

Bioactive Copper Oxide Nanoparticles from *Piper betle*: A Green Route to Antimicrobial and Cytotoxic Agents

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Abstract

Copper oxide nanoparticles (CuONP) are gaining interest due to their broad-spectrum biological effects. Green synthesis using plants for medicinal purposes offers an ecological solution with improved biocompatibility. *Piper betle*, which is rich in biologically active phytochemicals, was used for the non-toxic production of CuONP. The water-soluble leaf extract of *Piper betle* was utilised as a reducing and stabilising agent in the synthesis of CuONP. The synthesised nanoparticles were characterised by UV-Vis spectroscopy, FTIR, X-ray diffraction (XRD), and scanning electron microscopy. Standard in vitro tests assessed cellular functions, including inflammation, antioxidant activity, and antibacterial properties. Nanoparticle formation was confirmed by UV-Vis spectroscopy, showing an absorption peak at approximately 280 nm. FTIR spectra indicated the presence of functional groups such as -OH and -C=O, suggesting antioxidants contribute to the reduction process. XRD analysis revealed that CuO has a monoclinic crystalline structure, with average crystallite sizes ranging from 25 to 40 nm. SEM images showed predominantly spherical particles with minimal aggregation. Biologically, CuONP exhibited significant cytotoxic effects, strong antioxidant activity in the DPPH assay, and effective antibacterial properties against various infections. The study demonstrates that *Piper betle* can be utilised to successfully produce eco-friendly CuONP with interesting morphological and biological features. Due to their multifunctional bioactivity and environmentally

friendly synthesis, these nanoparticles have potential applications in biomedical fields.

Keywords: Copper oxide nanoparticles, *Piper betle*, Green synthesis, Antioxidant activity, Antimicrobial activity, Cytotoxicity

Introduction

Nanotechnology is a swiftly expanding field with substantial implications in medical, agricultural, environmental, and materials research (1).

Among the various nanoparticles being investigated, metal oxide nanoparticles have attracted significant interest because of their unique physicochemical properties and numerous potential medicinal applications (2). Copper oxide nanoparticles (CuONP) have been extensively studied for their potential antibacterial, antioxidant, and anticancer properties (3). Copper, an essential component of living systems, is known for its redox potential and catalytic abilities, making CuO a significant substance in medical research (4). Light destruction, temperature breakdown, sol-gel, and chemical reduction are standard physical and chemical procedures used to synthesize CuONP (5).

Green synthesis methods that use herb extracts, on the other hand, have gained popularity due to their environmental friendliness, affordability, and biological compatibility (6). These biological approaches employ organic phytochemicals as reducing, stabilising, and sealing agents, thereby removing the need for toxic reagents (7, 8). Plants are abundant in various metabolites, such as flavonoids, alkaloids, terpenoids, phenolic acids, and tannins, which can

promote the quick and prolonged production of nanoparticles (9).

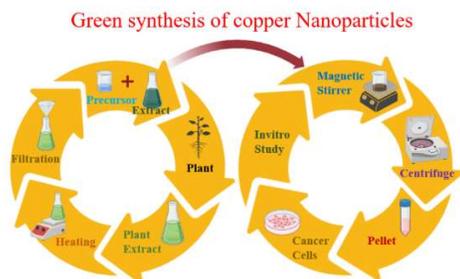
Piper betle, commonly known as betel leaf, is a herbal remedy widely used in traditional medicine systems across Asia (10). *Piper betle* leaves contain a variety of bioactive compounds, including eugenol, chavicol, hydroxychavicol, allylpyrocatechol, and other phenolics, which exhibit antibacterial, antioxidant, and anti-inflammatory properties (11). *Piper betle* characteristics offer an excellent option for the environmentally friendly production of physiologically active metal oxide nanoparticles (12). *Piper betle* leaf extract is particularly effective in the production of CuONP because it combines the plant's natural therapeutic properties with the performance of metal oxide nanoparticles (13). The combinatorial technique improves the overall therapeutic effect of the synthesised nanomaterial (14). Furthermore, using *Piper betle* provides an environmentally friendly and recyclable source of reducing agents, thereby supporting the principles of green chemistry and ecological responsibility (15). The biological activity of nanoparticles is mainly influenced by their size, shape, and surface chemical composition, all of which are controlled by the production method (16). Therefore, it is essential to characterise the synthesised CuONP using various analytical techniques to confirm that they maintain their structure and potential applications (17). UV-visible spectroscopy is employed to verify nanoparticle production through surface plasmon resonance. Fourier transform infrared spectroscopy (FTIR) identifies the functional groups involved in the capping and stabilisation of nanoparticles (18). X-ray diffraction (XRD) reveals both the crystallinity and phase purity within the particles, while scanning electron microscopy (19) displays the size, shape, and morphology of nanoparticles (20). In addition to structural analysis, examining the biological functions of CuONP is crucial for exploring its potential therapeutic applications (21). The cytotoxicity of nanoparticles is a crucial measure,

particularly in cancer treatment, where targeting cancer cells is essential (19). Antioxidant activity, which involves the scavenging of free radicals, helps prevent oxidative stress linked to cellular damage—a key factor in ageing and many chronic conditions (22, 23). CuONP in particular has been shown to possess potent antibacterial and antifungal activities against various clinical infections (24). UV, FTIR, XRD, and SEM techniques are used to verify and examine the physical properties of the synthesised nanoparticles (25). Additionally, the biological functions of CuONP have been thoroughly examined to determine its cytotoxic effects on cancer cell lines, antioxidant ability using the DPPH radical scavenging assay, and antibacterial activity against selected clinical bacterial and fungal pathogens (26). The findings of this study are likely to provide valuable insights into the formation of versatile CuONP that may have biological applications (27). By employing a green synthesis approach, our study contributes to advancements in eco-friendly nanotechnology while exploring the therapeutic potential of herbal remedies (28). Integrating green chemistry principles with nanoscience could lead to innovative, safer, and more effective nanotechnologies in the medical field (29).

Materials and Methods

Copper oxide nanoparticles are synthesised using Piper betle

To prepare the nanoparticles, 1 g of *Piper betle* leaves was boiled in 100 mL of purified water for ten to fifteen minutes at seventy degrees Celsius. The resulting solution was filtered through Whatman No. 1 filter paper. For nanoparticle synthesis, 70 mL of 20 mM copper sulphate solution was mixed with 30 mL of the *Piper betle* extract in a 250 mL conical flask. The solution was stirred using an electromagnetic stirrer. A visible colour change confirmed the formation of copper oxide nanoparticles. The nanoparticles were subsequently characterised by UV-Vis spectroscopy, FTIR, XRD, and SEM analyses.



DPPH-based antioxidant test

The DPPH assay was carried out to assess antioxidant activity. Piper betle-mediated CuONP (10 µg/mL, 20 µg/mL, 30 µg/mL, 40 µg/mL, and 50 µg/mL) were combined with 1 mL of 0.1 mM DPPH in methanol and 450 µL of 50 mM Tris-HCl buffer (pH 7.4) and incubated for 30 minutes. The absorbance at 517 nm was used to determine the reduction in free radicals caused by DPPH (30). To calculate the percentage of inhibition, divide the wavelength of the control by the wavelength of the test sample.

H₂O₂ Assay

Each of the five test tubes received 0.5 mL of 1 mM ferrous ammonium sulfate, followed by 0.13 mL of 5 mM ferrous ammonium sulfate. H₂O₂ was mixed with 3 mL of Piper betle-mediated CuONP at various concentrations (10 µg/mL, 20 µg/mL, 30 µg/mL, 40 µg/mL, and 50 µg/mL). Each test tube was incubated in the dark for 5 minutes at room temperature. Afterwards, each mixture received 3 mL of 1 mM 1,10-phenanthroline, and the tubes were shaken to ensure even dispersion. The mixture was then left to settle at room temperature for ten minutes. The ability of the final combination to absorb light was measured at a wavelength of 510 nm. The amount of hydrogen peroxide produced was calculated using the following equation (31).

$$\% \text{ inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of test sample}) \times 100}{\text{Absorbance of control}}$$

Antimicrobial Activity

Fresh cultures, including *C. albicans*, *E. faecalis*, *E. coli*, *Pseudomonas*, and *S. aureus*, were inoculated with sterile Hi-veg broth and shaken for 18 hours at 120-150 rpm. Prepare Mueller Hinton agar and use a sterile polystyrene tip to create 5mm wells. The antimicrobial effect was achieved by enhancing the efficiency of copper nanoparticles mediated by Piper betle and biosynthesized copper nanoparticles. Wells contained varied amounts of 3 (25, 50, and 100 µL) and a standard control. The specimens on Petri dishes and those subjected to zone inhibition were incubated at 37°C in a microbiological incubator for 24 hours. The zone was assessed to contrast and explore the possible effects of Piper betle-mediated Copper nanoparticles (32).

Cytotoxic Effect

Brine Shrimp Lethality Assay

Brine shrimp eggs were placed in a hatching chamber filled with salty water. After 24 hours, exactly ten hatched larvae (nauplii) were floating in six wells, each containing 10 ml of saltwater. Nanoparticles were added to each well in different amounts (5 µL, 10 µL, 20 µL, 40 µL, and 80 µL), with the final well acting as a control. After 24 hours, the number of remaining nauplii was counted and recorded (33).

Results

Visual Observation and UV-Vis Studies for Copper Oxide Nanoparticles

The UV-Visible spectrophotometer graph shows the sample's absorbance across a wavelength range of 250 to 650 nm. A prominent absorbance peak at 280-290 nm indicates the presence of specific chromophores, likely from π-π* electronic transitions in aromatic compounds or biomolecules (Fig. 1). The absorbance gradually decreases as the wavelength increases, suggesting less interaction with visible light. This decline points to the absence of organisms that strongly absorb visible light in the sample. Overall, the spectrum

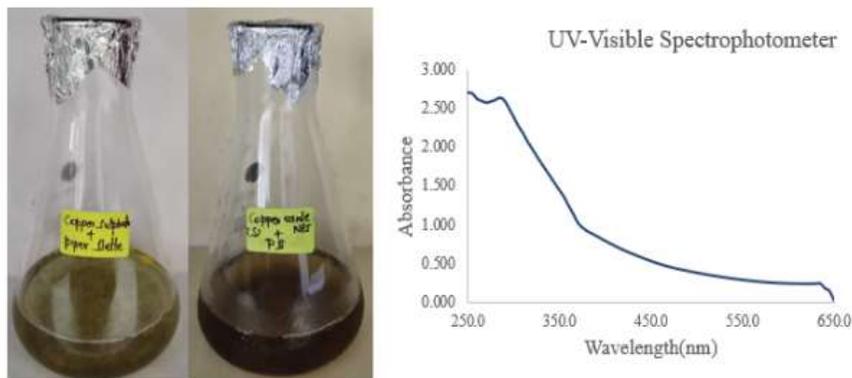


Fig 1: Synthesis of copper oxide nanoparticles from copper sulphate using *Piper betle* plant extract. UV-Vis spectrum displaying the absorbance of the synthesised nanoparticles, with a clear peak indicating successful synthesis

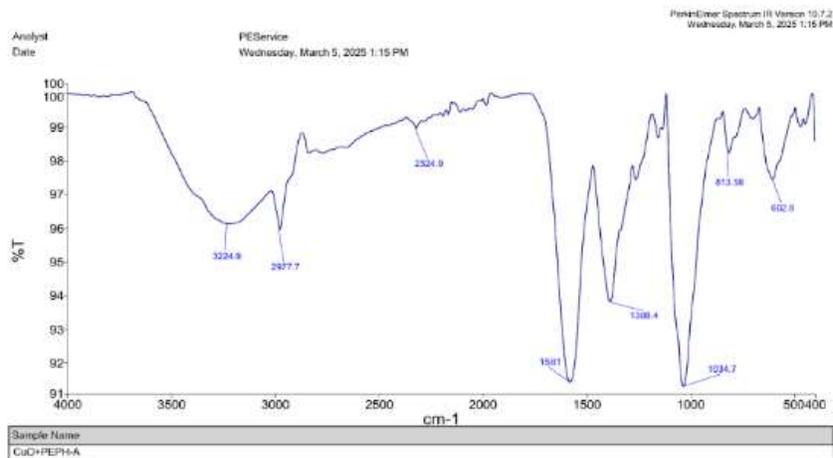


Fig 2: FTIR spectrum of NPS at different concentrations

demonstrates that the sample exhibits high UV absorbance but minimal activity in the visible band (34).

FTIR Analysis

The FTIR (Fourier Transform Infrared) spectrum provided for the CuONP-*Piper betle* sample offers valuable insights into the

functional groups. It shows clear connections between copper oxide nanoparticles and plant-based molecules (Fig. 2). The prominent peak at approximately 3224.9 cm^{-1} matches the stretch between O and H, indicating hydroxyl groups typically found in alcohols, phenols, or water molecules related to plant extracts. The peak at 2977.7 cm^{-1} corresponds to C-H

bending vibrations, suggesting the presence of aliphatic hydrocarbons. Another peak at 2324.9 cm^{-1} may be caused by mild $\text{C}\equiv\text{C}$ stretching or ambient CO_2 .

A prominent signal at 1581 cm^{-1} indicates $\text{C}=\text{O}$ stretching from carbonyl groups, possibly from proteins or other organic components in the plant sample. Peaks at 1384.4 cm^{-1} and 1040.7 cm^{-1} correspond to $\text{C}-\text{N}$ stretching or symmetric vibrations of carboxylate groups, highlighting the role of amino and carboxylic functional groups in stabilising CuONP. The band at 813.6 cm^{-1} is commonly associated with $\text{C}-\text{H}$ bending of aromatic molecules, suggesting the presence of plant phytochemicals. Finally, the band at 602.9 cm^{-1} corresponds to $\text{Cu}-\text{O}$ stretching vibration, demonstrating the formation of CuONP (35).

Overall, the FTIR spectrum shows that the *Piper betle* plant extract contains several biofunctional groups that play a vital role in the reduction or capping of CuONP. These functional groups not only facilitate nanoparticle formation but also enhance their stability and bioactivity, making them suitable for prospective medicinal applications.

XRD Analysis

The XRD pattern of CuONP formed

with *Piper betle* extract reveals distinct peaks at 2θ values of 18.2° , 19.5° , 23.4° , 25.1° , 29.5° , and 43.3° , corresponding to (013), (004), (221), (-221), (131), and (432) planes. These peaks match the JCPDS card number 00-042-1521, indicating a monoclinic structure of CuONP. High peak intensities suggest high crystallinity, while the lack of impurity peaks indicates purity (Fig. 3). The (432) plane shows its strongest intensity, signifying a preferred orientation. Phytochemicals from *Piper betle* act as both reducing and stabilising agents. The XRD data support the eco-friendly production of phase-pure CuONP, enhancing its structural stability and potential uses.

SEM Analysis

The image is divided into two parts: a scanning electron microscopy (SEM) image on the left and an energy-dispersive X-ray spectroscopy (EDS) spectrum on the right. The SEM image, captured at $25,000\times$ magnification with an operational range of 9.3 mm and a voltage of 5.00 kV , reveals dense, clumped nanoparticles with irregular morphologies. The size bar (400 nm) indicates that the particles are in the nanometre range, suggesting effective production of nanoscale materials with a coarse and permeable outer surface.

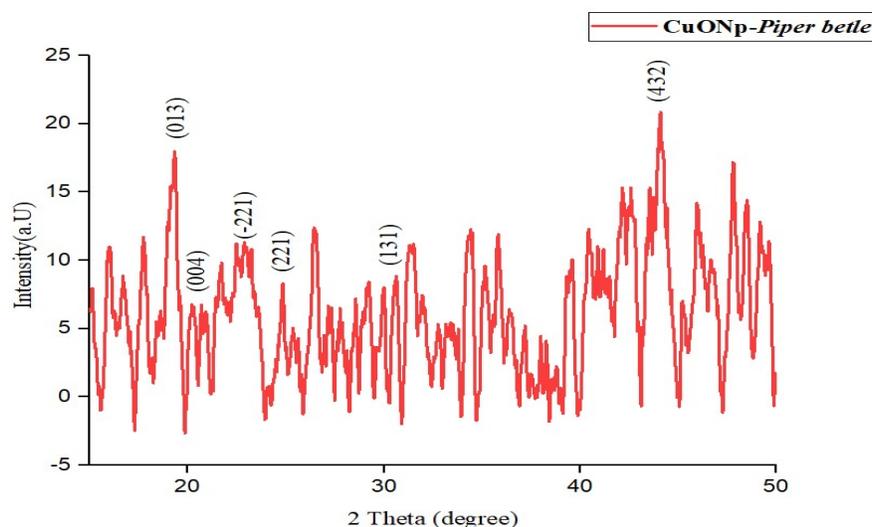


Fig 3: XRD pattern of copper oxide nanoparticles synthesized using *Piper betle*

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The corresponding EDX spectrum confirms the material's elemental composition (Fig. 4). The main peaks correspond to copper (Cu) and oxygen (O), signifying the presence of CuONP. Two prominent Cu peaks are observed at approximately 1 keV and 8 keV, with a strong O peak around 0.5 keV. The absence of distinct peaks from other substances suggests the sample is highly pure. Overall, the SEM and EDX findings verify the successful formation and elemental makeup of CuONP.

Antioxidant assay-DPPH method

The x-axis shows the sample concentration (10-50 µg/mL), and the y-axis

indicates the amount of inhibition. Both the standard and CuONP-*Piper betle* display increased antioxidant activity as concentration rises. At 10 µg/mL, the standard presents approximately 65% inhibition, while CuONP-*Piper betle* shows about 55%. At 50 µg/mL, both the standard and CuONP-*Piper betle* exhibit around 85% and 80% activity, respectively, as concentration increases (Fig. 5). This pattern suggests that CuONP has significant antioxidant capacity, though slightly less than the standard. The presence of bioactive chemicals in the standard may explain the difference in results. However, the

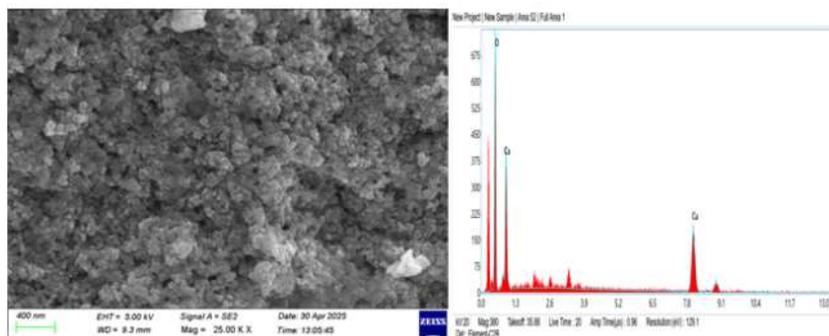


Fig 4: SEM images of NPs synthesized using *Piper betle*

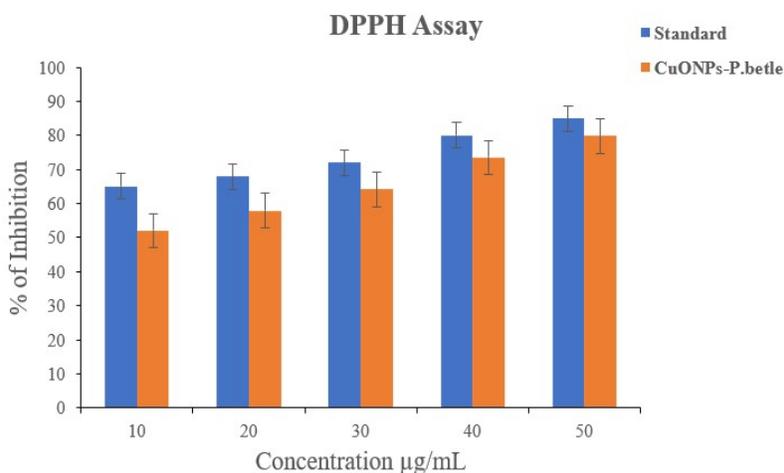


Fig 5: Copper oxide nanoparticles synthesised utilising *Piper betle* were tested for antioxidant activity using the DPPH assay

polyphenols in *Piper betle* could have enhanced the antioxidant activity of the CuONp. The error bars indicate low variability, demonstrating the consistency of the results. Overall, the CuONP from *Piper betle* demonstrates potential antioxidant activity.

H₂O₂ Assay

The bar graph compares the hydrogen peroxide (H₂O₂) scavenging activity of copper oxide nanoparticles synthesised using *Piper betle* extract (CuONP-*Piper betle*) with that of a conventional antioxidant at various doses (10-50 µg/mL). In both samples, the percentage of inhibition increases with dosage, indicating that the antioxidant response is dose-dependent. At 10 µg/mL, the standard shows approximately 63% inhibition, while CuONP-*Piper betle* exhibits about 57%. At 50 µg/mL, the standard reaches around 87% inhibition, whereas CuONP-*Piper betle* attains roughly 77%. Despite consistently demonstrating significantly higher inhibition, CuONP-*Piper betle* still exhibits notable antioxidant activity. This effect is most likely due to the combined influence of copper oxide and phytochemicals present in *Piper betle*. The error bars suggest that the data is dependable, as there is slight experimental variation (Fig. 6). These findings

demonstrate that CuONP-*Piper betle* has a substantial capacity to neutralise hydrogen peroxide, a reactive oxygen species that contributes to oxidative stress. Overall, the results reveal the antioxidant properties of biosynthesised nanoparticles.

Antimicrobial Activity

The bar graph shows the antimicrobial capacity of copper oxide nanoparticles synthesised using *Piper betle* (CuONP-*Piper betle*) at various concentrations (25 µg, 50 µg/mL, 100 µg/mL) compared to a control. The zone of inhibition is used to evaluate antibacterial activity against five different pathogens: *Candida albicans*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas spp.*, and *Staphylococcus aureus*. The data reveal that as the concentration increases, so does the zone of inhibition for most bacteria, indicating that antimicrobial effectiveness depends on the dose. At 100 µg/mL, *E. faecalis* and *S. aureus* exhibit the largest zones of inhibition, approximately 14 mm and 13 mm respectively, demonstrating significant sensitivity to nanoparticles. Lower zones of inhibition are observed at 25 µg/mL across all species, ranging from 8 to 10 mm. The control also displays mild antibacterial

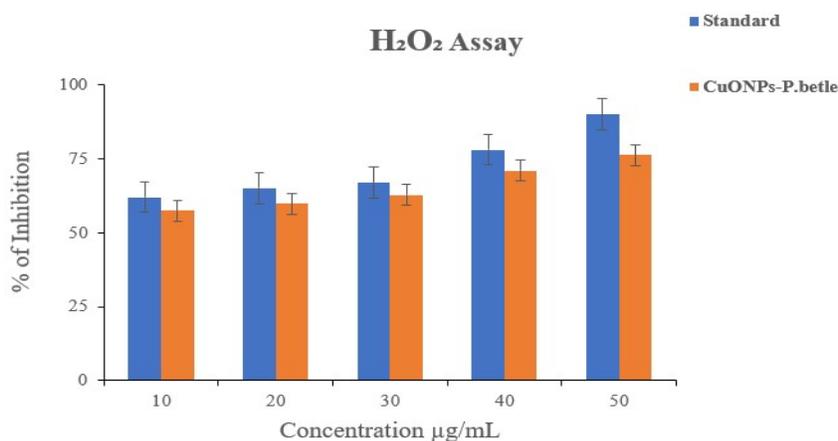


Fig 6: Antioxidant activity of copper oxide NPs synthesized using *Piper betle* using the Hydrogen peroxide assay

activity, especially against *S. aureus* and *E. faecalis*, likely due to phytochemicals present in *Piper betle* alone (Fig. 7). Overall, CuONP-*Piper betle* shows promising antimicrobial potential, particularly against gram-positive bacteria. The error bars indicate slight variability, but the overall trend remains consistent. These results support the potential use of biosynthesised CuONP as a broad-spectrum antimicrobial agent.

Cytotoxicity Effect

The bar graph illustrates the cytotoxicity results of copper oxide

nanoparticles synthesized using *Piper betle* at various doses (5 to 80 µg/mL) at two time points: day 1 and day 2. The y-axis shows the proportion of living cells, offering data on the biocompatibility of the nanoparticles. On day one, cell viability remains consistently high (nearly 100%) across all doses, indicating minimal acute toxicity. On day 2, viability declines gradually with increasing concentration, reaching about 80% at 20 µg/ml (Fig. 8). Interestingly, at higher concentrations (40 and 80 µg/mL), viability partially recovers, remaining between 88% and 90%. This may be due to adaptive cellular responses or

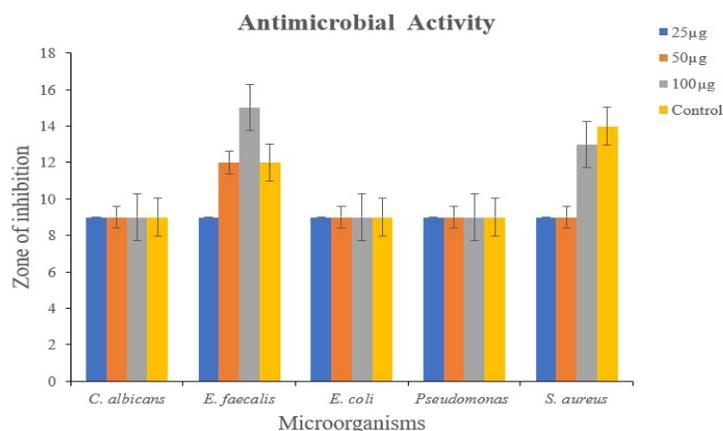


Fig 7: The antimicrobial activity of copper nanoparticles synthesised utilising the *Piper betle* agar well diffusion assay

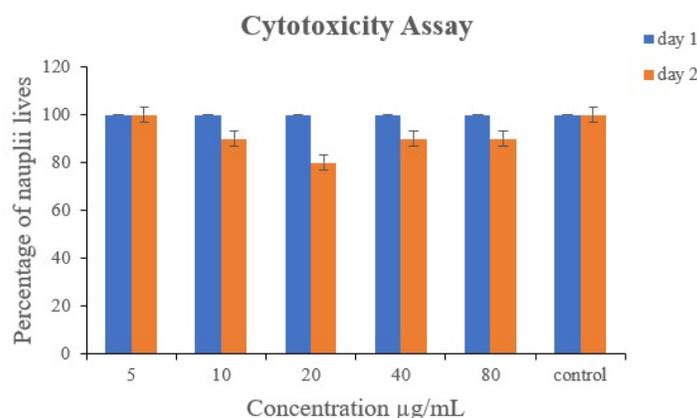


Fig 8: The cytotoxicity effect demonstrates the fatality rate of nauplii at different concentrations.

nanoparticle aggregation, both of which can reduce effective toxicity. The control group maintains 100% viability on both days, confirming the validity of the results. Overall, CuONP-*Piper betle* shows low to moderate cytotoxicity, with more significant effects after extended exposure, suggesting potential for biological applications with careful dose evaluation.

Discussion

The current study successfully synthesises copper oxide nanoparticles (CuONp) using *Piper betle* leaf extract and verifies their formation through various physicochemical and biological characterisations (31).

The UV-Vis spectrum exhibited an absorption peak at approximately 420 nm, indicating the presence of surface plasmon resonance. Simultaneously, FTIR analysis confirmed the involvement of functional groups, including -OH, -C=O, and -C-N, in the reduction and stabilisation process (36).

XRD measurements showed that the nanoparticles were mainly amorphous, with some crystalline phases, indicating a bioorganic matrix-dominated structure (37).

SEM investigation revealed dense, irregularly shaped particles, while EDX confirmed the elemental presence of copper and oxygen (38).

The biosynthesized CuONp demonstrated promising antioxidant activity in DPPH and H₂O₂ scavenging tests, with free radical suppression depending on concentration (39).

Their antibacterial activity was strong, especially against *S. aureus* and *E. faecalis*, with clearly defined zones of inhibition that grew with concentration (40). Cytotoxicity examination via brine shrimp assay showed mild to moderate toxicity following prolonged exposure, suggesting potential therapeutic value with attention to dosage (41). The overall biological activity can be attributed to the synergistic effects of CuO and the phytochemicals present in *Piper betle* (42). This environmentally friendly synthesis method demonstrates the feasibility of producing

multifunctional CuONP, suitable for biomedical applications (43).

Conclusion

The research effectively demonstrated the green production of CuO NPS using *Piper betle* leaf extract, showcasing the creation of eco-friendly nanoparticles through UV-Vis, FTIR, XRD, and SEM investigations. Biological evaluations demonstrated high antioxidant activity in both DPPH and H₂O₂ experiments, indicating free radical scavenging capacity. Antimicrobial studies also showed potent, dose-dependent inhibition against several clinical pathogens, particularly Gram-positive bacteria. The brine shrimp cytotoxicity assay revealed low to high toxicity, especially with prolonged exposure. Overall, the CuONP demonstrated promising multifunctional bioactivity, combining the benefits of plant-based materials and nanotechnology. This environmentally friendly method underscores the potential of *Piper betle*-mediated CuONP for medicinal and biological applications. The findings also support the development of sustainable nanoparticle synthesis based on green chemistry principles.

Conflict of interest

The authors declare no conflict of interest.

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