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Biosynthesis and antibacterial activity of *Passiflora incarnata* mediated copper oxide nanoparticles

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Abstract---Plant mediated nanoparticle synthesis is a rapidly growing field of study around the world. Plant biomolecules make non-toxic, stable, and cost-effective copper oxide nanoparticles. In this study, eco-friendly CuONPs were prepared through a biosynthetic approach using *Passiflora incarnata* L. leaf extract. The synthesized CuONPs were characterized using UV-Visible spectroscopy (UV-Vis), scanning electron microscope (SEM), X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), and Dynamic light scattering (DLS). Furthermore, the antibacterial activity was evaluated against *Listeria monocytogenes*. The SEM results indicated that rod-shaped CuONPs with an average particle size of 49.8 nm (size range of 39-63 nm) were synthesized and shown good antibacterial activity against *Listeria monocytogenes*. CuONPs produced from *Passiflora incarnata* leaf extract could be a potential antibacterial agent for treating foodborne infections caused by *Listeria monocytogenes*. However, more research is needed to determine how it works on the target microorganism.

Keywords---passiflora incarnata, biosynthesis, antibacterial activity, listeria monocytogenes.

Introduction

Copper oxide nanoparticles (CuONPs) have gotten attentions because of their unique chemical and physical features compared to other nanoparticles (Rathnakumar et al., 2019; Sau & Rogach, 2010). In comparison to other metals such as platinum, silver, and gold, copper is commonly utilized in

optical, antibacterial, and electrical applications because of its easy availability, inexpensive cost, and suitable oxidation properties. In addition to these uses, copper has been proven to be harmless to mammals (Fleming, Trevors, & Pollution, 1989). CuONPs have many uses, including high-temperature superconductors (Yang, Liu, Li, Jin, & Yu, 2006), energy storage devices (Nwanya, Ndipingwi, et al., 2019), photo catalysis (Kaviyarasu et al., 2020), batteries (Wang, Wu, Shu, Guo, & Wang, 2010), antimicrobial agents (Nwanya, Razanamahandry, et al., 2019), and bio sensors (Kaviyarasu et al., 2019). On several biomedical devices, CuONPs have been used as antibacterial and antibiofilm agents (Chari, Felix, Davoodbasha, Sulaiman Ali, & Nooruddin, 2017; Lewis Oscar et al., 2015; Nabila & Kannabiran, 2018). It has a longer shelf life and is more stable than other organic antimicrobials (Raffi et al., 2010; Ren et al., 2009). CuONPs' antibacterial action is related to their size and surface volume ratio and their peculiar crystal shape, which allows CuONPs to engage easily with bacterial cell membranes and kill the bacterial cell by metal ions released inside the cell (Laha et al., 2014; Sathiyavimal et al., 2018).

Precipitation (Mayekar, Dhar, Radha, & Research, 2014; Phiwdang, Suphankij, Mekprasart, & Pecharapa, 2013; Rahimi-Nasrabadi, Pourmortazavi, Davoudi-Dehaghani, Hajimirsadeghi, & Zahedi, 2013), sonochemical (Silva, Ramirez, Diaz, Garcia, & Hassan, 2019), thermal breakdown of copper salts (Shahsavani, Feizi, & DEHNO, 2016), electrochemical (Katwal, Kaur, Sharma, Naushad, & Pathania, 2015), heat treatment (Baquer et al., 2018), nonionic water-in-oil microemulsions (Dodoo-Arhin, Leoni, & Scardi, 2012), and sol-gel synthesis methods (Dorner et al., 2019) are some of the ways to make CuONPs. Although some of the approaches listed above are simple and dependable, the majority of them employ harsh, toxic, and expensive chemicals that have more negative side effects in medical sciences, biosciences, and environmental concerns (Xie et al., 2014). The biosynthetic strategy outperforms the physical and chemical approaches, which typically use a green extract of leaves and bark from various plants as a complexing agent for the safe and simple creation of nanoparticles.

The best way to make nanoparticles is to use plants or components of plants. It is a more environmentally friendly, cost-effective, biologically safe, and stable method of nanoparticle manufacturing (Balazs, Emrick, & Russell, 2006; Chatzimitakos & Stalikas, 2016; Shameli et al., 2012). Plant biomolecules make non-toxic, stable, and cost-effective copper oxide nanoparticles (Laha et al., 2014; Lewis Oscar et al., 2015; Nabila & Kannabiran, 2018; Ren et al., 2009). *Cynodon dactylon* and *Cyperus rotundus* Grass Extracts (S. Suresh, 2020), *Ailanthus altissima* leaf extract (Akl M. Awwad1, 2020), *Asparagus racemosus* roots extract (Pammi5, 2019), *Populus ciliata* (Muhammad Hafeez*1, 2019), fenugreek leaves and fruit (ARDEEP KUMAR1, 2019), *Terminalia bellerica* (AKHTER, 2019), *Terminalia bellerica* (Ahmad2, 2019), Sugarcane juice (A.P. Angeline Mary a, 2019), *Ruellia tuberosa* (Seerangaraj Vasantharaj et al., 2018), *Malva sylvestris* Leaf Extract (Awwad, 2015), and *Acalypha indica* (Rajeshwari Sivaraj a, 2014) are some of the plants being used to synthesize CuONPs.

Passiflora incarnata L. (*P. incarnata*) leaf extract. *P. incarnata* (Passionflower) is a woody, hairy climbing vine that has long been employed in herbal treatments, primarily in the form of a liquid tincture. Anxiety, restlessness, sleep difficulties,

and nervous tension are all treated with *P. incarnata* (Bradley, 1992; Bruneton, 1995). *P. incarnata* L. extracts extracted in methanol and ethyl acetate showed good antibacterial activity against oxacillin-resistant *S. aureus* and *E. coli* (Patil, 2010).

Therefore, considering its potential herbal remedies and antimicrobial application, CuONPs were prepared using *P. incarnata* extract, and its antibacterial activity was tested against *L. monocytogenes*. The novelty of this work is that *P. incarnata* aqueous extract was used to synthesize CuONPs for the first time. Advanced techniques were used to characterize the prepared CuONPs, and antimicrobial activity was also evaluated.

Materials and Methods

Plant material collection and authentication: The entire fresh *P. incarnata* L. plant (Figure 1) was purchased from Godawari Farms and Services and Nursery, Greater Noida, and the plant authentication certificate was obtained from CSIR-National Institute of Science and Communication Information Resources (Ref. NISCAIR/RHMD/Consult/2018/3292-93). All the chemicals, solvents, and reagents used in the experiment were of analytical quality, and all of the solutions were prepared freshly with distilled water.



Figure 1. *Passiflora incarnata* plant

Preparation of plant extracts: Fresh plant leaves were thoroughly cleaned with tap water and then rinsed again with distilled water to remove dust particles. The cleaned leaves were dried in the oven to remove any remaining moisture. A mixer grinder (Sujata) was used to grind the dry plant material into fine powder. The plant material was then extracted by dissolving 5 grams of plant powder in 50mL of distilled water. In a water bath, the solution was boiled for 30 minutes at 65°C, and the extract was filtered using WhattmanNo. 1 filter paper after cooling (Figure 2). The filtrate was then kept at 4°C in the refrigerator for subsequent testing.



Figure 2. Plant extract preparation

Copper oxide nanoparticle (CuONPs) synthesis: CuONPs were made by transferring 1 mL of plant extract dropwise to a 20 mL aqueous solution of copper nitrate (0.1 M) while stirring. The solution's original blue color soon turned green. Then it was kept in the darkroom overnight by wrapping the solution in aluminum foil. By visually seeing the color shift of the solution to green (Figure 3), nanoparticle production was preliminarily validated. When the solution turned green, it was then centrifuged at 10,000 rpm for 15 minutes. CuONPs were rinsed with distilled water after removing the supernatant, which contained unreacted herb extract.

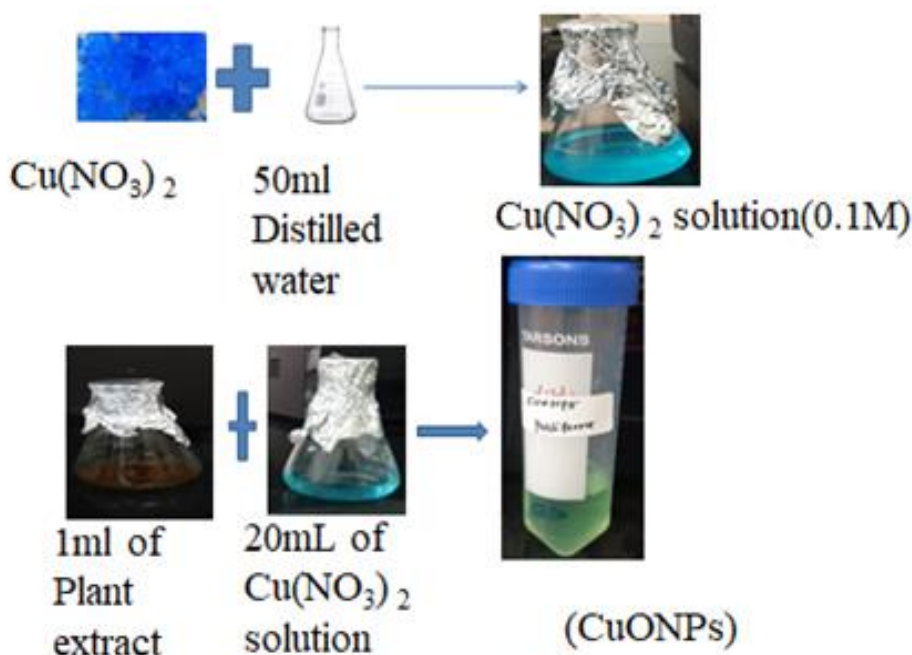


Figure 3. CuONPs synthesis using plant extract

Nanoparticle Characterization: The synthesized CuONPs optical properties, plant biomolecules involved during its synthesis, crystallinity, surface charges, nanoparticle size and shape were characterized by different techniques. The production of CuONPs was confirmed by a UV-Vis spectrophotometer (Shimadzu, UV 1800). It was submitted to UV-Vis spectroscopic examination between 200-800nm spectral ranges. The wavelength on the X-axis and the absorbance on the Y-axis were plotted to determine the peak of CuONPs' surface plasmon resonance (SPR). The hydrodynamic diameter and zeta potential of the produced CuONPs were determined using Dynamical Light Scattering (DLS). Azeta sizer Dynamic Light Scattering equipment was used to examine the charge, average size, and distribution pattern of green produced CuONPs.

Fourier-transform infrared spectroscopy (FTIR) analysis was done by FTIR Spectrometer (Agilent, Cary 630 FTIR-ATR including choice of KBr or ZnSe engine), an analysis of the probable functional groups in leaf extract involved in synthesizing and stabilizing CuONPs. The spectra were collected between 650 and 4000 cm^{-1} . CuONPs' morphological characteristics (shape and size) were studied using NOVA NANOSEM-450 FEI, (Netherlands) High-resolution field emission scanning electron microscope (HR FESEM). The crystallinity of CuONPs was measured using Rigaku Smart Lab Guidance X-Ray Diffractometer (Rigaku Company) at JMI University. Cu K radiation was used in the X-ray diffractometer (Rigaku SmartLab X-Ray Diffractometer) at a voltage of 40 kV and a current of 30 mA. The information gathered was examined with the help of origin pro (Version 2019b). After determining the width of XRD peaks, the Debye-Scherrer equation was used to calculate the average crystallite diameter based on the half-width of the diffraction peaks:

$$D = k\lambda / \beta \cos\theta$$

Where D is the mean particle size, λ is the wavelength of CuKa, β is the full width at half maximum (FWHM), θ is the Bragg diffraction angle, and k is a constant.

Antibacterial Activity: The CuONPs antibacterial activity was investigated using *L.monocytogenes* (MTCC 1143) bacterial culture. The bacterial culture was purchased from Microbial Type Culture Collection and Gene Bank (MTCC), CSIR - Institute of Microbial Technology, Sector 39-A Chandigarh-160036, India. Before usage, the bacteria were kept onto BHI media (Hi-Media, India) and sub-cultured. Both agar wells and discs were used to investigate the antibacterial capability of CuONPs.

Disc diffusion test: BHI agar (5.2gm) was dissolved in 100 ml of distilled water and autoclaved at 121°C for 15 minutes. After cooling, approximately 20 mL of medium was aseptically transferred to sterile Petri plates and allowed to solidify. By two-fold serial dilution from an overnight culture of the test organism (*L.monocytogenes* MTCC 1143) in sterile distilled water, bacterial suspension with 0.5McFarland turbidity standard (10^8 CFU mL^{-1}) was prepared. The inoculum was evenly distributed over the surface of BHI agar plates using a sterile cotton swab. Then, on the surface of the BHI agar plate, which was divided evenly into four quadrants, sterile filter paper discs (6mm in diameter) loaded with CuONPs, plant extract, copper nitrate, and chloramphenicol were placed. Before incubation at 37°C for 24 hours, plates were kept for 20 minutes at room temperature to allow diffusion of the solutions outward from the discs, establishing a concentration gradient. The plates were then incubated at 37°C for 24 hours, and the inhibitory zone (diameter in mm) was measured using a scale. Triplicates of the test were run. CuONPs inhibition zones were compared to a positive control (Chloramphenicol). Antimicrobial activity was expressed as the mean inhibition zone (mm) \pm SD.

Agar well diffusion method: Inoculation of bacterial suspension with 0.5McFarland turbidity standard (10^8 CFU mL^{-1}) prepared by two-fold serial dilution from an overnight culture of the test organism (*L.monocytogenes* MTCC 1143) was done on BHI media using a sterile cotton swab. Four wells were punched into the agar plate that was well separated and equidistant using a 6 mm cork borer. After that, 10 μ L of CuONPs, chloramphenicol, Cu(NO₃)₂, and plant extract solutions were poured into each well. After allowing samples to diffuse into the agar by keeping them at room temperature for 20 minutes, the plates were incubated for 24 hours at 37°C. After 24 hours, the diameter of the zone of inhibition was evaluated and measured in mm with a scale. The test was performed in triplicate, and the inhibition zones of CuONPs were compared with the positive control (Chloramphenicol). Antimicrobial activity was expressed as the mean inhibition zone (mm) \pm SD.

Determination of minimum inhibitory concentration (MIC): The strength of antibacterial activity can be assessed by evaluating antibacterial inhibition at a particular time point. It can be calculated using MIC, the lowest concentration at which no growth occurs in nutritional media. Micro broth dilution was used for MIC determination. CuONPs in various concentrations (17.862, 8.927, 4.463, and 2.231, 1.115, 0.558, 0.279, and 0.138mg mL^{-1}) were prepared by two-fold serial

dilution using distilled water and 100 μ L from each was transferred into a 96 well microtitre plate. An equal volume (100 μ L) of overnight grown bacterial cultures adjusted to 0.5McFarland turbidity standard was added to each well containing different concentration of CuONPs and the total volume in each well became 200 μ L. Chloramphenicol, Cu (NO₃)₂, and plant concentrations were all studied in the same way. As a control, BHI broth with CuONPs and bacterial culture without sample was introduced to separate wells. The plates were incubated in incubator at 37°C for 24 hours. The plates were observed for bacterial growth (turbidity) in the wells. The lowest concentration of CuONPs which inhibited visible growth of the bacteria (having no turbidity) was recorded as the MIC value. All the experiments were carried out in triplicates.

Statistical Analysis: Data analysis was done using one-way analysis of variance (ANOVA), and p values of < 0.05 were considered statistically. All the experiments were done in triplicate.

Results and Discussion

UV-Vis analysis: CuONPs production can be preliminarily demonstrated by the colour shift of the solution to green after the reaction of plant extract with copper ions. This result closely agrees with the findings of other research that have been done before (Gebremedhn, Kahsay, Aklilu, & Pharmacology, 2019), (Shaheen, Fouda, Salem, & Research, 2021), (Sepasgozar, Mohseni, Feizyazadeh, & Morsali, 2021). Furthermore, the absorbance was measured, and the peak value of CuONPs was recorded at a wavelength of 270.5nm (Fig. 4). Surface plasmon resonance (SPR) at 270.5nm validated CuONPs synthesis using *P. incarnata* plant biomolecule as a reducing agent, and this finding is similar to the findings of earlier research. CuONPs with UV absorption peaks of 270nm were synthesized from *Carica papaya* (Bhavika Turakhia, 2020), *Psidium guajava* (Sreeju, Rufus, & Philip, 2017), *Adiantum lunulatum* (Sarkar et al., 2020), and *Origanum vulgare* (R. Sankar, Prasath, et al., 2014) plant extracts. CuONPs made from *Pterospermum acerifolium* leaf extract have UV absorption at 274 nm (Saif, Tahir, Asim, & Chen, 2016) while CuONPs having 275nm absorbance were prepared from *Tinospora cordifolia* leaf extract (Nethravathi et al., 2015).

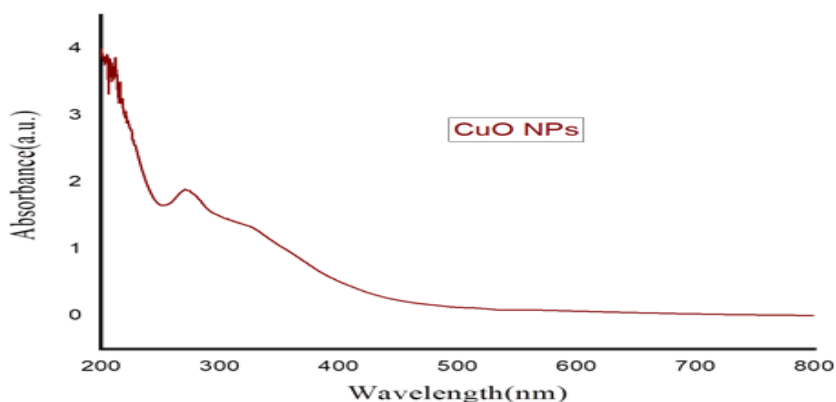


Figure 4. UV-Vis absorption spectrum of prepared CuONPs

SEM analysis: The morphological features (size and shape) of the produced CuONPs were determined using FE-SEM images. Rod-shaped CuONPs with an average particle size of 49.8 nm (size range of 39-63 nm) were synthesized, according to the findings (Fig. 5). CuONPs of various sizes mediated by plants have already been reported. The size and shape of plant mediated CuONPs could be influenced by Plant extract constituent phytochemicals (Mittal & Chisti).

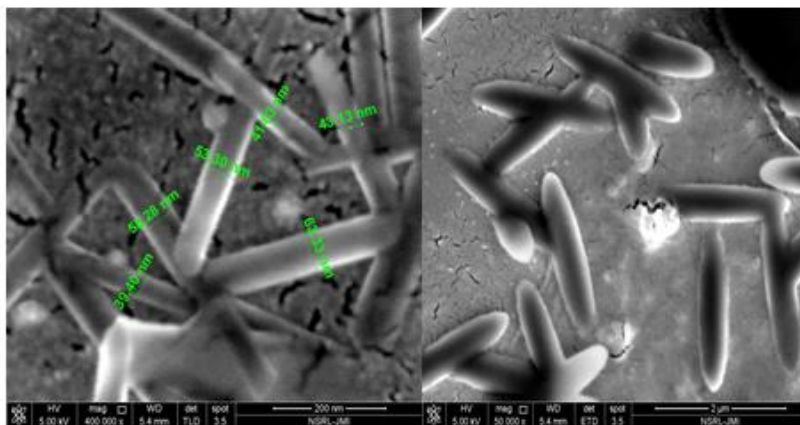


Figure 5. SEM images of CuONPs at different magnifications

FT-IR characterization: This technique was applied to analyse the plant phytochemicals involved in reducing copper ions to CuONPs and subsequent capping. The peaks in the spectra of *Passiflora incarnata* leaf extract are 3335 cm^{-1} , 1652 cm^{-1} , 1637 cm^{-1} , 884 cm^{-1} , 840 cm^{-1} , and 806 cm^{-1} (Fig. 6A). O-H has a peak at 3335 cm^{-1} , C=O stretching at 1652 cm^{-1} , C=O stretching vibration at 1637 cm^{-1} , and C-H bending has a peak at 884 cm^{-1} . The CuONPs have peaks at 3357 cm^{-1} , 3331 cm^{-1} , 1637 cm^{-1} , and 1398 cm^{-1} corresponding to the *P. incarnata* leaf extract peaks (Fig. 6B). The -OH group in the *P. incarnata* leaf extract involved in converting copper nitrate to copper oxide nanoparticles is represented by the peaks at 3357 and 3331 cm^{-1} . C=C and C=O stretching have peak at 1637 cm^{-1} , while C-H stretching has a peak at 1398 cm^{-1} .

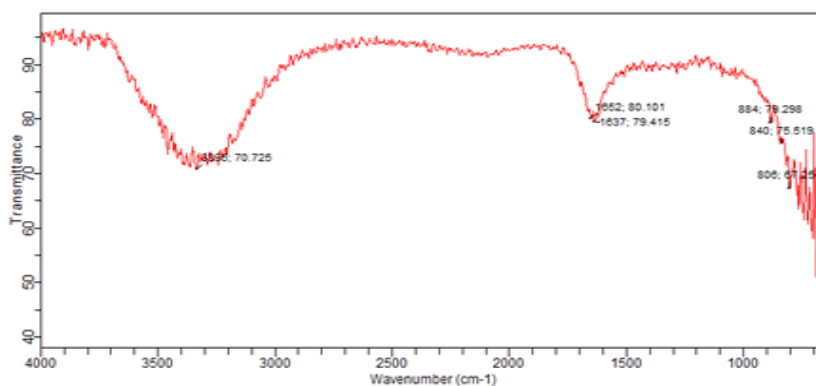


Figure 6A. FT-IR spectrum of *P. incarnata* leaf extract

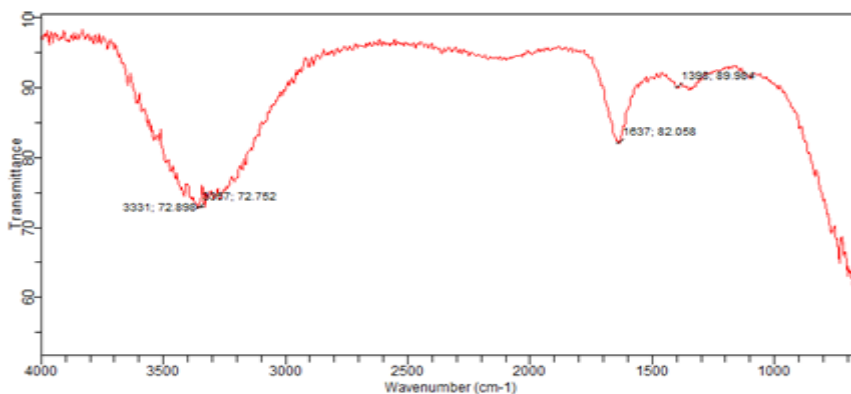


Fig.6B. FT-IR spectrum of prepared CuONPs

XRD Analysis: Figure 6 shows the XRD analysis of copper oxide nanoparticles generated with *P. incarnata* leaf extract. Diffraction peaks were found at $2\theta=12.88^\circ$, 25.8° , 29.66° , 33.36° , 44.06° , 58.6° , 62.82° , and 65.94° (Fig. 7). CuONPs produced by reducing Cu ions by *P. incarnata* leaf extracts are crystalline, as seen by strong peaks in X-ray diffraction patterns. Using Jade software, the miller indices and peaks of CuONPs were compared to the JCPDS database and with those published pieces of literature and found to be relatively similar. The (111), (111), (213), (202), (022) planes of the crystalline phase of cupric oxide (CuO) are related to the diffraction peaks positioned at 2θ values 25.80, 33.360, 58.60, and 65.940, respectively.

The result agrees with powder CuO obtained from Joint Committee on Powder Diffraction Standards (JCPDS file no. 44-0706), confirming the formation of copper oxide, while the peaks positioned at 2θ values of 29.66° and 62.82° are assigned to the (110) and (220) planes of the crystalline phase of cuprous oxide (Cu₂O) respectively which matches with the cubic phase of Cu₂O values with the corresponding JCPDS card no. 34-1354). Furthermore, the 2θ value at 12.88° is assigned with the (110) crystalline plane, and it agrees with the reported literature (AKHTER, 2019). The existence of peaks in the XRD spectra corresponding to CuO and Cu₂O suggests that both oxides are present in the crystalline phase of CuONPs (AKHTER, 2019). According to the Scherrer equation, the crystalline size of green produced copper oxide nanoparticles made with *P. incarnata* leaves extract was about 15.02 nm.

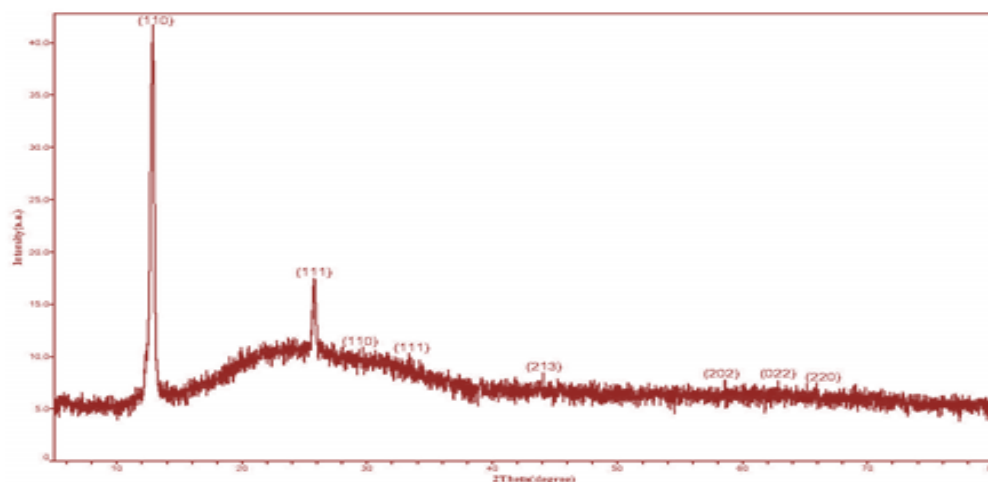


Figure 7. XRD pattern of prepared CuONPs

DLS study: DLS analysis was used to determine nanoparticles' size and surface charge through colloidal solutions. According to the DLS results, the CuONPs had an average particle size of 524 nm (Fig. 8A). Because DLS evaluates the hydrodynamic size of the produced nanoparticles, the particle size is bigger than that reported by SEM (Pallela et al., 2019). Biomolecules and the aqueous layer covering nanoparticles' surfaces were included in the measurement (Bhakya et al., 2015). DLS analysis revealed that *Origanum vulgare* plant synthesized CuONPs having a particle size of 551 nm (R. Sankar, Prasath, et al., 2014). CuONPs synthesized from *Ficus religiosa* and *O. vulgare* leaves were measured at 577 nm and 551 nm, respectively, using dynamic light scattering analysis (R. Sankar, Maheswari, Karthik, Shivashangari, & Ravikumar, 2014). The charge distribution and size improve CuONPs' biological activities (Sankar et al., 2014a). According to the findings, CuONPs with a positively charged surface had a zeta potential of +15 mV (Fig. 8b). This positive charge is sufficient and specifies a strong repelling force among the particles, preventing them from interacting and maintaining the sample's particle size (Pallela et al., 2019; Renu Sankar et al., 2013). If the hydrosols have a high negative or positive zeta potential, they will resist each other, preventing the particles from clumping together. On the other hand, if the particles have low zeta potential levels, there will be no force to keep them from colliding and flocculating. (Sorbiun1 & 2018).

Antibacterial activity: The antibacterial activity of the CuONPs against *L. monocytogenes* was investigated using an agar well and disc diffusion assay (Fig. 9). For the agar well and disc diffusion procedures, respectively, a 29.17 mm and 21.60 mm zone of inhibition was found after 24 hours of incubation with the CuONPs (Table 1). The result indicates that a higher inhibition zone was recorded in the agar well diffusion method than in the disc diffusion technique. Different researches have shown similar results with greater inhibition zones in agar wells diffusion than in disk diffusion test (Gunalan, Sivaraj, & Rajendran, 2012; Hamelian, Hemmati, Varmira, & Veisi, 2018). The synthesized CuONPs were found to have a MIC of 0.558 mgmL⁻¹ (Table 2). The antibacterial properties of the synthesized CuONPs against the target microbe may be due to the shape of the

nanoparticles. This is because rod-shaped nanoparticles present a large surface area to volume ratio that enhances their interaction with the bacterial cells and therefore helps to inhibit its growth (Qamar, Rehman, Chauhan, Tiwari, & Upmanyu, 2020). In contrary to this, spherical CuONPs prepared from *Camellia sinensis* extract and *Prunus africana* bark extract exhibited potent antibacterial activity (Ssekatawa et al., 2022). In addition to this, the antibacterial activity of the *P. incarnata* plant-mediated CuONPs may be due to its positive zeta potential/charge (+15mV). The bacterial cell membrane exhibits a net negative charge that greatly attracts positively charged materials. (Ssekatawa et al., 2020). Because of this electrostatic interaction, adsorption and consequently penetration of bacterial cells by CuONPs is enhanced (Ssekatawa et al., 2022). CuONPs synthesized from *Rosmarinus officinalis* L., *Syzigium aromaticum* and *Cinnamomum verum* (Osaili et al., 2019), *walnut shells* (Mehdizadeh, Zamani, & Abtahi Froushani, 2020), *Capsicum frutescens* [196], and *Lemon Extract* (Tshireletso, Ateba, & Fayemi, 2021) have shown antibacterial activities against *L. monocytogenes* at different concentrations. Different strains of *L. monocytogenes* have been inhibited by CuONPs synthesized from *Rosmarinus officinalis* L., *Syzigium aromaticum* and *Cinnamomum verum* (Osaili et al., 2019), *walnut shells* (Mehdizadeh et al., 2020), *Capsicum frutescens* (K, S, P, S, & S, 2021), and *Lemon Extract* (Tshireletso et al., 2021).



Figure 9. Antibacterial activity of CuONPs against *L. monocytogenes*: (a) agar well diffusion and (b) disc diffusion technique [Where, A = chloramphenicol, B = CuONPs, C = Plant extract, D = $\text{Cu}(\text{NO}_3)_2$].

Table 1
Inhibition zone of CuONPs against *L. monocytogenes*

Copper nanoparticles	Zone of inhibition (mm)	
	Well	diffusion
Disk diffusion		
CuONPs	29±1.73	
21± 0.60		
Cu (NO ₃) ₂	19.66±2.51	
15±0		
Chloramphenicol	33.67 ± 0.57	
25.33±0.57		

Data are means of triplicates ($n = 3$). NA: No Activity

Table 2
MIC value of CuONPs against *L.monocytogenes*

Copper nanoparticles	MIC value (mgmL ⁻¹)
CuONPs	0.558
Cu (NO ₃) ₂	2.344
Chloramphenicol	0.015
Plant extract	NA

Data are means of triplicates ($n = 3$).

Conclusion

Copper oxide nanoparticles were successfully synthesized using *P.incarnata* leaf extract. The prepared nanoparticles were characterized using UV-Vis spectroscopy, FTIR, SEM, DLS, and XRD. Both disc diffusion and agar well diffusion methods were used to investigate the antibacterial activity of the prepared CuONPs. These nanoparticles have shown good antibacterial activity against *L.monocytogenes*. The synthesized CuONPs could be an alternative antibacterial agent against multidrug-resistant *L.monocytogenes*. Although few research works are available, further research is required to understand the mechanism of CuONPs' action fully.

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