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Optimal biosynthesis and characterization of broad-spectrum antibacterial cupric oxide nanoparticles using bee glue

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Abstract: This work describes a simple and effective approach for producing broad-spectrum antibacterial cupric oxide nanoparticles (CuO NPs) using bee glue. The volumetric ratio of the reactants (copper salt and aqueous propolis extract) was 40 ml to 20 ml. The pH of the reaction was 8. The optimum temperature for the reaction was 70 °C, while the fastest time for the formation of these nanoparticles was 13 minutes. Ultraviolet-visible ray absorption spectra indicated surface plasmon resonance at around 300 nm, proving the synthesis of CuO NPs. The Fourier-transform infrared spectra study confirmed the presence of the CuO nanoparticles at 533-582 cm-1, as well as the responsible biomolecules for the capping and stability of these nanoparticles. X-ray powder diffraction and transmission electron microscopy determined the shape and size of the CuO NPs nanoparticles. These nanoparticles have a monoclinic shape and an average size of 31.75 nm. The antibiotic activity assay results of CuO NPs were impressive and promising against six different strains of microbes.

Keywords: CuO NPs; Staph. aureus; Escherichia coli; Candida albicans; optimal parameters.

1. Introduction

The emergence of viruses, serious diseases, and epidemics prompted scientists to target and improve the efficiency of drugs or antibiotics. The development of a broad-spectrum antibacterial efficacy capable of killing both Gram-positive and Gram-negative bacteria was critical to achieving this goal. The nanotechnology revolution has attracted attention as one of the potential solutions (Dodevska *et al.*, 2022; Shroff *et al.*, 2022; Yah & Simate, 2015).

Nanotechnology has occupied a prominent position in the path of modern materials science, as it is one of the most attractive research fields (Saleh *et al.*, 2022). The unique properties of metal nanoparticles, such as their optical, electronic, mechanical, magnetic, and chemical properties, have been successfully invested in many different applications, such as electrochemical processes and the production of hydrogen, electronic devices and communication systems, sensor design, and medical imaging technologies (Mohamed *et al.*, 2021).

Specifically, the wide uses of nanoparticles in various fields such as the chemical, health, and electronic industries are due to their unique and very important property, which is the high surface area to volume ratio, in addition to their size, distribution, and morphology (Letchumanan *et al.*, 2021; Thamer, 2018).

There are many methods used in the synthesis of nanoparticles, which include chemical, physical, electrochemical, optical, and biological methods (Dhand *et al.*, 2015; Jamkhande *et al.*, 2019). Despite the great success achieved by these methods in the synthesis of nanoparticles in a pure and well-defined form, the shortcomings of these methods, such as their high cost and environmental risks, made them inferior (Fentie *et al.*).

Hence the importance of green methods in synthesis, which have received great attention in recent years in this most vital field (Samuel, 2022).

As we mentioned earlier regarding the widespread use of metal nanoparticles, it has become necessary to search for environmentally friendly synthetic methods away from the use of chemicals that pose a threat to the environment and human health (Amaliyah *et al.*, 2020; Çalhan & Gündoğan, 2020; Tavakoli, Kharaziha, & Ahmadi, 2019).

Biomaterials, including bacteria, fungi, yeast, viruses, microalgae, vitamins, sugars, and plant extracts, have been widely used for the biological synthesis of nanoparticles (Priya & Iyer, 2022). Among all these biomaterials, plant extracts exploited for nanoparticle synthesis have received much attention due to their availability, renewable nature, simplicity, efficiency, stability of the nanoparticles synthesized, and low cost rate (Ghareib *et al.*, 2019; Shiny *et al.*, 2019).

The prominent role of plant extracts in the synthesis of nanoparticles lies in reducing metal ions and supporting the stability of nanoparticles formed as a result of the presence of metabolites such as sugar, terpenoids, polyphenols, alkaloids, phenolic acids, and protein (Gunawan & Sardjono, 2022; Ocsoy *et al.*, 2018).

Copper and its compounds have played a prominent role for centuries due to their wide and varied uses. Copper has been used in water purifiers, algaecides, fungicides, and antibacterial agents (Akintelu *et al.*, 2020).

Nowadays, it has been found that the unique properties possessed by metal oxide nanoparticles (NPs) such as copper oxide (CuO), iron oxide (Fe_3O_4), and zinc oxide (ZnO) have drawn attention because of their unique optical, physical, and biological properties (Nandiyanto *et al.*, 2023; Petrova *et al.*, 2021; Suleiman *et al.*, 2013).

The advantages of synthetic nanoparticles of copper oxide, such as its very high conductivity and its cheapness compared to silver and gold, made it of great importance and the focus of attention of researchers, so it was exploited in many fields such as gas sensors, waste handling, catalysis, batteries, food preservation, high-temperature superconductors, solar energy conversion, dyeing fields, and agricultural emissions (Andualem *et al.*, 2020; Begum *et al.*, 2019; Diamond *et al.*, 2007). Ongoing developments in the biosynthesis of metallic nanoparticles by plants remain a focus and area of interest for researchers .

Honey bees produce a resinous mixture called propolis, or bee glue, that has effective antimicrobial properties (Almuhayawi, 2020; Anjum *et al.*, 2019). Its ingredients generally consist of waxes (30%), resins (50%), balms (10%), essential oils and aromatics (5%), pollen, and natural bioactive compounds, such as polyphenols (phenolic acids, phenolic esters), flavonoids, and caffeic acid, with its esters, clerodanes, lupeones, propolones, and prenylated benzophenones (Anjum *et al.*, 2019; Simionatto *et al.*, 2012).

The pharmacological activity such as antioxidant, antibacterial, anticancer, antifungal, anti-inflammatory, wound healing, and antiviral activity shown by propolis is due to its different chemical components mentioned above (Cortés-Higareda *et al.*, 2019; Sangboonruang *et al.*, 2022).

Moreover, propolis' chemical composition and properties depend on its country or location and the types of plants or trees found there (Kurek-Górecka *et al.*, 2022).

By reviewing past research under the same title, the biosynthesis of copper oxide nanoparticles utilizing propolis, we discovered only a few studies that did not surpass the number of fingers on a hand (Bayrami, 2020; Hajizadeh *et al.*, 2022; Seyyed Hajizadeh *et al.*, 2023). This study adds significant and novel concepts to the production of these nanoparticles utilizing propolis. The most basic of these concepts is to employ propolis aqueous extract in the synthesis process. This procedure is simple and more practical because it took less time than those prior studies, which employed ethanolic extract. As well as the low side effects of the aqueous extract due to the simple chemical reaction between water and propolis, as compared to ethanol (Hu *et al.*, 2005).

The most important idea in this research is the detailed study of the factors influencing the process of synthesizing copper oxide nanoparticles using propolis. This idea was not clearly presented in previous research, and this enhances our work on the optimal synthesis of these nanoparticles.
The third contribution of this work is the wide variety of strains of microbes that were used in the antimicrobial activity study compared to past works.

2. Materials and methods

2.1 Materials

The propolis powder was purchased from the market, and the copper (II) nitrate ($Cu(NO_3)_2.3H_2O$) was purchased from Loba Chemie, an Indian company. The other chemicals were obtained from Merck. Deionized water was used to prepare all solutions.

2.2 Methods

2.2.1 Preparation of propolis aqueous extract:

According to our previous work for preparing the propolis aqueous extract (Osman *et al.*, 2023), briefly, about 2 g of fine propolis powder was mixed with 400 mL of DI water, and heated at 60 ^oC for up to 60 minutes with steady magnetic stirring. This mixture was allowed to cool at room temperature. Filter paper (Whatman No. 1) was then used to filter the mixture and remove any solid material. Finally, the filtrate, a brownish solution, was kept for further use.

2.2.2 Biosynthesis of copper oxide nanoparticles:

Several sets of experiments were conducted to reach the optimal biological synthesis of copper oxide nanoparticles using propolis extract by following the method published in our previous works (Gebremedhn, Kahsay, & Aklilu, 2019; Gunalan, Sivaraj, & Rajendran, 2012). For optimal biosynthesis of CuO NPs, firstly, a certain volume of 0.1M copper nitrate was added to a specific volume of aqueous propolis extract, which was then completed with deionized water to 100 mL. Secondly, the mixture was heated and stirred constantly. Thirdly, drops of ammonia were slowly added to the solution while vigorously stirring it to control the pH. This stirring continued for 2 hours until the mixture's color changed from a light blue to a dark brown precipitate. Fourthly, the centrifuge was used to collect the precipitates and wash them many times with ethanol and water before they were dried at 70 ^oC for for 9 hours in an oven. Fifthly, the dried sample was ground to a fine powder and transferred to an ash oven, where it was annealed at 600 °C for 2 h. Finally, the obtained black-

brown powder was stored in suitable containers and used for further characterization to confirm the formation of CuO nanoparticles. The sets of experiments to obtain optimal CuO nanoparticles were as follows:

1. ***The Volume Ratio****:* Experiments were carried out to establish the ideal volumetric ratio by varying the volume of the copper salt solution and the propolis aqueous extract. So that five samples with different volume ratios, as shown in **Table 1**, were prepared.
2.

Table 1: The volume ratios of reactants

No.	Volume ratio	Symbol
1	Cu-salt 10ml +propolis extract 50ml + DI-water 40ml	C1
2	Cu-salt 20ml +propolis extract 40ml + DI-water 40ml	C2
3	Cu-salt 30ml +propolis extract 30ml + DI-water 40ml	C3
4	Cu-salt 40ml +propolis extract 20ml + DI-water 40ml	C4
5	Cu-salt 50ml +propolis extract 10ml + DI-water 40ml	C5

The pH of the samples was adjusted to 7 using ammonia drops before being heated to 60 ^{o}C. Every experiment lasted for two hours, and the time of formation of the dark brown precipitate was recorded.

The collecting, washing, drying, grinding, and calcination procedures for the obtained precipitates were carried out as described in the previous section. Finally, the resultant powders were examined using the UV absorption technique to confirm the formation of CuO NPs and then choose the best volume ratios for the biosynthesis of CuO NPs.

3. ***The pH Factor***: The pH factor experiments were carried out according to the following steps: Five samples were prepared using the fixed concentrations and the optimal volumetric ratio of the reactants, which were obtained from the previous section. The pH values of the prepared samples were 6.4, 8, 9.2, 10, and 11.4, which were adjusted by adding different drops of ammonia. Then the same procedures as previously were followed before conducting the same technique to monitor the formation and choose the optimum pH value for the biosynthesis of CuO nanoparticles.
4. ***The Biosynthesis Temperature:*** The following experiments in this section were conducted to study the reaction temperature effect on the biosynthesis of CuO nanoparticles. Four samples were prepared by fixing the concentrations, volume ratios, and pH of the reactants. Then, these samples were magnetically stirred and heated at different temperatures (60, 70, 80, and 90 ^{o}C). The formation time of the dark-brown precipitate was monitored for every sample. Then, the resultant powders after washing, drying, and calcination were studied using ultraviolet-visible spectroscopy to determine the optimum temperature for the biosynthesis of CuO NPs.
5. ***The Formation Time:*** The formation time of CuO nanoparticles was studied based on the previous optimal parameters that were obtained, as shown above. Taking into account that the period of all the experiments conducted in the above sections was two hours, during this period the time for the formation of copper oxide precipitate in the solutions was observed and recorded. Finally, the shortest time for the dark-brown precipitate formation was selected, and this was considered the optimal time for the biosynthesis of CuO nanoparticles.

2.3 Characterization of Biosynthesized CuO NPs:

The following characterization studies were performed to find out the structure and properties of the biosynthesized CuO-NPs using different and suitable methods.

The formation of the green synthesis of CuO NPs was monitored by UV-visible spectroscopy using a Shimadzu ultraviolet spectrometer 2700 to obtain the optimal sample of CuO NPs using propolis.

Then the Infrared spectra of the optimal biosynthesized CuO NPs were recorded using an Fourier transform infrared (FTIR) spectrometer (IR Prestige 21, Shimadzu) to identify the functional groups responsible for the stabilization and reduction processes. FTIR spectra were measured in the range of 500–4000 cm^{-1}, operating at a resolution of 1 cm^{-1}.

The X-ray diffraction (XRD) patterns of the optimal CuO-NPs were recorded in the 2θ range (10°–80°) using a powder X-ray diffractometer (PANalytical X'Pert-PRO Powder X-Ray Diffractometer) at a wavelength of 1.5406 nm. The diffracted intensities were recorded by continuous scanning at 45 kV and 30 mA. According to the line width of the maximum intensity reflection peak, particle size calculations were obtained utilizing the diffraction patterns of the optimal CuO NPs .

For determining the structure and morphology of the optimal CuO NPs, a transmission electron microscope (TEM) operating with a 70 kV accelerating voltage, the JEOL GEM-1010, was used. On a copper grid, a drop of the particle-containing solution was deposited and allowed to dry by evaporating water at room temperature.

2.4 Antimicrobial Activity Experiments:

Using the agar diffusion technique (Espinel-Ingroff *et al.*, 2007), the antimicrobial activity of the biosynthesized CuO NPs was assessed. A volume of the microbial inoculum was typically spread over the full agar surface. A sterile cork borer was then used to aseptically punch two holes with a diameter of 6 to 8 millimeters. Then, a 20-µl volume of the CuO NPs aqueous solution at a concentration of 10 ppm and a 20-µl volume of the Gentamycin solution, as a control, were added to the wells. Bacillus subtilis (ATCC 6633), Staph. aureus (ATCC 6538), Escherichia coli (ATCC 8739), Pseudomonas aeruginosa (ATCC 90274), Candida albicans (ATCC 10221), and Mucor were the six microbes used in these experiments. These test microorganisms were then properly placed on the agar plates, as mentioned, and they were incubated for 24 or 48 hours, depending on the microbes' needs. The CuO NPs and Gentamycin hampered the diffusion of the tested microbiological strains through the agar medium. Three rounds of the tests were conducted, and the average diameter of the inhibition zones caused by the CuO NPs and Gentamycin against these microbes was determined.

3 Results and Discussion

Copper nitrate $Cu(NO_3)_2$, the precursor, was mixed with propolis extract, the reducing ligand, to synthesize CuO nanoparticles. The propolis extract is composed of phytochemicals, or phenolic compounds, that are capable of reducing the Cu^{2+} ion by donating electrons and capping, as well as stabilizing the formed nanoparticles (Gebremedhn, Kahsay, & Aklilu, 2019; Gunalan, Sivaraj, & Rajendran, 2012). In addition, this reducing ligand acts as a controller for the size of particles.

3.2 The UV-Visible Analysis:

UV-vis spectroscopy was used to monitor the bio-reduction of copper ions to generate CuO NPs by the bioactivity of propolis extract. The development of a distinctive surface plasmon resonance (SPR) peak verified the synthesis of CuO NPs by changing the hue from blue to dark brown. In another

sense, surface plasmon resonance (SPR) plays a prominent role in identifying the formed nanoparticles, as the change in color of the reaction mixture results from surface plasmon resonance during the reduction and formation of CuO-NPs. It should be noted that the difference in surface plasmon absorbance values reported in the literature using different plant extracts is attributed to the difference in the size, shape, and properties of the CuO NPs and the surrounding environment of the metallic material (Kütük, 2022; Nasrollahzadeh, Maham, & Mohammad Sajadi, 2015).

The green synthesized CuO NPs were dark brown in an aqueous solution due to the excitation of electrons and changes in electronic energy levels, which reflect the reduction of Cu^{2+} ions into the Cu element (Nwanya *et al.*, 2019; Waris, 2021). The different parameters, like temperature, pH, formation time, and volume ratios of copper nitrate and propolis extract, affect the synthesis of CuO NPs.

3.1.1 The Volume Ratio: The formation of copper oxide nanoparticles was confirmed by the UV-vis absorption of diffused scattering reflectance spectra, as shown in **Figure 1**.

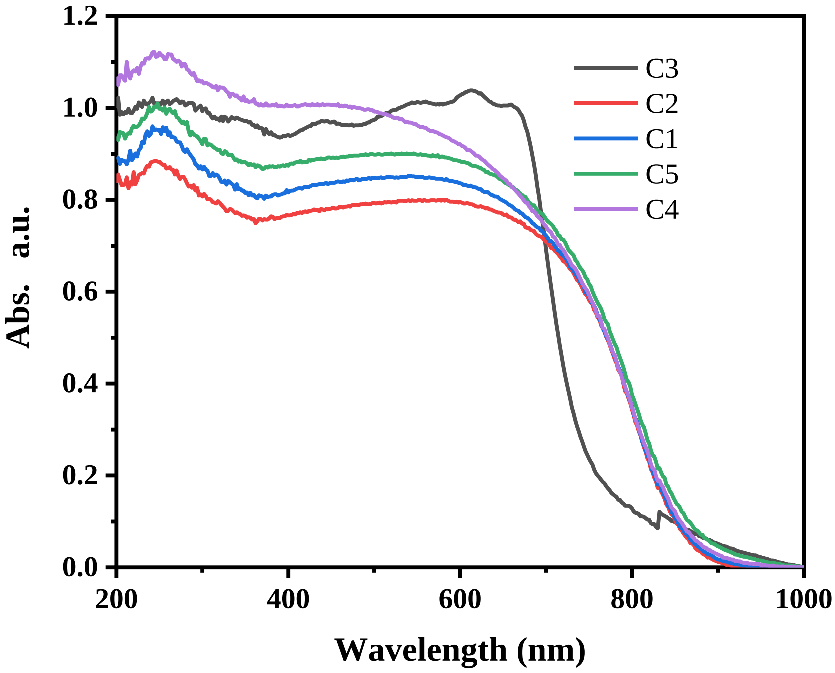

Figure 1: The UV-DRS absorption spectra for the biosynthesized samples according to the volume ratios

The results show that the volumetric ratio (40 ml of copper nitrate salt solution with 20 ml of aqueous extract of propolis) related to sample C4 is the optimal ratio, with a high absorbance in the range of 200 to 600 nm when compared to the spectra of the other samples. More precisely, a higher absorption band was observed, which represents SPR at approximately a maximum wavelength in the range of 298–305 nm, which is in good agreement with previous literature (Fentie *et al.*; Ghareib *et al.*, 2019; Long-Bao Shi, 2017). This high-intensity spectrum shown by the aforementioned sample indicates the high percentage of particles in the precipitate obtained, in addition to the large size of nanoparticles (Birla *et al.*, 2013; Dang, 2011; He *et al.*, 2005). The various sizes and shapes of CuO nanoparticles can be used to explain why the UV-vis absorption spectrum of the C3 sample differs from the other samples (Keabadile *et al.*, 2020; Woźniak *et al.*, 2017). In other words, it can be said that the formed size of CuO NPs at sample C3 was smaller than other samples due to its shorter cut-off wavelength (Jeyarani *et al.*, 2016; Ke, 2012).

3.1.2 The pH factor: Adjusting the pH is one of the most important factors in producing copper oxide nanoparticles (Mohamed et al., 2021). Moreover, the shape and size of nanoparticles can be controlled by a change in pH (Ahmed M. Eid & Amr Elkelish, 2023).

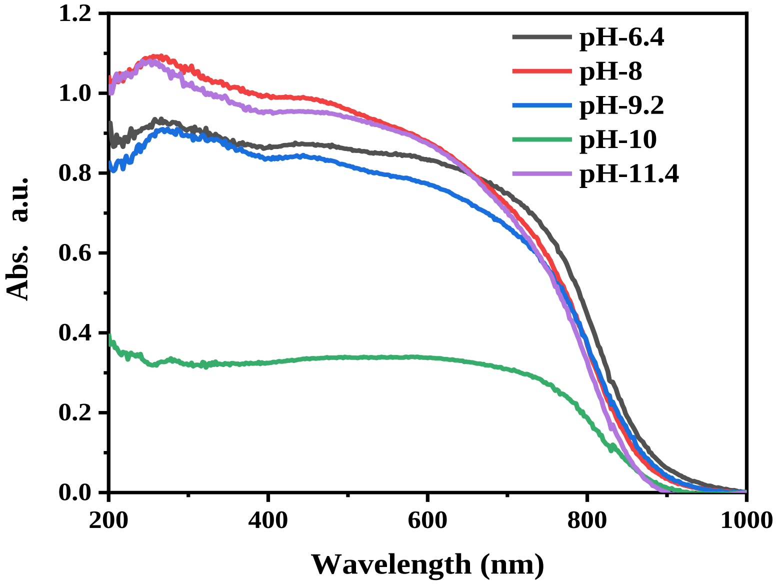

Figure 2: The UV-absorption of CuO NPs under the pH effect.

The results shown in **Figure 2**, which contained five mixtures with different pH values, clearly showed the effect of changing the pH in favor of pH 8, which gave high absorption with a maximum wavelength of about 300 nm (Ahmed M. Eid & Amr Elkelish, 2023), so this pH was considered the optimal one, which was used in the preparation of copper oxide nanoparticles by propolis extract.

It should be noted that the SPR peaks shift towards the longer wavelengths in the basic medium (8 and 9), while they shift towards the shorter wavelengths in the acidic medium, which is consistent with our work. More clearly, studies indicate that the displacement towards longer wavelengths in the alkaline medium results in a somewhat larger size of the copper oxide nanoparticles compared to neutral and acidic conditions. In addition, the increase in pH value increases the intensity of the SPR peak (Melkamu & Feleke, 2022).

3.1.3 The Biosynthesis Temperature: Temperature is one of the prominent factors affecting the biosynthesis of metal oxide nanoparticles. Through previous studies, it was found that the recommended temperature for the synthesis of CuO NPs, as well as nanoparticles of other metals, using plant extracts must be above room temperature (Patra & Baek, 2014). The reported literature showed that the biosynthesis of copper oxide nanoparticles and other metal oxides using plant extracts is fast and complete at higher temperatures(Attou, Jaber, & Ez-Zahraouy, 2018; Rajendran & Sen, 2016), which is consistent with the results of our study depicted in **Figure 3**, which includes four temperatures.

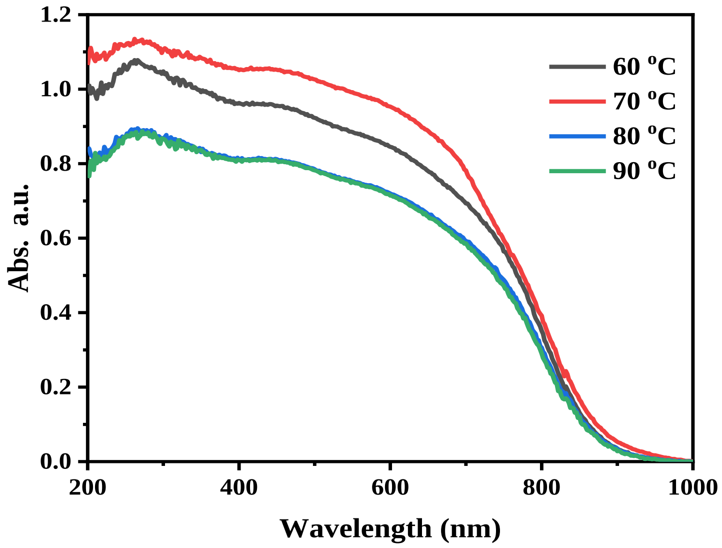

Figure 3: The UV absorption of CuO NPs under the effect of temperature.

It was clear from the results that the optimum temperature for the production of nanoparticles is 70 °C, which had the highest absorption compared to other temperatures (80 and 90 °C), which inhibit the vital molecules responsible for the reduction of metal ions and then weaken the synthesis of nanoparticles (Akintelu et al., 2020).

3.1.4 The Formation Time: During the period of all experiments, the change in formation time was studied by observing the reaction of $Cu(NO_3)_2.3H_2O$ solution with propolis extract to form the biosynthesized CuO NPs and their stability. The formation time is considered the shortest and fastest time for the color change from light blue to dark brown, as in **Figure 4**.

Figure 4: The color change for the biosynthesis of CuO NPs.

The main observation from all the previous experiments was that the formation time for the optimal biosynthesis of CuO nanoparticles decreases gradually; in the first set of experiments, the time was around 90 minutes, but after obtaining the optimal values for the influencing factors, the optimal time became 13 minutes. As expected, the increase in the formation time affects the size and the aggregation number of the CuO nanoparticles, which were formed from the conversion of divalent copper ions into primary nanoparticles (Balavandy *et al.*, 2014; Katwal *et al.*, 2015). This optimal formation time for the biosynthesized CuO nanoparticles using propolis extract is considered very short and saves money and effort, and this gave the current work a remarkable superiority compared to the reported works (Çalhan & Gündoğan, 2020; Mbewana-Ntshanka, Moloto, & Mubiayi, 2021; Mustafa *et al.*, 2013; Wongpisutpaisan *et al.*, 2011).

3.2. FT-IR Analysis:

As we mentioned above, propolis extract, like plant extracts, plays a double role in the reduction process of metal ions as well as a capping agent due to the presence of biomolecules that contain many functional groups that can be examined in the propolis extract and CuO nanoparticles by using FT-IR spectroscopy.

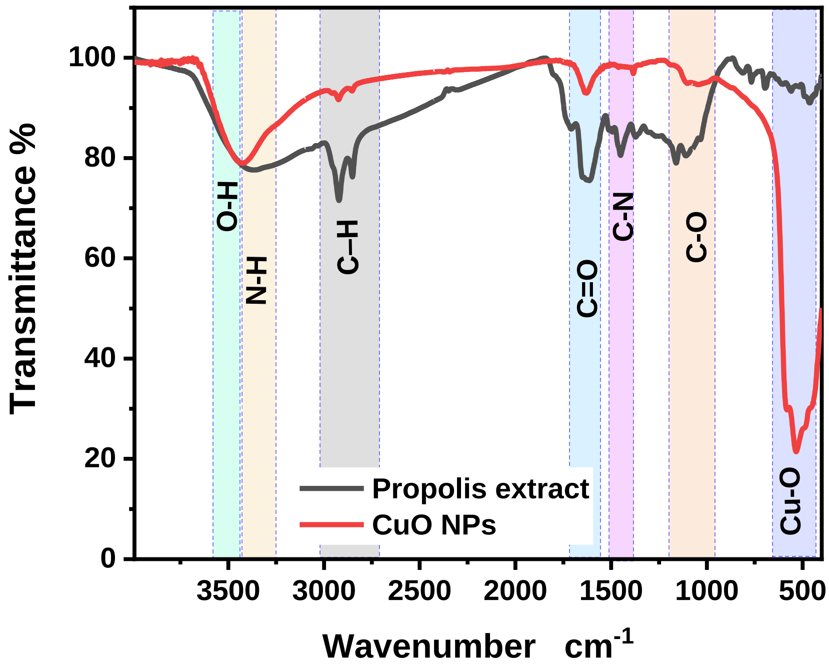

Figure 5: FT-IR spectra of propolis extract and CuO NPs

When studying the FTIR transmittance spectra of the optimal CuO NPs and propolis extract, as in **Figure 5**, it must be noted that there are various organic functional molecules/groups around CuO NPs(Veisi *et al.*, 2021), which are specified by the peaks observed as follows:

The IR spectrum of CuO NPs using propolis extract showed a broad peak corresponding to O-H stretching frequencies of 3455 and 3510 cm^{-1} confirming the presence of the OH group coming from alcohols and phenol (Kanavi *et al.*, 2022; Pansambal *et al.*, 2017; Raul *et al.*, 2014). However, there is an indication that the peak at 3369 cm^{-1} is attributed to the stretching vibration of the N-H group. The band in the range 2854–2924 cm^{-1} revealed the presence of C–H stretching vibrations of an aromatic aldehyde (Altikatoglu *et al.*, 2017; Fatoni *et al.*, 2021).

In addition, the absorbance band at 1627 cm^{-1} was assigned to the bending vibrations of the carbonyl group, indicating the presence of flavonoids adsorbed on the surface of biosynthesized CuO NPs (Saleh *et al.*, 2022). Further, the stretching vibration of C-N derived from the amine group was found at 1384 cm^{-1} (Rao & Venkatesha, 2014; Xie *et al.*, 2020). The peaks at 1046 and 1101 cm^{-1} were due to C-O stretching vibrations of the carboxylic group and flavanones, which are in close agreement with the reported work (Amaliyah *et al.*, 2020). A Cu-O stretching frequency in the range 533-582 cm^{-1} indicated the formation of CuO NPs, which is consistent with the reported CuO NPs and the preparation of CuO NPs from Piper retrofractum extract (Amaliyah *et al.*, 2020).

Figure 5 shows that, despite the annealing process, there are residues of biological molecules or functional groups (OH and C=O) on the copper oxide nanoparticles, and these functional groups are involved in the reduction process, capping, and stability of these CuO nanoparticles (Gebremedhn, Kahsay, & Aklilu, 2019).

3.3 XRD Analysis:

The XRD pattern of the propolis-synthesized CuO nanoparticles is plotted in **Figure 6**.

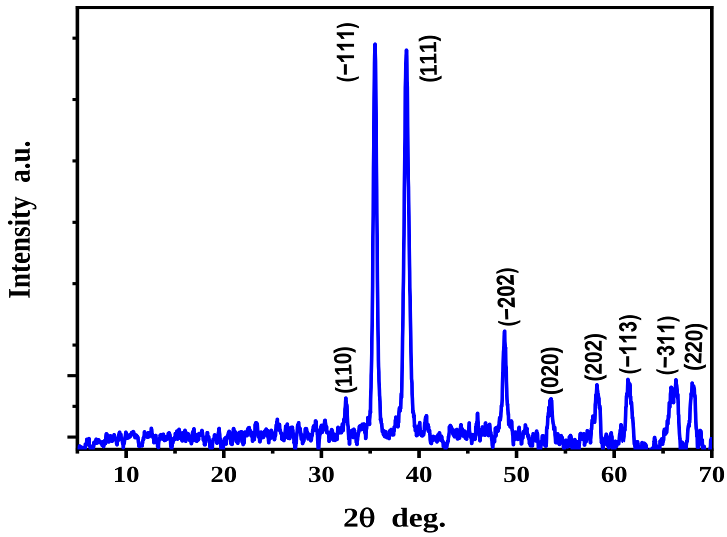

Figure 6: XRD pattern of the biosynthesized CuO NPs using propolis extract

The resulting sharp peak positions in the XRD pattern were at 32.54°, 35.47°, 38.76°, 48.73°, 53.54°, 58.34°, 61.55°, 66.27°, and 68.13°, which referred to the following atomic planes:(1 1 0), (−1 1 1), (1 1 1), (−2 0 2), (0 2 0), (2 0 2), (−1 1 3), (−3 11), and (2 2 0). These findings are remarkably coincident with the monoclinic phase of CuO according to JCPDS Standard No. 01-080-0076 (Nagajyothi *et al.*, 2017).

The Scherrer formula was used to obtain the average crystalline size (D) of CuO nanoparticles, as follows:

$$D = \frac{k\lambda}{\beta\cos\theta} \qquad \textbf{Eqn. 1}$$

where β is the full width half maximum (FWHM), λ is the X-ray wavelength, θ is the angle subtended in the peak, and k is a constant of shape (~0.94) (Scherrer, 1912). The computed average crystalline size for CuO nanoparticles was 29.77 nm.

3.4 TEM Analysis:

TEM analysis is the most common method for identifying and determining the size, shape, and size distribution of nanoparticles. The TEM analysis clearly confirmed the crystalline nature of the biosynthesized CuO NPs. TEM micrographs of all CuO NPs prepared by propolis extract showed that the particles are monoclinic, which is in close agreement with the published work (Sepasgozar *et al.*, 2021).

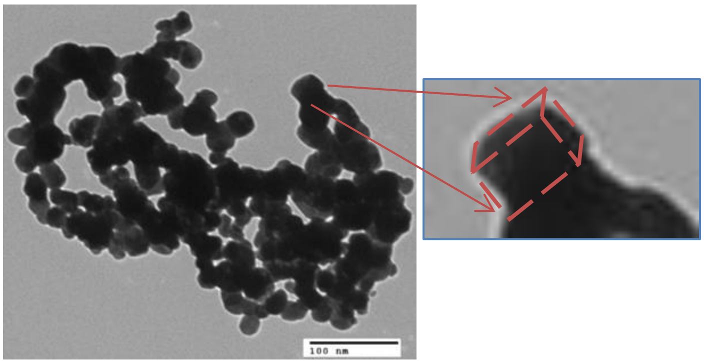

Figure 7a. A TEM micrograph

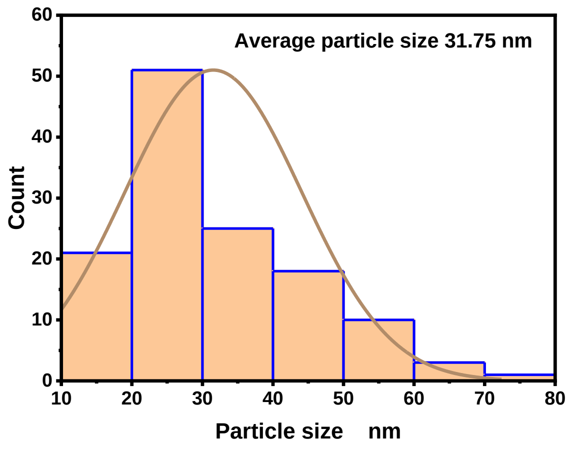

Figure 7b. The size distribution of the green synthesized CuO NPs using propolis extract.

The micrographs shown in **Figure 7** suggest particle sizes of around 10 to 80 nm, with the average size being 31.75 nm, and this is in good agreement with the XRD results for crystal size.

3.5 Antimicrobial Activity of CuO NPs:

The antimicrobial potential of the biosynthesized CuO NPs using propolis extract is examined against the pathogens listed in **Table 2** and shown in **Figure 8**.

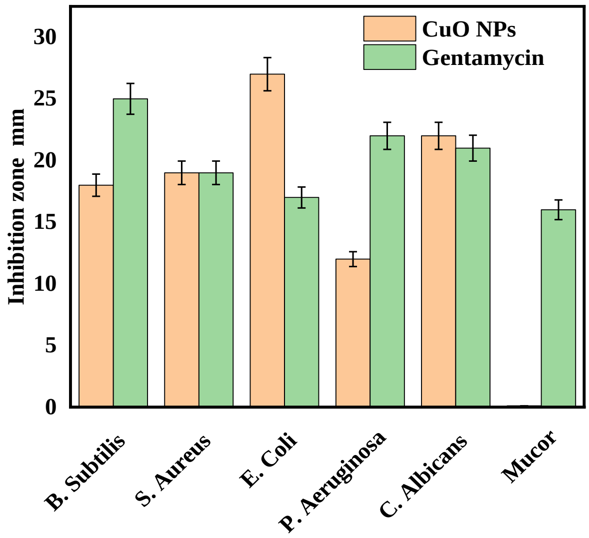

Figure 8: The antimicrobial activity of CuO NPs through the inhibition zone.

The results obtained by the biosynthesized CuO NPs display an antimicrobial effect on all the tested bacterial strains and fungi, except Mucor. Moreover, the inhibition activity of CuO NPs was found to be higher for Gram-negative bacteria than for Gram-positive bacteria and was based on the difference in the structural composition of Gram-positive and Gram-negative bacteria.

Table 2: Antimicrobial activity of CuO NPs using propolis extract.

Mucor	***Candida albicans***	***Pseudomonas aeruginosa***	***Escherichia coli***	***Staph. aureus***	***Bacillus Subtilis***	**Type of pathogen**
NA	22	12	27	19	18	**Zone of stability of CuO Nps**
16	21	22	17	19	25	**Zone of stability of**

	Gentamycin

The results presented in **Table 2** show that the biosynthetic copper oxide nanoparticles derived from propolis extract have inhibitory activity against the tested pathogens and almost appear to be higher than the standard antibiotic. In the case of *Escherichia coli*, it is observed that the inhibition zone is 27 mm, which is a remarkable value when compared to the standard antibiotic's value of 17 mm. On the other hand, the inhibition zone of copper oxide nanoparticles generated from propolis extract was shown to be smaller than that of the conventional antibiotic (Gentamycin) against the pathogens *Bacillus subtilis* and *Pseudomonas aeruginosa*. However, the CuO NPs with propolis extract still has high inhibition activity compared to previous literature(Atri *et al.*, 2023; Ezealisiji & Nwodo, 2023; Sathiyavimal *et al.*, 2018).

The results of antimicrobial activity do not show any effect of these nanoparticles in inhibiting pathogens of fungi (*Mucor*), while a remarkable inhibitory activity against *Candida albicans* was higher than that of the antibiotic as well as from previously published works(Atri *et al.*, 2023; Imani *et al.*, 2020; Samuel, 2022). Based on the aforementioned, there are several mechanisms that may explain the inhibitory activity of nanoparticles, which are most potentially used by CuO NPs to resist these pathogens and can be as follows:

It is believed that there are repulsive electrostatic forces resulting from the presence of positive charges on bacteria strains of the gram-positive and the charges present on copper binary ions liberated from CuO NPs, which makes this strain less sensitive than the gram-negative. In contrast, this negative strain shows attraction and adhesion with these ions on the walls of the membranes, which impair membrane permeability, osmoregulation, respiration, and electron transport(Zafar *et al.*, 2018). Another proposed mechanism is that the Cu^{2+} ions released from the CuO NPs may attach to the negatively charged bacterial cell walls, producing reactive oxygen species (ROS) inside the cell, leading to its rupture and then denaturing the cell protein, eventually causing cell death (Raul *et al.*, 2014; Samuel, 2022).

In general, the antibacterial mechanism of all nanoparticles can be described as follows: Bacterial cell membranes, consisting of polymeric peptidoglycans, contain sugars and amino acids, which facilitate the entry of CuO nanoparticles into cell membranes and the destruction of cell membrane enzymes (Fatoni *et al.*, 2021).

4. Conclusion

The following conclusion paragraphs highlighted significant findings that were revealed after discussing all of the study's findings.

The developed method for biosynthesizing copper oxide nanoparticles utilizing bee's glue (propolis) was safe, simple, green, and economical.

This green-developed method was implemented in a more accurate and systematic manner, and the optimal parameters for the synthesis of CuO NPs were obtained, which gave the method an advantage compared to the reported works.

This research strengthened the outstanding connection between eye observations and the various measurements of spectroscopy techniques, such as UV-vis absorption, Fourier-transform infrared, and X-ray diffraction, to clearly demonstrate the formation of copper nanoparticles.

The crystalline phase of CuO NPs was revealed to be a monoclinic nanostructure, as demonstrated by TEM micrographs. Moreover, there was also a great concordance between the XRD calculations and TEM measurements for the size of CuO NPs.

This work emphasized the antibacterial activity of CuO nanoparticles against six different microbes and showed their high impedance, allowing them to be developed and used as an alternative to broad-spectrum antibiotics. In fact, the increased inhibitory ability of CuO NPs against *E. coli, S. aureus, and Candida albicans*, in particular, was a fantastic and promised observed result, and this gives the current research work a clear distinction compared to the reported works.

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