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Synthesis, Characterization and Antimicrobial Activity of Copper Nanoparticles from *Lavandula Stoechas L.* by Green Synthesis Method

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Abstract

Metal nanoparticles (copper (Cu), silver (Ag), gold (Au), platinum (Pt), zinc (Zn)) have a wide antimicrobial activity against different types of microorganisms such as gram negative-gram positive bacteria and fungi and are alternatives to antibiotics. Green synthesis is particularly preferred among synthesis methods because it is simple, environmentally friendly, cost-effective, and yields products quickly. In this study, copper nanoparticles (CuNps) were synthesized using Lavandula stoechas extract as a stabilizing agent, leveraging the properties of this medicinal and aromatic plant.

The synthesized CuNps were characterized, showing that they were spherical and less than 50 nm in size. Their antibacterial activity was assessed using both broth dilution and disc diffusion methods. The minimum inhibitory concentration (MIC) values for the bacterial strains were as follows: 250 µg/mL for *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Salmonella enteritidis*; and 500 µg/mL for *Enterococcus faecalis* and *Escherichia coli*. In the disc diffusion test, the inhibition zone diameters increased with higher CuNps concentrations across all Gram-negative and Gram-positive strains. The highest inhibition zones were recorded as 15 mm for *B. subtilis*, 16.5 mm for *S. aureus*, 14 mm for *E. faecalis*, 19.5 mm for *P. aeruginosa*, 16.5 mm for *S. enteritidis*, and 13.5 mm for *E. coli*.

In summary, this study demonstrates that CuNps can be successfully synthesised using *Lavandula stoechas* extract and exhibit significant antimicrobial properties. These findings suggest that CuNps could serve as effective alternatives to traditional antibiotics, potentially helping to address the growing issue of antibiotic resistance.

Keywords: *Lavandula stoechas L*; green synthesis; copper nanoparticles; antimicrobial activity

1. Introduction

Throughout history, scientists have battled against disease-causing microorganisms, with antibiotics becoming a key weapon against bacterial infections since the 1940s (Tenover 2006; Sengupta 2013). Despite this, infection-related morbidity and mortality remain alarmingly high (Lagedroste et al. 2019; Canlı et al. 2019). The excessive and indiscriminate use of antibiotics has led to a crisis of antibiotic resistance, marked by multidrug-resistant “superbugs” and biofilm formation (Lagedroste et al. 2019; Beyth et al. 2015). Consequently, there is an urgent need for alternative antibiotic treatments, with nanoparticles (NPs) emerging as a promising option (Lagedroste et al. 2019; Canlı et al. 2019).

Traditional antibiotics generally target bacterial cell walls, protein synthesis, or DNA replication mechanisms (Tenover 2006; Wang et al. 2017). In contrast, nanoparticles directly interact with the bacterial cell wall without entering the cells, making it difficult for bacteria to develop resistance. While the antibacterial mechanisms of NPs are not fully understood, one proposed mechanism involves metal ions from the NPs attaching to bacterial cell walls through transmembrane proteins, thereby obstructing

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transport channels and altering cell membrane structure. Once inside the cell, these ions cause cell death (Prabhu et al. 2012; Dizaj et al. 2014). Additionally, reactive oxygen species (ROS) produced by metal NPs damage essential cellular structures, including the peptidoglycan layer, cell membranes, DNA, mRNA, ribosomes, and proteins, contributing significantly to their antibacterial effects (Raffi et al. 2008; Pelgrift and Friedman 2013). Metal ions can also bind with thiol groups in enzymes, inactivating them, and they can disrupt DNA by binding to purine and pyrimidine bases, breaking hydrogen bonds and destroying DNA integrity (Jung et al. 2008; Hoseinzadeh et al. 2017; Shahzadi et al. 2018).

Copper nanoparticles (CuNps) have become popular in recent years because they have a high surface-to-volume ratio, work very well as catalysts, and kill microbes very effectively. They are also cheaper than noble metals like silver, gold, and platinum (Olajire et al. 2018). The antimicrobial activity of CuNps is attributed to the release of copper ions (Mott et al. 2007). Although various physical and chemical methods exist for NP synthesis, these methods are often costly and generate toxic by-products. Additionally, they make it difficult to precisely control NP surface chemistry, size, and structure.

Given these limitations, green synthesis has gained attention as an affordable, environmentally friendly, and non-toxic alternative. This method uses living things like plants, algae, bacteria, yeasts, and fungi to change inorganic metal ions into metal nanoparticles by using proteins and metabolites to break them down (Manikandan et al. 2017; Kumar et al. 2017). Plants, which are rich in phytochemicals like flavonoids, terpenoids, tannins, and alkaloids, are especially popular for green synthesis.

In this study, *Lavandula stoechas* L., a medicinal and aromatic plant, was chosen as the reducing and stabilizing agent for NP synthesis due to its abundance of natural polyphenols, flavonoids, glycosides, saponins, and essential oils. Using the green synthesis method, this study aims to determine the antimicrobial activity of NPs synthesized from *L. stoechas*, avoiding toxic and costly chemicals.

2. Materials and Methods

Preparation of plant extract and CuNps synthesis

CuNps were synthesized using an assisted green synthesis method with *L. stoechas* extract as a reducing and stabilizing agent, following a modified approach (Rajesh et al. 2018). Dried *L. stoechas* was washed with distilled water, and a 15 g sample was prepared in 400 ml of distilled water and incubated on a magnetic stirrer at 1000 rpm for 24 hours at room temperature. After centrifuging at 10,000 rpm and 24°C for 20 minutes, the supernatant was stored at 4°C. For CuNps synthesis, a 0.001 M copper acetate solution was added to the plant extract at a 10:1 ratio and incubated at 60–70°C for 2 hours. A color change, indicating CuNps formation, was observed. The mixture was then centrifuged, washed, and dried at 80°C for 24 hours. The dried CuNps were transferred to sterile tubes and stored in the dark at room temperature.

Characterization of CuNps

Np's size, shape, surface morphology, stability, crystallographic structure and functional groups transmission electron microscopy (TEM) (Hitachi HighTech HT 7700), scanning electron microscopy (SEM) (Zeiss Sigma 30), UV-Vis spectroscopy (UV-Vis), fourier conversion infrared spectrophotometer (FTIR) (Bruker Vertex 70v), and X-ray diffraction (XRD) (PANalytical Empyrean) has been characterized.

Antimicrobial assay

Antimicrobial activities of CuNps were tested using agar disc diffusion method and broth dilution method for *P. aeruginosa, B. subtilis, S. aureus, S. enteritidis, E. coli, E. faecalis*. Agar disc diffusion test was carried out according to Shende *et al.* (2015), the stock solution was prepared from Np as 250 µg/mL, 500 µg/mL, 750 µg/mL, 1 mg/mL and the application was made. Broth dilution test was performed according to Wiegand *et al.* (2008). Serial dilutions were made at concentrations ranging from 1000 to 1.95 µg/mL and the last tube without bacterial growth was considered as the minimum inhibitory concentration (MIC) value.

3. Results

Characterization of CuNps

TEM and SEM

TEM and SEM images of CuO NPs were given in Figure 1 and Figure 2. Both images show that the particles have different shapes and diameters. It has been determined that the shapes of CuNps's are spherical and their size are <50 nm. TEM images of CuNps have shown an organic coating layer around the Np. This layer is proof that the nanoparticles synthesized from the plant show an excellent dispersion in solution (Kahrilas et al. 2014).

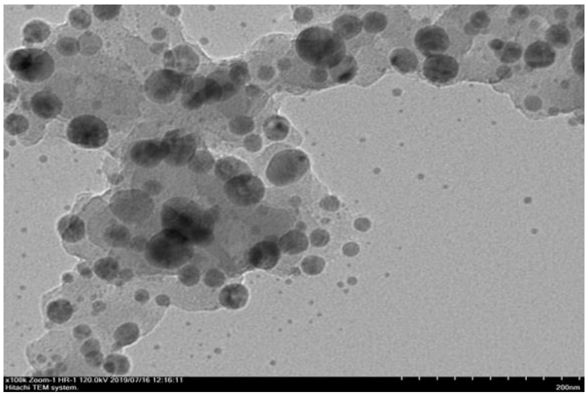

Figure 1. *TEM image of CuNps.*

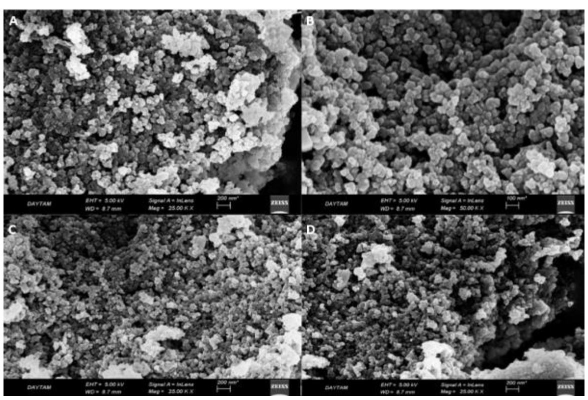

Figure 2. *SEM images of CuNps.*

UV-Vis spectroscopy

CuNps were measured at wavelength range of 200-875 nm. It shows that the maximum absorbance of the CuNps is at 310-320 nm according to the UV-Vis spectrum (Figure 3). The maximum peak value of 310-320 nm shows the reduction process and the formation of Np's. The decrease in the size of the nanoparticles leads to an increase in the UV-Vis bandwidth (Yeshchenko et al. 2012). In addition, metal nanoparticles can be agglomerated due to Van Der Waals interactions. For this reason, the absorbance values to be obtained may deviate from the expected (Hassanien et al. 2018).

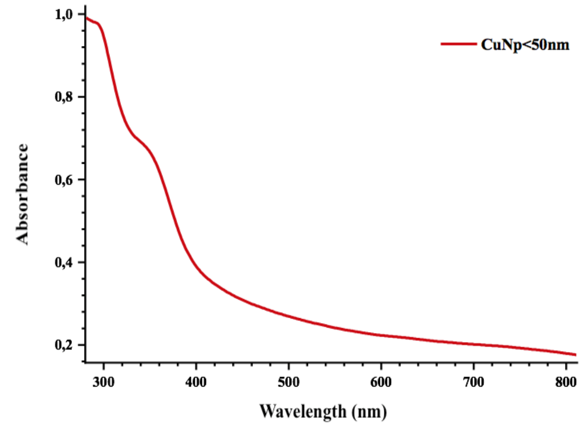

Figure 3. *UV-Vis spectrum of CuNps.*

FTIR

Nps were measured the range of 50/4000 cm^{-1} to obtain good signal to noise ratio. FTIR measurements performed to characterize the surface structure of CuNps is shown in Figure 4. FTIR spectra of CuNps have exhibited vibrations in the area of 500-600 cm^{-1}, which can be attributed to Cu vibrations that confirm the formation of CuNps's. An absorption band at 617 cm^{-1} was observed due to the vibrations of the Cu. The band at 3373 cm^{-1} corresponds to hydroxyl functional groups (Veisi et al. 2016). Also, according to the measured spectra, alkenes (C-H) at 675-781 cm^{-1}, C-O bonds at 1053 cm^{-1}, alcohol at 1219 cm^{-1}, ester, carboxylic acid, ester groups, 1412-1450 cm^{-1} aromatic ring (CH_2), aromatics at 1623-1728 cm^{-1} (C-O, C-H, C = C), alkynes at 2139 cm^{-1} (C = C), alkane stretches at 2918 cm^{-1} (C-H) and the presence of amines (NH, -OH) at 3373 cm^{-1} was confirmed by the standard IR-correlation table (Sulpizi et al. 2012; Sathish et al. 2012; Conrad et al. 2014; Save et al. 2015; Smith 2018). The emergence of these groups in the FTIR spectrum of CuNps obtained by green synthesis using *L. stoechas* confirms the presence of some metabolites such as some reducing sugars, amino acid residues, proteins, flavanones or terpenoids (Bar et al. 2009). These functional groups play a significant role in the synthesis of copper nanoparticles.

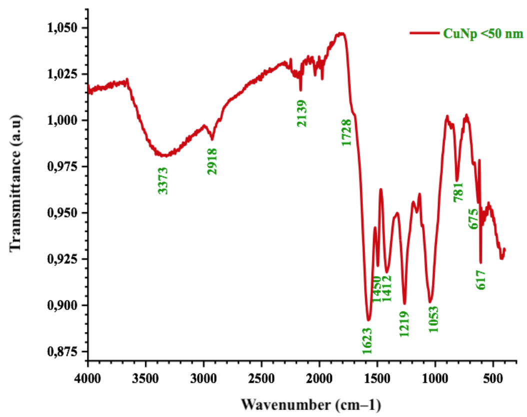

Figure 4. *FTIR spectrum of CuNps.*

XRD

The XRD pattern of the synthesized CuNps was analyzed with a step size of 0.02 in the range of 2θ between 10° and 90°. CuNps have been identified for XRD (Figure 5), which are considered as a key tool for evaluating the crystals and tertiary structures of particles at molecular levels (Sapsford et al. 2011; Li et al. 2015). Significant crystalline phases associated with CuNps with X-ray diffraction: metallic Cu, cuprite (Cu_2O) and tenorite (CuO). X-ray diffraction patterns of CuNps's were obtained in an angle of 2θ = 20–80. Diffraction peaks were observed at 36.46° (111), 42.69° (111), 50.5° (200), 61.49° (111) and 73.25° (200). The intensity of increases and decreases in the intensity of the peaks may also arise due to the presence of plant material (Berra et al. 2018).

In addition, oxidation was observed in the sample even though the samples were kept in a way that they would not get light and air. Because of the increased surface areas due to their size, the nanoparticles are much more sensitive than the bulk of the same material. So, nanoparticles that come into contact with oxygen react quickly and oxidize. It has been noted that the XRD spectrum of the oxidized CuNps (CuO Np) is 111.

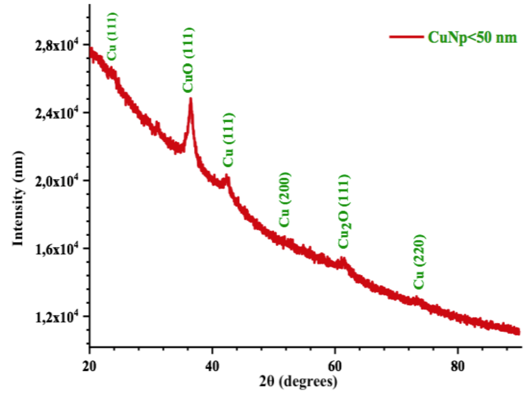

Figure 5. *XRD pattern of CuNps.*

Antimicrobial assay

In this study, the in vitro susceptibilities of CuNps synthesized from *L. stoechas* by the green synthesis method to gram-positive and gram-negative bacteria were determined by disc diffusion and broth dilution methods. While the maximum zone diameter of 19.5 mm was observed in P. aeruginosa in the disk diffusion test, CuNps at low concentrations did not form any zone diameter against *E. faecalis* and *E. coli* bacteria (Table 1). Differences in susceptibility and resistance to both gram-positive and gram-negative bacterial populations may be due to differences in cell structure, physiology, metabolism, or the degree of contact of organisms with Nps. In addition, other factors such as nanoparticle diffusion rate may affect the bacterial strain differently.

Table 1. *Antimicrobial susceptibility of CuNps to bacteria (μg / mL) by broth dilution method. "-" no bacterial reproduction, "+" bacterial reproduction.*

Bacteria	1000	500	250	125	62,5	31,25	15,62	7,81	3,90	1,95	Control
E. coli	-	-	+	+	+	+	+	+	+	+	+
P. aeruginosa	-	-	-	+	+	+	+	+	+	+	+
B. subtilis	-	-	-	+	+	+	+	+	+	+	+
S. aerous	-	-	-	+	+	+	+	+	+	+	+
S. enteridis	-	-	-	+	+	+	+	+	+	+	+
E. fecalis	-	-	+	+	+	+	+	+	+	+	+

Inhibition zone diameters (mm) formed around the discs are shown in Table 2 as a result of the agar disc diffusion test performed with the concentrations determined according to the MIC value. In addition, the graph of the inhibition zones formed by CuNps is given in Figure 6.

Table 2. *Zone diameters of CuNps in mm against bacterial strains with disc diffusion method. The zone diameter isn't formed for those indicate by "-".*

Bacteria	250 µg/mL	500 µg/mL	750 µg/mL	1000 µg/mL	$Cu(CH_3COO)_2$
B. subtilis	11.5	12.5	13	15	11
S. aureus	11	16.5	11.5	13	10
E. faecalis	-	14	-	12	9
P. *aeruginosa*	13	19.5	14	16	9
S. enteritidis	10	16.5	12	12	8
E. coli	-	13.5	-	11.5	9

The zone diameter increased as the CuNps concentration increased. The most effective stock solution in all gram negative and gram positive bacterial strains is 1000 µg / mL. Diameters of inhibition zones are seen at this concentration; 15 mm in *B. subtilis*, 16.5 mm in *S. aureus*, 14 mm in *E. faecalis*, 19.5 mm in *P. aeruginosa*, 16.5 mm in *S. enteritidis* and 13 mm in *E. coli*.
While the maximum zone diameter was observed in P. aeruginosa with 19.5 mm in agar disc diffusion test, CuNps at low concentrations applied did not create any zone diameter against *E. faecalis* and *E. coli* bacteria.

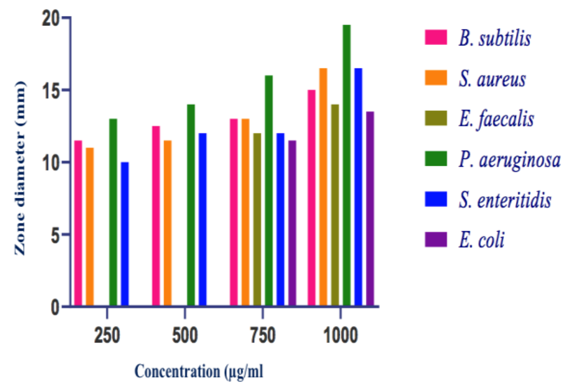

Figure 6. *Inhibition zones formed by CuNPs against various bacterial strains at different concentrations.*

4. Discussion

In recent years, traditional physical and chemical methods for synthesizing nanoparticles hazardous reducing agents and toxic organic solvents are increasingly being replaced by green synthesis techniques. This shift is due to the advantages of the green synthesis method - it is fast, clean, non-toxic, cost-effective and environmentally friendly. A preferred green synthesis approach uses plant extracts that can reduce metal ions thanks to their bioactive compounds, including flavonoids, terpenoids, tannins, alkaloids, proteins and other phytochemicals. These compounds act not only as reducing agents but also as stabilizers that limit Np growth. This green synthesis approach is easily scalable for industrial applications and offers a sustainable alternative to conventional methods due to its cost-effectiveness, low-temperature synthesis and reduced time requirements.
In this study, copper nanoparticles (CuNps) synthesized from Lavandula stoechas using green synthesis method showed potent antimicrobial activity against both gram-positive and gram-negative bacteria. The observed differences in antibacterial effects can be attributed to various factors such as bacterial cell structure, metabolic variations and the degree of contact with nanoparticles. In particular, the thick peptidoglycan layer in gram-positive bacteria may impede the penetration of nanoparticles, potentially resulting in lower efficacy (Azam et al. 2012). Furthermore, the lipopolysaccharide structure in the

outer membrane of gram-negative bacteria has been shown to allow better penetration of nanoparticles, leading to more effective results (Ruparelia et al., 2008). It was reported that CuNps synthesized and characterized using the extract of *Polyalthia longifolia* roots produced inhibition zones of 17.2 ± 0.2, 15.6 ± 0.2 and 13.7 ± 0.1 mm against *S. aureus*, *E. coli* and *C. albicans*, respectively, and can be used as an antibacterial and antifungal agent (Maulana et al. 2024). In another study evaluating the antibacterial activity of CuNps synthesized by green synthesis method against *S. aureus* and *E. Coli*, it was reported that 15.7 and 12.3 inhibition zones were formed, respectively (Maulana et al. 2023).

In the disk diffusion test, the zones of inhibition increased with higher nanoparticle concentrations in all bacterial strains. This shows the concentration-dependent microbicidal effect of CuNps. The antibacterial activity of CuNps may vary depending on microbial species, suggesting that the mechanisms of interaction of nanoparticles with bacterial cell membranes differ between bacterial species. The antibacterial activity of CuNps synthesized using *Curcuma longa* extract was tested against *B. subtilis* and *E. coli* and it was noted that the inhibition zone of *B. subtilis* was higher than that of *E. coli* (Jayarambabu et al. 2020). In another study, CuNps were synthesized using Artemisia plant, the antibacterial activity of these CuNps against *E. coli* and *B. Subtilis* was tested and similar results were obtained (Al-Khafaji et al. 2022).

The antibacterial properties of CuNps make them a promising alternative to conventional antibiotics. The global increase in antibiotic resistance has intensified the need for new and effective treatments against pathogenic bacteria (Hassan et al. 2018). In this context, CuNps synthesized via green synthesis from commonly available plants such as L. stoechas offer potential as a low-cost, eco-friendly and effective antimicrobial agent. Rajesh et al. (2018) reported that CuNps were particularly effective against multidrug-resistant bacteria, indicating that such nanoparticles may be promising in overcoming antimicrobial resistance.

In conclusion, the findings from this study suggest that *L. stoechas*-based CuNps could serve as a novel antimicrobial agent to address the antibiotic resistance crisis. Future studies should further investigate the efficacy of these nanoparticles against other pathogenic species and multidrug-resistant bacteria. Furthermore, studies on the biocompatibility and toxicological properties of these nanoparticles are crucial to ensure their safe and effective use in clinical applications.

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