Biocidal activity of CuO nanoleaves against AGS cells, *S. Aureus, E. Coli,* and *Micrococcus*

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Actividad biocida de nanohojuelas de CuO contra células AGS, E. Aureus, E. Coli y Micrococos

Activitat biocida de nanofulletes de CuO contra cèl·lules AGS, E. Aureus, E. Coli i Micrococos

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SUMMARY

In the present work, we carried out the synthesis of copper oxide nanoleaves and evaluate their biocidal activity. The resulting nanoparticles were characterized by X-ray Diffraction, Scanning Electron Microscopy, and Fourier Transform Infrared Spectroscopy. The antibacterial activity of the CuO nanoleaves particles was studied through the minimum inhibitory concentration method employing *Staphylococcus aureus*, *Escherichia coli*, and *Micrococcus* as model organisms. The results indicate that a concentration of 0.05 g mL^{-1} generate an inhibition zone of 20, 17 and 12 mm for *S. aureus*, *E. Coli*, and *Micrococcus,* respectively. Also, the copper oxide nanoleaves exhibited antitumor activity against human adenocarcinoma cells.

Key words: Copper-oxide; nanoleaves; antitumor; antibacterial; biocidal activity

RESUMEN

En el presente trabajo, llevamos a cabo la síntesis de nanohojuelas de óxido de cobre y evaluamos su actividad biocida. Las nanopartículas obtenidas fueron caracterizadas por Difracción de Rayos X, Microscopía Electrónica de Barrido y Espectroscopía Infrarroja por Transformada de Fourier. La actividad antibacteriana de las nanohojuelas de CuO se estudió mediante el método de concentración mínima inhibitoria empleando *Estafilococo Aureus, Escherichia coli* y *Micrococos* como organismos modelo. Los resultados indican que una concentración de 0.05 g mL^{-1} genera una zona de inhibición de 20, 17 y 12 mm para *E. Aureus, E. Coli* y *Micrococos,* respectivamente. Además, las nanohojuelas mostraron actividad antitumoral contra células de Adenocarcinoma humano.

Palabras clave: óxido de cobre, nanohojuelas, antitumoral; antibacterial; actividad biocida.

RESUM

En el present treball, duem a terme la síntesi de nanofulletes d'òxid de coure i avaluem la seva activitat biocida. Les nanopartícules obtingudes van ser caracteritzades per Difracció de Raigs X, microscòpia electrònica de rastreig i Espectroscòpia Infraroja per Transformada de Fourier. L'activitat antibacteriana de les nanofulletes de CuO es va estudiar mitjançant el mètode de concentració mínima inhibitòria emprant Estafilococo Aureus, Escherichia coli i Micrococos com organismes model. Els resultats indiquen que una concentració de 0.05 g mL-1 genera una zona d'inhibició de 20, 17 i 12 mm per E. Aureus, E. Coli i Micrococos, respectivament. A més, les nanofulletes van mostrar activitat antitumoral contra cèl·lules d'Adenocarcinoma humà.

Paraules clau: Òxid de coure; nanofulletes; antitumoral; antibacterià; activitat biocida.

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INTRODUCTION

Nowadays, some harmful bacteria are becoming resistant to the available antibiotics. For example, *Staphylococcus* aureus now is resistant to multiple clinically important antimicrobial classes, but in the past, the infections caused by these bacteria were usually controlled by penicillin[1]. Thus, there is a global concern about the emergence of new strains of resistant bacteria. Therefore, the development of new methods focused to eliminate microbial contaminations is a topic of great importance[2]. In this sense, inorganic nanoparticles have attracted considerable interest as effective antimicrobial agents. Additionally, these nanostructures are stable, safe, and less toxic, in comparison to the traditional organic antibiotics[3]. Also, the development of these inorganic antimicrobial agents is advantageous since the pathogens are developing resistance against traditional organic antibiotics[4]. Also, these nanoparticles are comparatively more economical and stable than the conventional antibiotics[5,6]. Thus, the use of this kind of nanostructures with biocidal properties are emerging as new and promising area[7-10]. In this sense, the biological activity exhibited by CuO nanostructures has attracted a great interest due to their large surface to volume ratio and crystallographic surface structure, which increase their antimicrobial activity[11]. These nanostructures have been employed to sterilize foods, liquids, medical instruments, human tissues, among others[2, 12-17]. Also, CuO nanoparticles are stable, robust and have a longer shelf life compared to common organic antimicrobial agents[18, 19]. Thus, it is possible to find in the literature many works focused to synthesize copper oxide nanostructures. These nanostructures have been prepared hydrothermally[20], through microwave synthesis[21], by precipitation without employing any surfactant[22] and with surfactant[23], calcination[24], reverse microemulsion[25], electrochemical[26], chemical[27], combustion[28], template[29], laser ablation[30], and sol–gel synthesis[16] methods. However, the sol-gel method is considered as the simplest technique and has the ability to control the morphology and size[16]. Thus, considering the importance of the biocidal activity of CuO nanoparticles in different fields[31-35], in the present work, we carried out the synthesis of copper oxide nanoleaves by the precipitation method, and evaluate the biological activity of these copper oxide nanoparticles in order to gain a deeper insight into this system. We synthesized CuO nanoleaves by the economical sol gel technique and characterized the nanostructures by X-ray Diffraction measurements (XRD), Scanning Electron Microscopy (SEM), and Fourier Transform Infrared Spectroscopy (FT-IR). The biological activity of these CuO nanoparticles was analyzed against one kind of cancer cell, human adenocarcinoma (AGS) cells. Also, we tested the CuO nanoparticles against *Staphylococcus aureus*, *Escherichia coli*, and *Micrococcus* bacteria.

EXPERIMENTAL

Synthesis of CuO Nanoparticles

Cuo nanoparticles were synthesized following a similar methodology to the reported by Arora et al[36]. All the chemical reagents used in our experiments were of analytical grade and used as received without purification. 1.5 M NH_4OH was added dropwise to 0.5 M $Cu(NO_3)_2{\cdot}6H_2O$ solution to precipitate the copper as a light-blue hydroxide gel. The molar ratio of NH_4OH:Cu was (2.5:3). The hydrated precipitated gel was filtered and washed several times with deionized water until the pH = 7. The precipitated gel was transferred to a flask with a minimum amount of water (~5 mL) and fitted with a water condenser. The precipitated gel was stirred under reflux for 6 h at 100 °C. The color of the gel changed from light-blue to splendid black in reflux during which copper nano oxide was formed. The solid product, after refluxing, was filtered and washed with ammonium hydroxide and then deionized water several times. The obtained solid product was dried at 100°C overnight in an oven.

Characterization

XRD patterns were recorded by a Bruker D8-Advance Diffractometer using Cu Kα radiation (λ =1.5406A). FT-IR spectra were recorded on a Bruker spectrophotometer in KBr pellets. The surface morphology of the product was characterized by a LEO-1430.VP scanning electronic microscope with an accelerating voltage of 15 kV.

Cell culture

AGS cells were grown at 37 °C in an atmosphere containing 5% CO_2, with RPMI-1640 MEDIUM HEPES modified with L-glutamine and 25 mM HEPES (SIGMA-ALDRICH CHEMIE GmbH), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco), 2.7% sodium bicarbonate, and 500 mg/L ampicillin.

In Vitro Activities

The CuO nanoparticles were assayed for cytotoxicity *in vitro* against AGS cells. The cell lines were provided by the Pasteur Institute Laboratory of Natural and Biomimetics in Iran. The procedure for cytotoxicity studies is similar to that reported earlier[37]. Briefly, to calculate the concentration of each drug that produces a 50% inhibition of cell growth (IC_{50}), 190 mL of cell suspension (5×10^4 cell mL^{-1}) were exposed to various concentrations of the compound dissolved in sterile DMSO. The final concentration of DMSO in the growth medium was 2% (v/v) or lower, concentrations that do not affect cell replication[38, 39]. After incubation periods of 72 h for all cell lines, the cell concentrations were analyzed in both the control and drug-treated cultures. All experiments were carried out six times and in series.

RESULTS AND DISCUSSION

Characterization of structure and morphology

The reaction between copper (II) nitrate and NH_4OH to form crystalline copper oxide is as follows: The overall process involves three steps: formation of copper hydroxide, formation of copper oxyhydroxide polymer chains by oxidation of copper hydroxide via O_2 dissolved in air, and decomposition of this polymer into nano crystallite copper oxide[40-52].

$$Cu^{2+} + 2OH^- \longrightarrow (Cu(OH)_2 \quad (1)$$

$$3Cu(OH)_2 + 1/2O_2 \rightarrow Cu(OH)_2 + 2CuOOH + H_2O \quad (2)$$

$$Cu(OH)_2 + 2CuOOH \longrightarrow 3CuO + 2H_2O + 1/2O_2 \quad (3)$$

The yield percentage of CuO nanoparticles obtained by this method is aprox. 75%. Room temperature powder XRD for the product was performed to identify the crystalline phase present in the sample and is shown in Figure 1. The peaks in the XRD pattern (Fig. 1) correspond to the CuO phase (JCPDS-ICDD standard card no. 45-0937). However, the peaks observed at 33.5° and 51.5° corresponds to the precursor $Cu(OH)_2$. All CuO diffraction peaks can be readily indexed to a pure monoclinic (Tenorite) structure (space group: $C_{2/c}$)[53] of CuO with lattice parameters (a = 4.688 Å, b = 3.422 Å, c = 5.131 Å, and β = 99.506°) that are close to the reported values (ICDD card no. 48-1548). No diffraction peaks arose from the phases Cu_2O, Cu, and $Cu(NO_3)_2$, etc. The broadening of the peaks indicated that the particles were in the nanometer range[54]. The average crystallite size (D_c) of the CuO nanocrystals was calculated using the Debye-Scherrer Equation (4) from the major CuO diffraction peaks:

$$D_c = \frac{K\lambda}{\beta \cos\theta} \quad (4)$$

where *K* is a constant equal to 0.9, λ is the X-ray wavelength (0.15405 nm), β is the full width at half maximum (FWHM) of the diffraction peak in radians, and θ is the Bragg angles of the main planes. The average crystallite size of the CuO nanocrystals was 20.44 nm.

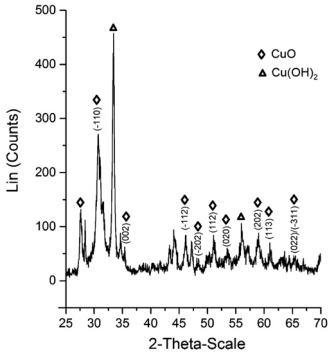

Fig. 1 *The XRD pattern of CuO nanoparticles synthesized in the present work.*

Also, the morphology, structure, and size of the samples were investigated by SEM and are shown in Figure 2. From this figure, note the presence of CuO nanoleaves with an average width of 20-24 nm. Although it is clear that the CuO nanoleaves are highly agglomerated, it is possible to note a high roughness, indicating a large contact area exhibited by the CuO nanoleaves synthesized in the present work, see Figure 2. Since, a large area of these CuO nanoparticles has been related to enhanced biocidal activity[11].

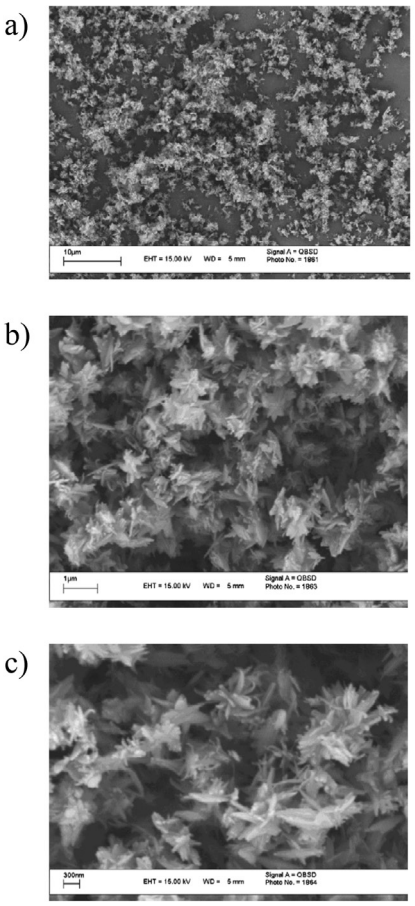

Fig. 2 *SEM images of CuO nanoparticles at different magnifications a) 10 mm, b) 1 mm and c) 300 nm.*

FT-IR spectrum has long been utilized as a powerful tool to provide supplementary data on the nature of copper oxides[55]. Figure 3, shows a FT-IR spectrum of the synthesized CuO nanoparticles. The abroad band at 3,440.96 cm^{-1} was assigned to both the ν_s(O-H) and ν_{ass}(O-H), and the less intensive band at 1,644.21 cm^{-1} was assigned to the bending vibration δ(HOH) of hydration water[56]. The absorption band of the (Cu(I)-O) appeared in the 623 cm^{-1} region as a singlet[41], but the absorption bands of the (Cu(II)-O) appeared in the 430-606 cm^{-1} region as a triplet[56-58]. Hence, the broad band at 524.05 cm^{-1} can be attributed to the stretching vibrations of (Cu(II)-O).

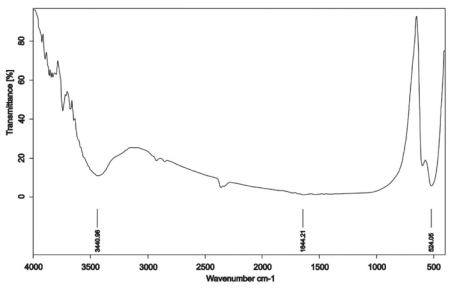

Fig. 3 *FT-IR spectra of CuO nanoleaves.*

Antimicrobial activity

The biocidal activity of CuO nanoleaves was tested against the bacterial species *Staphylococcus aureus*, *Escherichia coli*, and *Micrococcus*. These studies were carried out using Amikacin as a standard antibacterial agent by the Kirby-Bauer disc diffusion method[59]. The test solutions were prepared in DMSO. Diffusion[60, 61] was used to evaluate the antimicrobial activities of the tested compound as follows: 0.5 mL spore suspension (10^6 to 10^7 spore mL^{-1}) of each of the investigated organisms was added to a sterile agar medium just before solidification, then poured into sterile Petri dishes (9 cm in diameter) and left to solidify. Using a sterile cork borer (6 mm in diameter), wells were made in each dish, then 0.1 mL of the tested compound (dissolved in DMSO) were poured into three wells and the dishes were incubated at 37 °C for 24 h, where clear or inhibition zones were detected around each well. The results obtained indicated that at 0.01 and 0.03 g mL^{-1} of the concentration of sample there is not any inhibition zone, but a concentration of 0.05 g mL^{-1} generates inhibition zones of 20, 17 and 12 mm of diameter for *S. aureus*, *E. Coli*, and *Micrococcus,* respectively. Here, it is important to remind that an inhibition zone is indicative of the extent of the test organism's inability to survive in the presence of the test antibiotic. Moreover, the diameter of the inhibition zone is related to the level of sensitivity activity of a bacterium to an antibiotic. Thus, a bigger diameter is indicative of a major antimicrobial activity. On the other hand, if there is not an inhibition zone then the microorganism shows resistance to the test antibiotic. In the present case, a concentration of 0.05 g mL^{-1} of CuO nanoparticles, synthesized in the present work, exhibited a major antimicrobial activity against *S. Aureus* in comparison to *E. Coli*, and *Micrococcus.*

Cytotoxicity assays in vitro

Also, we tested the biological activity of the CuO nanoleaves against AGS cancer cells. The IC_{50} cytotoxicity values of the compound were compared to those found for the starting organic bases and some current anti-cancer agents, specifically cisplatin and oxoplatin compounds[45]. The accepted method for testing the anti-tumor properties of these kind of compounds is the standard testing method, which has been previously described. After 12 h of preincubation at 37 °C in a 5% CO_2 atmosphere and 100% humidity, the tested compounds were added in the concentrations 0.1 mM, 0.001 M, 0.01 M, and 0.1 M. The incubation lasted 72 h and, at the end of this period, the IC_{90} and IC_{50} values, the values that killed 90% and 50% of the total cells, respectively, were measured by Trypan blue. IC_{90} and IC_{50} values were determined both in control and in compound-treated cultures. The compounds were first dissolved in DMSO and then filtered. The concentration values that allow us to get IC_{50} and IC_{90} values are 0.1 mM and 0.1 M, respectively. From these results, it is clear that a concentration of CuO nanoparticles of 0.1 mM is able to kill the 50% of AGS cancer cells, but to kill the 90% of these cells is necessary to increase the concentration 100 times. Last results indicate that the CuO nanoparticles synthesized in the present work have biocidal activity against AGS cells. Moreover, note that a concentration of 0.1mM of CuO nanoparticles corresponds to 7.95 μg mL^{-1}, if one compares this value with a typical cisplatin concentration of 2.31 μg mL^{-1},[62] or 15 μg mL^{-1} of ethanolic extract of propolis,[63] for reach an IC_{50}, it is clear that CuO nanoparticles are a good and non-expensive option to attack AGS cancer cells.

CONCLUSIONS

CuO nanoleaves with biocidal activity were synthesized through an inexpensive, nontoxic and simple method. The presence of CuO was confirmed by the presence of functional groups in FT-IR spectra, while SEM and XRD studies demonstrated that the CuO crystallites were in the nanometer range. According to the results of the biological tests, CuO nanoparticles without purification showed antitumor activity against AGS cells and inhibited the growth of *Staphylococcus aureus*, *Escherichia coli*, and *Micrococcus* in agar.

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