

Research Article

Antimicrobial Effect of Copper Nanoparticles Synthesized by Chemical Method

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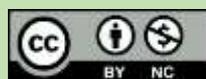
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Keywords:

Abstract

Copper nanoparticles were synthesized by chemical reduction of copper sulphate pentahydrate with sodium borohydride at ambient temperature in presence of PEG (Polyethylene Glycol) as protective agent and ascorbic acid as antioxidant. The synthesized copper nanoparticles were characterized by FT-IR to determine the presence of different functional groups attached to copper nanoparticles. Their optical property was determined by UV-Vis absorption spectrum where the peak was observed at 320 nm. The crystalline structure and surface morphology of copper nanoparticles were investigated by XRD and SEM respectively. The average crystalline size of nanoparticles calculated by Debye Scherer formula was found to be less than 10 nm having cubical shape. Microbial investigations by macro broth dilution, growth kinetics analysis and disc diffusion method confirmed antimicrobial nature of copper nanoparticles. The study reveals that concentration of copper nanoparticles at 128 ppm shows bacteriostatic effect. Their anti-fungal effect was evaluated by testing against *Alternaria* species. The maximum inhibitory effect was found when copper nanoparticle concentration was 400 ppm or more.

Introduction

In recent years, nanotechnology and nanoparticles have gained a significant attention from researchers because of their wide applications in the field of electronics, medicine, air purification, water filtrations, cosmetics and paintings and antimicrobial agent. Among the different metal, metal oxide nanoparticles, copper nanoparticles (CuNps) are of great interest due to their unique optical and electrical properties and lower cost than other metals like Au and Ag. Synthesis of copper nanoparticles can be achieved from various methods like physical, chemical and biological

methods (Umer et al., 2012). Among which chemical reduction method is one of the simpler, easier, faster and economical methods for obtaining the large amount of nanoparticle. In this article, we used sodium borohydride as a strong reducing agent for the complete reduction of copper (II) ion to copper nanoparticles.

Copper nanoparticles are expected to show better antimicrobial characteristics than bulk copper metal because of their larger specific surface area (Yoon et al., 2007), which allow them to interact closely with microbial

membranes (Ramyadevi *et al.*, 2012). Also, the growth kinetics studies of different microbial cultures performed in the presence of nanoparticles to observe their effect on the growth profile shows that copper nanoparticles have great promise as antimicrobial agent. (Ruparelia *et al.*, 2008).

Despite the various benefits, the easy oxidation of copper nanoparticles and formation of copper oxides in air under ambient atmosphere conditions in comparison to noble metals like Au and Ag are the disadvantages of copper, which must be overcome. Therefore, in order to prevent from oxidation and to avoid aggregations of particles during preparation and storage, various precautions have to be taken. In the present investigation, surfactant such as PEG is used as capping agent to prevent copper nanoparticles from agglomeration and ascorbic acid as an antioxidant to prevent copper nanoparticles from oxidation. Ascorbic acid and PEG not only prevent oxidation and aggregation but also help to control the particle size and morphology of copper nanoparticles.

Materials and Methods

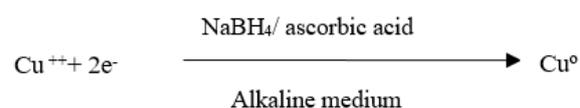
Materials

Copper (II) sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$; 99.5% purity), Ascorbic Acid, Sodium hydroxide (NaOH), Sodium borohydride (NaBH_4), Poly Ethylene Glycol (PEG-6000) and de-ionized water are the products of HiMedia laboratories (Hyderabad, India) and were purchased from Eureka International Pvt Ltd. Similarly, the culture media used during our work were Muller Hinton Agar, Nutrient Broth (NB), Nutrient Agar (NA), Potato Dextrose Agar (PDA) and they were also purchased from Eureka International Pvt. Ltd. at Kathmandu. Two bacterial culture of *Escherichia coli* (ATCC 25922) (Gram negative) and *Staphylococcus aureus* (Gram positive) were obtained from Department of Microbiology, NIST College. Gentamicin and tetracycline were purchased from Hi-media Mumbai, India. These antibiotics were used as standard for positive control. Aqueous solutions were all made in de-ionized water.

Synthesis of Copper Nanoparticles

Nanoparticles of copper were synthesized by chemical reduction method. The precursor $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.01M) salt was dissolved in de-ionized water with PEG 6000 (0.02M) and was vigorously stirred using magnetic stirrer. Then, ascorbic acid (0.02M) and NaOH (0.1M) were added simultaneously. Further, strong reducing agent NaBH_4 (0.1M) was added to the solution for complete reduction of copper salt. The solution instantaneously changed into blackish brown color when rapidly stirred for about 10 minutes more. For purification, the solution was centrifuged at 1500 rpm for 2 minutes at 4°C. The clear supernatant was discarded and the pellet of colloidal copper was washed 3 times with de-ionized water to remove impurities and the unbound extract components. To prevent the oxidation of synthesized CuNps, they were re-dispersed in de-ionized water and stored at room temperature. Half of the solution was dried in the hot air oven at 60°C for the characterization.

The copper (II) sulfate pentahydrate reduction reaction can be written as:



Characterization of CuNps

UV-Vis Spectroscopy

To examine the CuNps in the synthesized solution, we diluted the sample and absorbance in the wavelengths ranging from 200 to 800 nm was measured using Abarke SSI 2101 UV spectrophotometer with reference to de-ionized water as blank.

FT-IR Spectroscopy

For the FTIR analysis, FT-IR spectrum was recorded on Shimadzu IR prestige-21 instrument with Attenuated Total Reflectance (ATR) diamond crystal from 500–4500 cm^{-1} at spectral resolution of 1 cm^{-1} for each spectrum to identify the functional group present on the samples.

X-Ray Diffraction (XRD)

The XRD analysis of dried CuNps was carried out at NAST, Khumaltar, Nepal to examine the crystalline phase of synthesized nanoparticles. The diffraction pattern was recorded from diffraction angle from 20° to 80°.

Scanning Electron Microscopy (SEM)

The surface morphology and structural analysis was studied by Hitachi S-4800 field-emission scanning electron microscope operating at 10 kV in National Institute of Materials Science, Japan.

Antibacterial Assay

Determination of MIC

For the determination of MIC, two-fold dilution series of CuNps was performed in the range of 512, 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5 ppm. No CuNps was added to test tube labeled 0 ppm (Positive control). The inoculums were prepared by transferring the colonies of *E. coli* and *S. aureus* to the culture media (NB) and incubated with shaking at 37° C until the visible turbidity was equal to or greater than the 0.5 McFarland standards. Then, all dilutions of the test product(s) were inoculated with equal volumes of the specified microorganism. A positive (bacterial culture without CuNps) and negative control (Nutrient broth without bacterial culture and CuNps) tubes were included for every test microorganism to demonstrate adequate microbial growth over the course of the incubation period and media sterility, respectively. An aliquot of the positive control was plated and used to establish a baseline concentration of the microorganism used. The tubes were then incubated overnight at 37° C. Turbidity indicates growth of microorganisms and the MIC is the lowest concentration where no growth is visually observed.

Study of Bacterial Growth Kinetics

Spectrophotometer was used to maintain the bacterial culture concentration at 10^8 CFU/ml by taking absorbance at 600 nm. The working cell concentrations of 10^5 to 10^6 CFU/ml was prepared. Negative controls (Bacterial culture without CuNPs) for each bacterial strain and positive controls (media with CuNPs and without bacterial culture) at concentration starting from last two non-visible tubes to the last two visible tubes in MIC test were prepared. Test samples were similar to positive control except for the addition of bacterial culture. The absorbance of negative control (using nutrient broth as blank) and four test samples (using positive control of respective concentration as blank) was noted at 600 nm in different time intervals of 0, 2, 4, 6, 9, 12, 16, 20 and 22 hours. Then, the graphs were plotted according to the data obtained.

Antifungal Assay

Leaves samples showing symptoms of early blight were taken from infected tomato plants. The fungus, *Alternaria spp.* was isolated from them and then sub-cultured in PDA containing 0.25g/L chloramphenicol. The fungal samples were further inoculated in PDA media with varying concentration of CuNPs (0,100, 200, 400, 800 ppm). The effect of CuNPs solutions on the development of *Alternaria spp.* was studied by determining the diameter of fungal colonies after the incubation periods of 3 days, 6 days and 9 days respectively.

Results and Discussions

Visual Analysis

The final colloidal solution of CuNPs was blackish brown in color (Fig 1A.). After the centrifugation and dispersion in de-ionized water, the solution turned into pale yellow color (Fig 1B.) which was stored in air tight container to prevent oxidation.

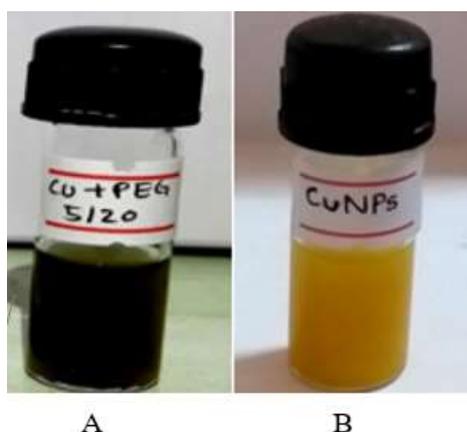


Fig. 1: Colloidal solution of CuNPs (a) before and (b) after purification

UV- Visible spectroscopy

The Fig. 2 shows the absorption spectra of the CuNPs dispersed in water. For the CuNPs, no absorption in the visible range was observed, while distinct peak was

observed at 320 nm. In this case, CuNPs showed no surface plasmon resonance, as shown in Fig. 2. This could be a consequence of a combination of small particle size or a thin copper oxide layer around the CuNPs. Usually, for CuNPs ranging between 10 and 40 nm in diameter, the plasmon resonance appears around 560 nm. However, the absorption spectra of small metal particles (diameter < 20 nm) depend only on the dipole oscillation and do not evidence surface plasmon resonance. These results are consistent with reports for the CuNPs in polar solvents (Díaz-Visurraga *et al.*, 2012). With the stabilizing agent, the UV spectra were shifted to lower wavelengths (~ 320 nm). This shift is probably due to the slight reduction of particle size by using PEG as stabilizer (Díaz-Visurraga *et al.*, 2012).

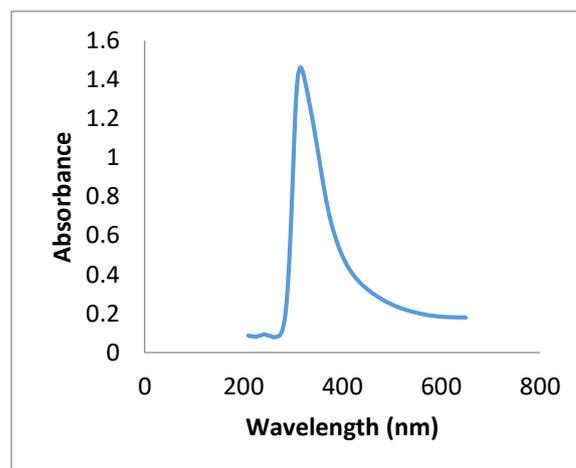


Fig. 2: UV-Visible absorption spectra of CuNPs

FT-IR Spectroscopy

The FT-IR spectra (resolution of 4 cm^{-1}) were recorded from 4500 to 500 cm^{-1} at room temperature. Fig. 3 represents the appearance of some different peaks. The appearance of peaks between 700 and 500 cm^{-1} wave number represents the formation of metal oxide bond indicating the presence of copper oxide particles in the sample.

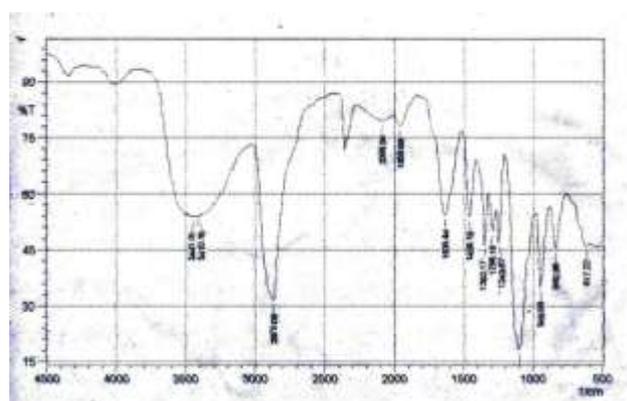


Fig. 3: FTIR spectra of synthesized CuNPs

X-Ray Diffraction Analysis

The solvent in CuNPs synthesized by chemical reduction method was evaporated at 80°C for 2 hours in hot air oven. After the sample had been dried, they were used for XRD analysis. Fig. 4 shows the XRD pattern of CuNPs. Data was taken for the 2θ range of 20 to 80 degrees with a step of 0.05 degree. The peaks observed at 2θ values of 42.379° and



Figure 6: Result of MIC of *E. coli*



Fig. 7: Result of MIC of *S. aureus*

Table 3: Result of MIC test

ppm	Test organisms	
	<i>E. coli</i>	<i>S. aureus</i>
0	+	+
0.5	+	+
1	+	+
2	+	+
4	+	+
8	+	+
16	+	+
32	+	+
64	+	+
128	-	-
256	-	-
512	-	-

Bacterial Growth Kinetics in Presence of CuNPs

Fig. 8 and 9 demonstrate the growth profile of the two representatives' bacterial strains treated with various concentrations of CuNPs. The results showed that for both bacterial strains, introduction of CuNPs affected the growth

kinetics as compared to the negative control (culture grown in absence of CuNPs). Bacterial growth was reduced with an increase in CuNPs concentration. At 512 ppm of CuNPs, visible growth of *E. coli* was not observed till 22 hrs and thus it represents the bactericidal concentration for *E. coli*. Similarly, for *S. aureus*, at 256 and 512 ppm, visible bacterial growth was not observed till 22 hrs and thus 256 ppm was considered as MBC for *S. aureus*.

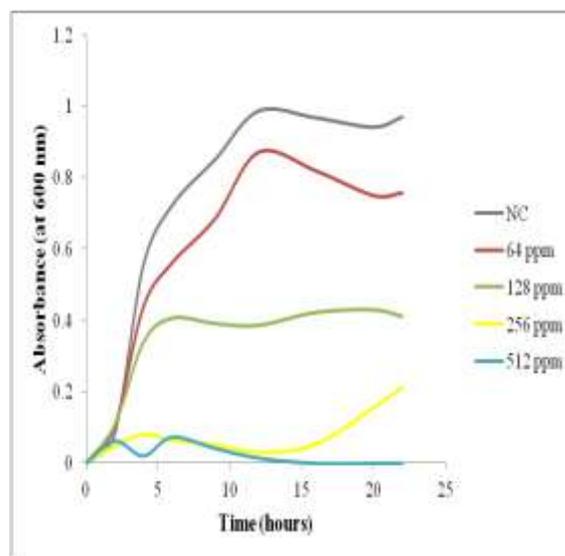


Fig. 8: Effect of CuNPs on growth kinetics of *E. coli*

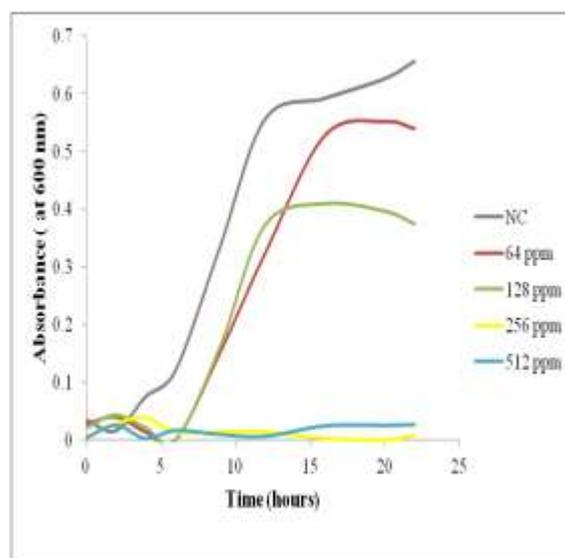


Fig. 9: Effect of CuNPs on growth kinetics of *S. aureus*

From these graphs, it indicates that, CuNPs are more effective to Gram positives than Gram negative bacteria. The value of MBC for *E. coli* was higher than that for *S. aureus*. This result can be explained because *E. coli* is gram negative bacterium and it is less susceptible to antimicrobials than Gram-positive bacteria. The Gram-negative bacteria tend to be more resistant to lipophilic and amphiphilic inhibitors than that of Gram-positive, including dyes, detergents, free fatty acids, antibiotics and chemotherapeutics agents (Nikaido, 1996).

Anti-Fungal Activity of CuNps

We surveyed the effect of CuNps solutions on the development of *Alternaria spp.* by determining the diameter of the fungal colonies on the samples with CuNps solution with the concentrations of 100 ppm, 200 ppm, 400 ppm and 800 ppm in reference to the control sample without CuNps. The diameter of the fungal colonies was determined after the incubation periods of 3 days, 6 days and 9 days respectively. It demonstrated that the diameter of colonies in all samples was supplemented with CuNps being smaller than the reference sample. The images of colonies according to incubation period at various CuNps concentrations are shown in figure 10.

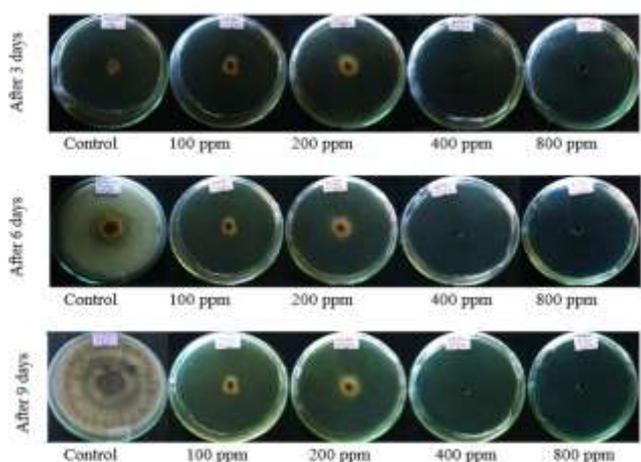


Fig. 10: The diameter of *Alternaria spp.* colonies according to incubation period at various CuNps concentrations

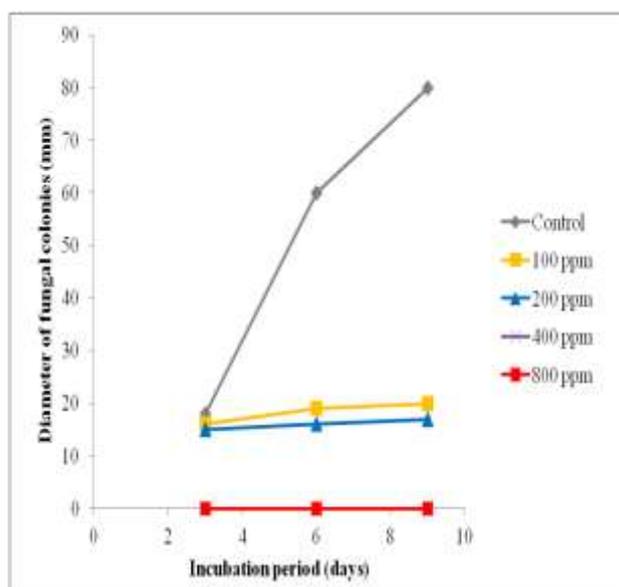


Fig. 11: Formation of the diameter of *Alternative spp.* colonies according to incubation period at various CuNps solution concentration

The Fig. 10 shows that CuNps inhibited the development of *Alternaria spp.* In 3-day incubation, the diameters of fungal colonies for additional formulations of 100 ppm, 200 ppm

of CuNps were measured about 16mm, 15 mm respectively but no fungal growth were observed in 400 ppm and 800 ppm in reference to control plate with 18mm. Diameter of fungal colonies of sample at 100 and 200 ppm CuNps concentration increased slightly till 9 days incubation, while that in 400 and 800 ppm, no fungal colonies were observed.

In addition, Fig. 11 shows the change of the diameter of fungal colonies at various incubation periods for various concentrations of CuNps solutions. The diagram of the diameter of the colonies with the CuNps concentration of 400 ppm and 800 ppm was a straight line. Therefore, this result demonstrated that the CuNps shows anti-fungal activity at 400 ppm and higher. It also shows that more the CuNps concentration less is the diameter of fungal colonies.

Conclusion

Thus, CuNps synthesized by chemical reduction method can be used effectively as anti-bacterial and anti-fungal agents.

Author's Contribution

All authors in this paper equally participated in all steps of research works, preparation & revision of the manuscript. Final form of manuscript was approved by all authors.

Conflict of Interest

The authors declare that there is no conflict of interest with present publication.

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