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Green Synthesis of Copper Oxide Nanoparticles using *Plumbago auriculata* Lam. Leaf Extract: Physicochemical Characterization, Antimicrobial Efficacy and Antioxidant Activity

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Abstract :

This study presents a green synthesis approach for the production of copper oxide nanoparticles (CuONPs) using *Plumbago auriculata* Lam. leaf extract. The synthesized CuONPs were characterized using various techniques, including UV-Vis spectrometry, X-ray diffraction (XRD), Fourier-transform infrared spectroscopy (FTIR), and transmission electron microscopy (TEM). The physicochemical properties of the CuONPs were thoroughly analyzed, revealing their unique biological and mechanical properties. Furthermore, the antimicrobial efficacy of the CuONPs was evaluated against several pathogenic bacterial strains and Candida albicans using the disc diffusion method and determination of minimum inhibitory concentration (MIC). The CuONPs exhibited significant antimicrobial activity, highlighting their potential as antimicrobial agents. Additionally, the antioxidant activity of the CuONPs was assessed through DPPH scavenging activity testing, indicating their ability to counteract free radicals and potential application in managing oxidative stress-related disorders. Overall, this study contributes to the development of eco-friendly and biocompatible CuONPs with potential applications in medicine, agriculture, and environmental remediation.

Keywords: nanotechnology, green synthesis, copper oxide, *Plumbago auriculata*, sustainable synthesis, eco-friendly, biomedical applications, environmental remediation.

INTRODUCTION

Nanotechnology has emerged as a prominent field encompassing the production, characterization, and application of nanoparticles (NPs)(Khan *etal*.,2018).NPs are nanoscale materials with dimensions ranging from 1 to 100 nm and have found diverse applications in various sectors such as medicine, agriculture, environment, and textiles(Nadeem *etal*.,2020). Green synthesis methods have gained considerable attention in nanoparticle production due to their eco-friendly nature and potential advantages (Sharma *etal*.,2009),(Iravani,2011).

Among the different nanoparticles, copper oxide nanoparticles (CuONPs) have attracted significant due to their unique biological and mechanical properties(Sankar *etal.*,2014). Traditional synthesis approaches involving chemical or physical methods often utilize hazardous chemicals and energy-intensive processes, posing risks to the environment and human health(Singaravelu *etal*.,2007). Therefore, the development of sustainable and eco-friendly synthesis routes for CuONPs is imperative.

Plant extracts have emerged as valuable resources for the green synthesis of nanoparticles. *Plumbago auriculata* Lam., commonly known as the Cape Leadwort, is a plant species rich in flavonoids, terpenoids, and phenolic compounds, which can act as efficient reducing and capping agents during nanoparticle synthesis(Vijayaraghavan *etal*.,2012). The utilization of *P. auriculata* leaf extract offers several advantages; including non-toxicity, sustainability, and control over nanoparticle size and shapes (Wu *etal*.,2015). Additionally, the presence of bioactive compounds like plumbagin in *P. auriculata* extract provides enhanced antimicrobial and antioxidant properties to the synthesized nanoparticles(Jayaprakash *etal*.,2007),(Yang *etal*.,2017).

Several studies have reported successful green synthesis of CuONPs using different plant extracts (Ghorbani and Rafiee-Moghaddam,2017),(Ghosh *etal*.,2016),(Gour and Jain,2019). However, further investigations are needed to explore the green synthesis of CuONPs using *P. auriculata* leaf extract and comprehensively characterize their physicochemical properties. Additionally, evaluating their antimicrobial and antioxidant activities is crucial for assessing their potential applications in biomedical and environmental domains (Vasantharaj *etal.*,2018).

This study aims to bridge these gaps by conducting a detailed analysis of the green synthesis of CuONPs using *P. auriculata* leaf extract. The physicochemical properties of the synthesized CuONPs will be thoroughly characterized using advanced techniques such as

Fourier-transform infrared spectroscopy (FTIR), transmission electron microscopy (TEM), UV-visible spectroscopy, and X-ray diffraction (XRD).

Furthermore, the antimicrobial efficacy of the green synthesized CuONPs will be assessed against the various human pathogenic bacterial strains and the fungal strain *Candida albicans* using the disc diffusion method and determination of minimum inhibitory concentration (MIC) (CLSI, 2012). This evaluation will provide valuable insights into the potential of CuONPs as antimicrobial agents. Moreover, the antioxidant activity of the CuONPs will be evaluated through DPPH scavenging activity testing, shedding light on their ability to counteract free radicals and their potential application in managing oxidative stress-related disorders(Salvamani *etal*.,2014).

The green synthesis of CuONPs using *P. auriculata* leaf extract presents a promising and sustainable approach for nanoparticle production. This study aims to contribute to the field by conducting a comprehensive analysis of the synthesized CuONPs, including their physicochemical properties, antimicrobial efficacy, and antioxidant activity. The outcomes of this research hold significant importance in advancing the development of eco-friendly and biocompatible CuONPs with potential applications in various sectors, including medicine, agriculture, and environmental remediation.

MATERIALS AND MATERIALS

Preparation of Plumbago auriculata Extract

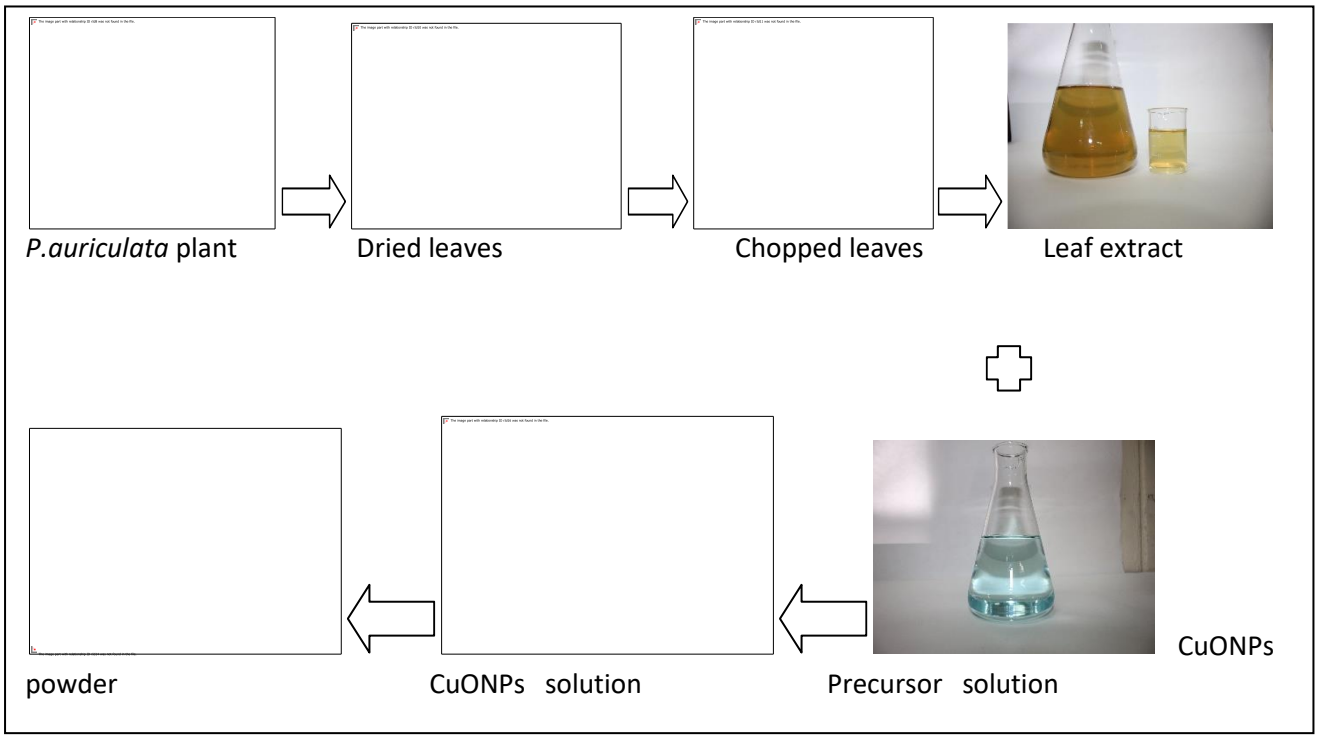
P. auriculata was collected from the Kerala Forest Research Institute (KFRI), Peechi, Thrissur, India. The collected leaves (100g) were thoroughly washed with running tap water (2-3 times) and then rinsed with distilled water. The leaves were air-dried at room temperature for 1 hour and finely chopped into small pieces. The finely chopped leaves were then boiled and vigorously stirred in 500 ml of distilled water at 60°C for 1 hour. The resulting leaf extract was filtered using Whatman filter paper and stored for further use in the synthesis of CuONPs (Anitha *etal*.,2015).

Synthesis of CuONPs using Plumbago auriculata Leaf Extract

Copper sulfate pentahydrate [CuSO4·5H2O] was used as the precursor solution, with a molecular weight of 249.68 g/mol. A solution of 10 mM CuSO4·5H2O was prepared. In a conical flask, 50 ml of the *P. auriculata* leaf extract was mixed with 450 ml of the 10 mM CuSO4·5H2O solution in a ratio of 1:9. The initial pH of the solution was noted to be 3.9, and it was adjusted to 8.2 by adding NaOH. The solution was then heated in a water bath at

80°C for 2 hours with continuous stirring. The change in color of the solution indicated the formation of CuONPs. After allowing the solution to cool, it was centrifuged at 10,000 rpm for 12 minutes, and the pellet was washed multiple times with distilled water. The washed pellet was then transferred to a petri dish and dried in a hot air oven at 80°C for a day. Subsequently, the obtained copper oxide nanoparticles [CuONPs] were calcinated at 300°C for 3 hours before further analysis (Bharathi *etal*.,2016).In this study, the formation of copper oxide nanoparticles was preliminarily detected by visual observation of a green-colored solution, which subsequently transformed into black-colored nanoparticles (Awwad and Amer,2020). The major steps involved in the green synthesised CuONPs using *Plumbago auriculata* Lamleaf extract are shown in Figure 1.

Figure 1. Major steps of the green synthesised CuONPs using *Plumbago auriculata* Lamleaf extract



Characterization of Green-Synthesized Copper Oxide Nanoparticles

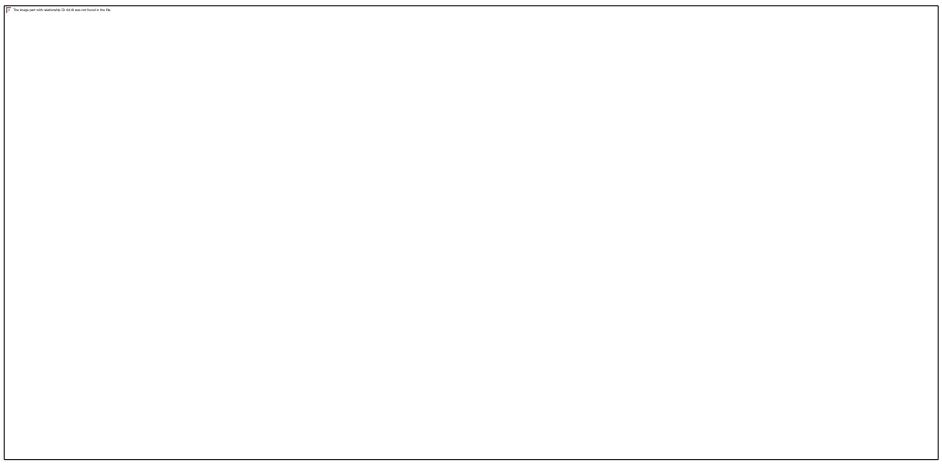
The green-synthesized copper oxide nanoparticles were characterized using various techniques, including UV-Vis spectrometry, X-ray diffraction (XRD), Fourier-transform infrared spectroscopy (FTIR), and transmission electron microscopy (TEM). UV-Vis spectrometry was employed to confirm the presence of the synthesized copper oxide nanoparticles. FTIR analysis was conducted to investigate the functional groups present in the nanoparticles, such as alcohols, aldehydes, amines, and aromatic compounds. XRD

analysis was performed to determine the size and nature of the nanoparticles, while TEM provided insights into their morphology, distribution, and size. It is important to note that the size, shape, and morphology of the synthesized nanoparticles can vary depending on the physicochemical factors involved in the synthesis process, including pH, time, temperature, and precursor concentration(Marooufpour *etal*.,2019).

Antimicrobial Activity - Disc Diffusion Method

The antibacterial activity of the green-synthesized copper oxide nanoparticles from *P. auriculata* was evaluated using the Mueller-Hinton agar disc diffusion method. Cultures of six different human pathogenic bacteria, including *Staphylococcus aureus* (MTCC 96), *Enterococcus faecalis* (MTCC 439), *Candida albicans* (MTCC 183), *Escherichia coli* (MTCC 452), *Serratia marcescens* (MTCC 97), and *Proteus vulgaris* (MTCC 426), obtained from the Microbial Type Culture Collection and Gene Bank in Chandigarh, were used. Mueller-Hinton agar medium was prepared and sterilized using an autoclave. Sterile standard discs impregnated with 1 mg/ml streptomycin and 1 mg/ml copper oxide nanoparticles (prepared by weighing and dissolving the nanoparticles in DMSO) were placed on the agar plates. After overnight incubation, the zones of inhibition were measured to assess the antibacterial activity of the synthesized CuONPs (CLSI, 2012). Details of the microorganisms used are given in Table 1.

Table:-1 Details of microorganisms used for Antimicrobial activity



Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of the copper oxide nanoparticles was determined using the micro-broth dilution method in microtiter plates, following the Clinical and Laboratory Standards Institute (CLSI) protocol. Stock solutions of CuONPs were prepared by dissolving 10 mg of nanoparticles in 1 ml of methanol. Different concentrations

of CuONPs ranging from 0.1 mg to 10 µl/ml were prepared from the stock solution. Bacterial and fungal suspensions were prepared in nutrient broth and Sabouraud dextrose broth, respectively. The microtiter plates were incubated overnight after streaking the different concentrations of CuONPs.The control bacterial and fungal suspensions were also included in the microtiter plates. The MIC was determined as the lowest concentration of CuONPs that showed no visible growth of microorganisms compared to the control (CLSI, 2012).

Determination of Antioxidant Activity by DPPH Assay

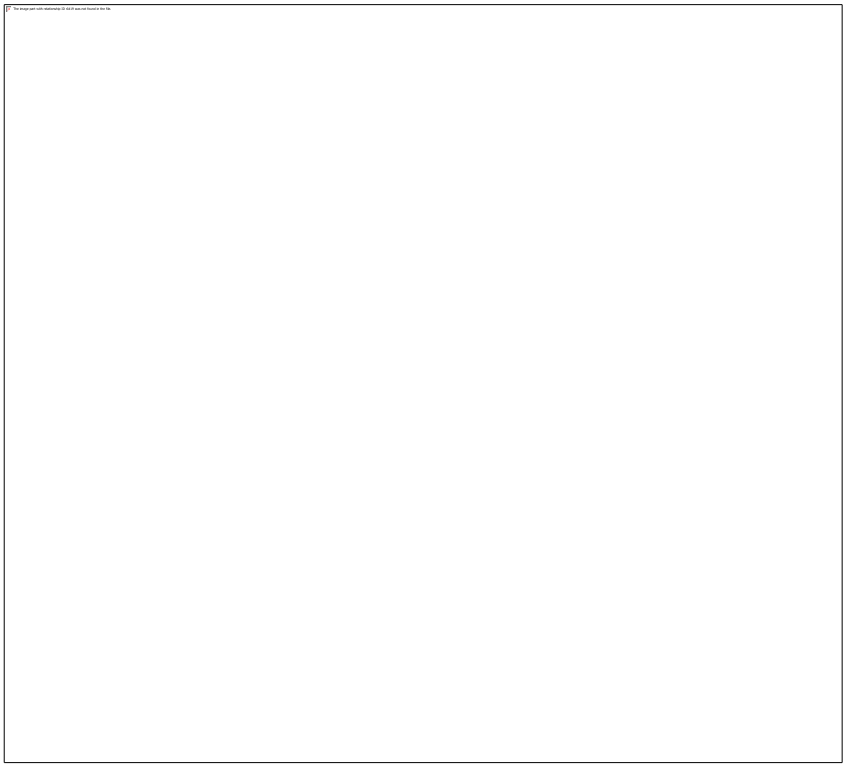
The antioxidant activity of the copper oxide nanoparticles synthesized using *P. auriculata* leaf extract was evaluated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method. A stock solution of CuONPs was prepared by dissolving 10 mg of nanoparticles in 1 ml of methanol and sonicated for 30 minutes. Different concentrations (1 mg, 2 mg, and 2.5 mg) of CuONPs were prepared by diluting the stock solution with methanol. Similarly, a control solution of ascorbic acid was prepared at the same concentrations. A 0.1 M DPPH solution was prepared using methanol. In test tubes, 1 ml of different concentrations of CuONPs or ascorbic acid was mixed with 2 ml of DPPH solution. The mixture was thoroughly mixed and incubated for 30 minutes in the dark at room temperature. After incubation, the absorbance of the solutions was measured at 517 nm using a UV-Vis spectrophotometer. The percentage of DPPH scavenging activity was calculated using the formula: %DPPH Scavenging activity = (Ac - As) / Ac × 100, where Ac represents the absorbance of the control and As represents the absorbance of the sample (Aromal and Philip,2012).

RESULTS AND DISCUSSION

UV-Visible Spectroscopy

The UV-visible spectroscopy analysis confirmed the formation of green-synthesized nanoparticles. The absorption spectra of the synthesized copper oxide nanoparticles were recorded in the range of 200-700 nm. The highest peak observed at 227 nm further confirmed the presence of copper oxide nanoparticles(Shah *etal*.,2022). Mali *et al*.,(2019) reported a similar absorption peak at 228 nm at pH 8. The surface plasmon resonance of CuONPs typically occurs in the range of 200-350 nm. (Figure 2)

Figure: 2 The UV-visible graph of synthesised nanoparticles



Transmission Electron Microscopy (TEM)

TEM analysis was conducted to determine the morphology and size of the nanoparticles. The TEM images revealed that the average size of the nanoparticles was 23.22 nm. The nanoparticles exhibited a monoclinic or irregular shape with agglomerate nature (Figure 3). TEM analysis is widely recognized as the most accurate technique for determining the morphology of nanoparticles(Huq and Akter,2021).

Figure 3: The Transmission Electron Microscopy (TEM) image of the CuONPs.

FTIR Spectroscopy

FTIR spectroscopy was employed to investigate the different functional groups responsible for the synthesis of green copper oxide nanoparticles. The FTIR spectra recorded the

wavelength region of the synthesized nanoparticles from 400 to 4000 cm^-1. Thirteen distinct peaks were obtained in the spectra. The absorption peaks at 3584.97 cm^-1 and 3560.90 cm^-1 corresponded to the –OH stretching of alcohols. The peak observed at 3378.39 cm^-1 was attributed to the N-H stretching of primary amines. The absorption peak at 1634.27 cm^-1 indicated the C=O stretching of ketones/acids. The broad peak at 1524.23 cm^-1 was associated with the N-O stretching of nitro compounds. The absorption peak at 1105.22 cm^-1 represented the C-O stretching of secondary alcohols. The absorption peak at 983.12 cm^-1 was assigned to the C-N stretching of aromatic amino groups. The peak at 943.38 cm^-1 corresponded to the C=C bending of alkenes. The absorption peak at 876 cm^-1 indicated the C-H stretching of aromatic compounds. The absorption peaks at 779.88 cm^-1 and 736.87 cm^-1 were related to the C-Cl stretching of alkyl halides. The narrow peak at 602.62 cm^-1 indicated the presence of C-Br stretching in aliphatic bromide. The sharp peak at 489.30 cm^-1 confirmed the formation of copper oxide bonds (Cu-O and M-O).The presence of alcohols (O-H), primary amines (N-H), ketones/acids (C=O), nitro compounds (N-O), secondary alcohols (C-O), aromatic amino groups (C-N), alkenes (C=C), aromatic compounds (C-H), alkyl halides (C-Cl), aliphatic bromide (C-Br), and metal oxides (Cu-O, M-O) were identified through FTIR analysis (Prasad *etal*.,2017),(Rafique *etal* ,2017),(Renuga *etal*.,2020). Figure 4 depicts the FTIR graph of CuONPs, and Table 2 presents the correlation table of FTIR peaks and corresponding functional groups.

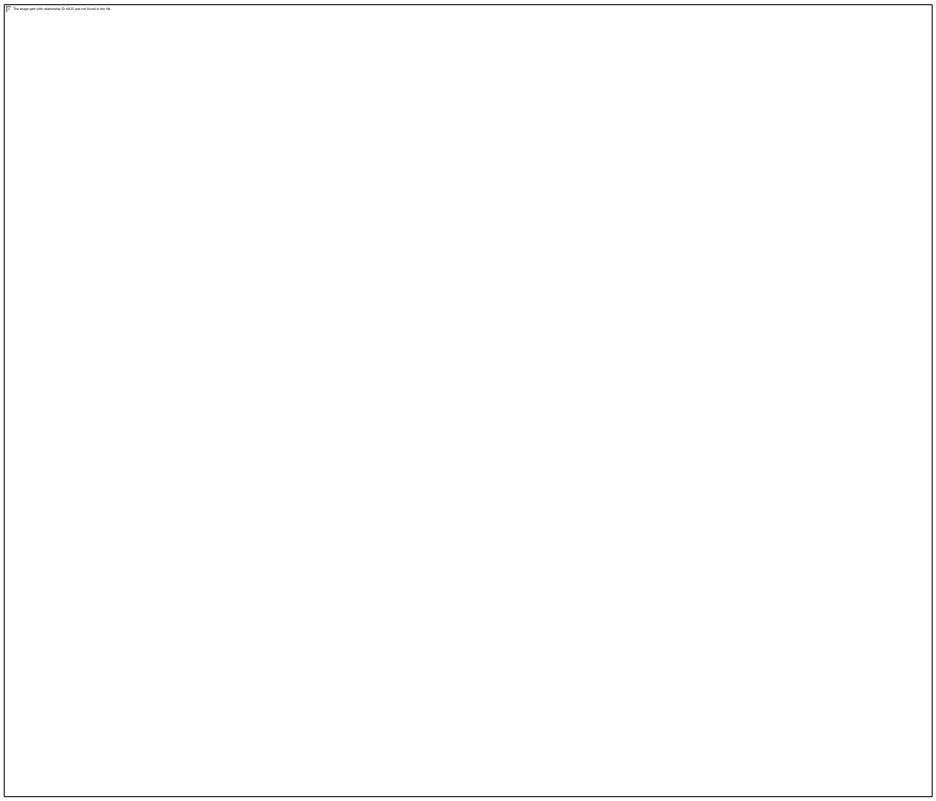
Figure4: FTIR graph of CuONPs

XRD Analysis

X-ray diffraction (XRD) analysis was used to determine the crystalline nature of copper oxide nanoparticles. The diffraction angles were recorded in the range of 10° to 80°. The obtained XRD pattern exhibited distinct peaks at 2θ values of 32.513°, 35.607°, 38.796°, 49.045°, 58.266°, 61.685°, 66.505°, 67.976°, 75.071°, corresponding to the crystallographic planes (110), (002), (111), (202), (202), (113), (311), (220), and (004), respectively(Fig .5). No impurity-related diffraction peaks were observed. These experimental results are in agreement with the reported diffraction patterns of CuO nanoparticles prepared by (Padil and Černík,2013) and (Shammout and Awwad,2021). The distinct and well-defined peaks in the XRD pattern provide evidence of the crystalline nature of CuO nanoparticles(Bashiri *etal*.,2018).

Figure 5: XRD graph of CuONP.

Table 2: FTIR correlation table



Antimicrobial Activity

The antibacterial activity of CuONPs was evaluated using the disc diffusion method, and the zone of inhibition was measured in millimeters. The CuONPs were tested against *Serratia marcescens*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Proteus vulgaris*, *Candida albicans*, and *Escherichia coli*, and the results were compared with a standard drug (streptomycin). The zone of inhibition for *Serratia marcescens* and *E. coli* was 7 mm, compared to the standard zone of 20 mm. Candida albicans exhibited a zone of inhibition of 6 mm, while *Proteus vulgaris*, *Staphylococcus aureus*, and *Enterococcus faecalis* showed zones of inhibition of 6 mm, 6 mm, and 5 mm, respectively, compared to the standardzones of 30 mm(Najaraj *etal*.,2019),(Padil and Černík,2013)(,(Renuja *etal.*,2022). The inhibitory effect of CuONPs on bacterial growth is attributed to their interactions with the bacterial cell membrane, leading to malfunction and ultimately cell death(Das *etal*.,2013).The enhanced antibacterial efficacy of CuONPs can be attributed to the presence of biomolecules, such as terpenoids, in the plant extract used for capping (Ali *etal*.,2019). Moreover, high concentrations of CuONPs are toxic to various bacterial pathogens in humans and plants (Applerot *etal*.,2012). Figure 6 illustrates the disc diffusion method for evaluating the zone of inhibition (Table 3 and Figure 6).

Table 3: Zone of inhibition of microorganisms.

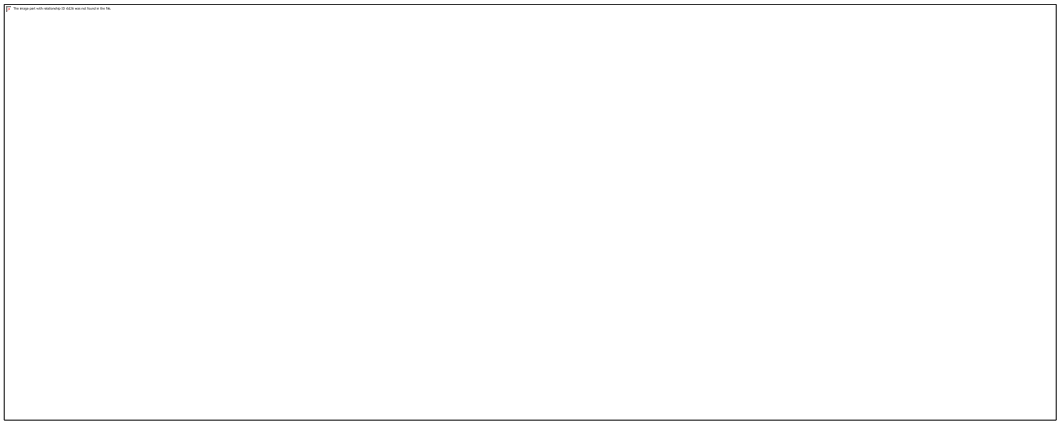


Figure 6: Diagram illustrating the disc diffusion method for evaluating the zone of Inhibition.

Serratia marcescens	*Proteus vulgaris*	*Staphylococcus aureus*
Escherichia coli	*Candida albicans*	*Enterococcus faecalis*

Determination of Minimum Inhibitory Concentrations

In this study, MICs were determined using the micro-broth dilution method, following the CLSI protocol. Different concentrations of CuONPs were prepared from the stock solution, ranging from 0.1 mg to 0.001 mg. The bacterial and fungal suspensions were incubated overnight in nutrient broth and Sabouraud dextrose broth, respectively. The various concentrations of CuONPs were added to the microtiter plates along with the bacterial and fungal suspensions, while control wells contained only the respective suspensions. The plates were then incubated, and the absence of visible growth was used to determine the MIC.

The results of the MIC determination showed that *E. coli* exhibited a MIC at 0.2 mg-20 µl, while *S. Marcescens*,*C. albicans*, and *E. faecalis* demonstrated MICs at 0.001 mg-1 µl. *P. vulgaris*, and *S. aureus*, exhibited MICs at 0.01 mg-10µl. These findings suggest that CuONPs possess antimicrobial properties against a range of bacteria and fungi. (Figure 7, Table 4).

The obtained MIC values align with previous studies. For instance,Renuja *et al*., (2022) confirmed the antibacterial activity of CuONPs against *S. aureus*, *C. albicans*, and *E. coli*. Similarly, Nagaraj *et al*.,(2019) and Padil and Černík (2013) reported the potential antibacterial activity of CuONPs against *S. aureus* and *E. coli*. These studies provide additional support for the antimicrobial efficacy of CuONPs observed in our study.

The mode of action behind the antimicrobial activity of CuONPs involves interactions with the bacterial cell membrane. The disruption caused by CuONPs leads to malfunctions in the bacterial cells, inhibiting their growth and ultimately resulting in cell death(Das *etal*.,2013). The precise mechanisms of action may involve oxidative stress, damage to cell components, and interference with essential cellular processes. The exact mechanisms are complex and require further investigation.

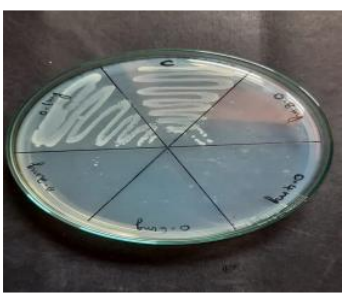
It is worth noting that the concentrations at which CuONPs exhibit antimicrobial activity are within a range that can be effectively utilized for potential applications. The use of plant extracts for the green synthesis of CuONPs provides an eco-friendly and sustainable approach. The presence of biomolecules in the plant extract, such as terpenoids, may contribute to the enhanced antimicrobial efficacy of CuONPs (Ali *etal*.,2019)

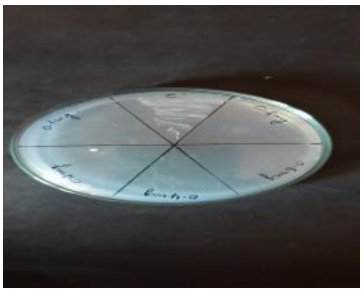
Figure: 7MICofdifferentmicroorganisms

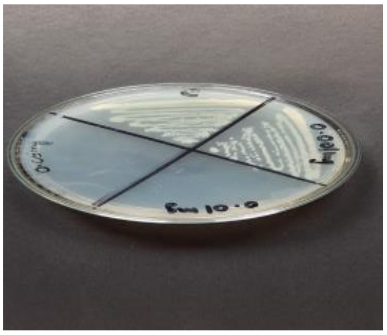
Escherichiacoli

Proteusvulgaris

Serratiamarcescens







Enterococcus faecalis

Staphylococcus aureus

Candida albicans

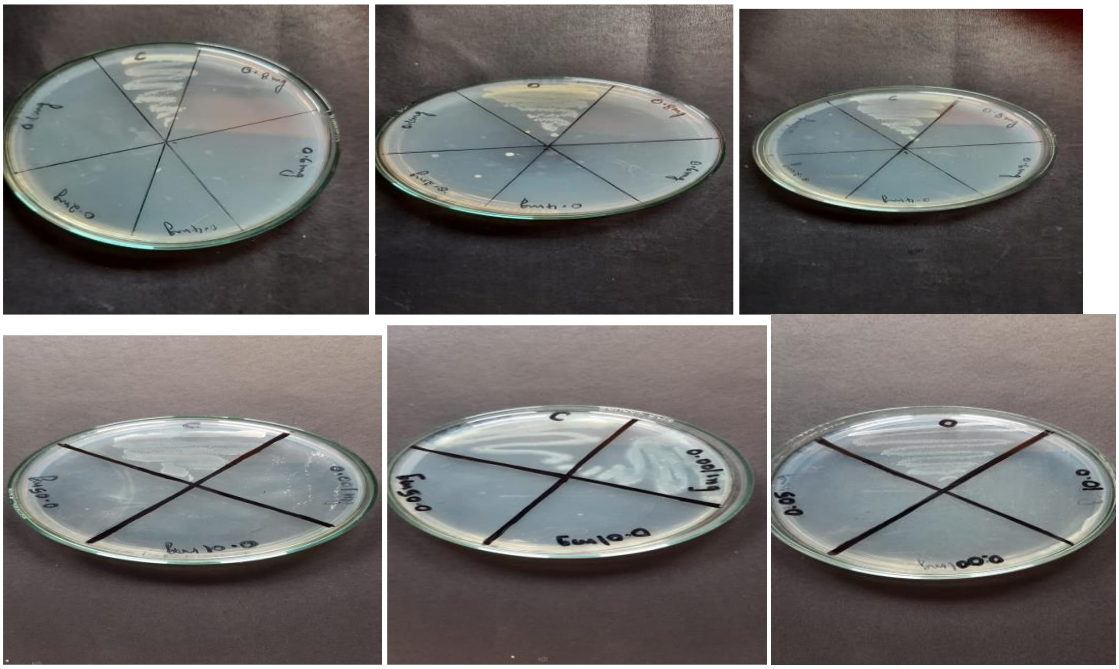
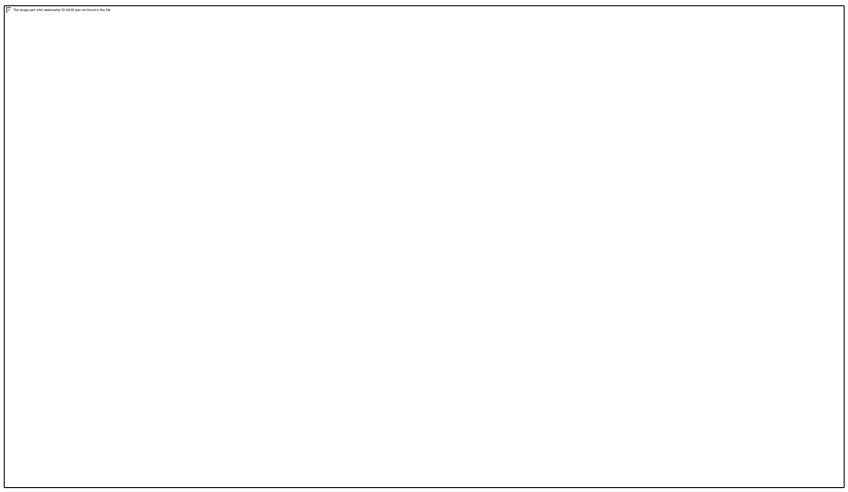


Table: - 4shows the MIC of microorganisms



Antioxidant activity

Green synthesized CuONPs were studied for their antioxidant activity against the DPPH radicalandcomparedtotheascorbicacid(Keshari *etal*.,2020).Thesynthesizednanoparticles'significantantioxidantactivities at different concentrations are 1mg/ml-74%, 2mg/ml-76%, and 2.5m g/ml-82%. Thestandardascorbicacidshows1mg/ml-97%,2mg/ml-97%,and2.5mg/ml-98 %.(Table 4-5, Figure 8-9).Theresultsconcluded that the CuONPs have significant antioxidant activity. The results of Antioxidant activity concluded that the CuONPs have significant antioxidant

activity. Antioxidants are natural or synthetic substances that may prevent or delay cell damage caused by oxidants (ROS, RNS, free radicals, and other unstable molecules)(Awwad and Amer 2020).These phenolic compounds possess hydroxyl and ketone groups, contributing to iron chelation and subsequently demonstrating a strong antioxidant property(Singh *etal*.,2017).

Table:5 Radical scavenging activity of Ascorbic acid

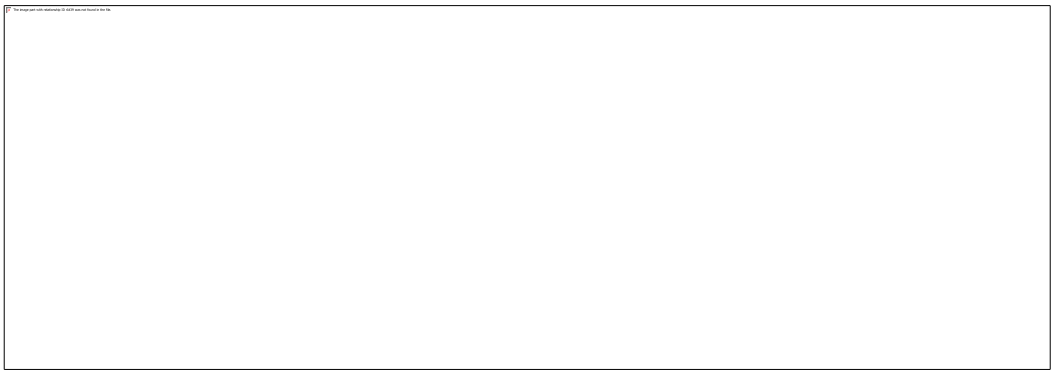
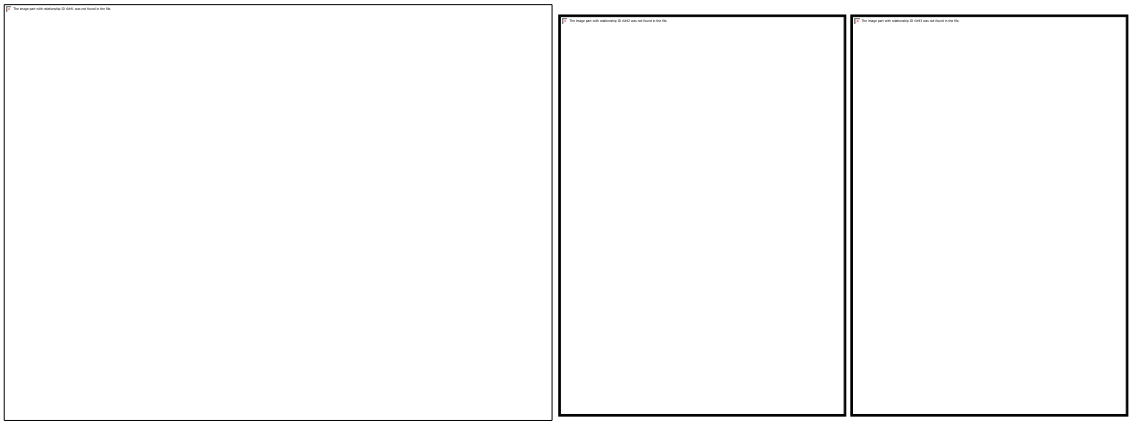


Table: 6 Radical scavenging activity of CuONPs.

Figure:-8 Antioxidant activity of CuONPs

Figure:-9 Visual observation of synthesized Nanoparticles (a) and standard absorbic acid (b).

a) b)



Conclusion

In conclusion, this study successfully synthesized green copper oxide nanoparticles (CuONPs) using a plant extract. The characterization of the synthesized CuONPs was carried out using various techniques. UV-visible spectroscopy confirmed the formation of CuONPs, with the highest absorption peak observed at 227 nm. TEM analysis revealed the morphology and size of the nanoparticles, showing an average size of 23.22 nm and irregular shapes with agglomerate nature. FTIR spectroscopy identified the functional groups involved in the synthesis of CuONPs, including alcohols, primary amines, ketones/acids, nitro compounds, secondary alcohols, aromatic amino groups, alkenes, aromatic compounds, alkyl halides, aliphatic bromide, and metal oxides. XRD analysis confirmed the crystalline nature of the CuONPs, with distinct peaks corresponding to the crystallographic planes of CuO nanoparticles.

The synthesized CuONPs exhibited significant antibacterial activity against *Serratia marcescens*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Proteus vulgaris*, *Candida albicans*, and *Escherichia coli*. The zone of inhibition observed in the disc diffusion method indicated the effectiveness of CuONPs in inhibiting the growth of these microorganisms. Furthermore, the MIC determination demonstrated the minimum inhibitory concentrations of CuONPs against these microorganisms.

Overall, this study contributes to the understanding of the synthesis, characterization, and antimicrobial properties of green-synthesized CuONPs. The results highlight the potential application of CuONPs as antimicrobial agents in various fields, including medicine and agriculture. Further research is warranted to explore the mechanisms of action and optimize the synthesis parameters for enhanced antimicrobial efficacy.

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