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Green Synthesis of Copper Oxide Nanoparticles from *Hybiscus rosa-sinensis* Extracts with Enhanced Bioactivity

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Abstract

Nanotechnology offers new opportunities in biomedical and environmental research, with copper oxide nanoparticles (CuONP) becoming popular due to their unique physical and chemical properties and diverse biological applications. In this study, CuONP were synthesised using *Hibiscus rosa-sinensis* leaf extract through a green synthesis method, with phytochemicals serving as natural stabilisers. A colour change indicated the formation of CuONP, which was then characterised using UV-Vis spectroscopy, FTIR, XRD, and SEM analyses. The UV-Vis spectra showed a strong absorption peak at 260-280 nm, confirming successful nanoparticle formation. FTIR identified hydroxyl, carbonyl, and alkyl groups in plant compounds, while XRD confirmed the monoclinic crystalline structure of CuoNp. SEM images displayed disorganised, porous nanoparticles, with elemental composition verified by EDS. The biological properties of the synthesised CuONP were evaluated through various assays. The nanoparticles effectively inhibited α-amylase, suggesting potential anti-diabetic activity. Anti-inflammatory effects were demonstrated using bovine serum albumin and egg albumin denaturation assays, which showed dose-dependent reduction compared to the standard. The DPPH radical-scavenging assay revealed strong antioxidant activity, surpassing the standard at higher concentrations. Antimicrobial tests indicated mild inhibition against *S. aureus, E. coli, Pseudomonas,* and *Candida albicans*. Cytotoxicity assessment via the brine shrimp lethality assay demonstrated excellent biocompatibility at lower concentrations and mild toxicity at higher doses. CuONP synthesised with *Hibiscus rosa-sinensis* show promising antioxidant, anti-inflammatory, anti-diabetic, and mild antimicrobial properties, highlighting their potential for biomedical and medicinal applications.

Keywords: Copper oxide nanoparticles, *Hibiscus rosa-sinensis*, green synthesis, antioxidant activity, anti-inflammatory activity, antimicrobial activity, cytotoxicity

Introduction

Nanotechnology is now recognised as a revolutionary field for biological and environmental research, offering innovative solutions for diagnostics, therapeutics, and microbial control (1). The use of metal oxide nanoparticles, particularly copper oxide nanoparticles (CuONP), has gained increasing interest among a wide range of nanomaterials due to their unique physical and chemical properties, affordability, and broad-spectrum biological functions (2). Copper, a vital trace element, displays strong redox activity that underpins the antimicrobial and cytotoxic properties of its oxide form when synthesised at the nanoscale (3). Traditional chemical methods for synthesising nanoparticles often involve hazardous

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substances and generate dangerous byproducts, highlighting the need for safer and greener alternatives (4, 5). Green synthesis, employing plant-derived compounds, has become a safe, environmentally friendly, and cost-effective method for nanoparticle production (6). This method utilises the diverse phytochemical properties of plants, which serve as natural agents for reducing and stabilising substances during nanoparticle synthesis (7). *Hibiscus rosa-sinensis*, commonly known as Chinese hibiscus, is a popular ornamental plant rich in bioactive compounds such as flavonoids, phenols, and organic acids (8) (9). These plant chemicals not only promote the breakdown of metal salts into nanoparticles but can also improve the biological properties of the resulting substances through surface functionalisation (10). The integration of plant-based biological molecules into CuONP could enhance its medicinal and antibacterial effectiveness (11). The CuONP synthesised using *Hibiscus rosa-sinensis* extracts has shown promising cytotoxic effects, particularly against cancer cells, due to its ability to induce oxidative stress and disrupt cellular structural integrity (12) (13). These nanoparticles may produce reactive oxygen species (14), causing dysfunctional mitochondria, DNA damage, and apoptosis in cancerous cells (15) (16). These processes show their potential for developing innovative anticancer agents, particularly in light of the increasing resistance to traditional chemotherapies (17). Plant-based production not only enhances biological compatibility, but it also decreases problems related to harmful effects and environmental persistence (18) (14). Despite its cytotoxic traits, the biosynthesized CuONP shows significant antioxidant and antimicrobial effects (19). The presence of plant chemicals on the outermost layer of the nanoparticle mainly accounts for its antioxidant capacity, as they can neutralise free radicals and prevent lipid peroxidation (20). Furthermore, these nanoparticles have shown substantial antimicrobial properties against various clinical infections, including drug-resistant bacteria (21). CuONP has the potential to damage bacterial membranes, interact with cellular enzymes, and induce oxidative damage, making it a useful tool in preventing infectious diseases (22). Generally, the plant-based production of CuONP with *Hibiscus rosa-sinensis* demonstrates an integrated method with notable medicinal advantages (23).

Materials and Methods

Copper oxide nanoparticles are synthesised using Hibiscus rosa-sinensis

To prepare the nanoparticles, 1 g of *Hibiscus rosa-sinensis* leaves was heated in 100 mL of distilled water for ten to fifteen minutes at 70℃. The extract was filtered using Whatman No. 1 filter paper. In a 250 mL conical flask, 70 mL of 20 mM copper sulphate solution was mixed with 30 mL of *Hibiscus rosa-sinensis* extract to produce nanoparticles. The mixture was stirred with an electromagnetic stirrer. A noticeable colour change indicated the formation of CuoNp. The nanoparticles were characterised using UV-Visible spectroscopy, FTIR, XRD, and Scanning Electron Microscopy (24).

Alpha amylase activity

Mix 100 µL of α-amylase solution (1 U/mL) with 10-50 µL of NPs and incubate at room temperature for thirty minutes. Then, add 100 µL of starch solution at 1% (w/v) concentration. The sample is then kept at room temperature for ten minutes. To stop the reaction, add 100 µL of 96 mM 3,5-dinitrosalicylic acid solution and boil it in a water bath for 5 minutes. As a control, a similar amount of sodium phosphate buffer (pH 6.9) was used instead of the enzyme concentrate. The absorbance was measured at a wavelength of 540 nm (25).

Bovine Serum Albumin Assay

To assess the anti-inflammatory activity, 0.05 mL of the CTLA nanogel was added to 0.45 mL of a 1% aqueous solution containing bovine serum albumin at concentrations of 10 µg/mL, 20 µg/mL, 30

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µg/mL, 40 µg/mL, and 50 µg/mL. A small amount of 1N hydrochloric acid was used to adjust the solution's pH to 6.3. The extracts were then incubated at room temperature for 20 minutes, followed by heating in a water bath at 55°C for thirty minutes. After heating, the samples were allowed to cool before measuring with a spectrophotometer at 660 nm. Diclofenac sodium served as the standard for evaluation. In this study, dimethyl sulfoxide (DMSO) was used as the control (26).

The following formula was used to calculate the percentage of protein denaturation: Percentage of reduction = (Absorbance of control - Absorbance of sample) / Absorbance of control × 100.

Egg albumin Denaturation assay

The samples for this experiment included 0.2 mL of fresh egg albumin, 2.8 mL of pH 6.4 phosphate-buffered saline (PBS), and 0.6 mL of nanogel at various concentrations, dispersed in 0.2% DMSO. The nanogel concentrations in the reactivity solution ranged from 10 to 50 µg/ml. The samples were incubated for 10 minutes at 37°C before being heated at 70°C in a water bath for an additional 20 minutes to induce denaturation of the egg albumin. The absorbance at 660 nm was measured after the mixture had cooled. The study included negative controls such as 0.2 mL of fresh egg albumin, 0.6 mL of 0.2% DMSO, and 2.8 mL of PBS. Diclofenac sodium was used as a positive control in the research (27).

The following equation was used to determine the amount of protein denaturation inhibition, which indicates the substance's anti-inflammatory activity: % Inhibition = (As/Ac - 1) × 100 (As = absorbance of sample, Ac = absorbance of control).

Antioxidant Assay

DPPH Method

Hibiscus rosa-sinensis-mediated CuONP (10 µg/mL, 20 µg/mL, 30 µg/mL, 40 µg/mL, and 50 µg/mL) was mixed with 1 mL of 0.1 mM DPPH in methanol and 450 µL of 50 mM Tris-HCl buffer (pH 7.4) for 30 minutes. The absorption at 517 nm was used to assess the decrease in free radicals caused by DPPH (28). To determine the level of inhibition, divide the control wavelength by the test sample wavelength.

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 sterilised polystyrene tip to create 5mm wells. The antimicrobial effect was achieved by enhancing the efficacy of copper nanoparticles mediated by *Hibiscus rosa-sinensis*. Copper nanoparticles are produced through the process of biosynthesis. The wells included varying amounts of 3 (25, 50, and 100 µL) and a standard control. The samples were placed on the Petri dishes, and zones of inhibition were incubated in a microbiological incubator at 37°C for 24 hours. The zone has been assessed to compare and investigate the potential effects of *Hibiscus rosa-sinensis*-mediated CuONP (29).

Cytotoxic Effect

Assay for Brine Shrimp Lethality

Brine shrimp eggs were placed in a saltwater-filled growing container. After twenty-four hours, 10 newly hatched larvae (nauplii) swam in 6 pools, each containing ten millilitres of saltwater. Micron-sized particles were added to each well in various amounts (5 µL, 10 µL, 20 µL, 40 µL, and 80 µL), with the final well acting as a control. After another twenty-four hours, the number of surviving nauplii was counted and recorded (30).

Results

Visual inspection and UV-Vis analyses for CuONP

The UV-Visible spectrum displays a strong absorption peak between 250-300 nm, indicating the presence of $\pi \rightarrow \pi^*$ or $n \rightarrow \pi^*$ electronic transitions, which are typical of substances with conjugated double bonds or aromatic rings (Fig. 1). Beyond 300 nm, the absorption gradually diminishes, suggesting minimal chromophore activity in the visible

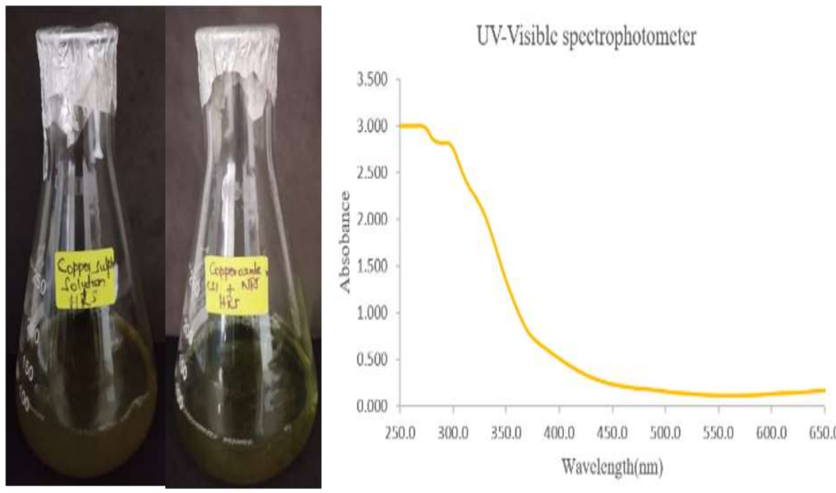

Fig 1: Synthesis of copper oxide nanoparticles using copper sulphate and *Hibiscus rosa-sinensis* extract from plants. UV-Vis spectrum of the synthesised nanoparticles, with a distinct peak indicating successful synthesis

spectrum. The highest ultraviolet absorbance (~3.0) around 260-280 nm could point to biomolecules or nanoparticles with strong UV absorption characteristics. After 400 nm, the spectrum levels off, showing almost no absorbance in the visible range. Overall, the UV-Vis profile suggests that the sample exhibits strong UV absorption but limited visible light absorption.

FTIR Analysis

The image below shows an FTIR spectrum, which is used to identify functional groups in a sample by analysing its characteristic absorption bands. Many peaks with different wavenumbers are visible, indicating various molecular vibrations (Fig. 2). The absorption band at 3440 cm^{-1} represents the stretching vibrations of hydroxyl (-OH) groups, implying the presence of alcohols or phenolic compounds. Peaks at 2920 cm^{-1} and 2850 cm^{-1} result from C-H stretching vibrations in alkanes, indicating alkyl chains. The sharp peak near 1630 cm^{-1} corresponds to C=O stretching vibrations, typically associated with

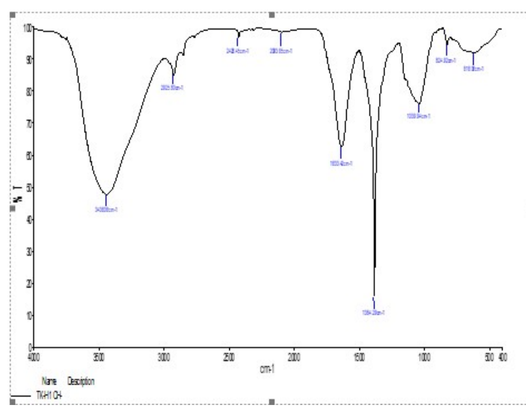

Fig 2: FTIR spectrum of NPS at different concentrations

carbonyl groups such as ketones, aldehydes, or carboxylic acids. The peaks around 1380 cm^{-1} suggest C-H bending vibrations, while the band near 1050 cm^{-1} indicates C-O stretching, confirming the presence of alcohols, ethers, or esters. Peaks below 800 cm^{-1} generally indicate out-of-plane bending vibrations, revealing aromatic ring

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substitutions. Overall, the FTIR spectrum shows the presence of hydroxyl, carbonyl, alkyl, and possibly aromatic groups, helping to determine the chemical composition of the sample.

XRD Analysis

The XRD pattern reveals the crystalline structure of copper oxide nanoparticles (CuONPs) synthesised from *Hibiscus rosa-sinensis* extract (Fig. 3). The diffraction peaks at 2θ values correspond to the planes (240), (112), (-151), (400), (601), (350), (-152), (261), (152), and (441), indicating the formation of highly crystalline CuONP. The distinct and sharp peak shapes suggest a well-defined crystalline structure with minimal amorphous content. The findings demonstrate that the bioactive compounds in *Hibiscus rosa-sinensis* extract acted as effective reducing and stabilising agents during nanoparticle synthesis. The diffraction peaks align with standard JCPDS data for monoclinic CuO, confirming phase purity. The absence of impurities further supports the success of green synthesis. Overall, the XRD analysis validates the structural integrity and crystalline quality of the synthesised CuONP.

SEM Analysis

This SEM image shows the surface structure of a substance at a high magnification (20,000x). The image reveals a rough, porous surface with irregularly shaped particles scattered across the frame. The presence of pores and cavities, which can increase surface area and influence the material's physical or chemical properties, indicates its porous nature. This morphology is often useful in applications such as catalysis, adsorption, and biomedical coatings. The magnification and scale bar (500 nm) suggest that the features observed are nanometer-sized. A secondary electron detector (SE2) was used to capture the surface topography, providing detailed insights into its structure. This type of analysis helps in understanding the material's properties,

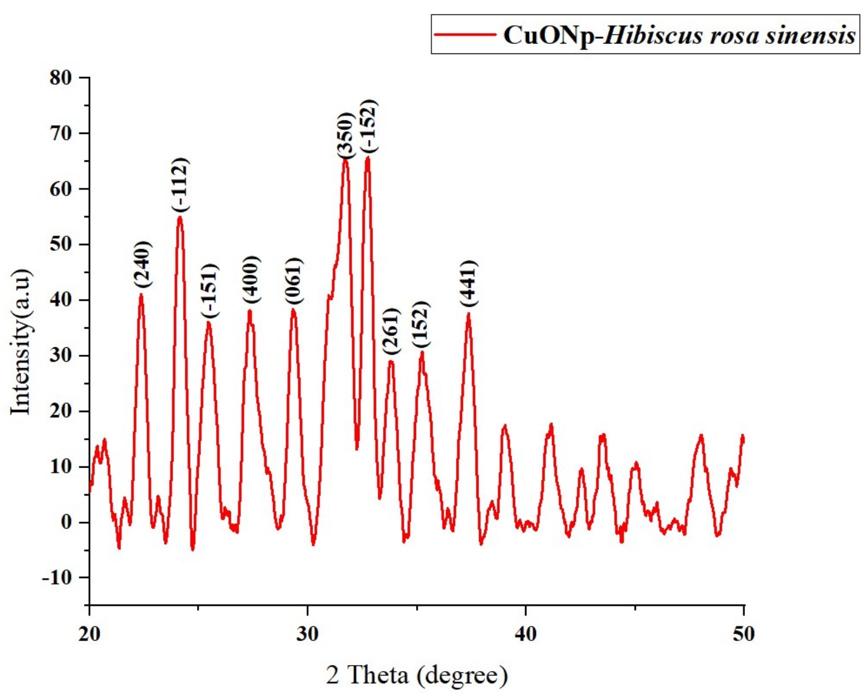

Fig 3: XRD pattern of copper oxide nanoparticles synthesized using *Hibiscus rosa-sinensis*

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which are essential for optimising its performance in specific applications (Fig. 4).

The EDS spectrum is used to analyse the elements in a sample. The spectrum displays two prominent peaks representing Oxygen (O) and Copper (Cu), indicating these are the main constituents in the examined area. The strong oxygen peak signifies a significant oxide content, likely resulting from copper oxide formation or surface oxidation. The presence of copper peaks indicates that the material has a metallic base. Minor peaks may also be present, but the dominant O and Cu signals mask them. The peak intensities reflect the relative amounts of each element. This analysis provides vital information about the material's elemental makeup, helping to better understand its functional and structural properties.

Alpha amylase activity

The chart shows the alpha-amylase inhibitory activity of CuONP synthesised with *Hibiscus rosa-sinensis* extract in comparison to the standard. From 10 to 50 µg/mL, both the standard and CuONP show a steady increase in inhibition. The inhibition is moderate at lower concentrations but rises considerably at higher concentrations. The standard exhibits slightly greater inhibition than CuONP across all tested concentrations, though the difference is minimal. This indicates that the synthesised CuONP possesses strong alpha-amylase inhibitory activity, suggesting potential antidiabetic properties (Fig. 5).

Bovine Serum Albumin Assay

The visualisation shows the Bovine Serum Albumin (BSA) assay results for CuONP synthesised from *Hibiscus rosa-sinensis* extract in comparison to the standard. As concentrations increase from 10 to 50 µg/mL, the percentage of inhibition rises in both samples. However, the standard consistently shows greater inhibition than CuONP at all tested concentrations. Nonetheless, CuONP significantly inhibits protein denaturation in a dose-dependent manner. The results suggest that the synthetic CuONP exhibits notable anti-inflammatory properties, although slightly less than those of the standard (Fig. 6).

Egg albumin assay

The graph shows the Egg Albumin Assay for CuONP synthesised with *Hibiscus rosa-sinensis* extract, compared to the standard. Inhibition percentages consistently increase with higher concentrations (10-50 µg/mL) in both samples (Fig. 7). The standard shows slightly higher inhibition values than CuONP, but the difference is minimal at all concentrations. CuONP inhibits protein denaturation in a concentration-dependent and potent way. These results indicate that the synthetic CuONP exhibits excellent anti-inflammatory activity, similar to the standard.

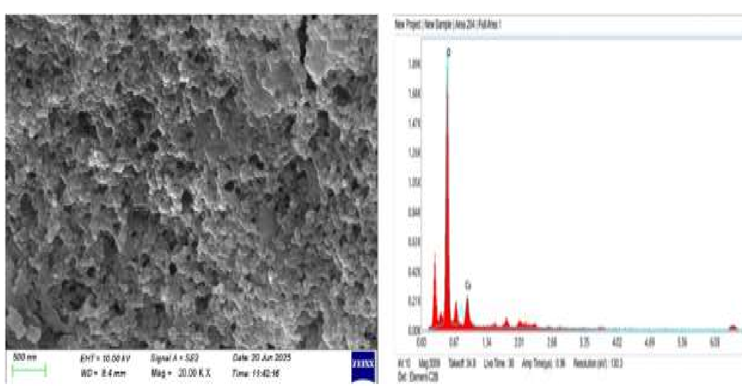

Fig 4: SEM images of NPs synthesized using *Hibiscus rosa-sinensis*

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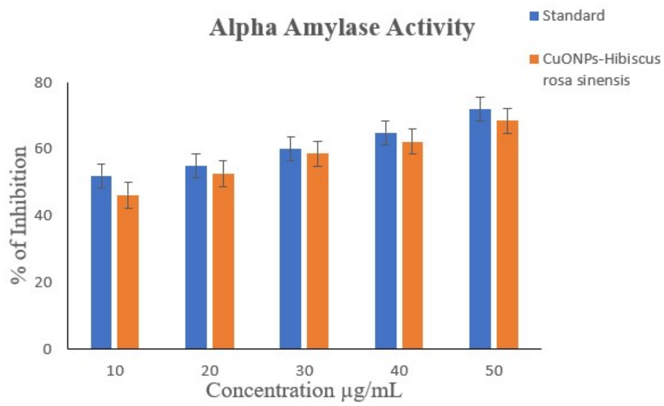

Fig 5: CuONP synthesised with *Hibiscus rosa-sinensis* demonstrated alpha-amylase inhibitory activity when compared to the standard at various concentrations

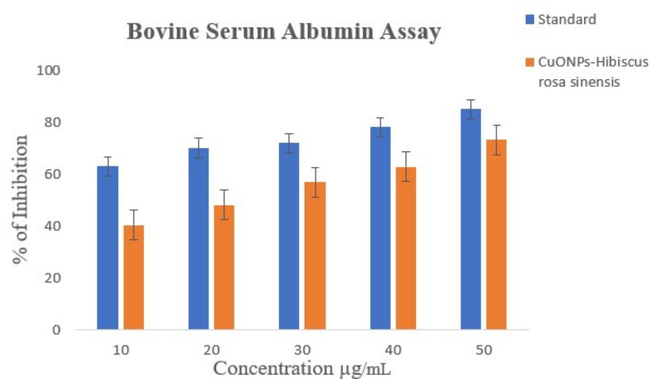

Fig 6: CuONP synthesised with *Hibiscus rosa-sinensis* showed inhibitory activity against bovine serum albumin (BSA) at various concentrations when compared to the standard

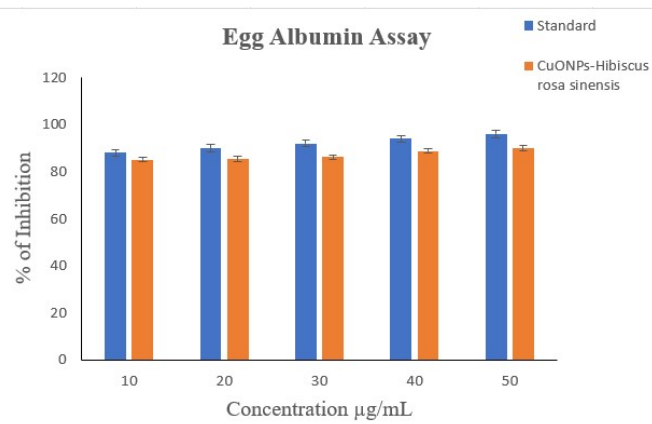

Fig 7: CuONP synthesised with *Hibiscus rosa-sinensis* demonstrated egg albumin inhibitory activity when contrasted with the standard at various concentrations

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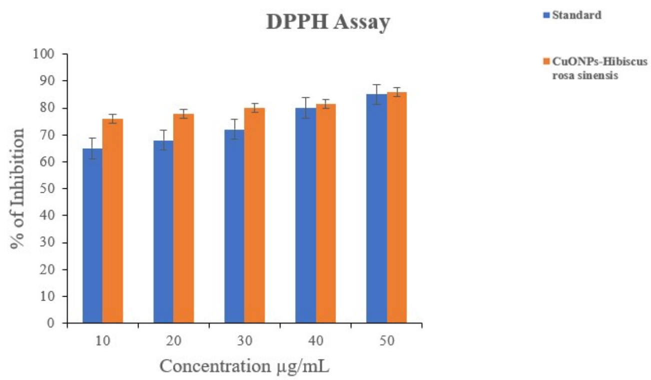

Fig 8: DPPH radical scavenging activity of standard and CuONP synthesised at various concentrations with *Hibiscus rosa-sinensis*

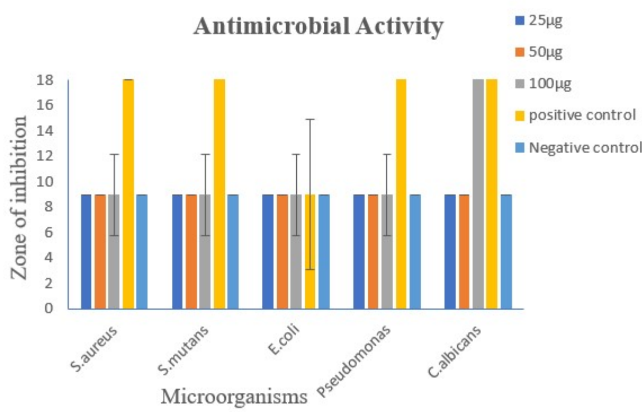

Fig 9: Antimicrobial activity of samples at different concentrations against microorganisms with zones of inhibition

Antioxidant Assay (DPPH Method)

The illustration compares the results of the DPPH assay for CuONP synthesised from *Hibiscus rosa-sinensis* extract to the standard. As concentrations increase from 10 to 50 µg/mL across the two samples, the percentage of inhibition gradually rises. Moreover, the CuONP demonstrates slightly greater inhibition than the standard at each tested dosage (Fig. 8). This suggests that the synthesised CuONP possesses significant antioxidant activity and may efficiently neutralise free radicals. The findings indicate their potential usefulness in reducing oxidative stress-related damage.

Antimicrobial Activity

The bar chart shows the antimicrobial activity of various concentrations (25 µg, 50 µg, and 100 µg) against different microorganisms, compared to positive and negative controls. The inhibition zone remained almost constant for all tested concentrations of *S. aureus, S. mutans, E. coli, Pseudomonas,* and *C. albicans*, measuring around 8-9 mm. The positive control produced significantly larger inhibition zones (~17-18 mm), indicating strong antimicrobial effectiveness. The negative control showed no notable inhibition, confirming that the observed activity is due to the sample (Fig. 9). Overall,

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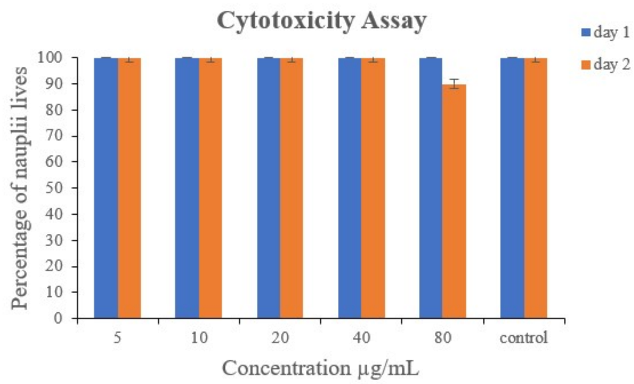

Fig 10: Cytotoxicity assay demonstrating the percentage of viable nauplii at various concentrations on days 1 and 2

the results imply that the sample displays only mild antimicrobial properties, in contrast to the positive control.

Cytotoxic Effect *(Brine Shrimp Lethality Assay)*

The graph illustrates the cytotoxicity assay of CuONP synthesised from *Hibiscus rosa-sinensis* extract, showing the percentage of nauplii survival at various concentrations over two days. Lower concentrations (5-40 µg/mL) resulted in high nauplii survival rates, indicating minimal cytotoxicity. Survival decreases slightly at 80 µg/mL, suggesting mild, concentration-dependent toxicity (Fig. 10). The control group had nearly 100% survival, confirming the assay's validity and reliability. These results imply that synthesised CuONP is generally beneficial and safe at lower concentrations.

Discussion

The present study demonstrates the successful green synthesis of copper oxide nanoparticles (CuONP) using *Hibiscus rosa-sinensis* leaf extract, highlighting the environmentally friendly and cost-effective nature of plant-based nanoparticle production (31). Analysis using FTIR revealed the presence of hydroxyl, carbonyl, and alkyl groups on the nanoparticle surface, indicating that phytochemicals in the extract, including flavonoids, phenolics, and organic acids, acted as benign agents that reduced and stabilised the nanoparticles (32). UV-Vis spectroscopy confirmed nanoparticle formation through a distinctive absorption peak at 260-280 nm, while XRD patterns revealed a monoclinic crystalline structure with high phase purity (33). SEM images revealed disorganised, porous structures, and EDS confirmed that copper and oxygen were the most dominant elements (34). These chemical and physical characteristics enhance biological activity because increased surface area and phytochemical functionalisation improve interactions with biological molecules and infectious agents (35). The nanoparticles showed significant antioxidant activity, with DPPH radical scavenging results indicating slightly higher inhibition than the standard at increased concentrations, demonstrating strong free-radical neutralising capacities (36). The anti-inflammatory activity, determined using BSA and egg albumin denaturation assays, showed significant concentration-dependent inhibition, comparable to that of diclofenac sodium (37). Inhibiting alpha-amylase demonstrated promising antidiabetic potential, while antimicrobial assays showed modest activity against *S. aureus*, *E. coli*, *Pseudomonas*, and *C. albicans* (38). The brine shrimp lethality assay showed good biocompatibility at lower concentrations and minimal harm at higher doses (39). These results collectively demonstrate that incorporating bioactive phytochemicals into

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CuONP enhances therapy effectiveness while ensuring safety within recognised guidelines (40).

Conclusion

The present investigation effectively synthesised copper oxide nanoparticles using a green approach with *Hibiscus rosa-sinensis* leaf extract, and confirmed their structural, physical, and biological properties. Cuonps showed significant antioxidant, anti-inflammatory, and antidiabetic activities, along with mild antimicrobial potential and biocompatibility at low doses. The incorporation of plant-derived chemicals was essential in enhancing the biological functions of the nanoparticles while ensuring their sustainability and safety. Given these promising results, *Hibiscus rosa-sinensis*-mediated Cuonps have potential for various therapeutic and medicinal uses. Future studies could explore their in vivo effectiveness, mechanisms of action, and possible applications in targeted drug delivery and pharmaceutical formulations.

Conflict of interest

The authors declare no conflict of interest

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