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Green Synthesis of Copper Nanoparticles by *Citrus sinensis* Extract, Characterization and Antibacterial Activity

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Abstract
Citrus sinensis seed extract was used as a capping and reducing agent in a novel, straightforward green chemistry process to create copper nanoparticles (CuNPs). The antibacterial activity of copper nanoparticles against Staphylococcus aureus, Streptococcus pneumoniae, Klebsiella pneumoniae, and Escherichia coli has been studied, and the successful formation of copper nanoparticles has been confirmed by UV–Vis, FTIR, EDAX, SEM, and TEM analysis. The outcomes imply that the produced CuNPs have effective antibacterial activity.

Keywords: Citrus sinensis seed extract, copper nanoparticles (CuNPs), antibacterial activity, UV–Vis, TEM.

1. Introduction

Since nanotechnology is being used in both science and technology, it is becoming a rapidly expanding sector [1]. Nanoparticles and their many applications in bio sensing and chemotherapy, energy-saving devices, drug delivery systems, optoelectronics, optochemistry, catalysis, optoelectronics, optoelectronics, and pharmaceutical therapeutics like antibacterial, anticancer, and antimicrobial agents have been the subject of numerous studies [2]. Due to their unique optical, mechanical, catalytic, electrical, and thermal conduction properties—all of which differ from those of their bulk metal counterparts—and low cost, copper nanoparticles, or CuNPs, have garnered significant attention recently. For the past many years, the synthesis of CuNPs has received particular attention because of their possible uses [3]. CuNPs' strong cytotoxicity, excellent selectivity, biocompatibility, and production make them potentially useful agents. A vital component of numerous biological processes, copper is one of the co-factors for numerous enzymes [4]. To address the unique requirements of various applications, a variety of technologies, including chemical, photochemical, electrochemical, and biological processes, have been employed to synthesize nanoparticles in varied sizes and shapes [5–9].

Chemical processes also result in a large number of dangerous by-products that harm the environment. As a result, "Green Synthesis" of nanoparticles is required; this synthesis method does not require high temperatures, pressures, energies, or hazardous chemicals, and it is safe, clean, and environmentally friendly [10–12]. Numerous bioactive substances found in citrus fruits, such as vitamins, flavonoids, essential oils, and phenolics, have anti-oxidant, antiinflammatory, anti-tumour, and anti-microbial qualities. In the field of cancer research, the combination of the isolates, polar and non-polar extracts, and their formulations demonstrated immuno-pharmacological activity [13].

The main nutrients identified in a recent assessment of published research on the nutritional and bioactive characteristics of Citrus sinensis, or common orange seeds, were fatty acids, fibers, minerals, and vitamins [14–16]. Citrus sinensis: The oil content of orange seeds ranges from 34.92 to 41.66%. Oleic and linoleic acids, two types of important unsaturated fatty acids, are abundant in this oil [17]. Furthermore, the seeds have beneficial substances such flavonoids and carotenoids [18]. Due to their antibacterial, antioxidant, and deterrent properties against free radicals, these substances may have positive health impacts [19]. CuNPs, silver, and gold were synthesized environmentally using citrus species. The antioxidant, antibacterial, antifungal, and antiproliferative qualities of these produced nanoparticles were further investigated [20].

This study describes an undocumented green chemistry method for making copper nanoparticles with an extract from citrus sinensis seeds and their antibacterial properties.

Materials and Methods

A. Materials Used

Orange fruits were bought in Madurai, Tamilnadu, India, from the local market (Fig.1). All of the glassware and chemicals used in this project were bought from United India Scientific Company in Madurai. Before being used, every glass item was thoroughly cleaned in sterile distilled water and dried in an air oven.

Figure 1: Common or Sweet orange fruit (Citrus sinensis) collected from the local Madurai market

B. Preparation of Aqueous solution of Powdered Citrus sinensis (Common Orange) seed extract

After removing the orange seeds from the fruit, they were chopped into little pieces, cleaned with water, and allowed to dry in the sun for five days. Using a mixer grinder, the dry seeds were finely ground (Fig. 2). A beaker containing about 6 g of powdered orange seeds was filled with 100 ml of distilled water and heated on a plate with a stirrer, cooled after around 30 minutes of boiling the ingredients. Following cooling, the extract was twice filtered through Whatman No. 1 filter paper, and the filtrate was saved for additional research.

Figure 2: Dried Orange seeds and powdered orange seeds

C. Preparation of Copper Nanoparticles

In 250 mL of distilled water, a 3 mL copper sulphate solution was made. After that, the copper solution was vigorously mixed for 30 minutes at room temperature using a magnetic stirrer, and 20 ml of orange (Citrus sinensis) seed extract was added dropwise (Fig. 3). The reaction mixture was heated to 60 degrees Celsius concurrently with the slow addition of 0.2 grams of sodium hydroxide pellets that had been dissolved in 5 mL of distilled water. After heating the reaction mixture once more until the colour turned brown, signifying the creation of copper nanoparticles, it was centrifuged for five minutes at 15,000 rpm and then cleaned with deionized water. After discarding the transparent solution, a viscous layer containing the copper nanoparticles was gathered.

Figure 3: Beakers containing copper sulphate solution, orange seed extract and synthesized copper nanoparticles

Characterization techniques

A. UV- Visible Spectroscopy

To determine the kinetic behaviour of copper nanoparticles, they were evaluated using a Perkin-Elmer UV-Vis spectrophotometer, Lambda-19. The sample was scanned at a speed of 480 nm/min within the range of 200–1100 nm. UV Win lab software was installed on the spectrophotometer in order to record and analyse data. Using a blank reference, the spectrophotometer's base line correction was completed. All of the samples' UV-Vis absorption spectra were noted, and numerical data were plotted in origin 6.5.

B. Infrared Fourier Transform Spectroscopy (FTIR)

Measurements using FT-IR spectroscopy are performed to pinpoint the individual proteins attached to the copper surface. A lyophilized sample was examined in the 400–4000 cm^{-1} spectra range.

C. Scanning Electron microscopy (SEM)

The sample was made by applying a drop of a colloidal copper sulphate solution to a copper grid coated with carbon, letting it dry in the air, and then moving it to a microscope running at a 130 KV accelerated voltage.

D. Transmission electron microscopy (TEM)

The size and form of copper nanoparticles were observed using TEM method. A drop of the particle solution was applied to the TEM grid, and it was then dried under an infrared lamp.

E. Energy dispersive x-ray spectroscopy (EDAX)

EDAX was used to confirm that elemental copper was present. An x-ray spectrometer with energy dispersive analysis makes use of the light's photon character. A single photon's energy is just enough in the x-ray range to create a detectable pulse of x-rays. The x-ray is detected by a semiconductor substance, which is also utilized for spectrum analysis and electrical processing. The instrument used to conduct the EDAX observations was connected to a SEM.

E. Antibacterial activity (Disc diffusion method)

Utilizing the Agar well diffusion method, antibacterial activity on nutritional Agar media was assessed. Six wells were bored using a sterilized cork borer after a swab of chosen bacteria was applied to the nutrient Agar media agar plates. 50 µL of copper metal solution and specific quantities of produced nanoparticles were added to the wells. Distilled water was utilized as the negative control and commercially available streptomycin (10 µg) as the positive control. For every sample, 50 milliliters were added to the corresponding well. The zone of inhibition on the plates was measured after a 24-hour incubation period at 37ºC.

Results and Discussion

A. UV Visible spectrum analysis

UV–Vis absorption spectra of Citrus sinensis aqueous seed extract (Fig.4) and the biosynthesized Cu nanoparticles (CuNPs) (Fig.5), show that the surface Plasmon resonance is responsible for the $CuSO_4$ solution's colour change from bluish green to brown (Fig.6). The particle's size and shape had a significant impact on the surface plasma resonance absorbance. Thus, the range of 361 nm, which is the typical absorption peak for copper nanoparticles, is where the SPR bands are found. It is known that the size and form of nanoparticles can be determined using UV-Vis spectra. Ashtaputrey et al. used the extract production method of Murraya Koenigii L leaves to observe that the absorbance peaks at 340nm for copper nanoparticles [21].

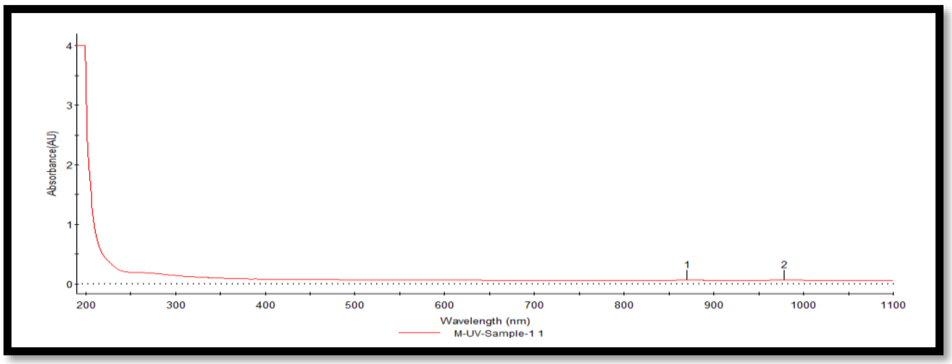

Figure 4: UV-Visible spectra of Citrus sinensis aqueous seed extract

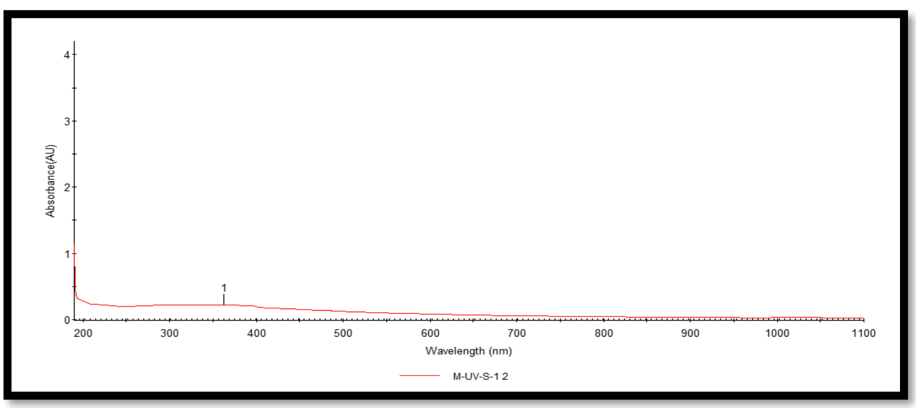

Figure 5: UV-Visible spectra of synthesized copper nanoparticles

Figure 6: Colour changes from blue to dark brown after adding citrus sinensis extract

B. Fourier Transform Infrared spectra analysis

The citrus sinensis aqueous seed extract and biosynthesised copper nanoparticles FTIR spectra were collected in the 400–4000 cm^{-1} range. The chemical shift brought about by the interaction of the functional groups present in the citrus extract was identified by FTIR analysis (Fig.7). Citrus extract's spectrum shows bands at 3375.93, 2854.73, 2925.21, 1746.62, 1465.33, and 1238.14 cm^{-1}. These bands correspond to the stretching of acid's O-H, alkane's C-H, alkane's CH_2 stretching, amide's C=O, aromatic C-C stretching in rings, thioether S=O stretching, and ether C-O stretching. Eleven bands were detected by FTIR in the green produced copper nanoparticles (CuNPs) (Fig.8). The copper nanoparticles span the absorption band at 3433.58, 2854.73, 2925.49, 1641.62, 1453.43, and 1384.87 cm^{-1}, which corresponds to the stretching of an acid's O-H bond, an alkane's C-H bond, an amide's C=O bond, an aromatic ring's C-C bond, and a thioether's S=O bond.

We determine that the two spectra are similar in their unique properties by comparing the spectrums of citrus extract and copper nanoparticles. This demonstrates that copper production and bio reductions were facilitated by the bioactive alkaloids and flavonoids.

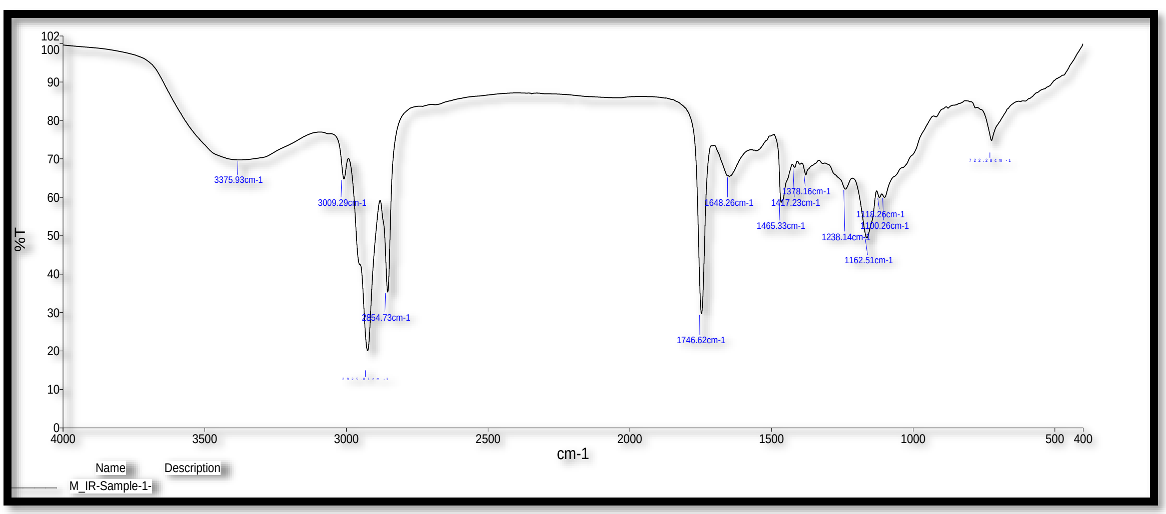

Figure 7: FTIR spectra of Citrus sinensis aqueous seed extract

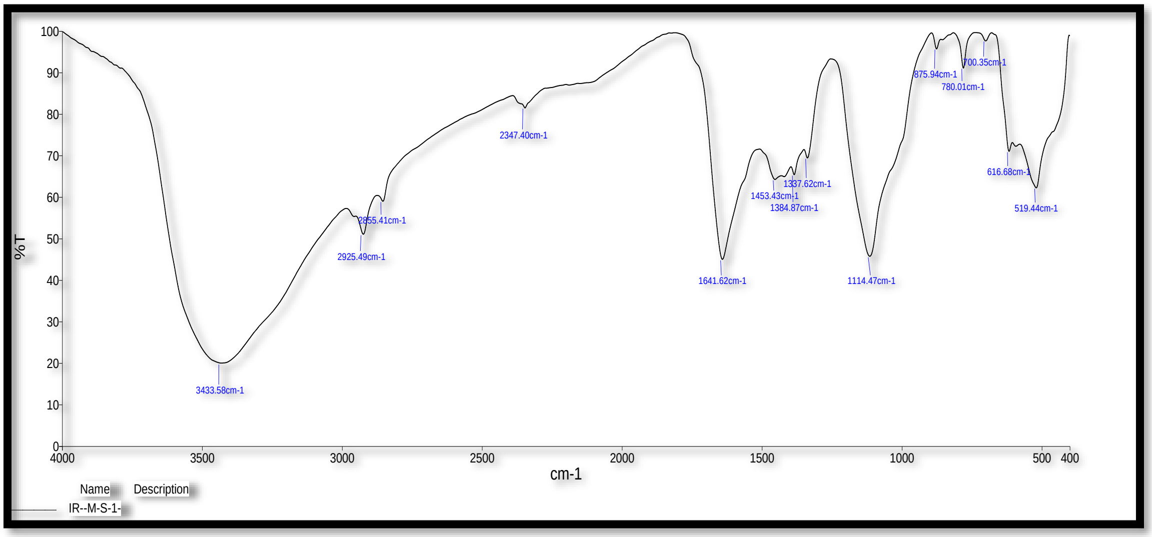

Figure 8: FTIR spectra of synthesized copper nanoparticles

C. Scanning Electron Microscopy (SEM)

Using aqueous (common orange) Citrus sinensis seed extract, polydispersed, versatile, and spheroid shaped Cu nanoparticles with considerable agglomeration owing to sampling preparation are shown in the SEM micrographs (Fig.9). Copper nanoparticles (CuNPs) that have been biosynthesized range in size from 20 to 30 nm. The addition of copper sulphate to Citrus sinensis seed extract does not alter the structure of the nanoparticles; nevertheless, it does increase their size, primarily at higher concentrations of 50%. According to Kupusamy et al. (2017), the nanoparticles aggregated into a relatively open, quasi-linear superstructure as opposed to a densely packed assembly, and their size ranges from 45 to 100 nm [22]. The copper nanoparticles have a spheroid form and are naturally polydispersed.

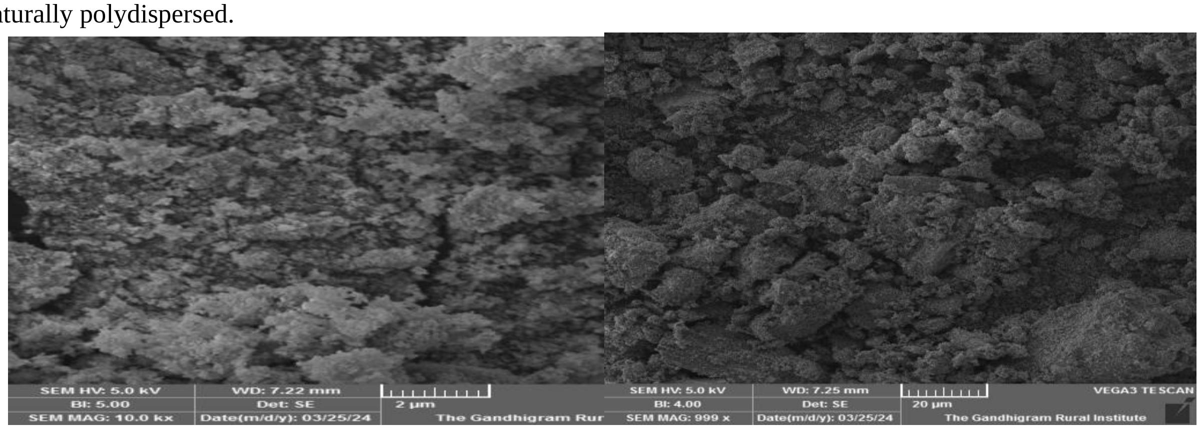

Figure 9: SEM Images of Synthesized copper nanoparticles

D. Transmission Electron Microscopy (TEM)

As seen (Fig.10), TEM images reveal that the particles are crystalline and widely distributed. Microscopy imaging at a greater resolution verified that the nanoparticles in the TEM image are not mixed, but rather evenly spaced out, with sizes ranging from 20 to 30 nm. Copper nanoparticles are spheroid and polydispersed in nature. The copper nanoparticles' floral shape is confirmed by the TEM image. This illustration shows how the phytochemicals in the plant extract limit the copper nanoparticles [33].

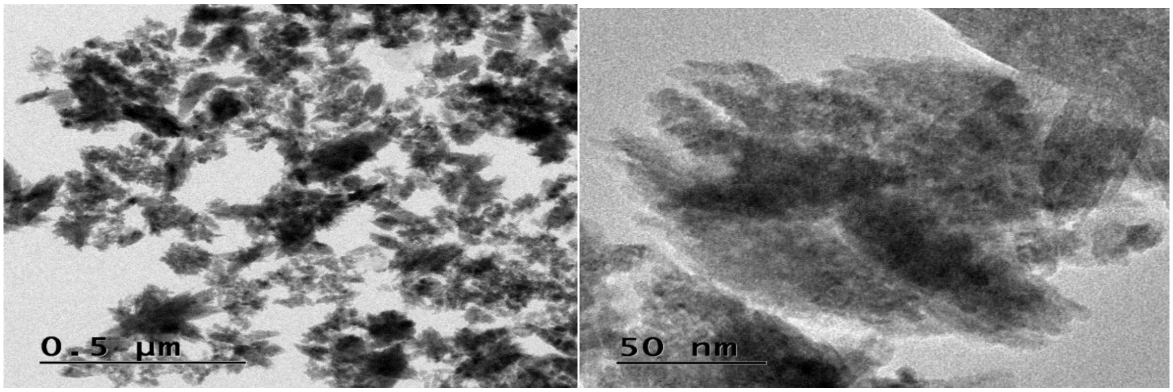

Figure 10: TEM Images of Synthesized Copper nanoparticles

E. Electron dispersive X-ray analysis

The elemental composition of the biosynthesized copper nanoparticles is clearly analysed by the energy dispersive spectrum of the particles that was captured with Oxford software on a SEM. The EDS spectra of biosynthesized nanoparticles placed on an aluminium sheet covered with carbon are displayed (Fig.11). Copper is present, as confirmed by the EDS spectrum signal. It suggests that Citrus sinensis seed extract reduces copper sulphate. Copper (Cu) has a weight composition of 62.33% and an atomic composition of 27.65%, respectively. Other contaminants, including carbon, oxygen, and nitrogen, were discovered as a result of the extract's interaction with the bioprocessing process. According to Patel et al., oxygen and copper had weight compositions of 75.42% and 4.45%, respectively. Carbon, potassium, and sodium are regarded as contaminants, and the atomic compositions were 1.29% and 86.65%, respectively [23].

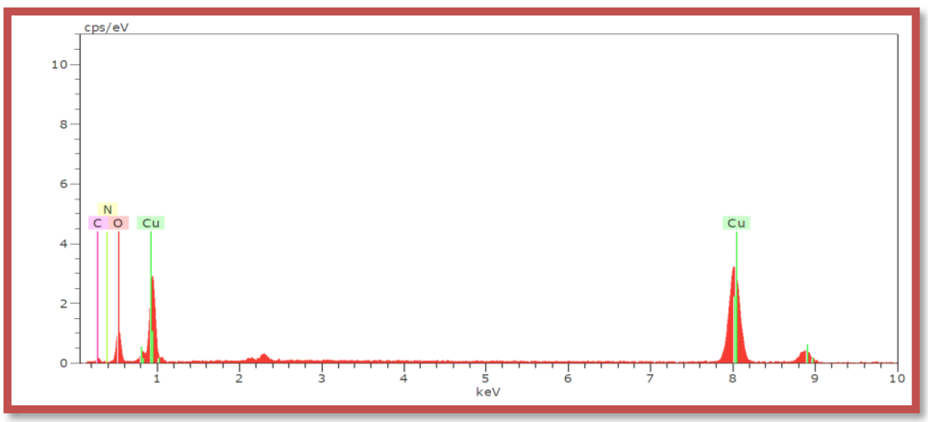

Figure 11: EDAX Spectra of synthesized copper nanoparticles

F. Antibacterial activity:

Using the agar well diffusion method (Fig.12), the antibacterial activity of the produced copper nanoparticles was evaluated against gram positive (S. aur eus, S pneumonia) and gram negative (Klebsiella pneumonia, E coli) bacterial strains. With the sterile swab, 100 mL of a fresh culture with 1×108 CFU/mL of bacteria was applied to the Mueller Hinton Agar (MHA) plates. The perti-plate was tested with copper nanoparticles dissolved in dimethyl sulphoxide (DMSO) at concentrations of 10 µl, 30 µl, and 60 µl. After that, zone of inhibition values (mm) were evaluated for 24 hours at 37 °C. The common antibiotic Streptomycin (10 µg disc) was utilized as a positive control.

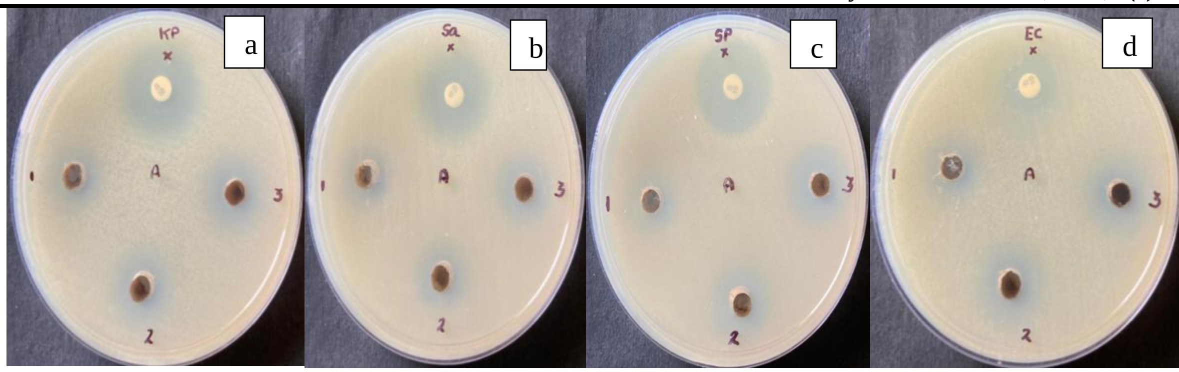

Figure 12: Antibacterial activity of copper nanoparticles against a) Staphylococcus aureus b) Streptococcus pneumoniae c) Klebsiella pneumoniae d) Escherichia coli

The zone of inhibition served as the basis for the analysis of copper NPs' antibacterial activity. Using the disc diffusion method, CuNPs demonstrated antibacterial efficacy against both gram-positive and gram-negative bacteria. These outcomes as shown in table 1 were contrasted with those of a typical antibiotic as well. Synthesized CuNPs exhibit strong and comparable antibacterial action against gram-positive and gram-negative bacterial strains, according to obtained experimental results.

Table 1: Effects of copper nanoparticles on microbial activity

Bacterial species	**Zone of inhibition diameter (mm)**			
	10 µl	**30µl**	**60µl**	**Streptomycin**
Staphylococcus aureus	12	13	14	21
Streptococcus pneumoniae	11	12	16	23
Klebsiella pneumoniae	11	12.5	16	23
Escherichia coli	15	16	17	20

Conclusion

In this work, we used Citrus sinensis seed extract to biosynthesise copper nanoparticles in an environmentally friendly manner. It's the easy, economical, and responsible way. The produced nanoparticles was examined by UV-Vis, FTIR, SEM, TEM and EDAX. The morphological features and the function of stabilizing agents during the synthesis of CuNPs were disclosed by the characterisation studies. The zone of inhibition approach was used to demonstrate the considerable antibacterial activity of the produced CuNPs.

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