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Research Article
Biosynthesis of Copper Oxide Nanoparticles and Their *in vitro* Cytotoxicity towards Nasopharynx Cancer (KB Cells) Cell Lines

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Abstract

Background and Objective: Copper oxide nanoparticles (CuO NPs) have attained extensive scientific interest because of their wide range of applications in nanotechnology. This study was conducted to establish green fabrication of copper oxide nanoparticles (CuO NPs) using *Arbutus unedo* leaf extract and examine the *in vitro* cytotoxicity against nasopharynx cancer cell lines (KB cells). **Materials and Methods:** CuO NPs were synthesized by mixing 25 mL of *Arbutus unedo* leaf extract with 5 mL of $CuSO_4$ (0.01M) aqueous solution and allowed for stirring for 10 min and heating at 100°C for 20 min to obtain brown color solution. The prepared CuO NPs have been studied by XRD, FT-IR, UV–Vis, EDS and TEM characterization techniques. The *in vitro* cytotoxicity against KB cells was determined using a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. **Results:** TEM and DLS analysis revealed the formation of spherical CuO NPs with mean particle size of 33 nm. The crystalline nature of the synthesized NPs was confirmed by XRD measurements of dried particles. The *in-vitro* cytotoxicity of *Arbutus unedo* extract mediated CuO NPs against nasopharynx cancer cell lines (KB cells) have confirmed their biocompatible nature. **Conclusion:** A facile and less expensive approach for the fabrication of CuO NPs using *Arbutus unedo* leaf extract is described. The CuO NPs showed their biocompatibility nature towards KB cells.

Key words: Copper oxide nanoparticles, *Coleus aromaticus*, *in vitro* cytotoxicity, nasopharynx cancer cell lines

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Nowadays, copper oxide nanoparticles (CuO NPs) have attained extensive scientific interest because of their wide range of applications in nanotechnology and science such as giant magneto resistance materials, superconductors, gas sensors, lithography, nanocomposites and in lubricants[1-5]. The CuO NPs exhibit a band gap of 1.7 eV with p-type semiconducting nature. On the other hand, the CuO NPs are being used in textiles, wall paints, plastic materials for killing microbes and also used against fouling[6]. Because of their excellent properties, CuO NPs have been used in the production of pesticides[7].

Various synthetic approaches have been developed for the production of CuO NPs, which includes the reduction by using chemicals[8], microwave reactions[9], electrochemical methods[10] etc. On the other hand, the CuO NPs synthesized by chemical methods suffers from the adsorption of some toxic chemicals onto their surface, which makes them not useful in biological applications. Hence, the requirement for the new environmental friendly approaches for the synthesis of CuO NPs is increasing demand.

The green synthesis of metal nanoparticles such as Au and Ag by using plant extracts and bacteria have already been reported[11,12]. For instance, plant polyphenols of gum karaya[13], *Albizia lebbeck*[14] and *Aloe vera*[15] were found to be used for the green fabrication of CuO NPs. Although, very few synthetic reports were available for the green synthesis of CuO NPs, the production of CuO NPs using *Arbutus unedo* leaf extract is not reported till date.

In the present research study, it was used the extract of *Arbutus unedo* plant for the biofabrication of CuO Nps. The morphology and size of produced nanoparticles was studied using different characterization techniques. Further, it was also investigated the *in vitro* cytotoxicity of the biofabricated CuO NPs against KB cells derived from nasopharynx carcinoma.

MATERIALS AND METHODS

Materials: Copper sulfate ($CuSO_4$), dimethyl sulfoxide (DMSO), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent and other chemicals, solvents used were obtained from Sigma-Aldrich chemicals Ltd. All the experiments were performed at Department of Otolaryngology Head and Neck Surgery, The First Affiliated Hospital of Shenzhen University, Shenzhen city, China.

Preparation of *Arbutus unedo* extract: The leaves of the *Arbutus unedo* plant were obtained from plants located nearby The First Affiliated Hospital of Shenzhen University in Shenzhen city in the month of June, 2016 and later dried under sunlight. The plant extract was prepared by adding 0.5 g of *Arbutus unedo* dried leaf powder to 50 mL of deionized water in a beaker and allowed to heat at 80°C for about 1 h. Then, the resulting hot mixture was filtered through a filter study (0.22 μm cellulose nitrate) to get a transparent extract solution, which further used for fabrication of CuO NPs.

Preparation of CuO NPs: Twenty five millilitres of freshly prepared *Arbutus unedo* leaf extract was mixed to 5 mL of $CuSO_4$ (0.01M) aqueous solution and allowed for stirring for 10 min. Then the solution was then heated on water bath at 100°C for about 20 min. The color change of reaction solution to brown after heating signifying the formation of CuO NPs within 10 min.

***In vitro* cytotoxicity and MTT assay:** The *in vitro* cytotoxicity of the fabricated CuO NPs was examined by studying the cell viability of KB cell lines derived from nasopharynx carcinoma. Cytotoxicity was studied after incubation of KB cells with CuO NPs at various concentrations of 0.05, 0.1 and 0.2 mg mL^{-1} in DMEM medium. Instead, a control test was conducted without the addition of CuO NPs in a complete growth DMEM culture medium. Further, the cytotoxicity was examined using the MTT reduction assay. The MTT assay was performed using a standard experimental procedure in a 96-well plate with minor changes in experimental procedure. The cells were seeded at a concentration of about 1×10^4 cells/well in a 96-well plate at and incubated for 24 h at 37°C. Later the solution was washed and a fresh medium was added incubated at 37°C for 24 h. Later a 10 μL of MTT (5 mg mL^{-1} in PBS buffer) reagent was added into 90 μL medium into each well. Then about 100 μL of DMSO containing soluble formazan crystals was added and kept at 27°C for 20 min and absorbance of all the wells were measured using a ELISA plate reader at a wavelength of 560 nm.

Characterization: To analysis the morphology of the synthesized CuO NPs, 100 μL of colloidal solution was taken and was made upto 1 mL using deionized water and was sonicated using ultrasonic bath. A drop of it was placed on the Cu grid and was allowed to dry in vacuum. The NPs were visualized using JEOL JEM 2100 HR-TEM at an acceleration voltage of 200 kV. The XRD studies, were carried out within the scanning range of 2θ from 10-90° at a scanning rate of 4° min^{-1} and with a step size of 0.02° using Bruker D8

Advance diffractometer with Cu Kα radiation (λ = 1.54A°). The instrument was calibrated using lanthanum hexaboride (LaB_6). Average size of CuO NPs was determined by DLS using Horiba Scientific Nanoparticci, Nanoparticle Analyzer, SZ-100. Each sample was analyzed for 3 times at 20°C at a scattering angle of 170°C. Zeta potential was collected through electrophoretic light scattering at 25°C at a voltage of 150 V. The CuO NPs was pulverized and analyzed for biomolecular capping studies using JASCO-FT-IR spectroscopy instrument.

Statistical analysis: All of the cytotoxicity experiments were performed 3 times for each concentration. Absolute values of each experiment were converted into percentages. All data points corresponding to the concentration versus cytotoxicity plot were shown as the arithmetic mean percent inhibition compared to the control standard error. A one-way analysis of variance (ANOVA) was used to determine the statistically significant differences between the mean values. A value of $p<0.05$ was considered as significant.

RESULTS AND DISCUSSION

In this study, a water extract of *Arbutus unedo* leaf was used as reducing agent for the biosynthesis of CuO NPs. The CuO NPs formation was visually observed after 20 min with the color change of the reaction solution from dirty yellow to brown. A similar experiment was performed without adding the plant extract and the reaction solution was found to be same without any change after 3 days, indicating the role of *Arbutus unedo* extract during the synthesis of CuO NPs.

The primarily indication of CuO NPs formation was known by color change of the reaction solution from dirty yellow to brown within 10 min. Further, the purified CuO NPs were studied using TEM. From the Fig. 1a, it is found that the NPs are spherical in shape and polydispersed in nature with the average diameter of 50 nm. On the other hand, Fig. 1b has showed the crystalline nature of the CuO NPs with circular bright spots. Further, the elemental composition of the obtained CuO NPs is studied by EDS analysis. Figure 1c showed the presence of both copper (Cu) and oxygen (O) elements in highest percentage. These results are in similar agreement with the CuO NPs prepared by using other plant extracts[13-15]. All these results confirmed the formation of CuO nanoparticles.

Figure 2 represents the X-ray diffraction of the prepared CuO NPs. XRD pattern shows the presence of a number of strong diffraction peaks at with corresponding planes of (110), (111), (200), (202), (020), (202), (113), (311), (220) and

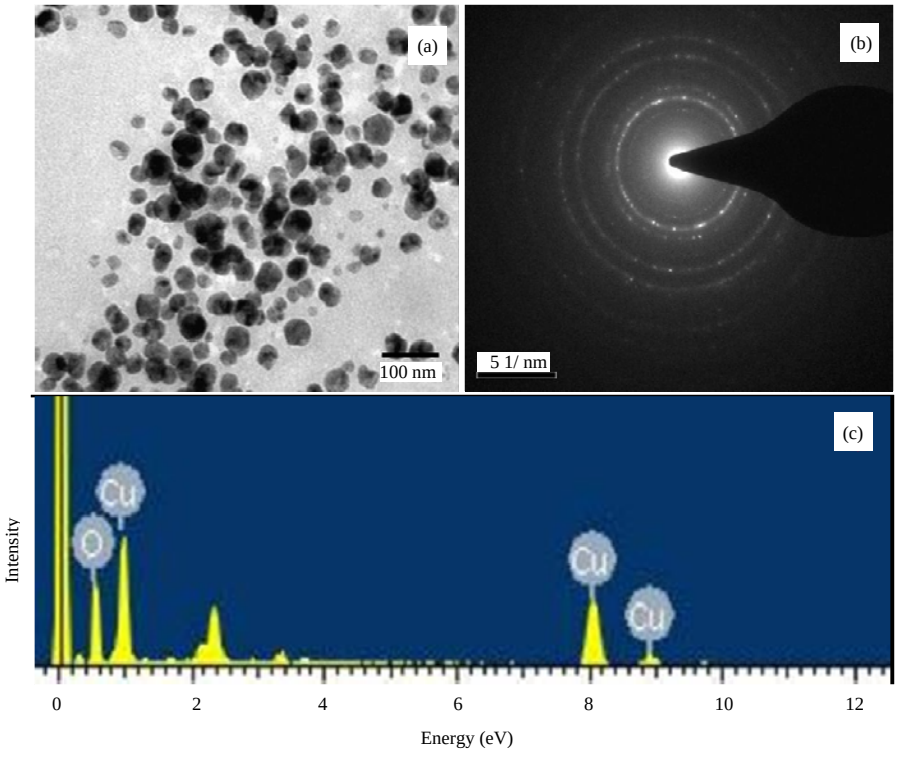

Fig. 1(a-c): (a) TEM image, (b) SAED pattern and (c) EDS spectrum of CuO Nps

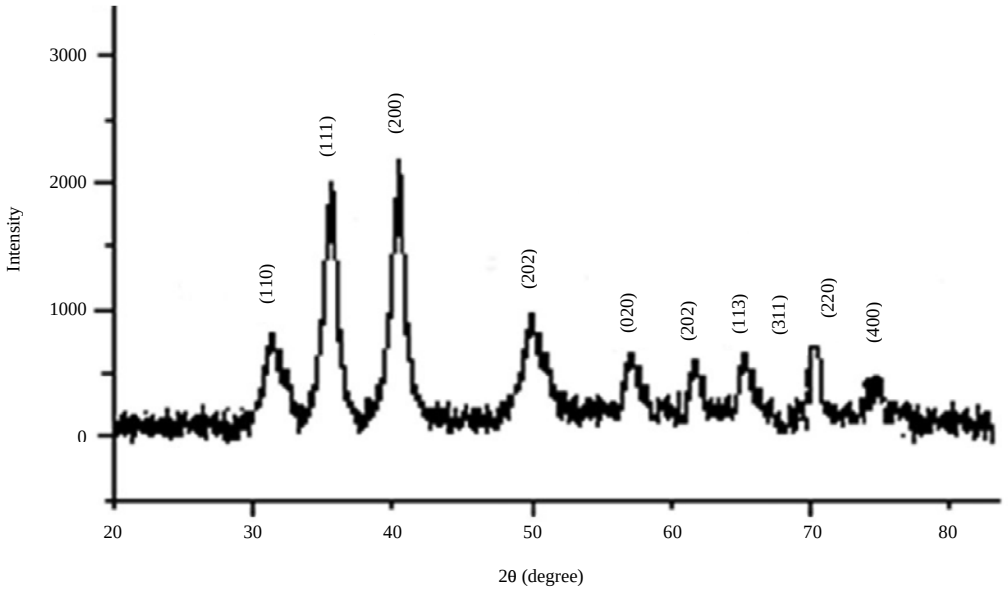

Fig. 2: XRD patterns of biosynthesized CuO NPs

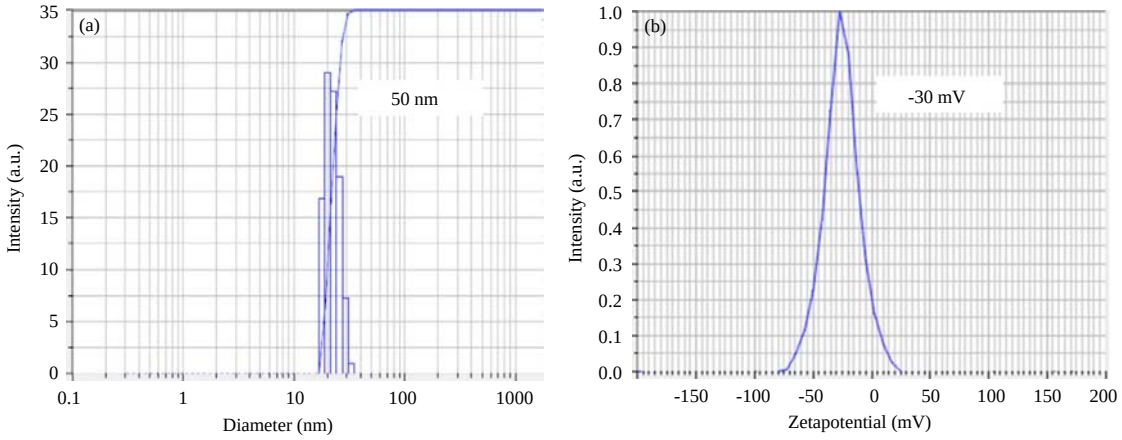

Fig. 3(a-b): (a) DLS and (b) Zeta potential of CuO NPs prepared by using *Arbutus unedo* extract

(400) represented the crystalline structure of the formed CuO NPs and lattice constants are in agreement with the Joint Committee on Powder Diffraction Standards (JCPDS No. 89-7102) database.

The size distribution and the average size of the prepared CuO NPs were known by DLS analysis. Figure 3 shows the DLS data of the prepared CuO NPs with an average diameter of 50 nm. On the other hand, the zeta potential of the NPs was found to be -30 mV. This negative zeta potential obtained was due to the capping of polyphenolic compounds found in the *Arbutus unedo* extract (also supported from FTIR results). This negative charge creates a strong repulsions between the neighboring NPs resulted in higher stability by preventing agglomeration.

FTIR analysis for both the plant extract and the prepared CuO NPs were performed to know the possible bio constituents accountable for the stabilization and reduction of CuO NPs. The FTIR corresponding to the plant extract and the prepared CuO NPs are represented in Fig. 4a and b. The *Arbutus unedo* extract showed vibrational bands corresponding to -OH groups at 3,400 cm^{-1} and bands at 1,625 cm^{-1} (olefinic band), 1,730 cm^{-1} (unsaturated ketone), 1,103 and 1,033 cm^{-1} (primary and secondary alcohols functionalities). Additionally, the bands at 1,400 and

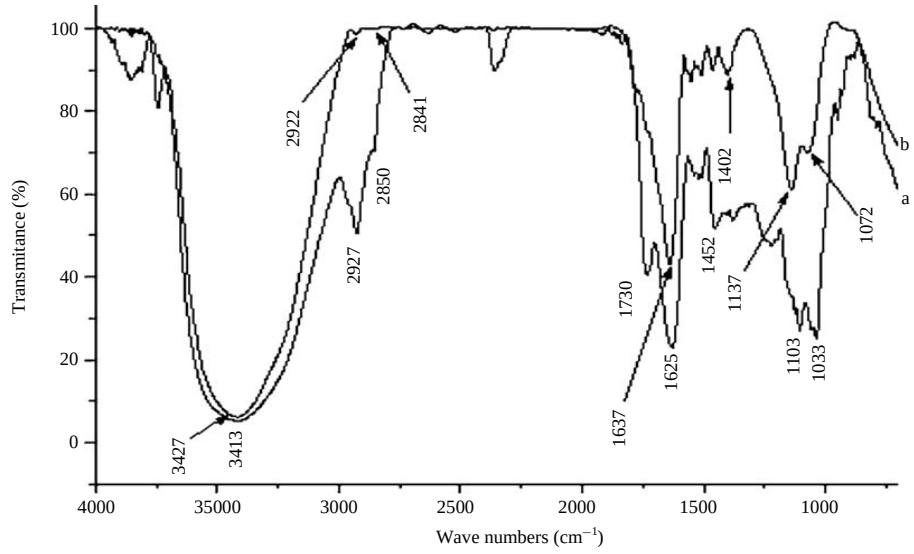

Fig. 4(a-b): FTIR spectrum of (a) Plant extract and (b) CuO NPs

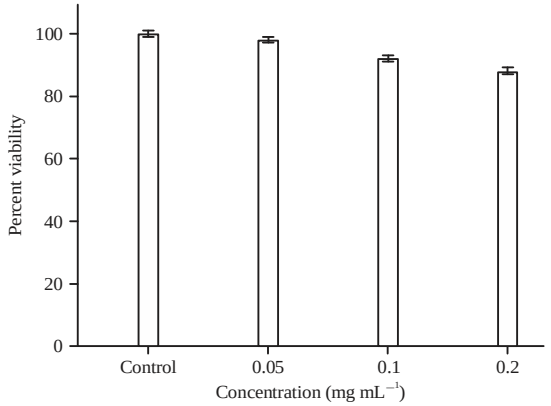

Fig. 5: Cell viability of KB cell lines induced by CuO NPs

3,000 cm^{-1} are corresponding to aliphatic C-H bending and stretching vibrational modes. On the other hand, the spectral lines of CuO NPs are similar to the spectrum of plant extract, indicating that the bio constituents present on CuO NPs are very close in composition to that of plant extract. It is also found that the vibrational peaks that are existed in the *Arbutus unedo* extract are found in the FTIR spectrum of CuO NPs with slightly changes in their position and intensity. The vibrational peaks of CuO NPs existed at 1,625 (C=C), 3,413 (O-H), 1,103 and 1,033 cm^{-1} (C-O) founding plant extract became narrow and moved to higher frequency regions. Similarly, the bands at 3,000 (C-H stretching) and 1,400 cm^{-1} (C-H bending) are moved to lower frequency regions with decreased peak intensity. However, similar kind of vibrational peaks were found in the nanoparticles synthesized by using plant extracts of *Terminalia arjuna* bark[11] and *Mimusops elengi* seeds[12].

The cytotoxicity of the fabricated CuO NPs is studied by using an MTT cell viability assay. The CuO NPs of various concentrations such as 0.05, 0.1 and 0.2 mg mL^{-1} were added to KB cell lines obtained from nasopharynx carcinoma and incubated. After about 24 h of incubation, the cell viabilities of KB cells are calculated to be higher than 85% for at the concentrations of CuO NPs (Fig. 5), indicating the non-toxic nature of fabricated CuO NPs with higher biocompatibility. On the other hand, the biocompatible nature of diastase-stabilized AgNPs towards mouse fibroblast (3T3) cell lines has already been reported[16]. The biocompatible nature of the fabricated CuO NPs is because of the capped biomolecules that exist on the surface of CuO NPs.

CONCLUSION

The study reported a low-cost, facile, eco-friendly strategy for the biofabrication of CuO NPs using *Arbutus unedo* extract. The FTIR analysis revealed the existence of biomolecules present in *Arbutus unedo* extract onto the CuO NPs surface. Further, TEM and DLS analysis confirmed the formation of spherical CuO NPs with average size of 30 nm. Additionally, *Arbutus unedo* extract mediated CuO NPs have exhibited good biocompatibility, making them useful as cell labeling agents.

SIGNIFICANCE STATEMENT

This study discovers the use of *Arbutus unedo* extract for the synthesis of CuO NPs and investigating their *in vitro*

cytotoxicity against KB cells that can be beneficial for the researchers studying on cancer treatment. This study will also help the researcher to uncover the critical areas of the use of nanomaterials in cancer therapy that many researchers were not able to explore.

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