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Research Article

CHEMICAL SYNTHESIS OF COPPER NANOPARTICLES AND ITS ANTIBACTERIAL EFFECT AGAINST GRAM NEGATIVE PATHOGENS

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ABSTRACT

In this study, copper nanoparticles (CuNPs) were synthesized using a chemical method involving the reduction of copper sulphate by sodium borohydride, while polyethylene glycol 6000 served as a stabilizer. Characterization of the synthesized nanoparticles was initially carried out by UV visible spectrophotometry and the maxima absorption was observed at 280 nm. Atomic absorption spectroscopy confirmed the concentration of the CuNPs in solution to be 19.60 ppm. SEM revealed the nanoparticles to possess a spherical morphology. The size of the nanoparticles was between 117nm to 873nm while their zeta potential was determined to be 0.2mV. Antimicrobial tests were done by the agar cup diffusion assay and the macro broth dilution assay. Antibacterial activity of the synthesized copper nanoparticles was tested against common Gram negative pathogens, *E.coli, S.typhi* and *V.cholerae*. The ratio of the minimum inhibitory concentration and minimum bactericidal concentration of the particles was equal to one for all three test organisms, indicating CuNPs to be a good bactericidal agent.

Keywords: Copper nanoparticles, Chemical synthesis, *E.coli, S.typhi*, V.cholerae, Antibacterial

1. INTRODUCTION

Copper, one of the first metals to be extracted and used by humans, has made a vital contribution in sustaining and improving our society. The antibacterial potential of storage of water in copper and brass vessels against common waterborne pathogens such as *Escherichia coli, Enterococcus faecalis, Salmonella* and *Vibrio cholerae* has been well documented [1,2]. One of copper's more recent applications includes its use in frequently touched surfaces such as brass doorknobs and handles, where its antimicrobial properties reduce the transfer of bacteria and disease [3].

The last decade has seen the beginnings of applications of nanotechnology in commercial products. Nanoparticles are of great scientific interest. The interesting and sometimes unexpected properties of nanoparticles are attributed largely due to their large surface area. Recently copper nanoparticles have been synthesized by several methods and their applications explored [4]. Copper nanoparticles have been tested for their use as antibacterial and antifungal agents when incorporated in coatings, plastics and textiles. They have been explored for use in copper diet supplements as well as in integrated circuits, super strong metals, alloys, nanowires and nanofibers. Additionally, they have been investigated to serve as catalyst in certain procedures. [5].

Previously, researchers have reported the antibacterial property of copper nanoparticles [6, 7]. In 2003, C.C. Trapalis et al. prepared composite copper containing silicate thin coatings (Cu/SiO2) on glass substrates and examined the antibacterial activity against *E.coli* [8]. In 2010, R. Usha et al. incorporated copper nanoparticles in several kinds of materials such as cloths. These cloths were sterile and could be useful in hospitals to inhibit or to minimize infection with pathogenic bacteria such *as S.aureus, E.coli* and *Aspergillus* [9].

Copper nanoparticles, due to their unique physico-chemical and biological properties could have far reaching industrial and medical applications.

The current research focuses on the development and characterization of chemically synthesized copper nanoparticles and further evaluating their antibacterial activity against common water borne pathogens *E.coli, S.typhi and V.cholerae.* The study also compares the antimicrobial of copper nanoparticles with that of copper sulphate which is known to possess disinfectant properties.

2. MATERIAL AND METHODS

2.1. Test microorganisms

Microbial suspensions of *E.coli, S.typhi* and *V.cholerae* were obtained from a single colony isolated on agar plates and inoculated in nutrient broth (HiMedia) for overnight cultures. After incubating microbial cells at 37^0C overnight, optical density (OD) of the suspension at 600 nm was adjusted to 1.0 using a colorimeter (ErmaInc.Colorimeter). The suspension was diluted with phosphate-buffered saline (pH 7.4) to 1:100 and suspended to final concentration of 1.0×10^7 cells/mL.

2.2. Synthesis of copper nanoparticles

In this study, copper nanoparticles were synthesized using a chemical reduction method involving the reduction of copper sulphate by sodium borohydride and the stabilizing agent being polyethylene glycol 6000.

The four-step preparation scheme for copper nanoparticles [Fig.1-4] was started by dissolving 0.01 M $CuSO_4$.$5H_2O$, in distilled water to obtain a blue solution. Next, 0.02 M PEG 6000 was dissolved in water and added to the aqueous solution containing the copper salt while vigorously stirring. In this step, the solution changed from blue to white. In the third step, ascorbic acid (0.02 M) and NaOH (0.1 M) was dissolved in water and added to the synthesis solution. Color change occurred in the aqueous phase from white to yellow. Finally, a solution of NaBH4 (0.1 M) in distilled water was prepared and added to the solution under continuous rapid stirring. An instant color change occurred in the aqueous phase from yellow to blackish green which indicated the presence of copper nanoparticles.

The nanoparticles thus prepared by both of the above methods were stored in air tight tubes as they readily oxidize in ambient conditions.

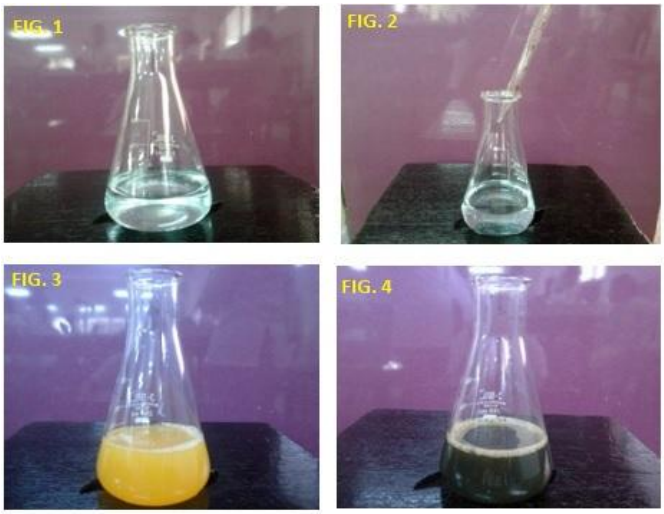

Fig. 1: $CuSO_4$.$5H_2O$ dissolved in D/W, Fig. 2: PEG 6000 was added to the aqueous solution, Fig. 3: Ascorbic acid and NaOH was added to the synthesis solution, Fig. 4: $NaBH_4$ was added to the solution. An instant color change occurred from yellow to black which indicated the presence of CuNPs

2.3. Characterization of the synthesized copper nanoparticles

To confirm the presence of copper nanoparticles, UV-visible spectrophotometer (Systronics 117) was used to measure the peaks exhibited by copper nanoparticles. Samples were analyzed by using the broth solutions as blanks.

Quantification of the prepared copper nanoparticles was carried out by atomic absorption spectroscopy (Perkin Elmer AA 700) using air- acetylene flame. Prior to analysis, the sample was digested using nitric acid on hot plate for 3hrs. The dry residue obtained was diluted with 5% nitric acid and filtered through 0.45µ filter and used for the analysis.

Further, the morphology of the nanoparticles was studied using scanning electron microscope equipped with an Energy Dispersive X ray Analyzer (Icon Analytical Pvt.Ltd).Each specimen was dispersed ultrasonically to separate individual particles before analysis.

The zeta potential and the particle size distribution were confirmed with a nanoparticle analyzer (HORIBA- SZ100Z).

2.4. Testing the antibacterial activity of copper nanoparticles and copper sulfate using Agar cup diffusion assay

Agar cup diffusion assay was performed on Mueller Hinton agar. The test cultures *E.coli, S.typhi* and *V.cholerae* were swabbed using sterile cotton swabs on the MH agar plates. Using a sterile cork borer wells were punched out and specified volume of the copper nanoparticles /copper sulphate was used to fill the wells. The plates were then incubated at 37°C/ 24 hrs and the zones of inhibition were observed on the following day.

2.5. Determination of Minimum inhibitory concentration of copper nanoparticles

Using stock solution of the prepared silver nanoparticles, various dilutions was prepared. 0.1 ml of the 24 hr old test culture was added to all dilutions. Positive and negative controls were maintained. Tubes were incubated at 37°C for 24 hrs and the lowest concentration that did not show growth corresponded to the minimum inhibitory concentration (MIC).

2.6. Determination of minimum bactericidal concentration of copper nanoparticles

About 0.1 ml of culture from the tube indicating minimum inhibitory concentration and all higher concentrations beyond it were surface spread on nutrient agar and incubated at 37°C for 24 hrs. The minimum concentration showing no growth corresponded to the minimum bactericidal concentration (MBC).

All microbiological experiments were done in triplicate and repeated three times. Mean values have been reported.

3. RESULTS AND DISCUSSION

The formation of chemically synthesized CuNPs was initially confirmed visually [Fig.5]. On visual inspection, the solution turned blackish green in colour due to surface plasmon resonance phenomenon indicating the formation of CuNPs in the reaction mixture.

The UV-Vis absorption spectrum showed a strong peak at 280 nm which indicated the formation of CuNPs (Fig. 6)

The band was observed only after addition of sodium borohydride. Since samples taken prior to the addition did not show any visible peak, it suggests that nanoparticles are not formed after the addition of NaOH.

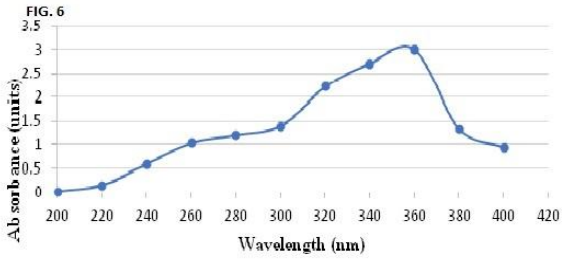

Fig.5: Synthesized CuNPs, Fig 6: UV visible spectrum CuNPs

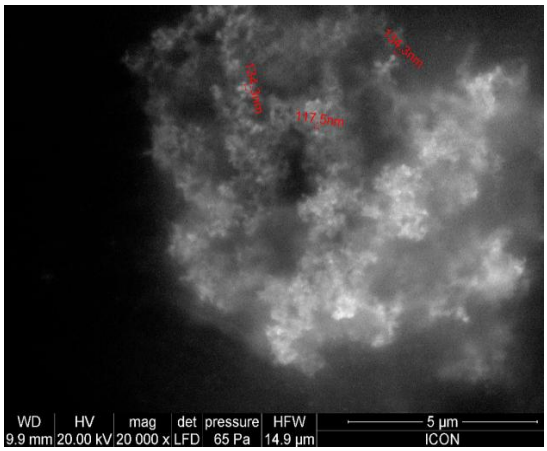

Fig. 7: SEM of CuNPs

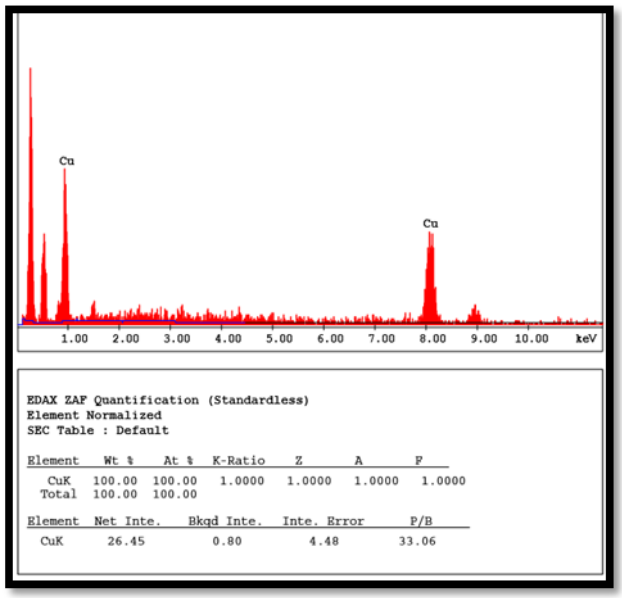

Fig. 8: EDX Spectra of CuNPs

Scanning electron micrograph revealed spherical nanoparticles (Fig. 7) Elemental analysis of the chemically synthesized copper nanoparticles were also carried out and the results showed the presence of copper, carbon and oxygen (Fig. 8).

Particle size and distribution are the most important characteristics of nanoparticles. They determine the in vivo distribution, biological fate, toxicity and the targeting ability of the nanoparticle systems. Though the exact reason is yet unknown, it has been observed that smaller the size of the nanoparticles, greater is their antibacterial activity.

Hence the synthesized nanoparticles were tested for their size distribution using the HORIBA Partica analyzer which uses the principle of dynamic light scattering (DLS) to test the samples. The size distribution of the CuNPs was within 117nm to 873nm. The zeta potential of the particles was found to be 0.2mV (Fig.9). This indicates that the particles were unstable which may be attributed to the presence of some agglomerates that sedimented at the bottom of the analysis tube. The concentration of CuNPs was found to be 19.60 ppm by atomic absorption spectroscopy.

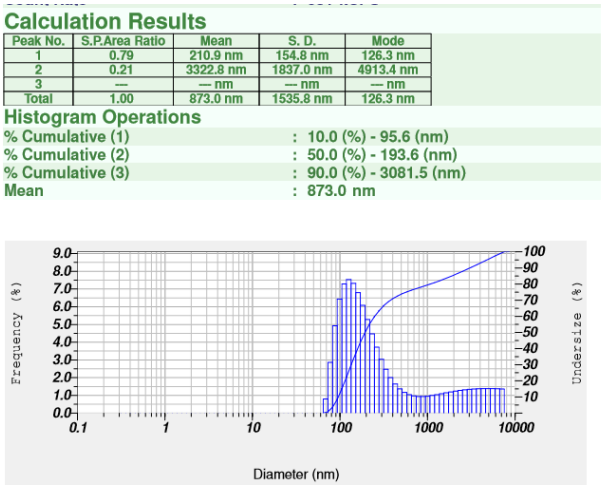

Fig. 9: Particle size analysis of CuNPs

The MIC and MBC of the synthesized copper nanoparticles for all three cultures namely, *E.coli, S.typhi, V.cholerae* was found to be 1.37×10^{-3} mg/ml. Since, the ratio of MIC to MBC was equal to one for each case, the prepared nanoparticles could be considered to possess potent bactericidal activity. The results of agar cup diffusion assay are depicted in Table 1.

***Table** 1: Agar cup diffusion test against bacterial cultures.*

Test organism	ZOI(mm)- CuNPs	ZOI(mm)– $CuSO_4$
E.coli	17±2	15± 1
S.typhi	20 ±3	12 ± 2
V.cholerae	19±2	17 ± 2

Copper sulfate is commonly used for the destruction of algal blooms in reservoirs and swimming pool water. It is also used as an antiseptic and a fungicide to prevent infections by incorporating it in the flooring mixture of swimming baths.

From the size of zone of inhibition (ZOI) observed by the agar cup method, it can be stated that the nanoparticles had significant antibacterial activity which was even more than that

observed with copper sulfate. The algicidal and fungicidal properties of these copper nanoparticles could be additionally evaluated. If was found to possess superior activity, copper nanoparticles may serve as a better option for use in public health and medicine in the future.

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