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Synthesis, Characterization, and Antimicrobial Activity of Copper Nanoparticles (CuNPs) Synthesized from *Alpinia officinarum* Rhizome Extracts

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Abstract: Nanoparticles can be synthesized from bacteria, fungi, and plant extracts and used in diverse fields as antimicrobial agents against various drug-resistant bacterial pathogens. Green synthesis of copper nanoparticles (CuNPs)is highly effective for developing drug candidates.The aim of this study is to synthesize CuNPsfrom *Alpinia officinarum* rhizome extracts and to confirm the antibacterial activity of the rhizome extracts. Green synthesis of CuNPs was carried out by adding copper sulfate in filtered rhizome extracts. Physicochemical characterization was carried out using Fourier Transform Infrared analysis (FT-IR), UV-visible spectroscopy, FE-SEM, EDX, DLS, and zeta potential. Synthesized CuNPsexhibited a good zone of inhibition, minimum inhibitory concentration, and minimum bactericidal activity, while biofilm activity showed high antibacterial efficacy against clinical strains of *Staphylococcus aureus*. This study revealed that CuNPssynthesized from *Alpinia officinarum* rhizome extracts have potential antimicrobial activity against clinical strains of *Staphylococcus aureus*.

Keywords: nanoparticles; Fourier transform infrared spectroscopy; UV-visible spectroscopy; *Staphylococcus aureus.*

1. Introduction

Nanotechnology is an emerging field of science, and the synthesis of metallic nanoparticles is increasing daily because of its wide applications in the medical and pharmacological fields [1-4]. Nanoparticles are cost-effective, and metallic nanoparticles are synthesized from bacteria, fungi, and plants. The biosynthesis of metallic nanoparticles does not cause any hazardous effect on the environment. Nanoparticles can be synthesized on a large scale in industries and can be used as an efficient antimicrobial, antioxidant, and anti-cancerous agent[5-7].The CuNPsaresynthesized by various physical, chemical, and biological methods. CuNPspossess antimicrobial, antioxidant, and anti-cancerous properties and are less toxic when compared with other metallic nanoparticles.CuNPsexhibit high catalytic activity and antimicrobial activity due to their close interaction with microbial membranes and the metal ions released in the solutions; nanoparticles cause alterations in the microbes' cell membrane, leading to cell death.CuNPswith high catalytic activity can be used as biosensors and electrochemical sensors [8-11]. *Alpinia officinarum* is widespread in Southeast Asia, and the plant originated in China. Hong Kong is a commercial center for the sale and distribution of *Alpinia*. This plant belongs to the ginger family, and it contains a high concentration of

phytocompounds such as quercetin, flavonol galangin, and labdane diterpene; these phytocompounds exhibit antimicrobial properties.

Rhizome extracts of this plant possess medicinal properties and are used to treat cold and body pain, and they are reputed to have stimulant and digestive effects [12,13]. *Staphylococcus aureus* is a major human pathogen that causes skin infection, bone infection, endocarditis, food poisoning, pneumonia, and toxic shock syndrome. *Staphylococcus* show resistance to various antibiotics. Strains of *Staphylococcus* produce enterotoxins, which cause food poisoning. A highly dangerous form of *Staphylococcus* emerged in the form of methicillin-resistant *Staphylococcus aureus*, which is resistant to a whole class of antibiotics called beta-lactams, which include methicillin, penicillin, amoxicillin, and oxacillin. In many Gram-positive bacteria, there is an additional bridge of glycines, which makes them resistant to antibiotics. The development of drug-resistant *Staphylococcus* is threatening, and the development of drugs is essential for such microbes[14].

In the present study, CuNPs were synthesized from rhizome extracts of *Alpinia officinarum, and the synthesis was confirmed* using various physicochemical techniques. The green synthesized CuNPswere used to study the antimicrobial activity in methicillin-resistant *Staphylococcus aureus* (MRSA), microbial type culture collection (MTCC), and multidrug-resistant (MDR) strains of *Staphylococcus aureus*.

2. Materials and Methods

2.1. Preparation of rhizome extracts of Alpinia officinarum.

The dry rhizome of *Alpinia officinarum* was powdered, and 10 g of powdered rhizome was taken in a conical flask added with 100ml of double distilled water, boiled for 20 minutes, and allowed for infusion for around 2 hours. The extract was filtered using Whatman No: 1 filter paper to get the aqueous extracts. After filtration, the solution was centrifuged for 15 minutes at 4°C at 4200 rpm, and then the obtained supernatant was stored at4°C for further use [15, 16].

2.2. Biosynthesis of CuNPs.

Rhizome extracts were mixed with 100mM copper sulfate in a 1:1 ratio, and the mixture was kept in a shaker with a frequency of 110 RPM (1.833333 Hz)for 48 hrs. The synthesis of CuNPswas observed by taking the spectrum in UV-Vis spectroscopy, between 300 nm and 800 nm and the change of color was observed from dark green to brown, which confirmed the synthesis of nanoparticles. Then, the solution was centrifuged at 15,000 rpm for 10 minutes at 4°C. The pellet was washed with sterile distilled water and dried in a thermostat [15, 16].

2.3. Characterization of CuNPs.

UV-visible spectroscopy confirmed the Synthesis of CuNPs, and characterization was performed using Fourier Transform Infrared analysis (FT-IR), FE-SEM, EDX, DLS, and zeta potential.

2.4. Antimicrobial assays.

MRSA, MTCC, and MDR clinical strains of *Staphylococcus aureus* were used in this study with ethical approval from the BSACIST ethical committee (Ref. no. BSAU: REG-OFF:

2016/02 SLS), *Staphylococcus aureus* causes nosocomial infection and skin infection. The increase in methicillin-resistant *Staphylococcus aureus* infection is alarming. *Staphylococcus aureus* causes foodborne diseases [25]. Synthesized CuNPs were assessed for antimicrobial activity by calculating the zone of inhibition using the agar well plate method. The antimicrobial potential of CuNPs synthesized from *Alpinia officinarum* was studied by using the agar well diffusion method. CuNPs were used in three different concentrations (75 µg/ml, 100 µg/ml, and 125 µg/ml), minimum inhibitory concentration, minimum bactericidal concentration, and biofilm assay in MRSA, MTCC, and MDR strains of *Staphylococcus aureus* as detailed in protocol Ranjani et al.[15] without any modification. All the experiments were performed in triplicates.

3. Results and Discussion

3.1. Synthesis and characterization of CuNPs.

Different biological methods are used for the synthesis of CuNPs. Plant extracts used for synthesis were more effective than bacteria and fungi-mediated synthesis since they are not industrially viable and require sterile conditions [17]. In the present study, CuNPs were synthesized from rhizome extracts of *Alpinia officinarium*, and their antimicrobial activity was evaluated. CuNps were characterized using UV-visible spectrum, FT-IR, FESEM, EDX, DLS, and zeta potential. CuNPssynthesized from *Alpinia officinarum* were characterized using UV-visible spectroscopy, which showed surface plasmon resonance peaks at 550nm and 400nm (Figure1A), confirming the CuNPs synthesis. Previous studies stated that CuNPs show surface plasmon resonance bands in the 350 to 800 nm range. Surface plasmon resonance bands are determined by different properties, like the solvent used during synthesis and the particle's size and shape, while bandwidth increases with a decrease in nanoparticle size [18].

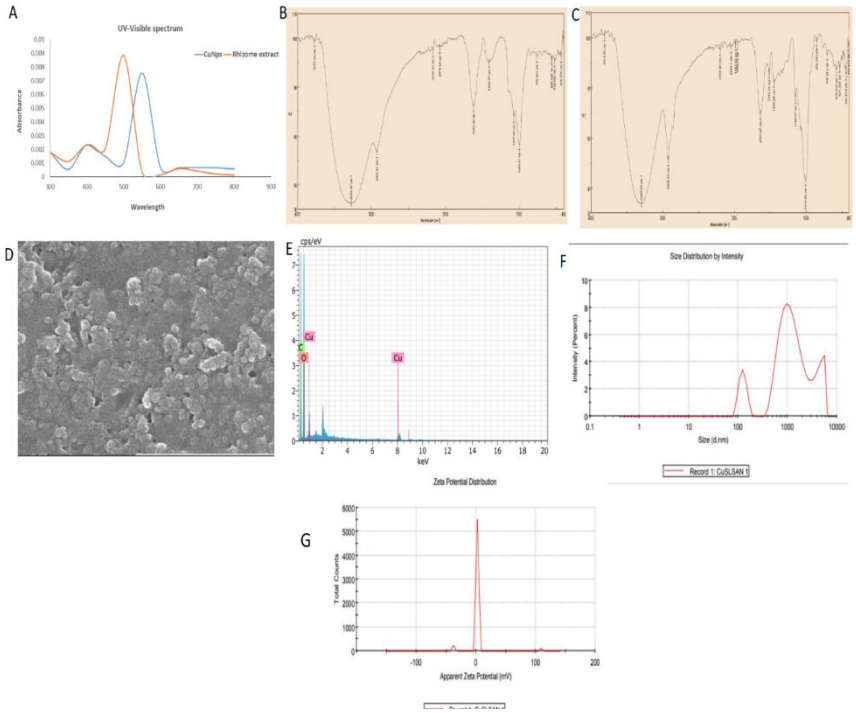

Figure 1. (A) UV-Visible spectrum of CuNPs and rhizome extracts of *Alpinia officinarum*; **(B)**FT-IR spectrum of *Alpinia officinarum* extracts;**(C)**FT-IR spectrum of CuNPs; **(D)**FESEM analysis of CuNPs;**(E)** EDX spectrum of CuNPs at 8 and 1 kev; **(F)** Dynamic light scattering analysis with the particle size distribution of CuNPs;**(G)** Zeta potential of CuNPs.

FTIR is performed to study the functional groups present in the CuNPs, which act as a capping and stabilizing agent, and the functional groups are shown in (Table 1) and (Figures 1B and C). Bands at 3276, 3772,2168, 2070,1618, 1410, 1077,1000,766 cm^{-1} attributed to O-H stretching, C≡C Stretching, C-H bending, C-O stretching, C-F stretching, C-Cl stretching in CuNPs whereas methyl group, aromatic ring, organic sulfates, aliphatic organohalogen, methylene group were found in a spectrum of rhizome extract. Similar functional groups, O-H stretching, C-H stretching, C≡C stretching, and C-F stretching, were also present in synthesized CuNPs. This confirms that these compounds act as a capping and reducing agent during the synthesis.

Table 1. FT-IR analysis and the functional group of CuNPs and *Alpinia officinarum* rhizome extracts [19].

Wave number (cm^{-1}) of CuNPs	Functional group of CuNPs	Compound class	Wave number (cm^{-1}) rhizome extracts	Functional of the group of rhizome extracts	Compound class
3276	O-H Stretching	Carboxylic acid	3297	O-H Stretching	Carboxylic acid
3772	O-H stretching	Alcohol and hydroxyl	2924	C-H stretching	Methyl group
2168	C≡C Stretching	alkene	2199	C≡C Stretching	alkene
2070	C≡C stretching	aromatic	2046	C≡C stretching	aromatic
1618	C=C stretching	Quinone or conjugated ketone	1637	C=C stretching	Quinone or conjugated ketone
1410	C-H bending	Carbonate ion	1515	C=C stretching	The aromatic ring (aryl)
1077	C-O stretching	Ether and oxy	1449	S=O	Organic sulfates
1000	C-F stretching	Phosphate ion	1149	C-F stretching	Aliphatic organohalogen
766	C-Cl stretching	Aliphatic	1010	Cyclohexane ring	Methylene

FESEM is used to study the shape, morphology, and topology of CuNPs. In this study, CuNPs are spherical, rod-shaped, and polydispersed, as shown in Figure 1D. The shape of the nanoparticles synthesized by plant extracts varies depending on the chemical compositions, concentrations, and pH of the media [20-22]. The EDX spectrum confirmed the presence of copper, oxygen, carbon, and CuNPs, and a peak was observed at 8Kev. EDX spectroscopy is used to evaluate the elemental composition of nanoparticles, as shown in Figure 1E.

Characterization using UV-VIS spectroscopy, FTIR, FESEM, EDX, DLS, and Zeta potential confirmed the presence, shape, morphology, size, and topology of the CuNPs. The CuNPsarea better option and were used in many industries and also used as an antimicrobial agent which inhibits the growth of Gram-positive and Gram-negative organisms [14-17]

The dynamic light scattering effect showed information about the size and distribution of the nanoparticles. The average particle size of CuNPs was 859.5 nm, and the PDI value was 0.554 nm, which showed an intercept at 0.865, as shown in Figure 1F. The Zeta potential value of CuNPswas 0.108 mV, the zeta deviation was 31.3 mV, and conductivity was 0.0358(mS/cm), as shown in Figure 1G. Zeta potential shows the positive charge of the CuNPs, which is polymorphic in nature. Zeta potential is a significant factor in understanding the surface charge of nanoparticles, and it predicts the stability of the dispersion. Previous studies reveal that nanoparticles have high stability if zeta potential values are higher than +25 and less than -25 [23,24].

3.2.Antimicrobial activity of CuNPs.

The antimicrobial potential of CuNPssynthesized from *Alpinia officinarum* was studied using the agar well diffusion method, and CuNPswere used in three different concentrations of (75µg, 100µg and 125µg/ml) which showed clear zones of inhibition and inhibition of microbial growth was observed with increase in the concentration of nanoparticles. Ampicillin (10 µg) was used as a positive control, and DMSO was also used as a control. Synthesized nanoparticles showed good antibacterial activity. The antimicrobial activity of CuNPswas due to its attachment to the cell membrane's surface, disturbing the cell's porosity [26].

3.3. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC).

MIC is the minimum concentration in which bacterial growth is inhibited; the treatment of CuNPsin 96 well microtiter plate reduced the growth of MRSA to 49.1 percent, 14.7 percent in MTCC, 20.3 percent in MDR clinical strains of *Staphylococcus aureus*. The highest bacteriostatic concentration of nanoparticles was found at 25µg/ml, and the lowest was found at 6.25µg/ml after 24 hours of treatment (Figure 2A).

Minimal bactericidal concentration was found at 100µg/ml for MRSA and MDR strains, while MBC of MTCC strains was 50µg/ml. The tolerance level of CuNPs was measured by the ratio of MBC versus MIC, which was found as 4µg/ml for MRSA strains, 16µg/ml for MTCC, and 8 µg/ml for MDR strains, which showed the bacteriostatic property of CuNPs(Table 2).

Table 2. Minimum bactericidal concentration, Minimum inhibitory concentration, tolerance level, and effect of CuNPs against *S. aureus* strains.

***S.aureus* strains**	**MBC CuNPs (µg/ml)**	**MIC CuNPs (µg/ml)**	**MBC/MIC**
MRSA	100	25	4
MTCC	100	6.25	16
MDR	50	6.25	8

3.4.Antibiofilm assay.

Biofilm assay was performed in 96 well microtiter plates, CuNPswas was serially diluted and inoculated with MRSA, MTCC, and MDR strains.

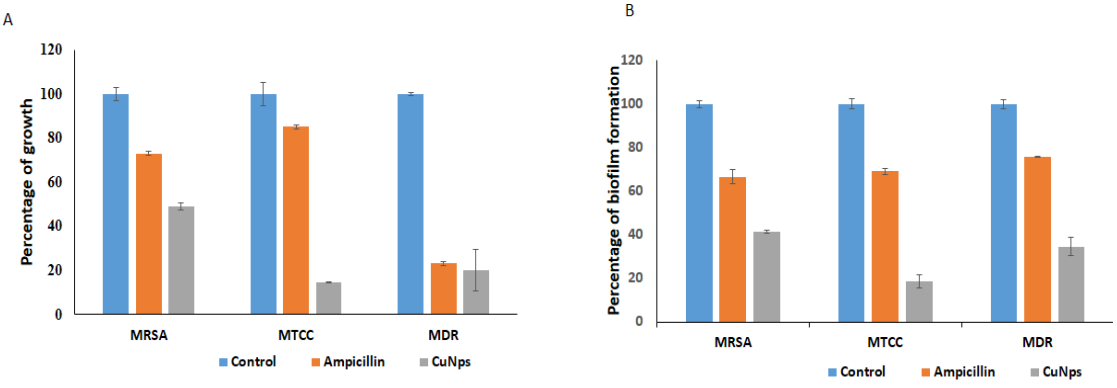

Figure 2. (A) The percentage of bacterial growth inhibited by CuNPs is compared with that of ampicillin and control; (B) the percentage of biofilm formation in S. aureus treated with CuNPs is compared with that of ampicillin and control.

After 48 hours of incubation, staining and destaining were performed, and the absorbance was measured at 517nm. CuNP treatment reduces biofilm formation when

compared with ampicillin and negative control. The percentage of biofilm formation was reduced to 41.2% in MRSA, 18.5 % in MTCC, and 34.6 % in MDR strains, which revealed good anti-biofilm activity in the CuNPs (Figure 2B). Copper ions also interrupt the biochemical processes and inhibit the growth of microbes, forming a clear zone of inhibition [27-30].

Previous studies revealed that nanoparticles penetrate the biofilm of the bacterial strains and stop exopolysaccharides secretion. The biofilm was a barrier that protected bacteria from antibiotics. CuNPs greatly penetrate the EPS layer and detach biofilm by numerous mechanisms [31- 36].

4. Conclusions

This study revealed that CuNPssynthesized from *Alpinia officinarum* rhizome extracts have potential antimicrobial activity against clinical strains of *Staphylococcus aureus*. Zone of inhibition was high in MTCC strains of *Staphylococcus aureus*. CuNPs minimized the growth of MRSA, MTCC, and MDR clinical strains. It also controls biofilm formation; after conducting a toxicity test, CuNPs can be synthesized in large quantities and used as an efficient antimicrobial agent against various Gram-positive and Gram-negative organisms.

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Conflicts of Interest

The authors declare no conflict of interest.

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