“Synergistic Research Into The Antimicrobial Behavior of Copper Oxide Nanoparticles (CuO NPs) against *Escherichia coli, Staphylococcus aureus,* and *Ascochyta fabae*”

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Abstract

The present study was performed to evaluate the antibacterial and antifungal activity of four different concentrations (25%, 50%, 75%, and 100%) of copper oxide nanoparticles (CuO-NPs) against three pathogens (*Escherichia coli, Staphylococcus aureus*, and *Ascochyta fabae*). There are various methods to synthesize metallic nanoparticles (NPs), including chemical, physical, and biological processes. In this study, Bacillus sp. FU4 was used as a biological source for the biosynthesis of CuO NPs. The synergistic antibacterial and antifungal activity of the copper oxide nanoparticles was tested on gram-negative and gram-positive bacteria, *Escherichia coli* (ATCC25922), *Staphylococcus aureus* (ATCC 43300), and the fungus *Ascochyta fabae* (ATCC38599) using the well diffusion, poison food and microdilution methods. It is known that the synergistic effects of transition metal-based nanocomposites have enhanced antimicrobial activities. CuO NPs were prepared using copper sulfate (CuSO4). CuO NPs were formed after the oxidation of Cu NPs. The average effect of CuSO4 concentration (0.1, 0.01, and 0.001 M), incubation, and cultivation time (48, 72, 96 hours) as three-level controllable factors were evaluated in the biosynthesis of CuO NPs. The characterization of CuO NPs was determined by Fourier transforms infrared spectroscopy (FT-IR). Furthermore, the antimicrobial properties of CuO NPs were investigated with *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 43300 as multidrug-resistant (MDR) bacteria. Although antibiotics can treat most bacterial infections, increasing microbial resistance is limiting the benefits of antibacterial agents in fighting infectious diseases. The threat posed by copper oxide nanoparticles prompts the search for alternative approaches to treating bacterial infections. Recently, copper oxide nanoparticles have been extensively studied for their application in combating microbial infections. This research attempts to briefly summarize the current studies on the antibacterial properties of copper oxide nanoparticles.

The following was evaluated: The size distributions of the NPs ranged from 2–41 nm with spherical shapes. The antimicrobial activities of CuO NPs were measured by inhibition zone diameter in disc diffusion assays of NPs dispersed in batch cultures. Two concentrations of CuSO4 (0.1 and 0.01 M) had an antibacterial effect on *E. coli* (330.57 and 6.2 mm). In the case of *S. aureus*, surprisingly, there were no signs of growth. This research shows that copper nanoparticles have strong antimicrobial activities and can be used to control and treat various infectious diseases in the future. The antifungal activity of ethanol extract of CuO nanoparticles was calculated against isolated phytopathogenic fungi by the evaluation using the poisoned food method. It emerged that the synthesized CuO nanoparticles were highly efficient against *Ascochyta fabae*. To understand the antifungal activity of CuO-based nanoparticles along with the detailed mechanism the morphological analysis using microscopic instruments is believed to play an important role. Various microscopic techniques like SEM, and TEM give detailed information about nanoparticles. Copper oxide nanoparticles (CuO-NPs) showed excellent antimicrobial activity against different bacterial and fungal strains (*Escherichia coli, Staphylococcus aureus, and Ascochyta fabae*). Possible mechanisms of the antimicrobial activity of CuO NPs should be further investigated. CuO NPs have an antibacterial activity that may be beneficial in the medical field in combating prominent pathogens such as *Escherichia coli* (ATCC25922), *Staphylococcus aureus* (ATCC 43300), and the fungus *Ascochyta fabae* (ATCC38599).

Keywords: Synergistic, Antifungal, Antibacterial, Copper Oxide Nanoparticles, Human Pathogens.

INTRODUCTION

Several chemical and organic compounds with antibacterial effects such as penicillin (lactam group) and natural substances kill bacteria or slow down their growth. Among them, nanoparticles (metal and semiconductor particles) have recently received more attention. Reactive oxygen species (ROS) such as superoxide anion (O-), hydrogen peroxide (H2O2), hydroxyl radicals (HO), and organic hydroperoxides (OHP), NP deposition on the surface of microorganisms and NP accumulation in the cytoplasm/ periplasmic area of bacteria, can be used for lead to the death of microorganisms. In the case of bacteria, ROS can damage cellular components such as lipids, peptidoglycans, proteins, and DNA by generating ROS through NPs and subsequently physically destroying them. Metallic and semiconductor NPs are important materials to study in the field of nanomedicine. This interest is related to size and shape based on physicochemical properties. The surface area to volume ratio of NPs is an important factor for these properties. In this case, copper oxide (CuO) and its nanometer-scale alloy are some of the most important materials used in the industry. Furthermore, these metallic NPs can be used as an alternative to silver and gold NPs. There are different methods to prepare CuO NPs; specifically characterized as a chemical, physical, and biological process. For example, as a physical method, proton irradiation and vacuum vapor deposition (VVD) can synthesize a wide range of metallic NPs. These methods have several disadvantages. Since the cost of these methods is higher and there is no approach to environmental protection.

Therefore, an environmentally friendly perspective on the fabrication of metal NPs using biological systems is essential. Plants, algae, yeast, fungi, and bacteria can be applied as a green approach for the biosynthesis of metal NPs. It is known that bacteria have mechanisms to survive under difficult conditions such as large amounts of toxic metals by converting toxic metal ions into their corresponding non-toxic forms (metal sulfides/ oxides). It is noteworthy that these mechanisms play a prominent role in NP biosynthesis. Also, NP biosynthesis by bacteria has several advantages compared to other organisms, such as B. Easy cultivation, extracellular NP production under mild experimental conditions (temperature, pH) and is affordable and not time-consuming. In this study, Bacillus sp. FU4 was used as a biological source for the biosynthesis of CuO NPs; In addition, XRD, FT-IR spectroscopy, and SEM have been employed as useful techniques to characterize CuO NPs. Finally, the antimicrobial activity of CuO NPs was examined by a well disk diffusion assay and minimum inhibitory concentration (MIC) of CuO NPs against various bacterial strains.

MATERIALS AND METHODS

Experimental design of the Taguchi methodology to optimize the experimental conditions, all combination experiments were performed using the assigned parameter values. Qualitek 4 software for the design and analysis of Taguchi experiments was used as the statistical method. Table 1 shows three controllable factors (CuSO4 concentration, incubation time, and cultivation time) and their levels in the design of the experiment.

MATERIALS

Copper (II) sulfate pentahydrate, 98% (CuSO4.5H2O), nutrient agar and Potato Dextrose Agar were purchased from Sigma-Aldrich and used without further purification for the synthesis of CuO NPs and measurement of antibacterial and antifungal activity.

CuO NPs BIOSYNTHESIS AND PRODUCTION OF SUPERNATIVE MATERIAL

Bacteria Bacillus sp. FU4 was obtained from a bacterial archive at Razi University, Kermanshah. The growth conditions were simple: growth in 0.5 nutrient broth (NB) medium at 37°C for three time steps (48, 72, and 96 hours). After bacterial growth at these times, culture media containing bacteria were centrifuged at 5000 rpm for 5 minutes. Then 5 ml of supernatant was added to CuSO4 in three concentration levels (0.1, 0.01, 0.001 M) in the Erlenmeyer flask in three replicates for each concentration level. Subsequently, these solutions were incubated with stirring (100 rpm) for three incubation stages (48, 72, and 96 hours).

CHARACTERIZATION

The structure, morphology, and elemental composition of the prepared tempered samples were characterized using XRD and SEM analysis tools. Crystallographic analysis was performed with an EQUINOX 3000 diffractometer in the 20-70 scan range (2) using Cu Ka radiation of wavelength 1.5406. A scanning electron microscope (model XL30, Philips, Eindhoven) was used to examine the morphology and size of NPs. The intensity of the absorption peaks of NPs was examined from 400 to 800 nm with a UV-Vis Spectrophotometer (Tomas, UV 331). In addition, Fourier transforms infrared spectroscopy measurements were performed with a spectrophotometer (Germany, Bruker, Model: ALPHA).

ANTIBACTERIAL EFFECTS

Escherichia coli ATCC 25922 and *Staphylococcus aureus* ATCC 45500 were used as multidrug-resistant bacteria to measure the effect of the antibacterial properties of CuO-NPs by modified well diffusion method. MDR bacteria were cultured on Muller Hinton agar plates (MHA); wells with a diameter of 5 mm were produced using a sterilized steel cork drill. Subsequently, different concentrations of CuO NPs (three levels with concentrations of 0.1, 0.01, and 0.001 M) were loaded into wells. The plates were then incubated at 37°C for 48 hours.

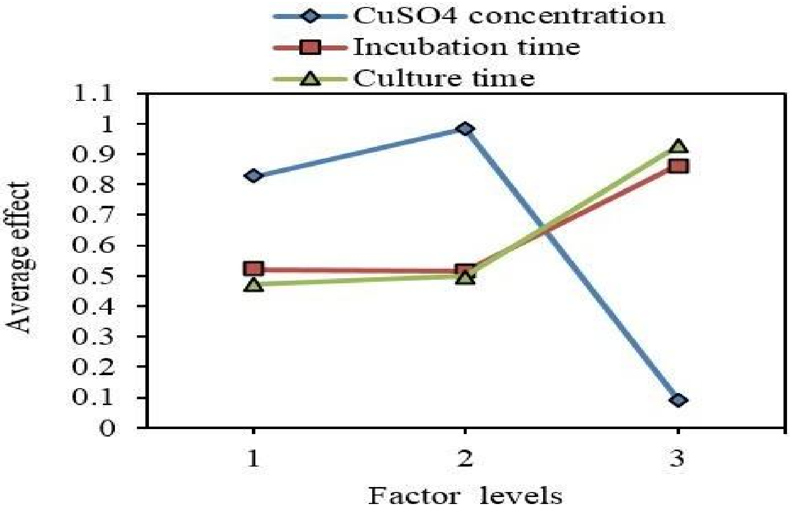

Figure 1. Taguchi results of the average effect of CuSO4, incubation time, and culture time.

UV-Vis analysis of CuO NPs biosynthesis

When cell-free supernatant from Bacillus sp. FU4 was added to the CuSO4 solution and incubated three times (48, 72, 96). The color reaction of the mixture changed from blue to light green. The UV-Vis spectroscopy spectrum absorption for this solution showed a clear absorption peak in the range of 700–800 nm.

ANTIBACTERIAL ACTIVITY

Antibacterial activity is defined as killing bacteria or reducing their growth without being generally toxic to the surrounding tissues of the body. There are several reports on the antibacterial properties of NPs. In this study, the antibacterial activity of CuO NPs was indicated by a well diffusion assay. Based on the assessment of antibacterial activity and maximum zone of inhibition, two major multidrug-resistant pathogenesis bacteria, *E. coli* ATCC 25922 and *S. aureus* ATCC 43300, were used. The results show that Green synthesis of CuO NPs (5-10 nm, spherical shape) showed antibacterial activity against S. aureus and *E. coli* in two CuSO4 concentration levels (0.1 and 0.01 M).

ANTIFUNGAL ACTIVITY OF CuO NANOPARTICLES

Copper Oxide nanoparticles (CuO NPs) have shown great antifungal activity against phytopathogenic fungi, making them a promising and affordable alternative to conventional fungicides. In this study, we evaluated the antifungal activity of CuO NPs against the fungus *Ascochyta fabae*. (Sharma and Chander 2021; Sharma et al, 2023)

The antifungal activity of CuO NPs against the fungus *Ascochyta fabae* was evaluated using the poisoned food method. Briefly, PDA was mixed with different amounts of CuO NPs. All treatments were carried out in triplicate. diameters of growing mycelia were measured after fifteen days after inoculation. The percentage of growth inhibition was calculated by measuring the average area of the fungal mycelia in the treatments and compared to the negative control. (Sharma and Chander 2020)

The disk of 6mm diameter of fresh fungal culture was placed into a sterile PDA medium already poured into sterile petri plates under sterile conditions. Four different concentrations of stock solution (15 g/ml) were prepared by serial dilutions, i.e., 25%, 50%, 75% and 100%. These solutions were already added to the PDA containing petri plates A, B, C, and D respectively. In this experiment, griseofulvin (15 g/ml) was used as a positive control agent, and ethanol as a negative control. The plates were incubated at 27°C in the dark for fifteen days. After fifteen days, inhibition zones were recorded to determine the minimum inhibition concentration.

DISCUSSION

The Taguchi method was used to optimize the adjustment of process factor values to improve quality characteristics and to identify the product factor values among the optimal process factor values. In this case, the Taguchi method uses orthogonal arrays as a specific design to explore the entire factor space with minimal experimentation. To analyze the quality characteristics, the Taguchi method uses three types of signal-to-noise (S/N) ratios. In this study, we used the S/N type "the higher the better". For the design experiment, three parameters (CuSO4 concentration, incubation time, and cultivation time) and their three levels were used. The results show a greater influence of CuSO4 concentrations than other parameters, which can be compared to previous reports. This color change illustrates the formation and oxidation of CuO NPs. In this case, Shantkriti Srinivasan and Palanisamy Rani (2014) reported a similar appearance of a green-colored solution upon the addition of CuSO4 to a flask containing Pseudomonas fluorescens. Due to specific particle properties such as size, shape, and capping agent, the exact position of the SPR band can shift. Copper oxide NPs synthesized from *Aspergillus clavatus* species confirmed the presence of copper oxide NPs at 300 nm. Compared to previous studies, the green synthesis of CuO NPs by Malva sylvestris and *Phyllantus amarus* plant leaves had a spherical shape.

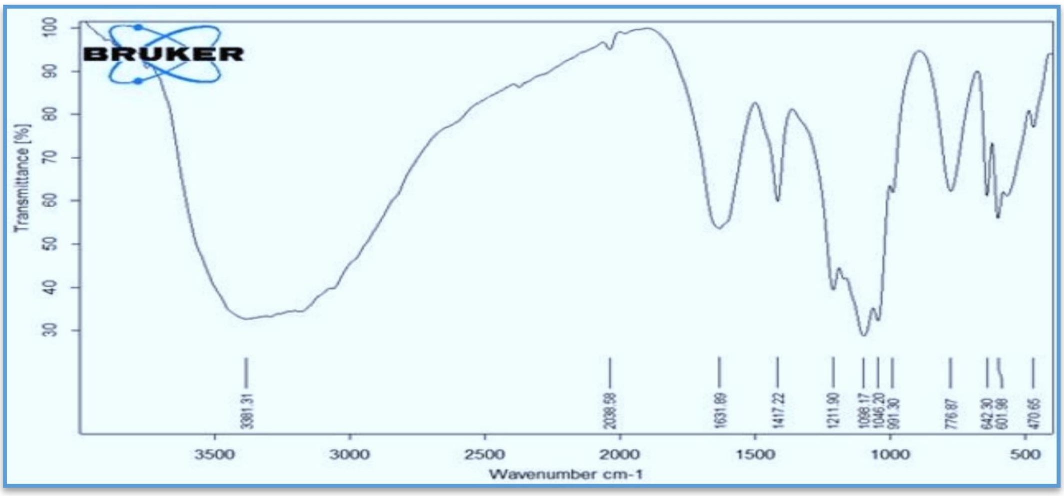

Figure 2: FTIR peaks of CuO NPs green synthesized by *Bacillus* sp. FU4.

The interaction between the CuO NPs and the medium gives rise to the corresponding vibrational binding, which shows a reaction between the Cu NP surface and the carbonyl and hydroxyl functional groups. As shown in a similar study, these functional groups can contribute to NP biosynthesis through their capping function. The grain size of CuO NPs (64.97 nm) was calculated using Scherrer's formula. Similarly, Sonia et al. (2016) report a grain size of 50 nm for copper oxide NPs. As shown in Figure 2, the quality and composition of biosynthesized CuO NPs were indicated by Fourier transform infrared spectroscopy (FTIR) in the 400–4000 cm-1 range. Similar studies revealed the Cu-O bond in CuO NPs at 430, 507, and 606 cm-1.

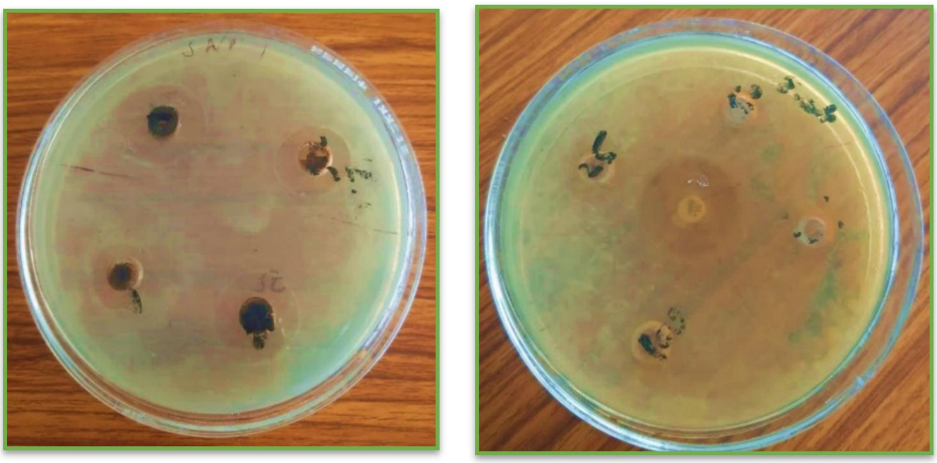

Figure 3. Bactericidal activity of CuO NPs on *E.coli* ATCC 25922, *S. aureus* ATCC 43300.

ANTIFUNGAL ACTIVITY

The synthesized CuO nanoparticles are very effective against this harmful phytopathogen. At different concentrations, these particles differentially inhibited the *Ascochyta fabae.* Table 1 below shows the zone of inhibition against *Ascochyta fabae* in mm. In this study, ethanol is the negative control, and griseofulvin (15 g/mL) is the positive control. Table 1 shows the effect of CuO nanoparticles against *Ascochyta fabae*. The ethanolic extract of CuO shows a maximum zone of inhibition at 100% concentration (15 g/ml), i.e., 16.0 ± 0.2 mm compared to the negative control. At the 75% concentration, the zone of inhibition is 13.0 ± 0.2 mm. The minimal zone of inhibition was recorded at a concentration of 25%, i.e., 8.0 ± 0.2 mm (Fig. 4) as compared to the negative control.

Table 1: Effect of CuO nanoparticles against *Ascochytafabae.*

Sr.No.	**Concentrations**	**Zone of Inhibition (mm)**
1.	25%	8.0 ± 0.2
2.	50%	10.0 ± 0.2
3.	75%	13.0 ± 0.2
4.	100%	16.0 ± 0.2
5.	Positive control	18.0 ± 0.2

Figure 4. Bactericidal activity of CuO NPs on fungus *Ascochyta fabae* (ATCC38599) with different concentrations.

Two types of bacteria: *Escherichia coli*, *Staphylococcus aureus* were used to evaluate the antibacterial activity of CuO NPs. This study showed that *E. coli* was more sensitive than second bacterial species at the highest concentration of CuO NPs (10 g/mL) with an inhibition zone of 26.0 x 1.00 mm. Sonia and coworkers (2016) investigated the antibacterial activity of CuO NPs at three different concentrations (12.5 g/mL, 25 g/mL, and 50 g/mL) using the agar diffusion method against four pathogenic bacterial species: *Serratia marcescens*, *Streptococcus pneumonia*, *Staphylococcus aureus*, and *Salmonella typhimurium*. For antibacterial mechanisms of action, physical perturbations and oxidative stress are the main cause of NP toxicity. Reactive oxygen species (ROS), including superoxide anions (O-), hydrogen peroxide (H2O2), hydroxyl radicals (HO), and organic hydroperoxides (OHP), can lead to the deposition of NPs on the surface of bacteria and the accumulation of NPs in the cytoplasm /periplasmic space lead to bacterial death3 (Manke et al.,2013). ROS can cause damage to cellular components (lipids, peptidoglycan, proteins, and DNA) by being released from NPs and subsequently entering bacteria (Manke et al.,2013).

CONCLUSION

In this study, CuO NPs with spherical shapes and average mean sizes ranging from 2–41 nm and crystal structure were grown in a green method from *Bacillus* sp. synthesized. FU4. UV-Vis, XRD, and FT-IR were used to characterize NPs. There are many studies on the biosynthesis of NPs by plants, fungi, and bacteria. Based on this study, the green method is a simple and environmentally friendly way to synthesize CuO NPs with relative purity of the NPs. Also, Bacillus sp. synthesized copper oxide NPs. FU4 extracellular and stabilization of CuO NPs were possible without using toxic capping agents. Also, these NPs have antimicrobial effects that can be used in medical aspects to combat prominent pathogens such as *E. coli* ATCC 25922 and *S. aureus* ATCC 43300 and *Ascochyta fabae* (ATCC38599). In general, this study presents simple, inexpensive, environmentally friendly, and high productivity in the fabrication of CuO NPs. Copper oxide nanoparticles (CuO-NPs) showed excellent antimicrobial activity against different bacterial strains (*Escherichia coli*, *Staphylococcus aureus*, and *Ascochyta fabae*). Possible mechanisms of the antimicrobial activity of CuO NPs should be further investigated. The copper oxide nanoparticles were tested for antimicrobial activity against human pathogens such as *Escherichia coli* (E. coli), *Staphylococcus strains* and Phytopathogen *Ascochyta fabae*. which has proven to be excellent. CuO NPs have an antibacterial activity that may be useful in the medical field in combating prominent pathogens such as *Escherichia coli* (ATCC25922), and *Staphylococcus aureus* (ATCC 43300). This approach is quite effective in the agriculture field against phytopathogen *Ascochyta fabae* (ATCC38599). The presented work proves that using Cu-NPs could be considered a highly efficient alternative with better antifungal properties than other formulations commonly proposed and commercially available fungicides.

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CONFLICT OF INTERESTS

The authors claim that there is no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

ETHICAL APPROVAL

Approved from all the ethical point of view.

DATA AVAILABILITY STATEMENT

Data will be made available on request.

ADDITIONAL INFORMATION

No additional information is available for this paper.

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