

Original Research Article

Green synthesis of CuO nanoparticles using *Cassia auriculata* leaf extract and in vitro evaluation of their biocompatibility with rheumatoid arthritis macrophages (RAW 264.7)

Long-Bao Shi, Pei-Fu Tang*, Wei Zhang, Yan-Peng Zhao, Li-Cheng Zhang and Hao Zhang

Department of Orthopedic Surgery, The General Hospital of The People's Liberation Army, Beijing 100853, China

*For correspondence: **Email:** peifutang@hotmail.com

Received: 3 September 2016

Revised accepted: 12 December 2016

Abstract

Purpose: To undertake green synthesis of copper oxide nanoparticles (CuO NPs) using *Cassia auriculata* leaf extract and evaluate their biocompatibility with rheumatoid arthritis macrophages (RAW 264.7 cell line).

Methods: CuO NPs were prepared by heating a mixture of 10 mL of 0.01 M CuSO₄ solution and 30 mL of *C. auriculata* extract at 80 °C for 1 h. The synthesized CuO NPs were characterized by x-ray diffraction (XRD), Fourier-transform infrared spectroscopy (FTIR), ultraviolet-visible spectroscopy (UV-Vis), energy-dispersive x-ray spectroscopy (EDS), scanning electron microscopy (SEM), transmission electron microscopy (TEM) and dynamic light scattering (DLS). The cytotoxicity of the NPs against RAW 264.7 cells was studied using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Results: The gradual change in color of the reaction solution from brownish yellow to dark brown indicated CuO NP formation. TEM images revealed spherical, polydispersed NPs (mean particle size, 23 nm). FTIR results indicated capping of polyphenols on the surface of the NPs. Most RAW 264.7 cells (> 95 %) remained alive following exposure to CuO NPs at concentrations of up to 200 µg/mL, indicating biocompatible with the cells.

Conclusion: An eco-friendly, low-cost, biosynthetic method for CuO NP preparation has been successfully developed using *C. auriculata* leaf extract. Furthermore, the nanoparticles are biocompatible with RAW 264.7 cell line.

Keywords: *Cassia auriculata* extract, Copper oxide nanoparticles, RAW 264.7 cell line, Rheumatoid arthritis

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Copper oxide nanoparticles (CuO NPs) have attracted significant interest due to their wide variety of applications, including in catalysts, gas sensors, high-T_c superconductors, giant magnet resistance materials, solar energy devices, and

in the preparation of organic-inorganic nanocomposites [1-5]. Additionally, CuO is used as an antifungal, antibiotic and antimicrobial agent when introduced into textiles and coatings [6].

Moreover, copper-related materials have demonstrated proficient biocidal properties, such

as those found in pesticide formulations [7]. Several methods are available for CuO NP preparation, namely, microwave irradiation [8], and sonochemical [9], electrochemical [10] and chemical reduction [11]. However, chemical synthesis of CuO leads to the adsorption of toxic chemicals on its surface, which may have harmful effects when used in biomedical applications. Hence, an environmentally friendly, cost-effective approach is required for CuO NP synthesis. Recently, biosynthesis of nanomaterials using green reducing agents and plants has been reported [12,13]. For example, nanomaterial biosynthesis with extracts of *Albizia lebbbeck* [14], gum karaya [15], *Aloe vera* [16], and algae [17] has been demonstrated.

The aim of the present work was to synthesize CuO NPs using dried clove extract of *Cassia auriculata* leaves and evaluate their *in vitro* cytotoxicity against RAW 264.7 cell line in order to ascertain if they are biocompatible with the cells.

EXPERIMENTAL

Materials

Copper sulfate (CuSO_4), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), potassium bromide (KBr), and other solvents were purchased from Sigma Aldrich Chemical Ltd., Shanghai, China.

Preparation of plant extract

Leaves of *Cassia auriculata* were collected from a plant located near the General Hospital of the People's Liberation Army (No. 28, Fuxing Road, Haiding District, Beijing 100853, China). Approximately 2 g of dried *C. auriculata* leaves were added to 30 mL of double-distilled water and heated in a water bath for about 2 h at 90°C. The solution was filtered to obtain a clear solution of *C. auriculata* extract.

Synthesis of CuO nanoparticles

An amount (10 mL) of 0.01 M CuSO_4 solution was added to 30 mL of aqueous *C. auriculata* extract and mixed well with mechanical shaking. The solution was then heated in a water bath at 80 °C for 1 h. The gradual change of color of the reaction solution from brownish yellow to dark brown indicated the formation of CuO NPs.

In vitro cytotoxicity test

RAW 264.7 cells procured from the American Type Culture Collection were initially cultured in RPMI1640 medium containing 1% (w/v) penicillin–streptomycin and 10% (v/v) fetal bovine serum (FBS) at 37°C in a 5 % CO_2 atmosphere. For cytotoxicity evaluation, the cells were initially seeded at 1×10^4 cells per well in 96-well titer plates. Cell growth proceeded for 24 h. After two cycles of washing with phosphate buffer solution (PBS; pH: 7.4), various concentrations of CuO NPs were added to each well and incubated for 24 h. After the incubation period, the cells were washed twice with PBS, followed by the addition of fresh culture medium. About 20 μL of MTT reagent (5 mg/mL in PBS) was then added to each well and further incubated for 4 h at 37 °C. The medium in each well was then discarded and DMSO was added to dissolve the cells. The optical density of each well was measured at 570 nm using a microplate reader.

Characterization

XRD analysis was performed using a Bruker D8 Advance diffractometer with $\text{Cu-K}\alpha$ radiation ($\lambda = 1.54 \text{ \AA}$) and a step size of 0.02° over the range from $10\text{--}80^\circ$ at a scan rate of about $4^\circ/\text{min}$. FTIR analysis of the prepared CuO NPs was carried out with a Jasco system to evaluate surface capping. A purified sample was dried and mixed well with KBr powder for pellet preparation for FTIR measurements. The surface morphology and size of the synthesized CuO NPs were analyzed using a high-resolution transmission electron microscope (JEOL JEM 2100 HR-TEM). The sample for TEM analysis was prepared by placing a diluted CuO nanocolloid on the copper grid surface, which was later dried under a vacuum. Additionally, the surface charge and size

distribution of the CuO NPs were examined using a Horiba Scientific SZ-100 Nanoparticle Analyzer. Samples for zeta potential analysis were prepared by diluting the CuO NPs with double-distilled water.

Statistical analysis

The results are presented as mean \pm standard deviation (SD). Statistical and analytical comparisons of the data were conducted using Student's t-test or one-way analysis of variance (ANOVA). $P < 0.05$ was considered to indicate statistical significance.

RESULTS

The UV-Vis spectra of aqueous dispersed CuO exhibited a strong surface plasmon resonance (SPR) absorption band at 285 nm, signifying the formation of CuO NPs. No optical absorption peak was observed in the UV-Vis spectra of the plant extract (Figure 1).

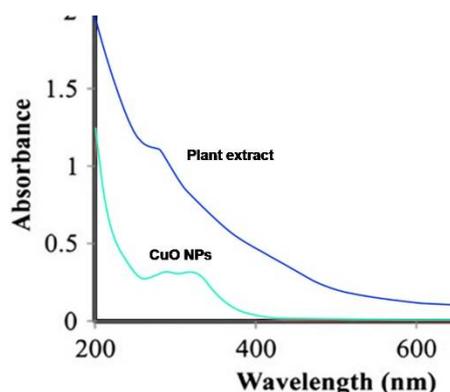


Figure 1: Ultraviolet-visible absorption spectrum of the synthesized CuO nanoparticles (CuO NPs)

The characteristic XRD peaks observed for CuO NPs prepared using *C. auriculata* extract are shown in Figure 2. Intense diffraction peaks at 32.54° , 35.56° , 38.77° , 48.74° , 53.53° , 58.37° , 61.56° , 66.29° and 68.17° , corresponding to 110, 0 02, 111, 202, 020, 202,113, 311 and 113 planes, respectively, indicated the formation of a typical monoclinic CuO NP structure without impurities. Sharp, well-defined CuO reflections in the XRD pattern confirmed the highly crystalline nature of the as-prepared CuO NPs (JCPDS card no. 801268).

Figure 3 shows field-emission SEM (FE-SEM) and high-resolution TEM (HRTEM) images of the biosynthesized CuO NPs; spherical CuO NP formation was revealed. The average particle size as indicated by TEM was 23.17 nm, in good agreement with the DLS results. The EDS spectrum of the CuO NPs showed the presence of O and Cu peaks only; there was no evidence of impurity peaks (Shown in Figure 4).

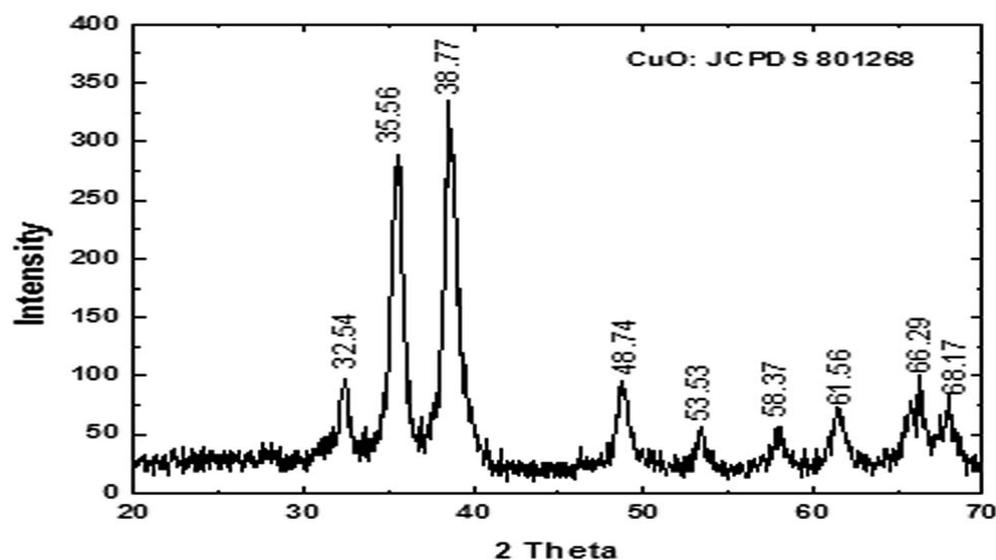


Figure 2: X-ray diffraction pattern of *Cassia auriculata* extract-mediated synthesized CuO NPs

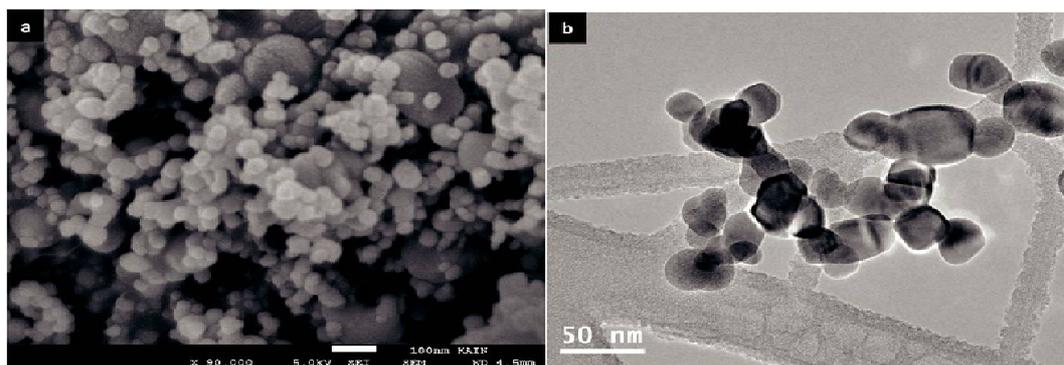


Figure 3: Field-emission scanning electron microscopy (a) and high-resolution transmission electron microscopy image (b) of *Cassia auriculata* extract-mediated synthesized CuO NPs

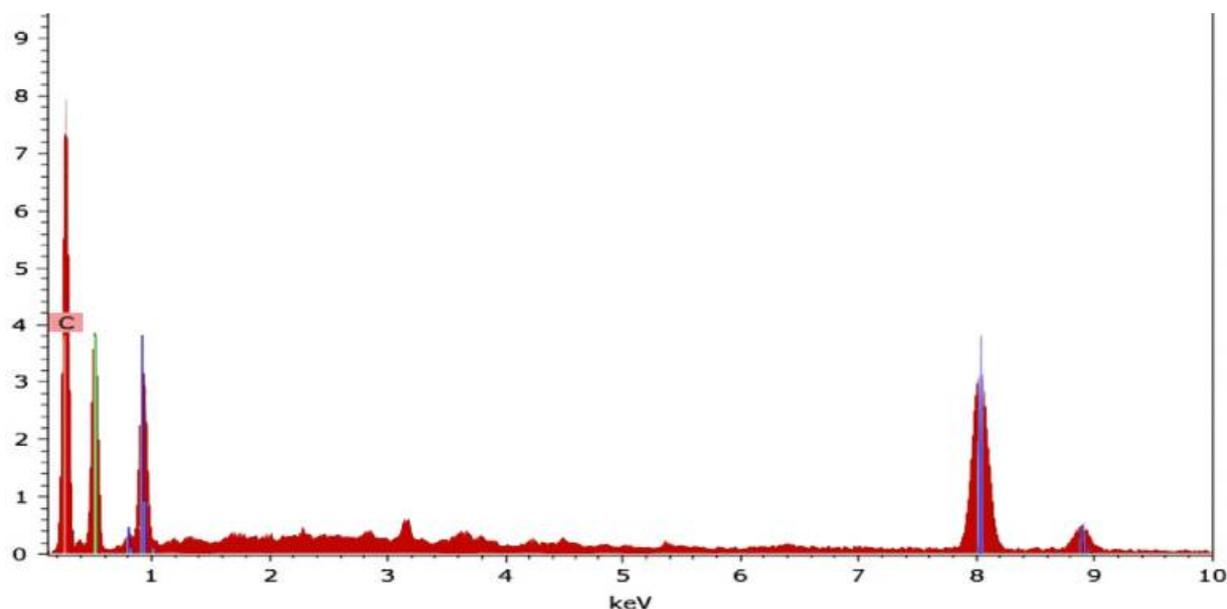


Figure 4: Energy-dispersive X-ray spectroscopy spectrum of *Cassia auriculata* extract-mediated synthesized CuO NPs

Additionally, DLS analysis was carried out to determine the size distribution of the formed CuO NPs. From Fig. 5A, the average particle size of the CuO NPs was 22.4 nm. Zeta potential analysis showed a negative surface zeta potential of -25.4 mV (Fig. 5B). It is well known that the surface charge and size distribution play key roles in the biological activity of prepared NPs.

Figure 5 shows the presence of bands in the FTIR spectrum of CuO NPs at 3385 , 1612 , and 1033 cm^{-1} corresponding to hydroxyl group (-OH) stretching, hydroxyl (-OH) bending, and C-O stretching, respectively. Additionally, the presence of bands at 2922 and 1720 cm^{-1} is associated with C-H stretching and ketone group

vibrations. The bands at 600 and 668 cm^{-1} are related to Cu-O absorption.

DISCUSSION

The formation of CuO NPs was indicated by the presence of a dark brown precipitate in the bottom of the flask. The *Cassia auriculata* extract filtrate containing 0.5 M CuSO_4 solution began to change color 30 min into the reaction and turned completely dark brown after 1 h. A control experiment performed without the addition of plant extract did not show color change or evidence of CuO NP formation.

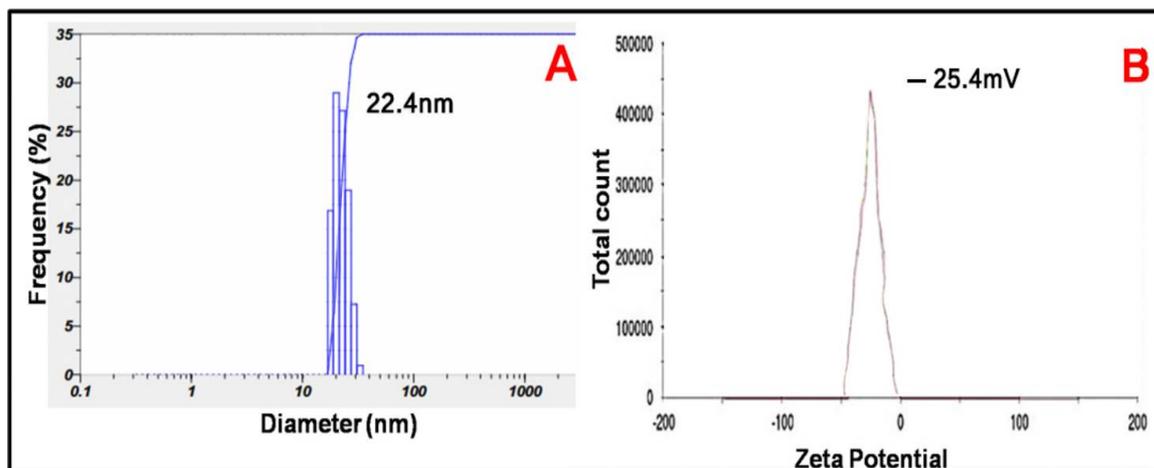


Figure 5: Dynamic light scattering (A) and zeta potential (B) analysis of *Cassia auriculata* extract-mediated synthesized CuO NPs

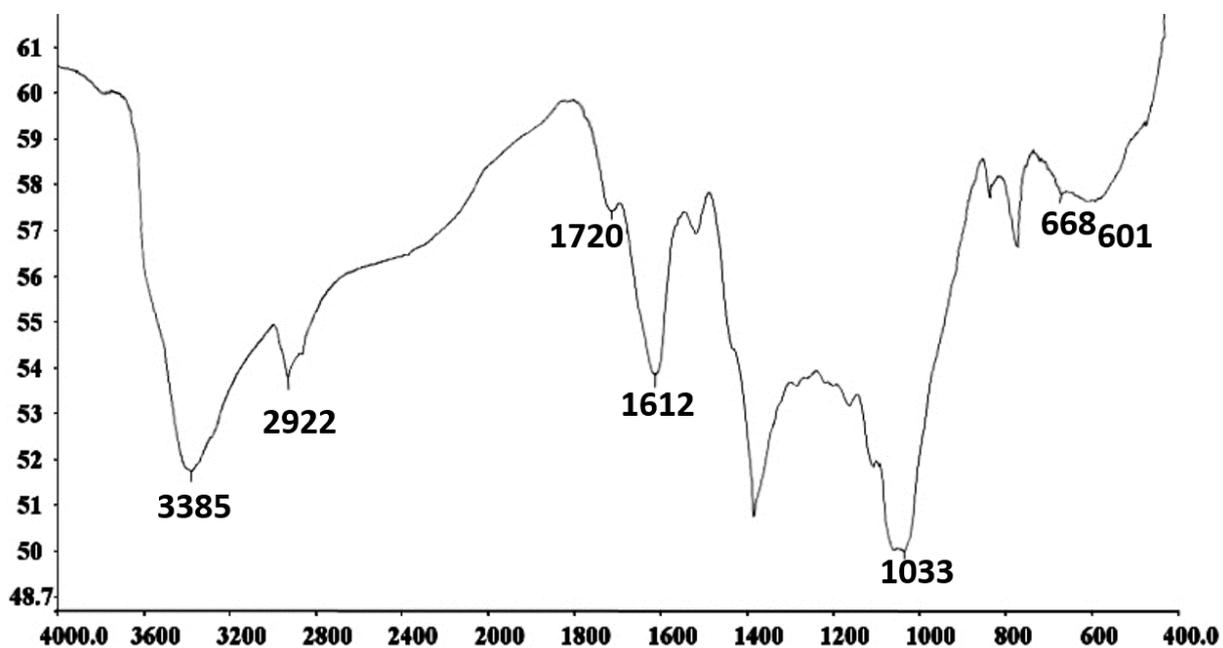


Figure 6: Fourier-transform infrared spectrum of *Cassia auriculata* extract-synthesized CuO NPs

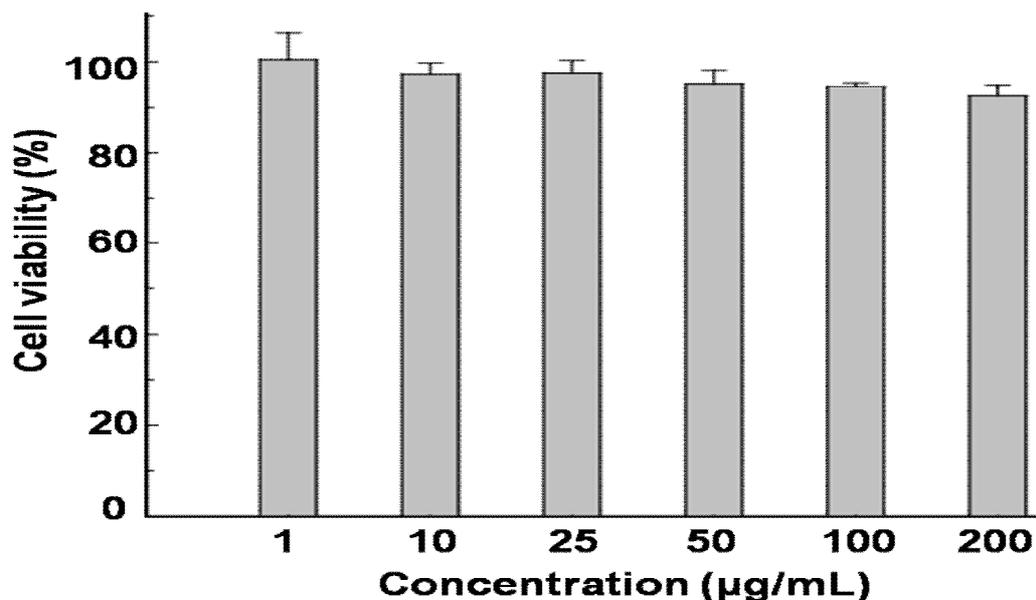


Figure 7: *In vitro* cytotoxicity of *Cassia auriculata* extract-mediated synthesized CuO NPs; the error bars in the graph represent standard deviation (n = 3)

The UV-Vis absorption spectrum confirmed CuO NP formation, showing a characteristic SPR band at 285 nm. SPR in the CuO NPs was attributed to the collective oscillation of free surface conduction electrons, excited by the incident electromagnetic radiation. XRD, EDS, TEM analyses indicated the crystalline nature of the plant extract-mediated synthesized CuO NPs. The EDS spectrum confirmed the presence of copper and oxygen; smaller peaks were attributed to biomolecules present in the extract solution. These results are similar to those of previous studies in which green synthesized CuO NPs were prepared using sublimated precursors [18,19].

Furthermore, the zeta potential of the as-synthesized CuO NPs indicated a negative surface charge, which may have been due to the stabilization of the biomolecular constituents of *Cassia auriculata* extract on the surface of the formed NPs after the reduction process. The negative surface charge of the synthesized CuO NPs further leads to the generation of strong electrostatic repulsion forces among the NPs; this stabilizes the NPs by reducing their aggregation. Notably, the negative surface charge of NPs fabricated using plant extracts has been reported in the literature [20].

The FTIR spectrum of the CuO NPs showed the existence of C-O and hydroxyl functionalities on the NP surface. The FTIR band present at 1720 cm^{-1} corresponded to ketone functional groups of oxidized polyphenols in the

extract of *Cassia auriculata*, signifying that the synthesized CuO NPs are capped by oxidized polyphenols. Additionally, the stretching bands at 600 and 668 cm^{-1} are related to Cu-O absorption, further indicating CuO NP formation. These results indicate that the synthesized CuO NPs are stabilized by biomolecular constituents present in the extract of *Cassia auriculata*.

Macrophages play an important role in rheumatoid arthritis. Synovial fibroblasts along with macrophages are involved in the production of a number of matrix-degrading enzymes, chemokines, and cytokines that mediate the interaction with endothelial and inflammatory cells. Subsequently, they are accountable for the successive destruction of bone and articular cartilage, taking into consideration the fact that synovial fibroblasts and macrophages mediate the most significant pathway of joint destruction. Hence, these macrophages would be important targets in current therapeutic approaches to reducing the destruction of bone and cartilage in rheumatoid arthritis [21,22]. In this regard, we have studied the cell viability of RAW 264.7 cell lines after their

treatment with CuO NPs to examine the ability of the prepared NPs for drug delivery during the treatment of rheumatoid arthritis.

To study the cytotoxicity of prepared CuO NPs, RAW 264.7 macrophage cell lines were incubated with different concentrations of NPs. Most of the cells (> 95 %) remained alive after exposure to a CuO NP concentration of up to 200 µg/mL, signifying their low cytotoxicity. Thus, CuO NPs prepared using *C. auriculata* extract may be suitable as a drug delivery vehicle for anti-rheumatic agents for rheumatoid arthritis treatment.

CONCLUSION

An eco-friendly, low-cost biosynthetic method for the preparation of CuO NPs using *Cassia auriculata* leaf extract is presented in this work. The *Cassia auriculata* extract-mediated CuO NPs exhibit very low cytotoxicity towards RAW 264.7 cell lines, thus indicating their biocompatibility with the cells and potential development rheumatoid arthritis therapy.

ACKNOWLEDGEMENT

The authors acknowledge the help of The General Hospital of the People's Liberation Army in providing the funds and lab facilities for this research.

REFERENCES

- Li L, Liang J, Tao Z, Chen J. CuO particles and plates: synthesis and gas-sensor application. *Mater Res Bull* 2008; 43; 2380–2385.
- Kruis FE, Fissan H, Peled A. Synthesis of nanoparticles in the gas phase for electronic, optical and magnetic applications—a review. *J Aerosol Sci* 1998; 29; 511–535.
- Cheon J, Lee J, Kim J. Inkjet printing using copper nanoparticles synthesized by electrolysis. *Thin Solid Film* 2012; 520; 2639–2643.
- Tarasov S, Kolubaev A, Belyaev S, Lerner M, Tepper F. Study of friction reduction by nano copper additives to motor oil. *Wear* 2002; 252; 63–69.
- Baik NS, Sakai G, Miura N, Yamazoe N. Preparation of stabilized nanosized tin oxide particles by hydrothermal treatment. *J Am Ceram Soc* 2000; 83; 2983–2987.
- Abramov OV, Gedanken A, Koltypin Y, Perkas N, Perelshtein I, Joyce E, Mason TJ. Pilot scale sonochemical coating of nanoparticles onto textiles to produce biocidal fabrics. *Surf Coat Technol* 2009; 204; 718–722.
- DeOliveira-Filho EC, Lopes RM, Paumgarten FJR. Comparative study on the susceptibility of freshwater species to copper based pesticides. *Chemosphere* 2004; 56; 369–374.
- Zhao Y, Zhu JJ, Hong JM, Bian N, Chen HY. Microwave induced polyol-process synthesis of copper and copper oxidenanocrystals with controllable morphology. *Eur J Inorg Chem* 2004; 20; 4072–4080.
- Wongpisutpaisan N, Charoonsuk P, Vittayakorn N, Pecharapa W. Sonochemical Synthesis and Characterization of Copper Oxide Nanoparticles. *Energy Procedia* 2011; 9; 404 – 409.
- Mandke MV and Pathan HM. Electrochemical growth of copper nanoparticles: structural and optical properties. *J Electroanal Chem* 2012; 686; 19–24.
- Avnish kumar A and ritu. Synthesis of nanosized copper particles: a simple and effective method. *Int. J. Chem. Sci.* 2013; 11; 1270-1276.
- Augustine R, Kalarikkal N, Thomas S. A facile and rapid method for the black pepper leaf mediated green synthesis of silver nanoparticles and the antimicrobial study. *Appl Nanosci* 2014; 4; 809–818.
- Tsibakhashvil N, Kalabegishvili T, Gabunia V et al. Synthesis of silver nanoparticles using bacteria. *Nano Stud* 2010; 2; 179–182.
- Jayakumarai G, Gokulpriya C, Sudhapriya R, Sharmila G, Muthukumaran C. Phytofabrication and characterization of monodisperse copperoxide nanoparticles using *Albizia lebbek* leaf extract. *Appl Nanosci* 2015; 5; 1017–1021.
- Thekkae Padil V, Cernik M. Green synthesis of copper oxide nanoparticles using gum karaya as a biotemplate and their antibacterial application. *Int. J. Nanomed.* 2013; 8; 889-898.
- Vijay Kumar PPN, Shameem U, Pratap K, Kalyani RL and Pammi SVN. Green Synthesis of Copper Oxide Nanoparticles Using *Aloe vera* Leaf Extract and Its Antibacterial Activity against Fish Bacterial Pathogens. *Bio Nano Sci.* 2015; 5; 135-139.
- Abboud Y, Saffaj T, Chagraoui A, El Bouari A, Brouzi K, Tanane O, Ihssane B. Biosynthesis, characterization and antimicrobial activity of copper oxide nanoparticles (CONPs) produced using brown alga extract (*Bifurcaria bifurcata*). *Appl Nanosci* 2014; 4; 571–576.
- Soofivand F, Salavati-Niasari M, Novel solvent-less synthesis of CuO nanoparticles by using sublimated precursors. *Mater Lett* 2013; 106; 83–86.
- Hame S, Sharma A, Dhaygude M, Joglekar S, Kodam K, Hudlikar M. Novel route for rapid biosynthesis of copper nanoparticles using aqueous extract of *Calotropis procera* L latex and their cytotoxicity on tumor cells. *Colloids Surf B* 2012; 15; 284–288.
- Susmila AG, Kotakadi VS, Sai Gopal DVR, Subba Rao Y, Varada Reddy A. Efficient and robust biofabrication of silver nanoparticles by *cassia alata* leaf extract and their antimicrobial activity. *J Nanostruct Chem* 2014; 4; 82.

21. Huber L, Distler O, Tarnier I, Gay R, Gay S, Pap T, et al. Synovial fibroblasts: keyplayers in rheumatoid arthritis. *Rheumatology* 2006; 45; 669–675.
22. Firestein GS. Invasive fibroblast-like synoviocytes in rheumatoid arthritis. Passive responders or transformed aggressors? *Arthritis Rheum.* 1996; 39; 1781–1790.