrum broad survey (B) Cu2p XPS spectrum (C) O1s XPS spectrum and (D) C1s XPS spectrum of CuO NPs.
In-Vitro cytotoxicity

In vitro cytotoxic effects of CuO NPs were evaluated against HepG2 cells at different concentrations, which exhibited a potential cytotoxic effect against the experimental cells. The obtained results showed the incidence of significant cell death at higher levels of concentration that was observed even after 24 h of post incubation. Figure 7 showed the changes in percentage cell viability of HepG2 cell lines that were exposed to CuO NPs at different time intervals. The cell viability was noticed to be decreased significantly in time and dose depended manner upon incubating the HepG2 cell lines with CuO NPs. Nearly 50% of cell proliferation (IC50) was inhibited at concentration of 75 μgm l⁻¹ CuO NPs after 48 h of treatment. However, dose dependent cytotoxicity of nanoparticles such as AgNPs and AuNPs have already been reported towards cancer cells [39–42].

3.3. Determination of ROS formation

Incubation of cancer cells with CuO NPs at increasing concentration of 25 to 100 μg ml⁻¹ for 48 h caused an increased ROS production when compared with control. As represented in figure 8, CuO NPs significantly increased the DCF florescence at concentrations of 50, 75 and 100 μg ml⁻¹ (p < 0.05). In addition, N-acetyl cysteine decreased the formation of ROS significantly in the cells treated with CuO NPs for 48 h at IC50 (75 μg ml⁻¹ concentration) (p < 0.05) (figure 8). An increased concentration dependent production of ROS by CuO NPs in liver HCC cell lines suggested that the generation of oxidative stress performed an important role in the toxicity of CuO NPs that was consistent with inducing oxidative stress with nanomaterials. On the other hand, biofabricated NPs are already been proved for their cytotoxic activity with ROS generation [39, 43].

Literature reports have demonstrated the cytotoxic potential of Cl [44–46] and the existence of chlorine content in the leaf extracts may be one among the reasons for their selective anticancer effects. The exchange of bicarbonates (HCO₃⁻) over the RBC plasma cell membrane is possible with the help of chloride ions which helps
in maintaining ionic balance called chloride shift [47, 48]. This can be a possible reason for the cytotoxic ability of CuO NPs towards HepG2 cancer cells.

The mitochondria are responsible for the major source of ROS generation inside the cell [49, 50]. Impression of toxic NPs on the respiratory chain may result in an increase of ROS generation in mitochondria [51]. The electron chain breaking through NPs in the mitochondria increases ROS production and reduces the synthesis of ATP [52, 53]. This suggests that the apoptosis mediated with CuO NPs can be termed as mitochondria-based mechanism that takes place with an intrinsic pathway. We have confirmed that the intracellular production of ROS is required for mitochondrial based apoptosis through pre-treatment of experimental cell lines with N-acetyl cysteine (NAC) and CuO NPs as ROS scavenger. As a result, it can be proposed that CuO NPs with the help of mitochondrial pathway provokes cell death via ROS. Furthermore, these results are in good agreement with the earlier reports [54, 55].

4. Conclusions

In conclusion, we reported a simple, cost-effective and environmental-friendly method to fabricate CuO NPs using M. cochinchinensis extract. FTIR results confirmed the existence of plant extract biomolecules onto the surface of CuO NPs. The formation of spherical CuO NPs with an average size of 30 nm was confirmed by DLS and TEM studies. Further, the cytotoxicity studies revealed the concentration-dependent decline in the cell viability with IC50 value of 75 μg ml−1. Additionally, CuO NPs induced ROS production in liver HepG2 cell lines. As per our observations, CuO NPs in the treatment of hepato cellular carcinoma could be viewed as possible chemotherapeutic agents in future.

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Data availability statement

The data that support the findings of this study are available upon reasonable request from the authors.

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