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Green synthesis of Copper Nanoparticles from *Coleus amboinicus* stem extract and its Characterization, Antibacterial potential study against *Staphylococcus aureus*

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Abstract

The green synthesis of nanoparticles is widely preferred due to their versatile nature, and this approach enabled the successful synthesis of nanoparticles in the laboratory. The plant extract from Coleus amboinicus possesses antibacterial properties, and copper, a natural antimicrobial element, has been used since ancient times. Combining them as nanoparticles enhanced their antibacterial activity, effectively inhibiting the growth of Staphylococcus aureus, which was confirmed by agar well diffusion, showing the largest zone of inhibition at the maximum dose of 40 µl, and nutrient broth assay, exhibiting 88% inhibition at the same dose. Biofilm disruption was demonstrated using the Congo red agar method, linked to reactive oxygen species generation. 3.8% SOD activity was shown by Superoxide dismutase assay, indicating oxidative stress and confirming ROS presence. These findings validate the antibacterial potential of Mexican mint–copper nanoparticles, highlighting their applicability in biomedical and environmental disciplines.

Keywords: Green synthesis; Copper nanoparticles; *Coleus amboinicus*; Antibacterial activity; *Staphylococcus aureus.*

Introduction

Nanotechnology comprises the synthesis, analysis and use of materials on nanoscale (1-100nm), which exhibits unique physical and chemical behaviour that differ from their bulk forms[1]. Among various nanomaterials, metal and metal oxide nanoparticles have gained considerable attention due to their high surface area-to-volume ratio and enhanced catalytic, antimicrobial, and antioxidant properties, making them suitable for biomedical and environmental applications[2].

Nanoparticles are commonly synthesized using physical, chemical, or biological methods. However, conventional physical and chemical approaches often require high energy input and toxic reagents, raising environmental and safety concerns[3]. In contrast, green synthesis offers an eco-friendly and sustainable alternative by employing biological systems as reducing and stabilizing agents. Plant-mediated green synthesis, in particular, is advantageous as it eliminates the need for microbial culture maintenance and enables rapid nanoparticle formation through phytochemical-driven processes[4].

Plants are rich sources of bioactive compounds such as phenolics, flavonoids, terpenoids, alkaloids, and polysaccharides, which play a key role in nanoparticle synthesis and stabilization[5]. These phytochemicals not only facilitate nanoparticle formation but can also enhance biological activity, resulting in synergistic antimicrobial and antioxidant effects. Consequently, plant-derived nanoparticles have been increasingly explored for applications in wound healing, diagnostics, regenerative medicine, and infection control[6]. Among metal nanoparticles, copper nanoparticles (CuNPs) have received significant attention due to their strong antimicrobial, antioxidant, and anti-inflammatory properties, combined with relatively low cost and acceptable biocompatibility[7]. CuNPs have demonstrated effectiveness against a broad spectrum of pathogenic microorganisms, including drug-resistant bacteria, highlighting their potential as alternative or complementary antimicrobial agents. *Coleus amboinicus* also known as *Plectranthus amboinicus* (Indian borage or Mexican mint), a medicinal plant belonging to the family Lamiaceae, is widely used in traditional medicine and is rich in phytochemicals such as phenolics, flavonoids, terpenoids, and tannins[8]. Several studies have reported its antibacterial, antioxidant, and anti-inflammatory properties, supporting its therapeutic relevance[9]. *Staphylococcus aureus*, a clinically significant pathogen, causing various infections was inhibited by the extracts of *C. amboinicus*[10].

Staphylococcus aureus is a major human pathogen associated with both community- and hospital-acquired infections, and its treatment is increasingly complicated by the emergence of multidrug-resistant strains such as methicillin-resistant *S. aureus* (MRSA)[11]. This has intensified the search for novel antimicrobial strategies.

In the present study, copper nanoparticles were synthesized using the stem extract of *C. amboinicus* via a green synthesis

approach. The synthesized CuNPs were characterized using UV–Visible spectroscopy, Fourier-transform infrared spectroscopy (FTIR), and X-ray diffraction (XRD) to confirm their formation, functionalization, and crystalline nature[12]. Furthermore, the antibacterial potential of the synthesized CuNPs against *S. aureus* was evaluated, highlighting the combined effect of copper nanoparticles and plant-derived phytochemicals as sustainable antimicrobial agents.

Materials and Methods

Sample Drying: The sample selected for the study was the stem of *Coleus amboinicus* (Mexican mint). Five stems, each approximately 15–20 cm long, were washed, cut into smaller pieces, and sun-dried for one day. Once dried, they were ground into powder and used for extraction.

Metabolite Extraction: The dried stem sample (6.43g) was extracted by boiling in distilled water (~150mL) using a water bath with intermittent stirring for 6–7 cycles. The extract was sequentially filtered through single and double-layer cheesecloth to remove coarse and fine debris. From the filtrate, 40mL was collected, while the remaining portion was stored as stock. Of the collected volume, 20 mL was retained as an aqueous control, and the remaining 20 mL was subjected to solvent extraction by adding an equal volume of 70% methanol, followed by incubation in a room-temperature water bath for 1.5h. After incubation, particulate matter was removed by filtration, and the extract was concentrated at 35–40°C until reduced to 5mL. The concentrate was reconstituted with 40mL of deionized water and subsequently used as the metabolite extract for copper nanoparticle synthesis.

Preparation of $CuS0_4.5H_2O$: Copper Sulphate was chosen as a metal salt to form NPs using plant extracts. 1.248g of $CuSO_4.5H_2O$ was added in 50mL of distilled water to obtain 0.1M concentration. 1.5mL of 0.1M $CuSO_4.5H_2O$ was diluted to 50mL with distilled water to get a 3mM concentration.

Mixing of extracted metabolites with copper salt solution: 45 mL of Mexican mint stem extract was added to the 50 mL of3mM $CuSO_4.5H_2O$ in culture jar,

Formation of Nanoparticles: The solvent was mixed with the extract, the culture jar was then introduced to a magnetic stirrerat 200 rpm and maintained at pH 6.8–7 for 36–40 hours.This allowed the formation of nanoparticles since constant stirring was required. The change in colour indicated the formation of nanoparticles.

Colour indication and centrifugation of formed nanoparticles: Formation of MS-Cu nanoparticles was initially confirmed by a visible colour change from a clear light green solution to dark green following overnight magnetic stirring, indicating nanoparticle synthesis. For spectral characterization, 2 ml of the colour-changed MS-Cu solution was subjected to absorbance measurement across different wavelengths. The synthesized nanoparticles were then recovered by centrifugation at 4000 rpm for 8 min, followed by washing with deionized water and re-centrifugation under the same conditions. The final pellet was resuspended in a minimal volume of deionized water and dried at 35°C in a hot air oven. Low-temperature drying was employed to preserve surface-bound phytochemicals and polyphenols, thereby maintaining nanoparticle bioactivity.

Collection of dried Nanoparticles: The dried MS-Cu content in the watch glass was scraped using a spatula and divided into 2 aliquots in an Eppendorf tube. To one of the tubes of each NPs, 2mL of deionized water was added and the NPs were dissolved by vortexing, which was utilized for applications[13]. Characterization was performed on the remaining aliquot of MS-Cu.

Characterization of synthesized Cu Nanoparticles: Zeta potential: The synthesized NPs were suspended in deionized water and treated with ultrasonication to ensure proper homogenization for evaluating the particle size distribution. The mixture was filtered and centrifuged at 5000 rpm for 10 mins at 25°C. The supernatant was diluted 4 to 5 times and analyzed using a computer-controlled particle size analyzer[14].

UV-Vis Spectroscopy: The synthesized Nanoparticles were subjected to estimate the spectra at 200 to 800 nm using a UV-Vis spectrophotometer.

XRD analysis: The green synthesized Cu Nanoparticles were analysed by X-ray diffraction for crystallinity and phase identification. The data were collected over a 2θ range of 20° to 50°.

FTIR analysis of NPs: To validate the conjugation of extract to NPs on the surface modification, the functional group and the chemical bond present, FTIR was performed in the wavelength ranging from 400-4000cm^{-1} using the KBr pellet method[15].

Antibacterial activity tests: Antibacterial Activity of MMS-Cu NPs by Agar Well Diffusion: The antibacterial activity of MMS-Cu NPs was evaluated using the agar well diffusion method. *Staphylococcus aureus* was selected as the test organism. Nutrient Agar (NA) was employed as the culture medium to determine the zone of inhibition.

To the Nutrient Agar a loopful of *S. aureus* was inoculated, mixed thoroughly, and left to solidify. Five wells were created using sterile 9 mm pipette tips. The first well served as a positive control, into which 20 µL of erythromycin was added. The MMS-Cu NPs were loaded into three wells with different volumes (10µL, 20µL, and 40µL) to test their antibacterial activity against *S. aureus*. The middle well was left empty and served as the negative control. The bacterial plate was incubated at 37°C for 24 hours. After incubation, the zones of inhibition were measured[16].

Antibacterial Activity of MMS-Cu NPs using Nutrient Broth: The minimum inhibitory concentration of MMS-Cu NPs was determined by using *Staphylococcus aureus* as a test organism and Nutrient broth (NB).

A loopful of *S. aureus* was inoculated into the nutrient broth (NB) and mixed well. The five test tubes were labelled as control, 1, 2, 3, and 4. 5mL of nutrient broth (NB) was added to each test tube. MMS-Cu NPs were added to test tubes 1, 2, 3, and 4 with different volumes: 10 µL, 20 µL, 30 µL, and 40 µL, respectively, to test antibacterial activity against *S. aureus*. Nothing was added to the control test tube, and all test tubes were incubated at 37°C for 24 hours. After incubation and bacterial growth, the absorbance of each tube was measured at 620 nm[17].

Biofilm Assay by Congo Red Agar (CRA) Method: The Biofilm Assay was evaluated using the Congo red agar (CRA) method with MMS-Cu NPs. *Staphylococcus aureus* was selected as a test organism.

The Congo red agar was carefully poured into each sterilized Petri plate. Out of the two Petri plates, one was marked as a positive control and the other as a test. In the positive control Petri plate, 10µL of erythromycin was added, while in the test Petri plate, 50µL of MMS-Cu NPs was added. The contents were mixed well and left to solidify. After solidification, a loopful of *S. aureus* was streaked onto both plates. The cultured plates were incubated at 37°C for 24 hours[18].

Superoxide Dismutase (SOD) Assay Using Nutrient Broth: Superoxide dismutase (SOD) assay was evaluated using[19] with some modifications. *Staphylococcus aureus* was selected as a test organism.

The four test tubes were labelled as control, positive control, and test. 10 mL of nutrient broth (NB) was added to each test tube. To the positive control tube, 10 µL of erythromycin was added. MMS-Cu NPs were added to the test tubes with different volumes (50µL and 100µL) to test the antibacterial activity against *S. aureus* and the control tube was included. The test tubes were incubated at 37°C for 24 hours. After bacterial growth, absorbance was measured at 620 nm.

Centrifugation and separation: Four washed centrifuge tubes were taken and labelled as control, positive control, and test tubes. To the respective tubes, the culture was added and centrifuged at 4,000rpm for 8 minutes. The supernatant obtained from each centrifuge tube was discarded, and the pellet was taken. The pellet obtained from each centrifuge tube was dissolved in 2mL of 2M KOH. The mixture was centrifuged again at 4,000rpm for 8 minutes. The supernatant obtained was collected in washed test tubes, which were named according to their corresponding centrifuge tubes. These collected supernatant samples were taken for estimation of superoxide dismutase (SOD).

Estimation of Superoxide Dismutase (SOD): Five washed test tubes were taken and labelled as blank, control, positive control, and test tubes. To the blank test tube, 1mL of distilled water, 1 mL of 0.01M Tris HCl, and 0.5mL of 0.01M pyrogallol were added. To the control test tube, 1mL of centrifuged culture, 1 mL of 0.01 M Tris HCl, and 0.5mL of 0.01M pyrogallol were added. The same protocol was followed for the positive control and test tubes; that is, 1mL of the corresponding sample, 1 mL of 0.01M Tris HCl, and 0.5mL of 0.01M pyrogallol were added. All the test tubes were incubated at 37°C for 20 minutes. After incubation, the optical density (OD) was measured at 420 nm.

Statistical analysis: All the experiments were done in triplicates and represented as value ± SD. ANOVA was used for analysis and the P value was remembered at 0.05.

Results and Discussion

Characterization of synthesized Cu Nanoparticles: Cu Nanoparticles are characterized by Zeta potential, UV-Vis spectroscopy, XRD and FTIR analysis.

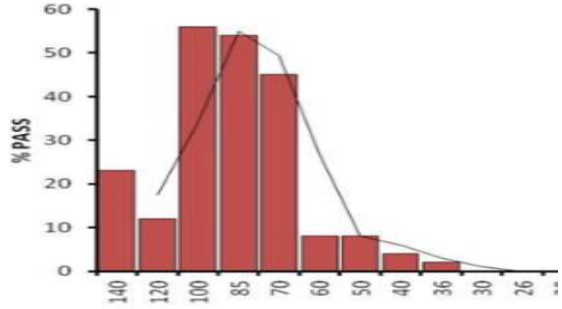


Figure-1: The graph represents the Size of synthesized Nanoparticles. Particle size analysis revealed that 56% of the synthesized nanoparticles possessed an average diameter of 100 nm, indicating a predominant size distribution within this range.

Zeta potential: The Zeta potential was found to be –2.23mV.

UV-Vis spectra: The Copper NPs showed absorbance at 270nm, which was clearly seen, shifted to 292nm with the formation of NPs with *C. amboinicus* extract (Figure-2).

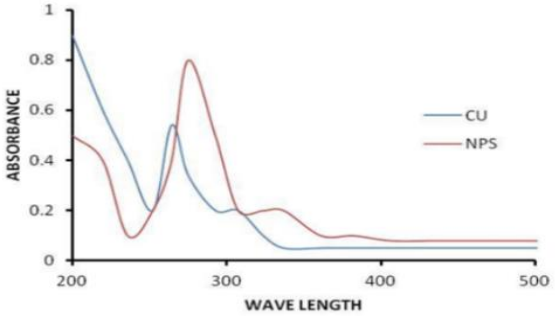


Figure-2: The samples synthesized were subjected to estimate the UV spectra at 200 to 800nm using a UV spectrophotometer (Shimadzu, 1800). The COPPER NPs showed absorbance at

270nm, which was clearly seen shifted from 292nm with the formation of NPs.

XRD analysis: The XRD diffraction data reveals the peaks at 36.8º, 38.2º, 48.6º, 58.6º corresponding to the (110), (110), (111), and (111) planes, respectively.

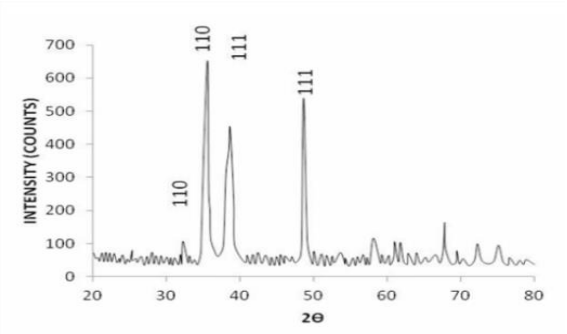


Figure-3: XRD spectra of green synthesized copper nanoparticles.

FTIR analysis of NPs: To validate the conjugation of extract to copper NPs on the surface modification of the synthesized NPs, FTIR (JASCO Ltd., Tokyo, Japan) was done in the wavelength range of 4000-400cm-1. Powdered sample was added to potassium bromide prior to evaluation**.**

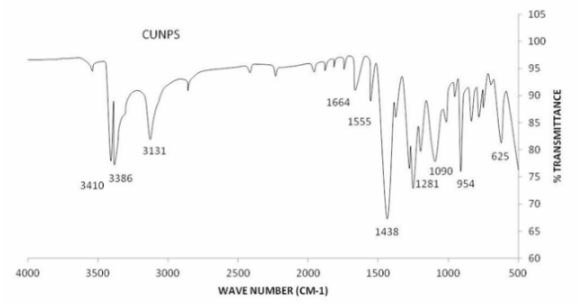


Figure-4: FTIR spectra of Cu nanoparticles.

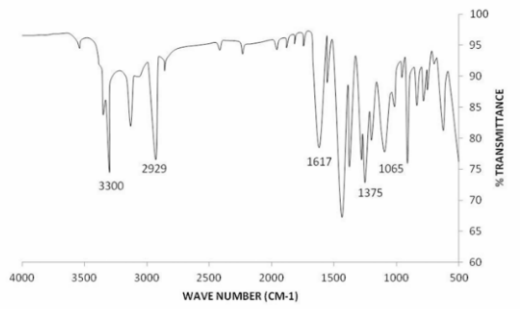


Figure-5: FTIR spectra of green synthesized Cu Nanoparticles.

FTIR verified that the conjugation of extract with Cu NPs was stable. Figure-5 displays the FTIR spectra of green synthesized Cu NPs from the stem of *C. amboinicus.* This could aid in identifying the possible biomolecules that could act as reducing and capping agents.

Bands at 3300cm^{-1}, 2929cm^{-1}, 1617cm^{-1} and 1375cm^{-1} correspond to plant extract. Bandat 3300cm^{-1}: O–H, -NH stretching vibration, Band at 2929cm^{-1}: C–H stretching vibration, Band at 1617cm^{-1}: COOH stretching, Peak at 1375cm^{-1} and 1065cm^{-1}: COO and C-N stretch for aromatic compounds[20].

Antibacterial Activity of MMS-Cu NPs by Agar Well Diffusion: The antibacterial activity of copper nanoparticles synthesized using Mexican mint stems was evaluated in comparison to the standard antibiotic, Erythromycin, by measuring the zone of inhibition. The nanoparticles showed a concentration-dependent response, producing inhibition zones of 0.8 cm at 10 µL, 1.4 cm at 20 µL, and 2.2 cm at 40 µL, indicating strong antibacterial activity at higher concentrations.

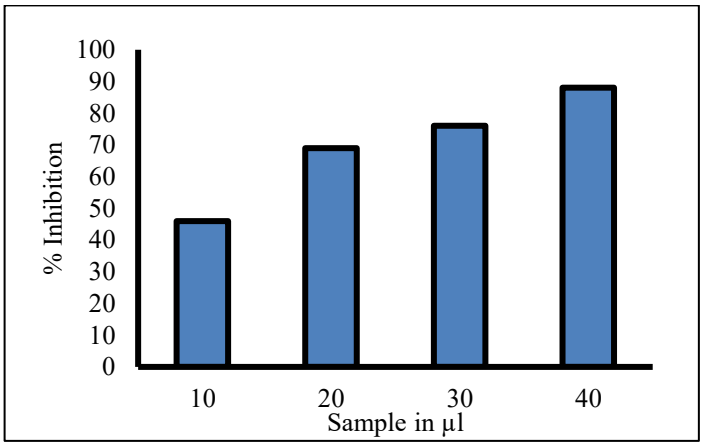
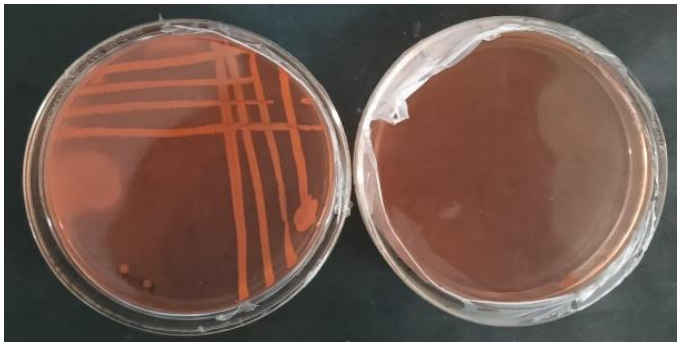


Figure-6: Antibacterial Activity of MMS-Cu NPs using Nutrient Broth.

The concentration dependent inhibition of bacterial growth from 46% to 88% was observed in the Nutrient broth assay. The MIC value was estimated using Linear Regression analysis between Concentration and Percentage Inhibition. The concentration corresponding to 100% inhibition was calculated to be approximately 47.7µL (Figure-6).



Test plate Positive plate

Figure-7: Biofilm Assay by Congo Red Agar (CRA) Method.

The Test plate showed bacterial growth, but not the biofilm formation due to presence of nanoparticle. The positive plate did not show bacterial growth since it has antibiotic (erythromycin).

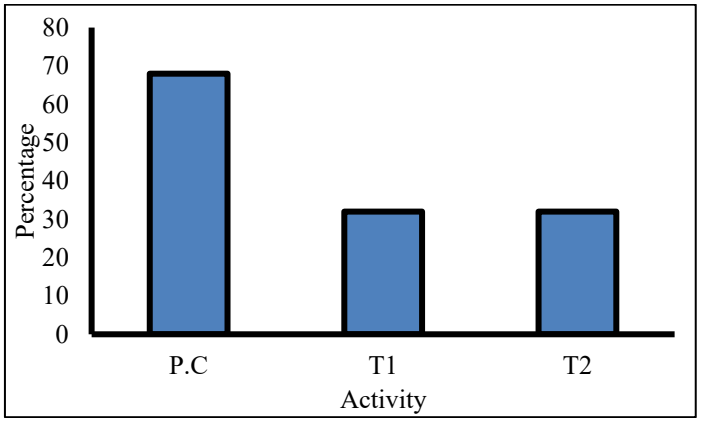


Figure-8: Estimation of Superoxide Dismutase (SOD) before centrifugation.

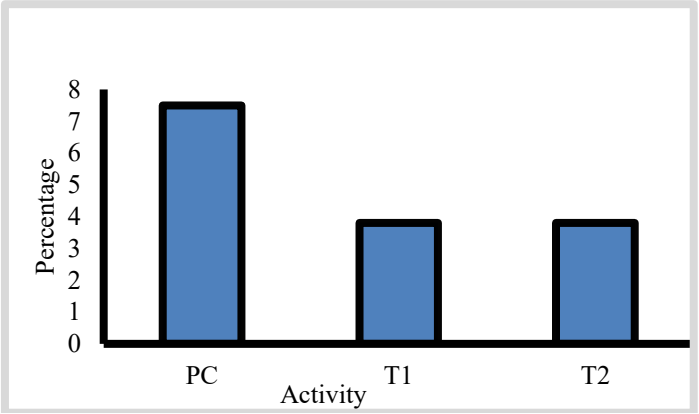


Figure-9: Estimation of Superoxide Dismutase (SOD) after centrifugation.

Before centrifugation, the positive control showed the highest antibacterial inhibition (68%), while test samples T1 and T2 exhibited similar inhibition (32%). After centrifugation, SOD activity confirmed stronger antibacterial effects in the positive control, with T1 and T2 showing similar activity.

The synthesized MMS-Cu nanoparticles exhibited significant antibacterial activity against *Staphylococcus aureus*, with an increase in nanoparticle concentration resulting in a corresponding increase in the clear zone of inhibition, indicating effective suppression of bacterial growth. The maximum inhibitory effect was observed at a concentration of 40 µL, showing the highest percentage inhibition of 88% ($P<0.05$). The optical density values were higher in test tubes containing nanoparticles compared to the control, confirming antibacterial activity in nutrient broth, with increased activity observed as nanoparticle concentration increased. On Congo red agar, *S. aureus* produced black, dry, crystalline colonies indicative of strong biofilm formation due to Congo red binding with extracellular polysaccharides. Upon treatment with nanoparticles, biofilm formation was disrupted, likely through mechanisms such as reactive oxygen species (ROS) generation, resulting in reduced or altered extracellular polysaccharides and a change in colony morphology from black to pink, signifying inhibition of biofilm production. In the SOD assay, the positive control showed high absorbance and SOD activity due to antibiotic-induced oxidative stress and ROS production, which activated antioxidant defence mechanisms in *S. aureus*. The test samples containing 50 µL and 100 µL of MMS-Cu NPs exhibited absorbance and percentage SOD activity values close to the positive control, indicating that similar oxidative processes occurred and further supporting the role of nanoparticles in inducing oxidative stress as a mechanism of antibacterial action.

Discussion: The leaves of Mexican mint are rich in phytochemicals and are well known for their antibacterial and antioxidant activities[21], the stem has been relatively less studied, and there are no reports on its antibacterial potential. The present study highlights the novel use of Mexican mint stem as an antibacterial agent against *Staphylococcus aureus.*

We have utilized a single strain of Staphylococcus aureus, as the primary objective was to evaluate the inhibitory potential of Mexican mint stem-Copper nanoparticles against the selected organism. Future studies are required to further investigate the antimicrobial efficiency of these synthesised nanoparticles against a wider range of bacterial cultures, including both Gram-positive and Gram-negative species. Hence, the present study specifically focuses on the inhibition of Staphylococcus aureus by the synthesised nanoparticles.

The plant extract has shown low or negligible activity when compared to the synthesised Nanoparticles and Positive control, therefore, it was omitted from the graph.

X-ray diffraction analysis was performed to determine the crystalline structure of the synthesised nanoparticle, and suggests the NPs to be of monoclinic cubic phase. FTIR spectroscopy was carried out to identify the functional groups present, revealing characteristic stretching and bending vibrations at different wavelengths. A band around 3300cm^{-1}corresponds to N-H stretching vibrations, characteristic of primary amine groups. The band at 2929$cm^{-1}$was attributed to C-H stretching of alkane groups. A strong absorption band at 1617cm^{-1} was assigned to C=C stretching, representing an α, β-unsaturated ketone. The band 1375$cm^{-1}$was due to O-H bending vibration, characteristic of phenolic groups. In addition, the band observed at 1065$cm^{-1}$is related to C-O stretching, suggesting primary alcohol groups. These functional groups (Figure-5) may play an important role in the functionality of the nanoparticles. Furthermore, from the UV-spectra, shift in the absorption peak was observed from 270nm to 290nm confirmed the formation of nanoparticles.

This study demonstrates that copper nanoparticles synthesized using *Coleus amboinicus* stem extract possess strong antibacterial and antibiofilm activity against *Staphylococcus aureus*. In agar well diffusion assays, the nanoparticles exhibited clear, concentration-dependent zones of inhibition, with the highest dose (40µL) producing a 22mm inhibition zone, closely comparable to the antibiotic control erythromycin (24mm). The Minimum Inhibitory Concentration corresponding to 100% inhibition was calculated to be approximately 47.7µL. The absence of inhibition in the negative control confirms that the antibacterial effect was solely due to the synthesized nanoparticles. These findings were further supported by quantitative Nutrient broth inhibition assays, which revealed a gradual increase in bacterial growth suppression from 46% to 88% with increasing nanoparticle concentration. In addition, the copper nanoparticles effectively disrupted biofilm formation. Congo Red Agar analysis demonstrated the loss of characteristic black, dry colonies associated with strong biofilm-producing *S. aureus*, indicating impaired extracellular polymeric substance production (Figure-7).

This antibiofilm activity is particularly significant, as biofilm-associated infections are known to exhibit heightened resistance to conventional antibiotics. The antibacterial and antibiofilm effects observed were closely associated with oxidative stress induction. Superoxide dismutase (SOD) activity in nanoparticle-treated bacterial cells approached levels recorded in antibiotic-treated controls, suggesting enhanced reactive oxygen species generation. This response indicates that the copper nanoparticles overwhelmed bacterial antioxidant defence mechanisms, contributing to membrane damage, biofilm disruption, and growth inhibition. In the present study, the synthesized nanoparticles exhibited a zeta potential value of –2.23 mV, which lies outside the conventional stability range (> +30 mV or < –30 mV) generally associated with colloidal stability. Despite the relatively low electrostatic repulsion, the nanoparticles demonstrated notable antibacterial efficacy against Staphylococcus aureus. This was evidenced by the concentration-dependent increase in the zone of inhibition, confirming their ability to suppress bacterial growth. Furthermore, disruption of biofilm formation was validated using the Congo red agar assay, highlighting the potential of these nanoparticles to interfere with microbial adherence and biofilm development. The observed biological activity may be attributed to phytochemicals from the plant extract employed in synthesis, which could contribute to partial stabilization and enhanced antimicrobial functionality. The reduced zeta potential may be influenced by factors such as pH fluctuations or proximity to the isoelectric point, conditions that favor aggregation. To improve nanoparticle stability, further investigations needs to be done, focusing on strategies such as the incorporation of steric stabilizers, pH adjustment, regulation of ionic strength, control of particle size distribution, and surface modification.

Although *C. amboinicus* has previously been explored for the synthesis of other metal nanoparticles, its application in copper nanoparticle production remains limited. The current investigation expands the utility of *C. amboinicus stem (Mexican mint stem)* as an effective reducing and stabilizing agent for eco-friendly copper nanoparticle synthesis. Overall, the integrated evaluation of growth inhibition, biofilm disruption, and oxidative stress response highlights the potential of *C. amboinicus*-derived copper nanoparticles as sustainable and multifunctional antimicrobial agents for combating *S. aureus* infections.

Conclusion

The green synthesis of nanoparticles using plant extracts presented an eco-friendly, sustainable, and cost-effective alternative to conventional chemical and physical methods. Through our experimental work involving the synthesis of copper nanoparticles using *Coleus amboinicus* extract, we successfully demonstrated the formation of stable and bioactive nanoparticles. Characterization techniques and assays confirmed the nanoparticles' effective antibacterial and anti-biofilm activities, validating their potential biomedical and environmental applications. This study highlighted the advantages of green synthesis, including biocompatibility, and simplicity of preparation without the use of harmful chemicals. Overall, our findings supported the viability of plant-mediated green synthesis as a promising method for producing functional nanoparticles with diverse applications, promoting sustainable nanotechnology development.

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