

Antibacterial and morphological studies of plant-mediated synthesized CuO nanoparticles using *Azadirachta indica* (neem) leaf extract

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Abstract

This paper deals with the synthesis of copper oxide (CuO) nanoparticles by the plant-mediated method using copper acetate monohydrate and neem extract. The formation of nanoparticles was confirmed by UV-Visible spectral studies. P-XRD studies revealed that the average particle size of synthesized nanoparticles was 11.30 nm which was in good agreement with TEM results. Morphology of synthesized nanoparticles was determined by SEM which revealed that CuO nanoparticles were spherical and some were agglomerated in nature. The EDX spectrum of nanoparticles exhibited three signals one signal at 0.9 keV and other signals at ~8 keV which is due to Cu and another signal of oxygen appeared at 0.5 keV this indicated that nanoparticles of copper have been formed as copper oxide. The synthesized nanoparticles were screened for their antibacterial activity *in vitro* against gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* by adopting the disk diffusion method. The results of antibacterial studies exhibited that CuO NPs were potential antibacterial agents.

Keywords: Antibacterial Activity; CuO Nanoparticles; Leaf Extract; Plant-Mediated Method; SEM.

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INTRODUCTION

In recent years, considerable attention has been paid by researchers to the synthesis of metal oxide nanoparticles (NPs) due to their diversified applications in different fields like optoelectronics [1], gas sensors [2], electrochromic materials [3], information storage, and catalysis [4, 5]. Among the various metal oxide NPs, copper nanoparticles have been of great interest due to their excellent physical and chemical properties and low cost of preparation [6]. Because of the high surface-to-volume ratio copper nanoparticles are considered more reactive. They can easily interact with other particles [7] and increase their antimicrobial efficiency. Green synthesis plays an important role in the synthesis of nanoparticles of metals [8, 9]. Biosynthesis of metallic nanoparticles is a single-step green method of synthesizing nanoparticles

in which plant extract contains novel metabolites such as phenolic acid, flavonoids, alkaloids, and terpenoids which are responsible for reducing and stabilizing agents [10-12] because of easy availability and safe to handle. In addition, the synthesis of nanoparticles using plant extract have many advantages such as the use of safer solvents, reagents, feasibility better control over size and shape of nanoparticles and their adaptability in use for medicinal and pharmaceutical applications [13]. Due to these advantages, several researchers aimed to explore different plant species their potential to synthesize nanoparticles. PEG is frequently used as the surfactant to prepare nanomaterials and as the stabilizer of metal colloids, because of its availability, low cost, and non-toxicity. PEG also works as a size controller by preventing the aggregation of the nanoparticles [14]. Ijaz *et al.* have reported the synthesis of CuO

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nanoparticles by using leaf extract of *Abutilon indicum* and Copper [II] nitrate trihydrate [15]. Kumar *et al.* have reported the synthesis of CuO nanoparticles by using copper nitrate and *aloe vera* leaf extract [13]. Sankar *et al.* have reported the synthesis of CuO nanoparticles by using cupric sulphate and papaya leaf extract [16].

The purpose of the present work is to extend an easy, rapid, and completely green method for synthesizing CuO NPs by using copper acetate monohydrate and plant extract and PEG as reducing and stabilizing agents in the aqueous medium. The synthesized copper oxide nanoparticles have been screened for their antibacterial activity *in vitro* against gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* by adopting the disk diffusion method.

MATERIALS AND METHODS

Collection of Plant materials and chemicals

Fresh neem leaves were collected from Khandari Campus, Agra. Copper acetate monohydrate, polyethylene glycol (PEG), and solvents were of analytical grade and bought from Sigma-Aldrich. All chemical reagents and solvents were used as available without any additional purification and distillation.

Preparation of neem (Azadirachta indica) extract

Fresh neem leaves were collected from Khandari Campus, Agra. Leaves were carefully washed in running tap water to remove the dirt and dust from the surface of the leaves and then again washed with distilled water. 25 g finely chopped leaves were kept in a beaker containing 150 mL distilled water and boiled for 20 min. The extract was cooled and filtered with Whatman filter paper No.1 and stored at 4 °C for further use.

Synthesis of copper oxide nanoparticles

In a 250 mL round bottom flask 4 g copper acetate monohydrate was dissolved in 25 mL distilled water. 20 mL aqueous solution of polyethylene glycol (0.5 g) was added dropwise and the solution was stirred for 15 min with heating at 70 °C. After that 100 mL, neem extract was dropwise mixed with stirring during constant heating at 70 °C. The color change from green to brown indicated the formation of nanoparticles. The obtained precipitate was centrifuged and washed several times with

deionized water to remove unreacted surfactant and precursors. It was dried at 150 °C for 1h and finally calcined at 250 °C for 2h to get copper nanoparticles.

CHARACTERIZATION TECHNIQUES

The UV-Visible spectrum of copper oxide nanoparticles was recorded by UV-Visible spectrophotometer LAB India in the range 200 to 600 nm. The P-XRD spectrum of nanoparticles was recorded on an XPERT-PRO X-Ray diffractometer at a voltage of 45 kV and a current of 40 mA with Cu K α radiation in a θ -2 θ configuration. The transmission electron microscopic measurements of synthesized nanoparticles have been performed by using the JEOL model JEM 2100. The scanning electron microscope was used to identify the morphology of nanoparticles by using model JSM6100 (Jeol) with Image Analyser. Energy-dispersive X-ray spectrometry was performed to analyze the elemental constituent of the nanoparticles. The biological activity of nanoparticles has been tested *in vitro* against gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* by adopting the disk diffusion method.

Antibacterial activity of CuO nanoparticles

Disk Diffusion Method

The synthesized nanoparticles were screened for their antibacterial activity against gram-negative bacteria *Escherichia coli* [17] and *Pseudomonas aeruginosa* [18] by the disk diffusion method [19, 20]. To cultivate bacteria, a standard Mueller Hinton agar medium was used. The culture media was sterilized in an autoclave at 15 pounds pressure for 30 min. After sterilization, media was spread smoothly on petri plates and then allowed to solidify. A cotton swab was dipped in bacterial cultural broth and applied on the surface of petri plates. Sterilized discs of 6 mm diameter were placed on the surface of petri plates. Thereafter, solutions of copper oxide nanoparticles were prepared in five different concentrations (100, 50, 25, 12.5, and 6.25 μ g/mL) in water. 6.25 μ g/mL aqueous solutions for standard antibiotic Amikacin was prepared in water. These discs were loaded with about 25 μ l of each concentration by using a micropipette. The petri plates were incubated at 37 °C for 24h. The obtained zones were measured in mm using the ruler scale method and compared with the zone of standard Amikacin.

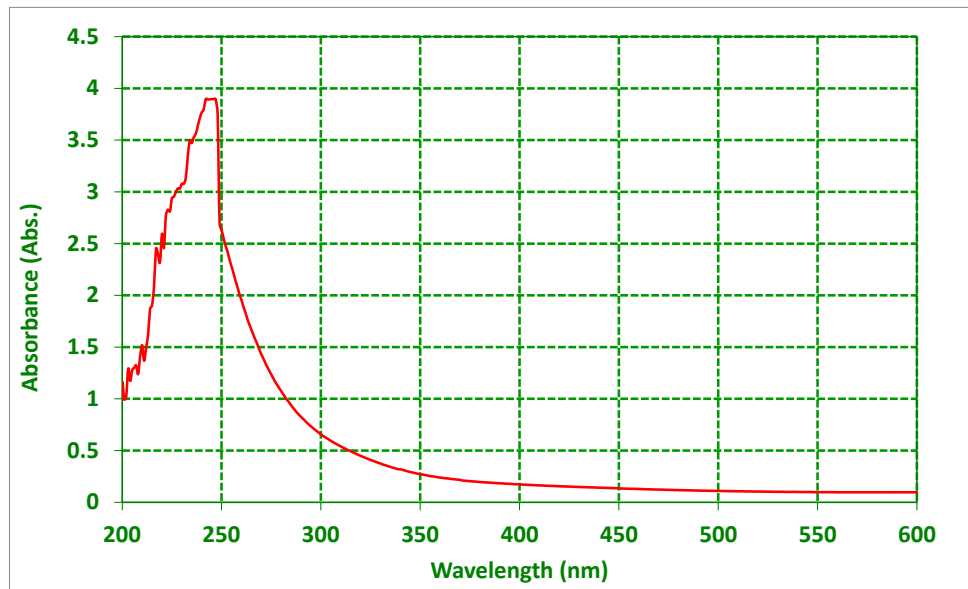


Fig. 1. UV-Vis spectra of copper oxide nanoparticles.

RESULTS AND DISCUSSION

UV-Visible Spectral Studies

The UV-Visible absorption spectrum (Fig. 1) of synthesized copper oxide nanoparticles exhibited an absorption peak at 247 nm which indicated the formation of copper oxide nanoparticles. Our results were in agreement with previous reports on synthesized copper oxide NPs [21] P-XRD studies

P-XRD analysis exhibited that the synthesized nanoparticles were crystalline. The size of nanoparticles was calculated by using the width of X-Ray peaks supposing they are free from non-uniform strains using Debye-Scherrer's formula [22],

$$D = \frac{k\lambda}{\beta \cos\theta}$$

Where,

D = Mean crystallite domain size, at right angles to the reflecting planes

k = Constant equals to unity

λ = X-Ray wavelength

β = Full width at half of the maximum intensity (FWHM) and

θ = Diffraction angle

P-XRD graph (Fig. 2) showed diffraction peaks at 32.4330, 35.4787, 38.6810, 48.7262, 61.4978, and 72.2898 corresponding to (110), (002), (111), (202), (113) and (311) planes respectively. These planes indicated face-centered-cubic structure of

CuO nanoparticles with a monoclinic phase. The observed diffraction reflections were comparable with JCPDS No. 45-0937 [23]. The average particle size of the synthesized copper oxide nanoparticles was found to be 11.30 nm [24].

Transmission Electron Microscopic Studies

The TEM images of copper oxide nanoparticles have been shown in Fig. 3(a, b). The transmission electron microscopic (TEM) images revealed that the particle size of synthesized copper oxide nanoparticles lies in the range of 3.02 to 27.66 nm with spherical shape. These observations are consistent with the previously reported studies [25].

Scanning Electron Microscope and EDX Studies

The SEM images Figs. 4 (a, b) of CuO nanoparticles indicated that most of the nanoparticles have been formed spherical and some were agglomerated in nature [26]. This result is consistent with the shape and uniformity of copper oxide nanoparticles [27]. The Effect of the viscous nature of plant extract is visible in SEM micrographs as some nanoparticles get agglomerated. In the EDX spectrum, (Fig. 5) of nanoparticles of copper one signal at 0.9 keV and other signals at ~ 8 keV may be due to the absorption of metallic Cu. However, the signal of the oxygen atom has appeared at 0.5 keV which indicated that nanoparticles of copper have been formed as copper oxide.

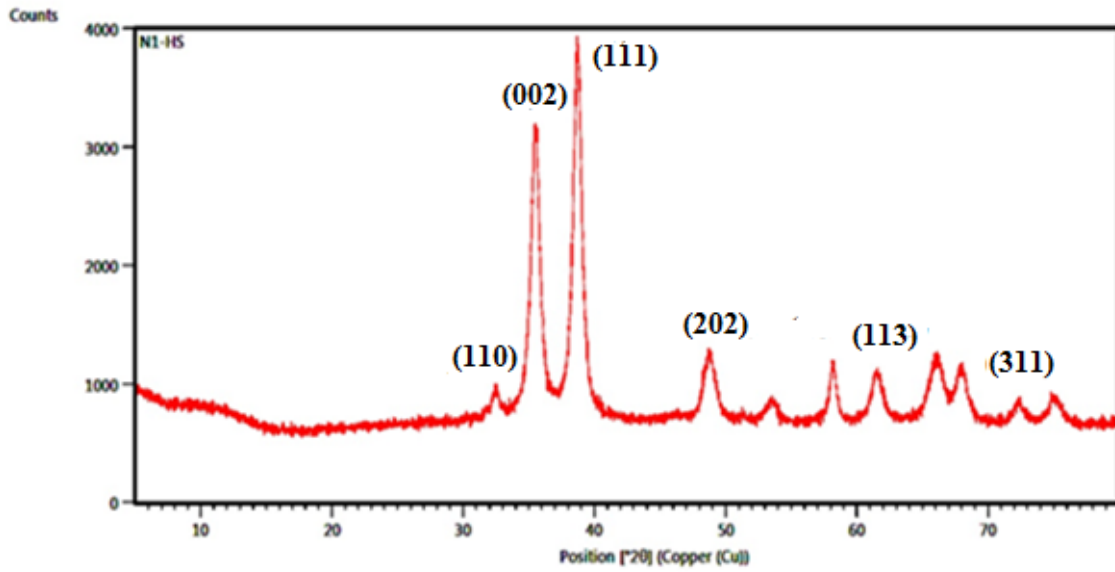


Fig . 2. X-Ray diffraction pattern of synthesized copper oxide nanoparticles.

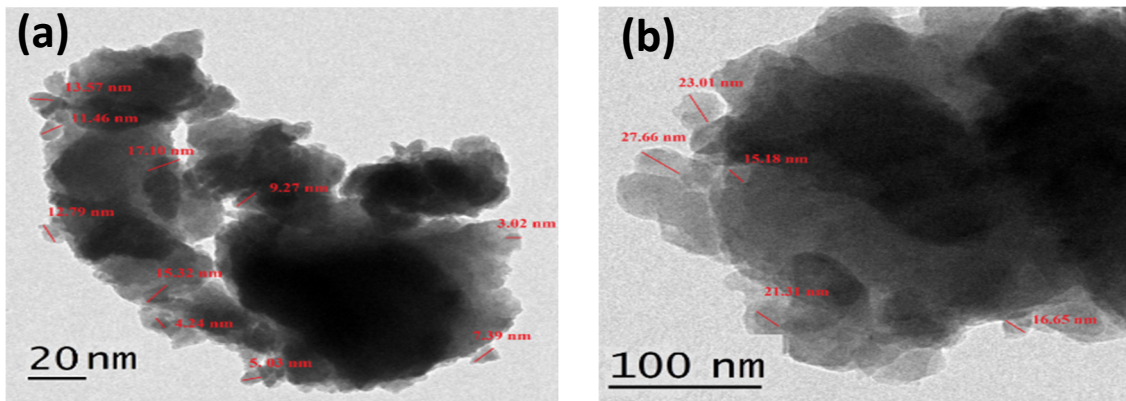


Fig. 3 (a, b). TEM images of synthesized copper oxide nanoparticles.

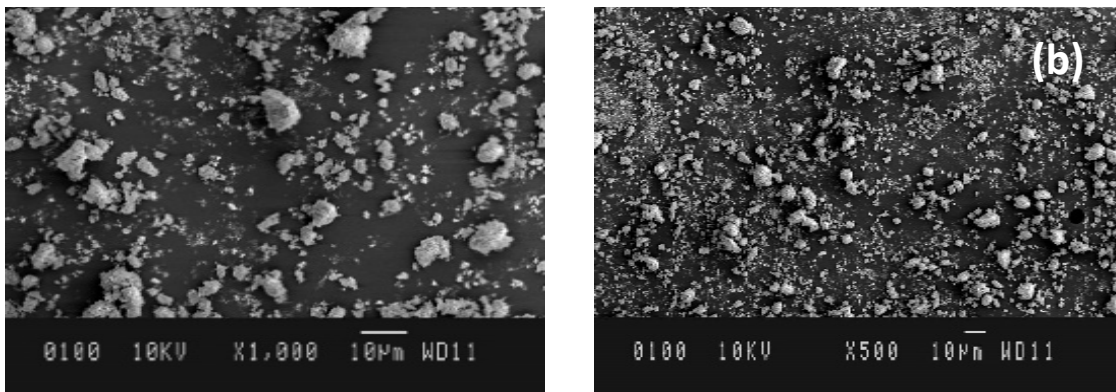


Fig. 4 (a, b). SEM images of synthesized copper oxide nanoparticles.

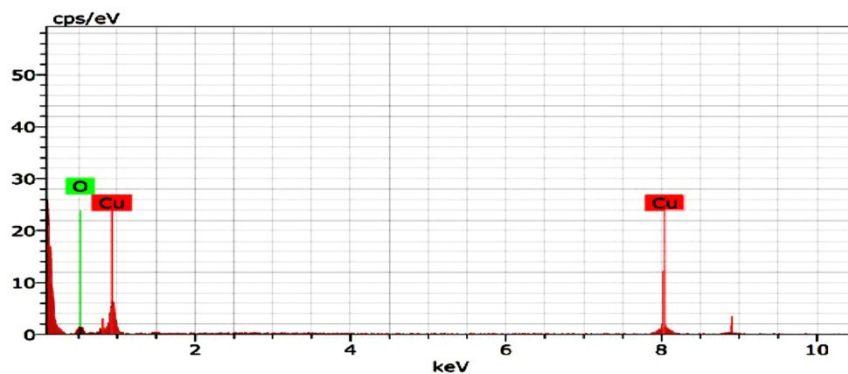


Fig. 5. EDX pattern of synthesized copper oxide nanoparticles.

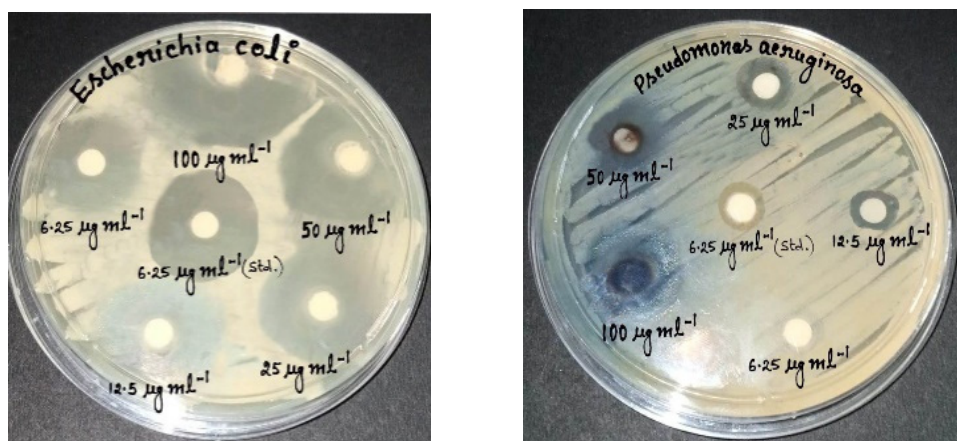


Fig. 6. Antibacterial activity of copper oxide nanoparticles against (a) *Escherichia coli* (b) *Pseudomonas aeruginosa*.

Table 1. Maximum zone of inhibition values for copper oxide nanoparticles against tested micro-organisms.

S. No.	Test Concentration (µg mL ⁻¹)	<i>Escherichia coli</i> (zone of inhibition in mm)	<i>Pseudomonas aeruginosa</i> (zone of inhibition in mm)
1.	100	23	21
2.	50	20	19
3.	25	18	17.5
4.	12.5	17.4	16
5.	6.25	14	13
Amikacin STD	6.25	16.5	16

Antibacterial Activity

The antibacterial activity of the synthesized copper oxide nanoparticles was evaluated against two gram-negative bacteria *Escherichia Coli* and *Pseudomonas aeruginosa*. The zone of inhibition (Fig. 6(a) Table 1) was found 23, 20, 18, 17.4, and 14 mm corresponding to the concentration 100, 50, 25, 12.5, and 6.25 µg/mL against the bacteria *Escherichia Coli*. The zone of inhibition (Fig. 6(b),

Table 1) was found 21, 19, 17.5, 16, and 13 mm corresponding to the concentration 100, 50, 25, 12.5, and 6.25 µg/mL against the bacteria *Pseudomonas aeruginosa*.

However, the zone of inhibition for standard drug Amikacin was observed at 16.5 mm and 16 mm at the concentration of 3.12 µg/mL against *Escherichia Coli* and *Pseudomonas aeruginosa* respectively [26].

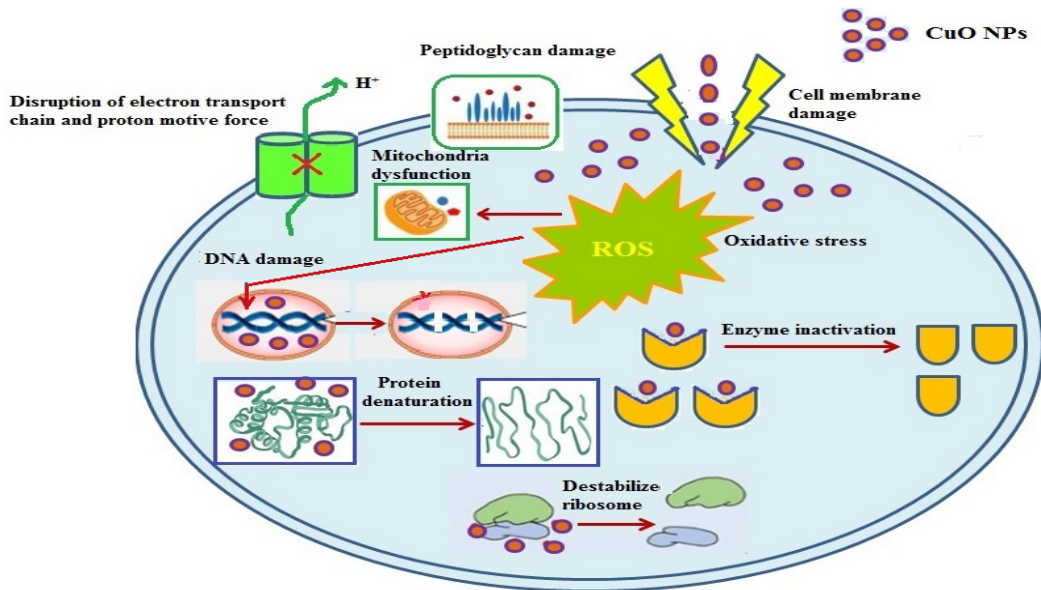


Fig. 7. Proposed Mechanism for antibacterial activity.

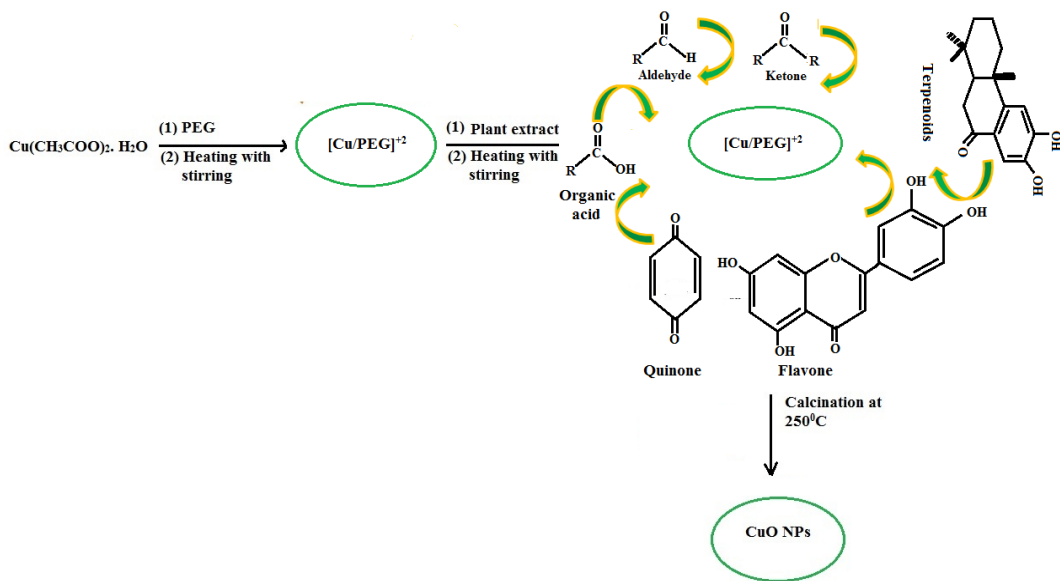


Fig. 8. Proposed Mechanism of CuO NPs nanoparticles synthesis.

Proposed Mechanism for Antibacterial Activity

The mechanism of antibacterial activity (Fig. 7) by which copper oxide NPs showed a vital action against bacteria may be due to induced oxidative stress produced by the generation of reactive oxygen species (ROS) which inhibit the growth of bacterial cells. Oxidative stress has been confirmed as the main supplier to changing the permeability of the cell membrane, which can

result in bacterial cell membrane damage [28]. Internalization of CuO NPs finally releases Cu^{2+} ions in the cytosol, leading to reactive oxygen species (ROS) accumulation in the bacteria and, subsequently, DNA and mitochondria damage.

Proposed Mechanism for the synthesis of CuO NPs

The phytochemicals present in Neem are namely terpenoids and flavanones, which act as

reducing as well as capping agents and help in stabilizing nanoparticles. First, the mechanism of synthesis involves the reaction of PEG with Cu^{+2} to form the complex [29] thereafter reduction [30] by phytochemicals of the neem leaf plant extract. Oxygen produced from either atmosphere or degrading phytochemicals links with the reduced metal ions. The electrostatic attraction will link metal oxide ions to each other and lead to the formation of nanoparticles. Nanoparticles are stabilized by some phytochemicals and PEG will also prevent agglomeration. The proposed mechanism has been presented in Fig. 8.

CONCLUSION

In this article, plant-mediated synthesis has been reported for the synthesis of copper oxide nanoparticles by using *Azadirachta indica* and characterized by UV, powder XRD, TEM, SEM. The biomolecules present in the leaf broth not only reduce the metal ions and also stabilize the metal NPs. The results of P-XRD data revealed that the average particle size of synthesized nanoparticles was 11.30 nm which was in good agreement with TEM results. SEM results revealed that most of the nanoparticles were spherical in shape and however some were agglomerated in nature. It was observed that the copper oxide nanoparticles had promising antibacterial activity against *Escherichia coli* and *Pseudomonas aeruginosa* bacteria. Hence, this green method could be used for the large-scale synthesis of nanoparticles as it has cost-effective antibacterial applications.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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