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Green Synthesis of Copper Oxide Nanoparticles Using *Aloe vera* Leaf Extract and Its Antibacterial Activity Against Fish Bacterial Pathogens

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Abstract The present study reports biologically oriented process for green synthesis of CuO nanoparticles by using eco-friendly and non-toxic *Aloe vera* leaf extract. Powder X-ray diffraction and transmission electron microscope analysis revealed that synthesized CuO nanoparticles are in monoclinic phase with average particle size of 20 nm. The antibacterial activity of green synthesized CuO nanoparticles was tested against three bacterial fish pathogens "viz:" *Aeromonas hydrophila*, *Pseudomonas fluorescens* and *Flavobacterium branchiophilum*, which are responsible for causing severe infectious diseases in fishes. CuO NPs exhibits enhanced antibacterial activity against all the fish pathogens even at lower concentrations, i.e. above 20 μg/mL.

Keywords Green synthesis · Fish pathogens · *Aloe vera* · Leaf extract · Antibacterial activity

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1 Introduction

Recent advances in the field of science and technology, particularly nanotechnology, have lead to the development a new concept of synthesizing nanosized particles of desired size and shape [1, 2]. Of the various processes available for the synthesis of nanoparticles, biosynthetic process plays a very important role in nanotechnology as it is cost-effective, is eco-friendly and is a better alternative to chemical and physical methods [3, 4]. Moreover, other chemical methods employ toxic chemicals, additives or capping agents and non-polar solvents in the synthesis procedure and are thus not suitable for their application in clinical and biomedical fields. Therefore, the need for the development of a clean, reliable, biocompatible, benign and eco-friendly process to synthesize nanoparticles forced many researchers to develop "green" chemistry and bioprocesses [5]. Green synthesis of nanoparticles (NPs) using plant extracts is an emerging area of research and is potentially advantageous over chemical or microbial synthesis as it eliminates the elaborate process and can also meet large-scale production [6]. Among various NPs, transition metal oxide NPs have wide application in various fields viz. chemistry, physics, material science, biotechnology, environmental technology, etc. [7, 8]. CuO is a p-type semiconductor material with a narrow band gap of 1.2 eV. In recent years, they are receiving lot of attention for their versatile properties and potential use as gas sensors, solar cells, lithium ion batteries, heterogeneous catalysts and antibacterial agents. Besides, CuO nanoparticles are stable, robust and have a longer shelf life compared to organic, antimicrobial agents [9–12].

From review literature, there are very few reports on plant-mediated synthesis of metal oxide nanoparticles [3, 13, 14]. During the present study, *Aloe vera* plant is used for the green synthesis of CuO NPs, as it contains about 75 potentially active ingredients which have been reported to possess immunomodulatory, anti-inflammatory, antiparasitic and wound or burnhealing properties [15]. Though there are reports on the antibacterial activity of CuO NPs against selected human pathogens [11], no studies are available against fish bacterial pathogens so far.

In India, fisheries sector has developed tremendously for the past few years and is contributing significantly to the national GDP. However, the major constrain facing the industry is sudden disease outbreak due to microbial pathogens. The excessive use of antibiotics for disease control has resulted not only in their accumulation in fish tissues but also in the development of resistance by these bacterial fish pathogens toward antibiotics. Besides, the accumulation of antibiotics in fish tissues gets carried over to humans who consume fish and cause severe health problems [16]. It is why the present study stands significant, as it deals with green synthesis of CuO NPs employing *A. vera* plant extract and testing their efficiency as antimicrobial agents against bacterial fish pathogens such as *Aeromonas hydrophila*, *Pseudomonas fluorescens* and *Flavobacterium branchiophilum*, which are reported to be responsible for causing haemorrhagic septicaemia and bacterial gill disease in cultured fish, especially carps.

2 Materials and Methods

Preparation of *A. vera* Leaf Extract To prepare the leaf extract of *A. vera* plant, leaves (25 g) were thoroughly washed, dried and finely chopped. The finely chopped material was allowed to boil for 5 min at 80 °C with 100 mL of de-ionized water in a 250-mL Erlenmeyer flask and then cooled down to room temperature. The resulting solution is passed through a filter paper to remove any solid particles and then again filtered through a Whatman filter paper of pore size 0.2 μm. The filtrate is stored at 4 °C as a stock for the synthesis of CuO NPs.

Green Synthesis of CuO NPs Fifty millilitres of 10 mM aqueous solution of copper nitrate (99.99 % purity, Aldrich) was added to 5-mL *A. vera* extract in a 100-mL Erlenmeyer flask with constant stirring on a magnetic stirrer at 100–120 °C. Colour change of the reaction mixture was observed from deep blue to colourless and then to brick red and dark red on vigorous stirring for 24 h. Then the resultant solution is centrifuged at 10,000 rpm for 10 min, at room temperature (using Beckman centrifuge with a Beckman JA-17 rotor), and the mixture is collected after discarding the supernatant. The collected CuO NPs are allowed to dry in a watch glass. The formed black precipitate is grinded for further characterization.

Characterization of Synthesized CuO NPs The morphological, structural and chemical composition of CuO NPs were analysed by employing SEM-EDS (JEOL JSM-6610-LV—with Oxford energy dispersive spectroscopy) and XRD (PAN analytical: XPERT-PRO) equipment. Transmission electron microscopic (TEM) analysis is done by using a TEM (JEM-1200EX, JEOL Ltd., Japan). For TEM analysis, a 3-mL sample is placed on the carbon-coated copper grid to make a thin film and the extra sample is removed with a blotting paper. The thin film is kept in the grid box sequentially for TEM analysis. Through FTIR (Shimadzu FTIR Prestige 21) and UV–Vis (Shimadzu UV–Vis 2450) spectroscopy spectral analysis, the optical properties of CuO NPs were examined.

Antibacterial Activity of Green Synthesized CuO NPs Green synthesized CuO NPs were tested for antibacterial activity against Gram-negative fish pathogenic strains like *A. hydrophila* (MTCC 646), *P. fluorescens* (MTCC 671) and *F. branchiophilum* (ATCC 35036) by agar well diffusion method. Minimum inhibitory concentration (MIC) of the CuO NP against each bacterial pathogen was determined by micro-dilution broth method. All bacterial dilutions were standardized to match the McFarland (turbidity) standard, showing a bacterial density of 1.5×10^8 CFU/mL. The modified

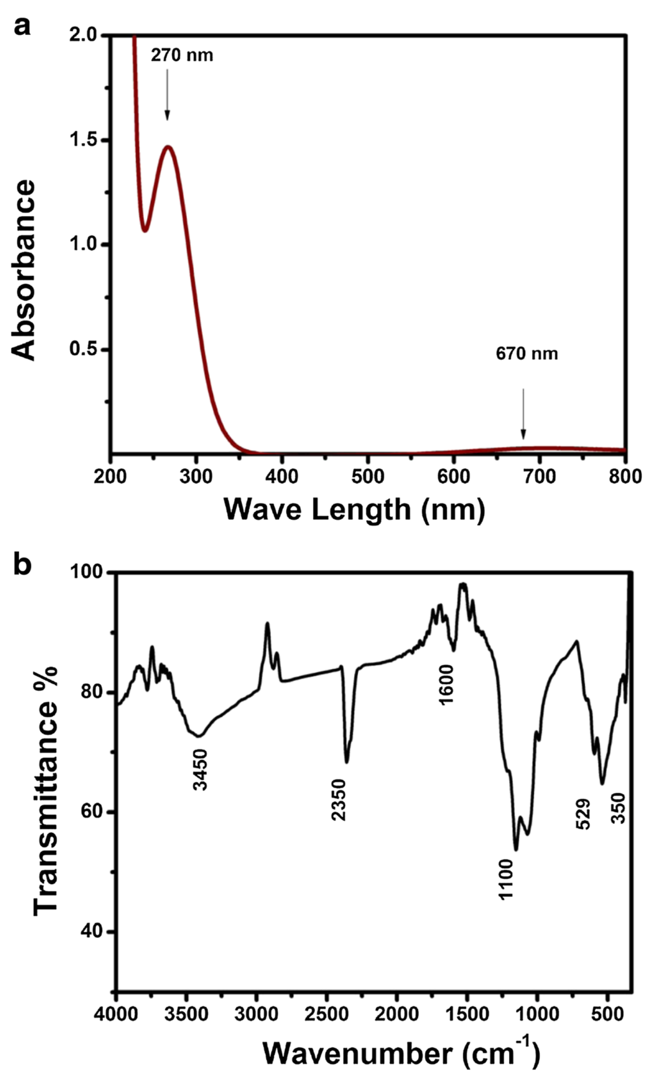


Fig. 1 **a** UV–Vis spectra and **b** FTIR spectra of green synthesized CuO NPs using *Aloe vera* leaves extract

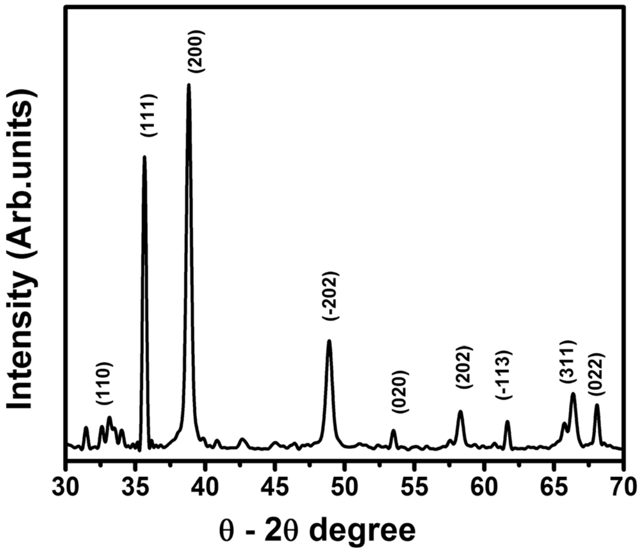


Fig. 2 XRD pattern of green synthesized CuO NPs using *Aloe vera* leaves extract

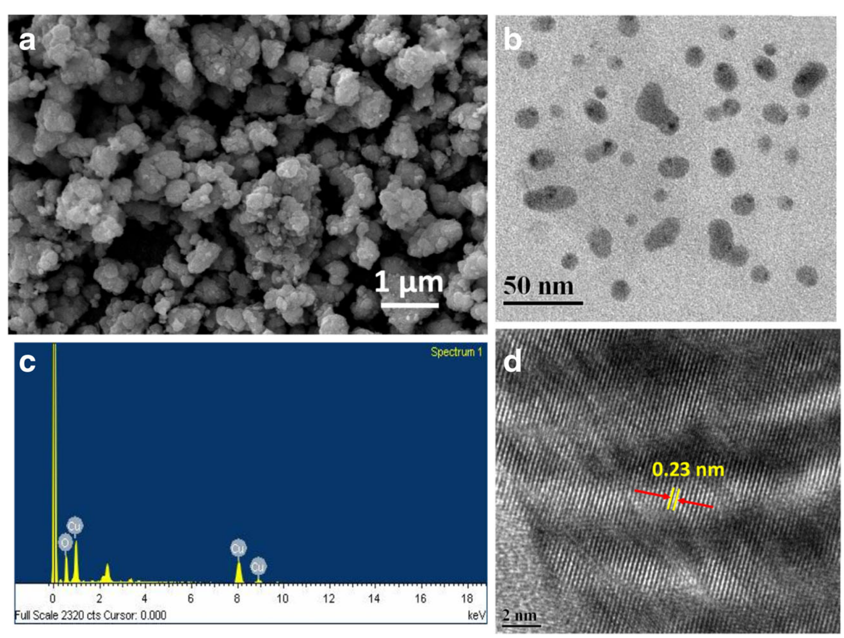
agar well diffusion method of Cappuccino and Sherman (1999) is employed to study the antibacterial activity [17]. For this, MHA medium is seeded with 24-h bacterial culture by spreading on the agar media plate with a glass spreader. The plates were allowed to stand for 10–15 min to allow the culture to get absorbed in the medium. Once the agar solidifies, four wells of 6-mm diameter were made in each Petri plate and are filled respectively with 50 μL (100 μg/mL) of CuO NPs dissolved in DMSO, *A. vera* leaf extract, antibiotic (sulphafurazole) as positive control and DMSO as a negative control. MIC of CuO nanoparticles is tested by preparing the concentrations in decreasing order of 100, 80, 60, 40 and 20 μg/mL. Plates were incubated for 24 h at 37 °C. Results were recorded based on the presence of inhibition zone and the size of the zone is measured.

3 Results and Discussion

Figure 1a shows UV spectra of biosynthesized CuO NPs using *A. vera* leaf extract. The colloidal suspension after reduction showed two strong resonances, one is in between 265 to 285 nm and another weak but broad resonance centred at about 670 nm, indicating the formation of CuO NPs. The peak at 260 nm is due to inter-band transition of core electrons of copper metal, while that of peak around 670 nm, corresponds band edge transition of cupric oxide (CuO). FTIR spectra were recorded in solid phase using the KBr pellet technique in the range of 4000–400 cm^{-1}. FTIR analysis (Fig. 1b) of green synthesized CuO nanoparticles revealed a strong band at 1100 cm^{-1}, whereas peaks at 529,350 cm^{-1} can be attributed to vibrations of CuO, confirming the formation of highly pure CuO nanoparticles [11]. The bands around 3450, 1600 and 2350 cm^{-1} show the presence of –OH, C–C and C=O stretching of hydroxyl groups, alkenes and presence of alkanes, respectively.

The XRD technique was used to determine and confirm the crystal structure of the nanoparticles. XRD analysis showed a series of diffraction peaks at 2θ of 32.41, 35.61, 38.81, 48.91, 53.31, 58.21, 61.61 and 66.31, which were assigned to (110), (111), (200), (−202), (020), (202), (−113) and (022) planes respectively. The XRD spectrum clearly suggested the crystalline nature of the CuO NPs synthesized from leaf extract of *A. vera*. The peak positions exhibited the monoclinic structure of CuO which was confirmed by the International Centre for

Fig. 3 **a** SEM, **b** TEM, **c** EDS and **d** HRTEM images of CuO NPs synthesized from *Aloe vera* leaves extract



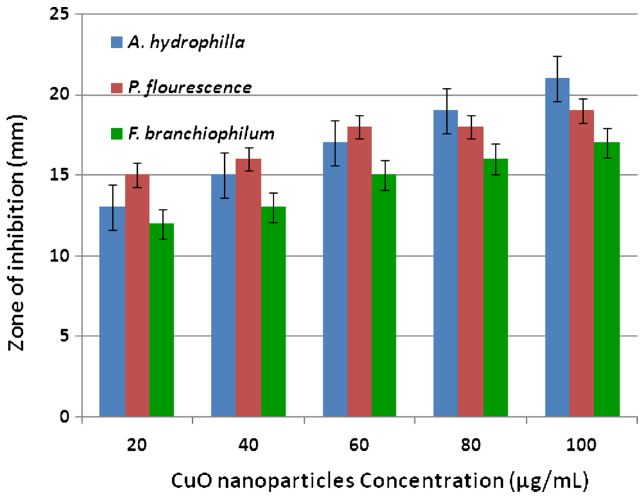


Fig. 4 Zone of inhibition of green synthesized CuO nanoparticles with different concentrations against various fish bacterial pathogens

Diffraction Data (ICDD) card no. 801916 (Fig. 2). All diffraction peaks can be indexed as typical monoclinic in structure and no other phases were observed. The average crystallite size of CuO nanoparticles was calculated using Scherrer formula, $D=0.9\ \lambda/\beta\cos\theta$, where λ is the wavelength of X-ray radiation, β is the full width at half maximum (FWHM) of the peaks at the diffracting angle θ. It was found to be about 22 nm indicating its nanocrystalline nature.

SEM image (Fig. 3a) of CuO nanoparticles showed the presence of some large particles, which can be attributed to aggregation or overlapping of smaller particles with sizes around 100 nm. The EDS analysis revealed the chemical composition of the nanoparticles having atomic percent of 54 % for Cu and 45 % for O (Fig. 3b). TEM image (Fig. 3c) showed a well-dispersed, versatile and spherical shape for green synthesized CuO nanoparticles with a size range of 20 to 30 nm. The HRTEM images exhibited inter-planar spacing of 0.23 nm indicating the preferred (200) orientation (Fig. 3d).

The bactericidal properties of green synthesized CuO NPs against three fish bacterial pathogens *A. hydrophila*, *P. fluorescens* and *F. branchiophilum* were studied. Concentrations ranging from 10 to100 μg/mL were used to evaluate the minimum inhibitory concentration of CuO NPs. MIC was observed at 20 μg/mL, with zone of inhibition at 13, 15 and 11 mm for *A. hydrophila*, *P. fluorescens* and *F. branchiophilum*, respectively. An increase in the size of inhibition zone was noticed for all the bacteria tested with increasing concentration of CuO NPs above 20 μg/mL (Fig. 4). It is interesting to note that the sensitivity of *P. fluorescens* is higher at lower concentrations (20–60 μg/mL) showing highest zone of inhibition when compared to other two fish bacterial pathogens. On the other hand, the sensitivity of *A. hydrophila* is higher at high concentrations of CuO NPs (60–100 μg/mL) with maximum zone of inhibition when compared to other bacterial pathogens. However, CuO NPs with 100 μg/mL concentration appears to be effective as there was no bacterial growth, with zones of inhibition for *A. hydrophila*, *P. fluorescens* and *F. branchiophilum* being 21, 19 and 17 mm, respectively (Fig. 5). Sulphafurazole (standard

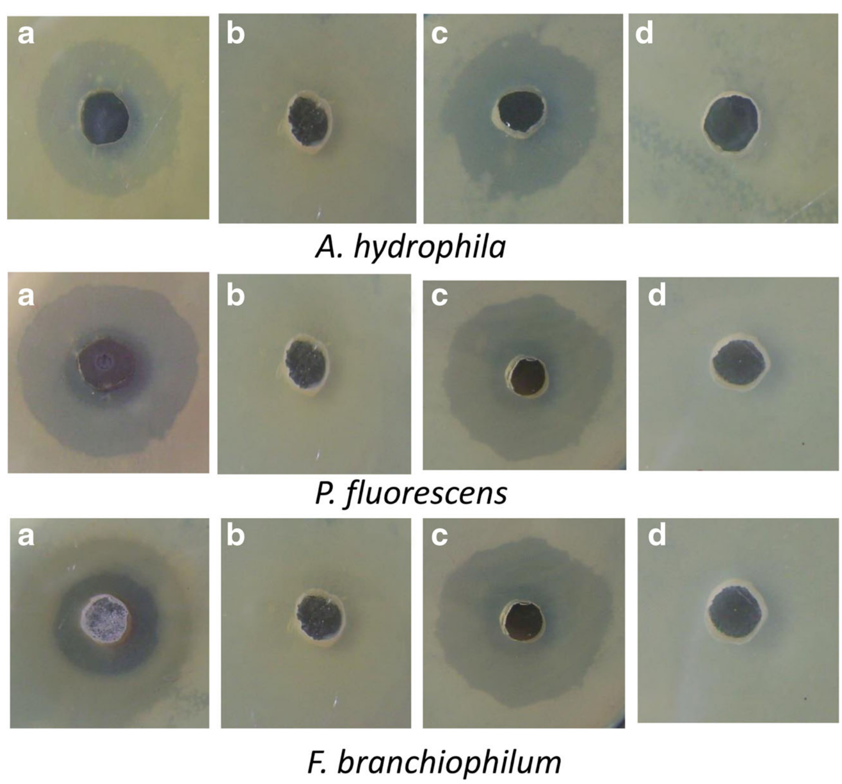


Fig. 5 Antimicrobial activity of green synthesized CuO nanoparticles against *A. hydrophila*, *F. branchiophilum* and *P. flourescens.* Note: **a** CuO nanoparticles, **b** leaf extract, **c** positive control (sulphafurazole), **d** negative control (DMSO)

antibiotic) was used as positive control. For comparison with standard antibiotic, we have tested zone of inhibition of sulphafurazole with 100 μg/mL concentration showing 17 mm for all the selected fish bacterial pathogens. Dissolving solution (DMSO) and extract were used as negative control which does not have any zone of inhibition. CuO NPs exhibited enhanced antibacterial activity against all the three fish bacterial pathogens even at lower concentrations, i.e. above 20 μg/mL (Fig. 4); however, the dose of 100 μg/mL was found to be effective for inhibiting the growth.

The probable mechanism involved for antibacterial activity can be attributed to disruption of cell membrane due to the release of copper ions from CuO nanoparticles, which attach to negatively charged bacterial cell wall and rupture it, thereby causing protein denaturation and cell death. Once entered into bacterial cell, it may bind to deoxyribonucleic acid molecules and get involved in cross-linking of nucleic acid strands, forming a disorganized helical structure. In addition, copper ion uptake by the bacterial cells also interrupts important biochemical processes [18].

4 Conclusions

In summary, the green synthesis of CuO NPs was carried out by using *A. vera* leaf extract singly without the involvement of any other chemical reagent. XRD and TEM analysis conformed that the CuO NPs show only monoclinic phase, to an average grain size of 20 nm. These green synthesized CuO NPs are cost-effective, biogenic molecules with the capability to serve as antimicrobial agents against fish bacterial pathogens.

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