Bull. Chem. Soc. Ethiop. **2024**, 38(6), 1653-1666.
DOI: https://dx.doi.org/10.4314/bcse.v38i6.12

ISSN 1011-3924
Printed in Ethiopia
Online ISSN 1726-801X

GREEN SYNTHESIS AND ANTIMICROBIAL ACTIVITY OF COPPER NANOPARTICLES (Cu-NPS) BY *PIPER LONGUM* FRUIT EXTRACT

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(Received April 18, 2024; Revised May 31, 2024; Accepted June 3, 2024)

ABSTRACT. Many applications of green synthesis in the field of environment and biomedicine aiming at decreasing the use of toxic chemicals through utilizing of plants which are safe. In this study, at 0.1 M concentration of copper sulfate ($CuSO_4.5H_2O$), CuNPs were prepared and identified by changing the blue color into green color (ethanolic extract) and from blue color to black color (water extract) and the spectrophotometric detection of ultraviolet and infrared spectra. X-Ray diffraction (XRD) pattern was utilized to confirm the crystal structure of CuNPs. The morphology and size of the CuNPs was measured by a high-resolution transmission electron microscope (HR-TEM). Energy dispersive X-ray (EDX) was used for identification of the elemental composition of CuNPs involving copper and oxygen. Field emission scanning electron microscopy (FESEM) was utilized to confirm the shape of prepared copper nanoparticles. In vitro antibacterial activity was tested using two methods including desk diffusion assay considering zones of inhibition and minimum inhibitory concentration (MIC) against six types of certain bacterial types (*Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Enterococcus faecalis*). The results showed potent inhibitory activity of copper nanoparticles (CuNPs) and water extract (Ext. 2) against all bacterial strains.

KEY WORDS: Copper nanoparticles, *Piper Longum*, Extract, Synthesis, Antibacterial

INTRODUCTION

Exploring new effective drugs is very important in treating bacterial infections, leading to overcoming the antibacterial resistance. Bacterial infections weren't going to surrender easily, as many of the early effective medications quickly developed resistance to them for example, *Staphylococcus aureus* producing lactamase led to the development of penicillin resistance, which results in reduced penicillin effectiveness where resistant strains are frequently discovered spreading to the community [1]. The bacterial resistance to antimicrobial treatments was initially resolved by developing new classes of medications, including macrolides, glycopeptides, aminoglycosides and structure modification of the antimicrobial molecules having weak antibacterial activity to enhance their antibacterial activity. Bacteria have a tremendous toolbox at their disposal to resist antibiotics. Without impacting the virulence or viability of a bacterial strain, a single genetic change can result in resistance. This kind of transformation is well-illustrated by resistance to medications such as streptomycin [2, 3]. There is an urgent need to find new potent antibiotics to prevent bacterial resistance to antibiotics [4]. Antibiotic stewardship and medication development would benefit greatly from being able to predict when and how

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antibiotic resistance will emerge and spread. For decades, plants have played an important role as resources for medications, and among these plants are the piper species [5, 6]. Herbal medicines have often retained their popularity despite the availability of modern medicines for historical and cultural reasons. The use of medicinal herbs is increasing globally due to concerns about the use of medicines manufactured using chemical methods [7]. *Piper longum* (pippali), also known as Javanese, is a plant in the Piperaceae family. *Piper longum*, also known as long pepper in India, is native to tropical and subtropical regions all over the world, including Sri Lanka, America, Middle Eastern countries, and the Indian subcontinent. It is widely grown for meals, medications, and other purposes [8-12]. Various nanomaterials are produced using nanotechnology by developing chemical reactions and synthesis which finally resulting in nanoparticles having sizes ranging from 1 to 100 nm [13, 14]. These nanoparticles are produced using various metals including silver, zinc, copper, magnesium, alginate, gold and titanium. Many applications of nanoparticles in various fields of life, from their use for medical purposes [15] and in the manufacture of medicines to their use in energy storage, in addition to the manufacture of clothing and cosmetics [16-19]. The chemical and biological processes can be used for the producing the nanomaterials. Biological methods for producing nanomaterials are very important including the use of enzymes [20], fungi [21], microorganisms [22] in addition to plants, which are less expensive and harmful to the environment [23]. On this basis, and as a result of the chemical effects on the environment, new methods are currently being rapidly developed to produce these materials using environmentally friendly materials such as plants in which do not have a negative impact on the environment [24]. At the present time, as a result of the increase in diseases and the difficulty of curing a large number of these diseases, there is a constant urgent need to develop medicines capable of combating these incurable diseases, which cause the loss of the lives of a large number of people in addition to large economic losses [25-30]. As part of our interest in the field of medicinal chemistry and for the many purposes for the use of long papper in medicine and meals, we are developing new methods that do not have a negative impact on the environment that lead to the production of nanomaterials or chemical agents that are capable of attacking serious diseases negatively on the environment [31-33]. Therefore we synthesize the copper nanoparticles (Cu-NPs) using long pepper extract and evaluate their antibacterial activity as well as the antibacterial activity of long pepper extract.

EXPERIMENTAL

Chemicals and reagents

Standard methods were used for drying the solvent and all procedures were achieved in an inert environment. Copper sulfate was obtained from the Department of Microbiology at Cihan University-Erbil. Long pepper (*Piper longum*) was purchased from Citadel's Bazar in Erbil. Fourier transform infrared (FTIR) spectra were recorded on a Sgimadzu 1800 infrared spectrophotometer (Iraq), and ultraviolet-visible spectra were recorded on a Shimadzu 1900 UV-VIS spectrophotometer (400-700 nm). Field emission scanning electron microscopy (FESEM) and energy dispersive analysis (EDX) images were taken using a Tescan Mira3 instrument (Iraq). Transmission electron microscopy (TEM) was recorded on Zeiss-EM10C with an accelerating voltage of 100 kV (Iraq). X-Ray diffraction (XRD) was recorded on the Phillips PW1730 instrument (Iraq).

Long pepper extraction preparation

The long paper fruits were collected after being thoroughly washed with distilled water (DW) to remove dirt and stuck-on dust, then drying it at 30 ºC for one week. Two extracts, one from water and one from ethanol, have been prepared as shown in Figure 1.

***Piper longum* Fruit** **Water Extract** **Ethanolic Extract**

Figure 1. *Piper longum* fruit, ethanol and water extracts.

Preparation of ethanolic extract (Ext. 1)

Long pepper fruit was extracted by grinding it into a powder, then dissolving 300 g of it in 600 mL of ethanol (96%) and stirring it for one day. Long pepper extraction was filtered using filter paper, and solvents were evaporated using distillation at 78 ºC. The oil extracts were stored away from light (at room temperature) in a dark and clean environment [34, 35]. The obtained extract yield was 1.5% (w/w) as a brown powder.

Preparation of water extract (Ext. 2)

The plant was collected after washing it well using DW to remove dirt and stuck-on dust, then drying it at 30 ºC and after that, the powder (100 g) was dissolved in 1 L of DW and heated at 60 to 70 ºC for 2 hours. Filtration of the mixture was carried out using filter paper and dried at 60 ºC using the oven for one day before being crushed into powder [34, 35]. The obtained extract yield was 4% w/w as black oil.

Preparation of Cu-NPs1 from ethanolic extract

An amount of $CuSO_4.5H_2O$ (0.1 M) is prepared as a precursor by mixing 2.5 g of copper salt ($CuSO_4.5H_2O$) in DW (100 mL) followed by stirring for 45 min while heating at 60 ºC, then cooling to room temperature, and storing in a clean and dark place. After adding the copper solution (25 mL) to the extract (75 mL) with stirring at 35 ºC (3 hours), the solution turned green. After 24 hours, the mixture turned dark green, and it was stored in a clean and dark place for two days. The mixture is placed under centrifugal force (3×10^3) for 20 min, then collected and washed several times with ethanol before being dried out for over 24 hours, yielding 250 mg of a green powder of Cu-NPs1 (Figure 2).

Figure 2. Copper nanoparticles synthesis from ethanol extract.

Preparation of Cu-NPs2 from water extract

An amount of $CuSO_4.5H_2O$ (0.1 M) is prepared as a precursor by mixing 2.5 g of copper salt ($CuSO_4.5H_2O$) in DW (100 mL) followed by stirring for 45 min while heating at 60 ºC, then cooling to room temperature, and storing in a clean and dark place. After adding the copper solution (25 mL) to the extract (75 mL) with stirring at 35 ºC (3 hours) resulted in a green color solution after 24 hours the mixture has become dark green and then store in a clean and dark place for two days. The mixture is placed under centrifugal force (3×10^3) for 20 min, then collected and washed several times with ethanol before being dried out for over one day, yielding 300 mg of a black powder of Cu-NPs2 (Figure 3) [36, 37].

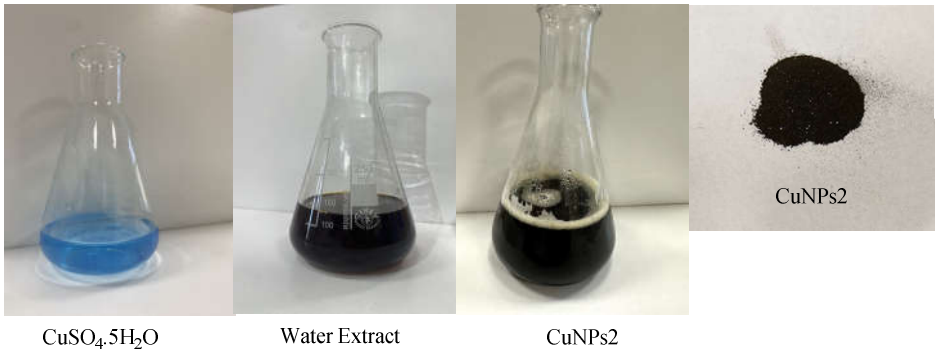


Figure 3. Copper nanoparticles synthesis from water extract.

Antimicrobial activity

Cultural media preparation

All the media used in the current study were prepared according to their manufacturing company's instruction. An autoclave was utilized to sterilize the vessel at 121ºC for 5 min.

Bacteria culture test

Microbiology Laboratory of the Medya Diagnostic Center (MDC) in Erbil has provided all bacteria strains including *Klebsiella pneumoniae* (13883), *Proteus mirabilis* (14153), *Escherichia coli* (25922), *Staphylococcus aureus* (25923), *Enterococcus faecalis* (29212) and *Streptococcus*

pneumoniae (6303). Antimicrobial activity was measured using two methods including micro broth dilution methods and disk diffusion. No ethical approval was needed by the Cihan University-Erbil as the bacterial strains were not taken from the patients.

Preparation of extracts and copper nanoparticles

10 mg of copper salt was dissolved in a solvent of dimethyl sulfoxide (1 mL). The biological acivity of the copper nanoparticles (Cu-NPs) and extracts (Ext. 1 and Ext. 2) including MIC was measured using micro broth dilution and while inhibition zone determination was investigated using the disk diffusion assay [34, 35].

Inhibitory activity determination by disk diffusion assay

The inhibitory activity of copper nanoparticles (Cu-NPs) and extracts (Ext. 1 and Ext. 2) against all the bacterial strains was measured using this assay. After verifying the purity of the strain, obtained typical colonies were suspended in physiological saline and vortexing suspension for a short time to make homogeneous suspension of bacteria. Incubate the broth at 30 °C to exceed the turbidity (0.5 MacFarland standard). Mueller-Hinton agar (MHA) plates were loaded with the suspensions and over these plates, sterile filter paper disks (6 mm in dimeter) were placed. Tested compounds (20 L, 10 mg/mL in DMSO) were used to impregnate the sterile disks. Amoxicillin was used as a positive control and sterile distilled water was used as a negative control. Incubation was carried out at 37 °C for one day and the inhibition zone was measured in millimeters [38-42].

Minimum inhibitory concentration determination (MIC)

A series of half-dilutions of the target nanoparticles and plant extract (long pepper) in sterile test tubes of 0.05, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, and 128 µg/L were prepared, and the tubes were inoculated with 0.1 mL (1.5 X 108 CFU/mL) of the bacterial suspension according to the multiplication dilutions. The test tubes were incubated at 37 °C for 24 hours as previously prepared and the obtained inhibitory activity results were compared to control model 1 (broth media inoculated with bacteria only) and control model 2 (broth media with plant extract but no bacteria) [38-42].

RESULTS AND DISCUSSION

Chemistry

Cu-NPs1 and Cu-NPs2 synthesis

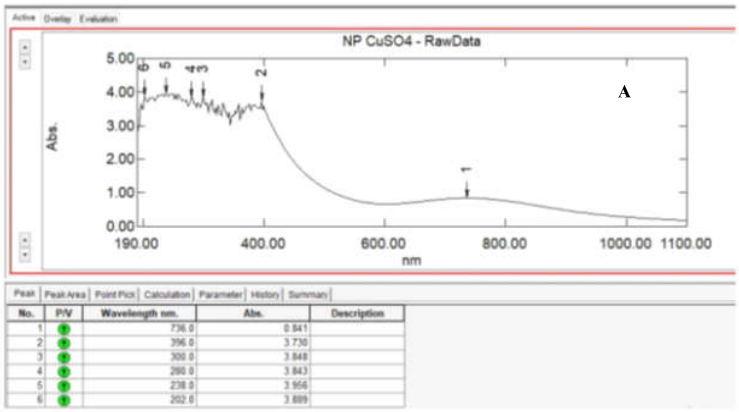
Cu-NPs1 was obtained as a green powder with 250 mg as a result of adding long pepper fruit extract (75 mL) to the 25 mL of $CuSO_4 \cdot 5H_2O$ solution (Figure 1) and Cu-NPs2 was obtained as a black powder with 300 mg as a result of adding long pepper fruit extract (75 mL) to the 25 mL of $CuSO_4 \cdot 5H_2O$ solution (Figure 2 and 3). long pepper fruit contains the flavonoids and phenolic compounds that are the main cause of the reduction of copper with oxidation state two into copper with oxidation state zero producing copper nanoparticles (Cu-NPs).

Ultraviolet–Visible spectrophotometer and Fourier transform infrared spectrophotometer

Ultraviolet spectra were investigated by UV-Vis spectrophotometer of both copper nanoparticles (Cu-NPs1 and Cu-NPs2) within 24 hours after color stability (Figure 4). UV-VIS spectra were utilized to investigate this reduction and an absorption band that appears at 736 nm due to the excitation of localized surface plasmon band (SPB) in copper nanoparticles [43] in comparison to the extract (396 nm). FTIR spectra of the copper nanoparticles (Cu-NPs1 and Cu-NPs2) were

measured in the range between 4000 and 400 cm^{-1} as shown in Figure 4, FTIR spectra revealed the presence of nine main peaks at (3417.86 Cu-NPs1, 3429.43 Cu-NPs2), (2924.09 Cu-NPs1, 2935.66 Cu-NPs2), (1624.06 Cu-NPs1, 1651.07 Cu-NPs2), (1554.63 Cu-NPs1, 1554.63 Cu-NPs2), (1446.61 Cu-NPs1, 1415.75 Cu-NPs2), (1299.87 Cu-NPs1, 1270.24 Cu-NPs2), (1107.14 Cu-NPs1, 1101.00 Cu-NPs2), (1041.56 Cu-NPs1, 1040.26 Cu-NPs2) cm^{-1} which represent functional groups OH (stretching, alcoholic or phenolic), CH (asymmetric stretching), C=C (stretching), C=C (stretching), C=C (aromatic, stretching), COH (stretching), COH (bending) and COH bending (Figure 4). The appeared peaks show the absorption of phenolic compounds of the long pepper extract on the prepared copper nanoparticles (Cu-NPs1, Cu-NPs2) surface via electron interaction [44]. Furthermore, the use of the carbonyl and hydroxyl bonding in the long pepper extract leads to the reduction of copper salt into copper nanoparticles [45]. The increase in the stability of copper nanoparticles is due to the absorption of flavonoids and phenolic compounds (capping agents) on the copper nanoparticle's surface. For 24 hours, the nanoparticles remained stable.

NPs cuso4



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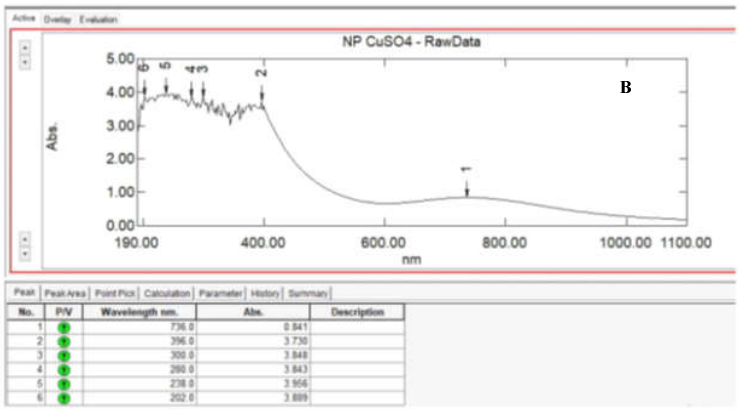

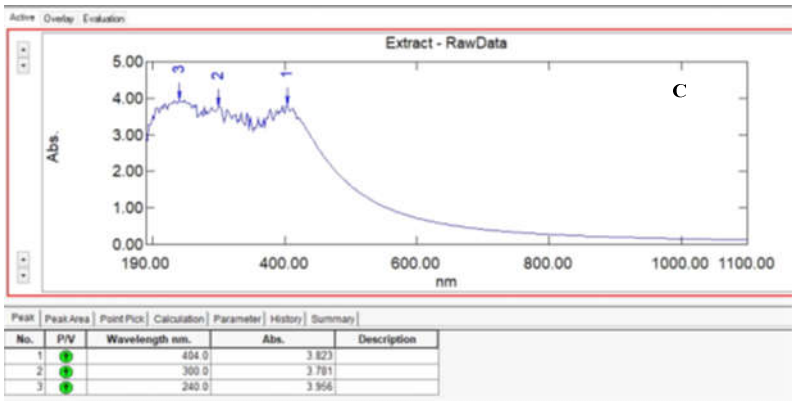
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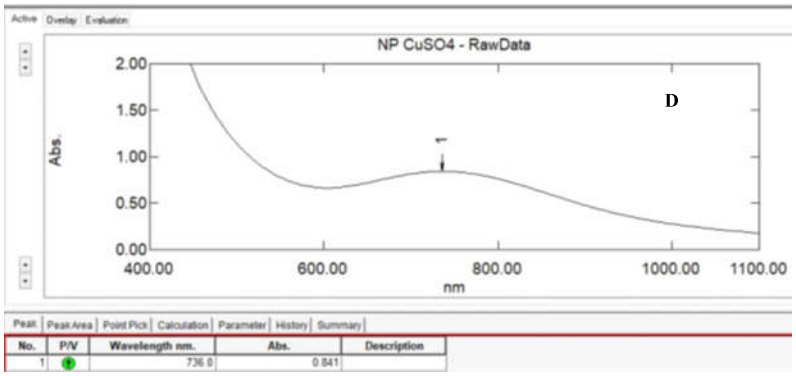
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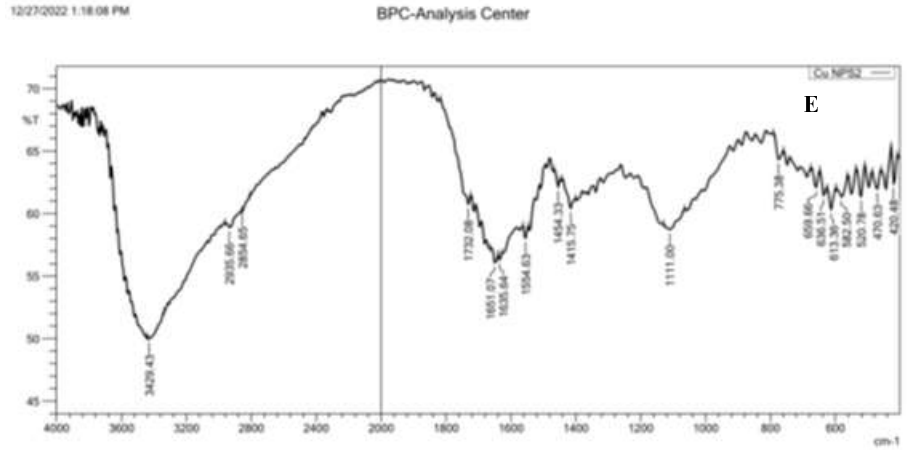
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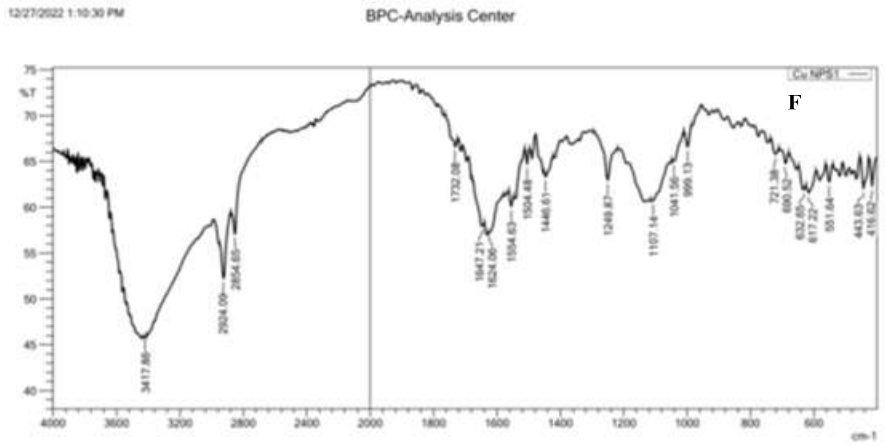

Figure 4. Wavelength (UV-VIS) of A) ethanolic extract of Long Pepper; B) CuNPs1; C) water extract of Long Pepper; D) CuNPs2; FT-IR for E) CuNPs1; F) CuNPs2.

Transmission electron microscopy (TEM)

TEM technique was utilized to confirm the shape of synthesized copper nanoparticles (Cu-NPs1 and Cu-NPs2) as shown in Figure 5. The TEM images showed the uniform dispersion of copper nanoparticles with 100 nm in diameter. The nanoparticles appeared tangled and imbricated. Based on these obtained measurements, with regard to shape and homogeneity, it appears that there is a high degree of compatibility with copper nanoparticles (Cu-NPs1 and Cu-NPs2) that have synthesized from other plant extracts via green methods [46].

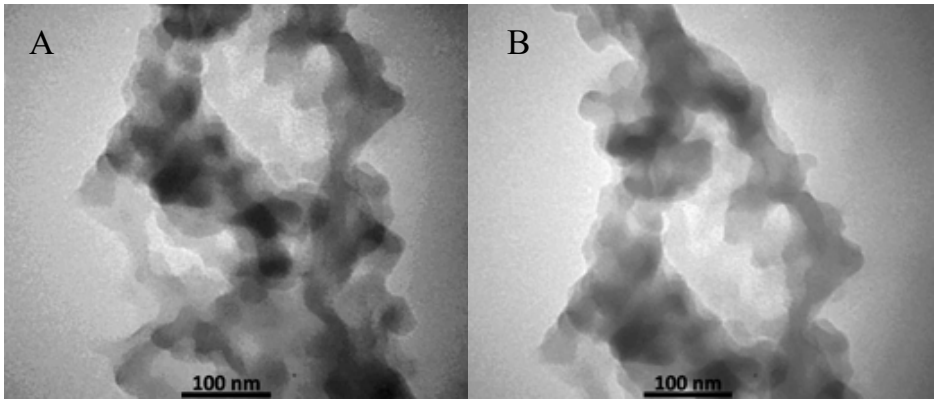

Figure 5. TEM of (A) Cu-NPs1 (B) Cu-NPs2.

X-Ray diffraction studies

The X-ray diffractometer is used to analyze XRD patterns and measure Cu-NPs (Figure 6). The XRD analysis confirmed that the copper nanoparticles were successfully synthesized. XRD

analysis shows that the synthesis of copper nanoparticles was succeeded and the primary elemental copper diffraction peaks were identified at 2θ = 31.7340, 42.3409, and 56.922°, which belongs to the (111), (200), and (220) copper crystal faces [47]. The average size of crystalline copper nanoparticles (Cu-NPs1 and Cu-NPs2) is calculated using Debye-Scherrer's equation as follows [48]: $D = k\lambda/\beta\cos\theta$

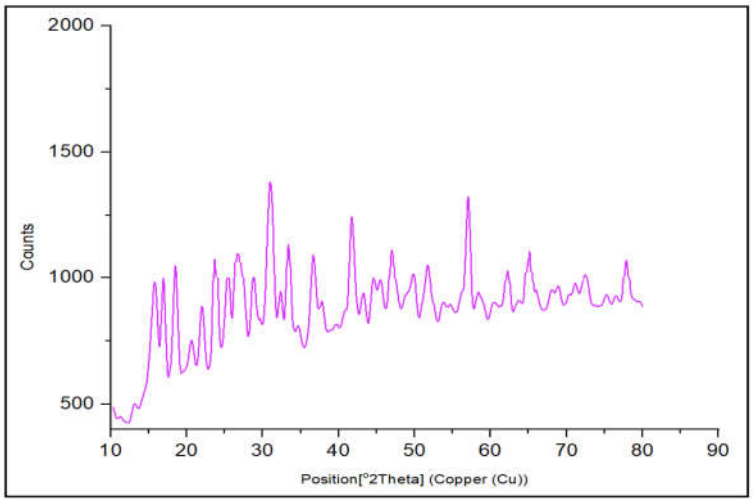

Figure 6. XRD spectrum of the copper nanoparticles (Cu-NPs1 and Cu-NPs2) synthesized from Long Pepper extract.

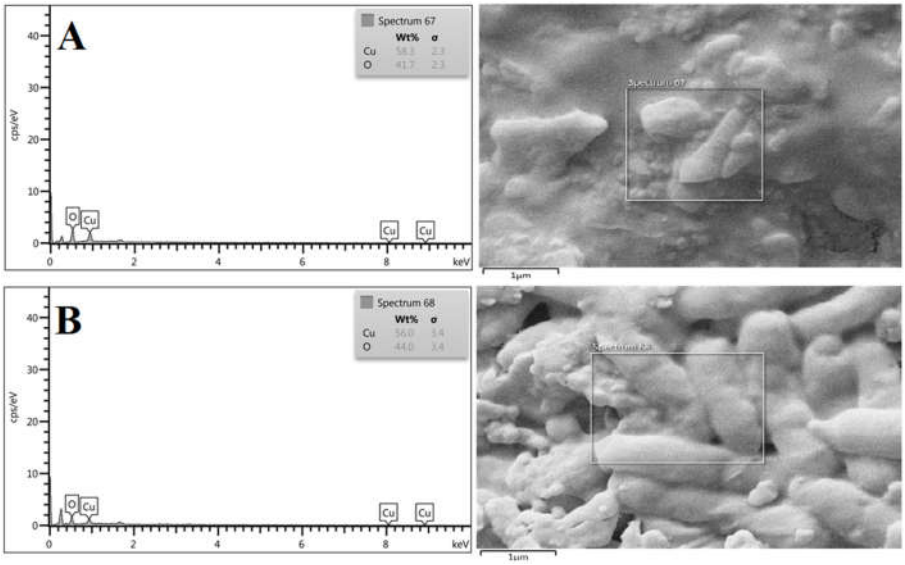

Figure 7. Scanning electron microscopic with energy-dispersive X-ray analysis (SEM-EDX) evaluating the chemical composition (spectra) and the element distribution (elemental mapping) of (A) Cu-NPs1 (B) Cu-NPs2.

Energy-dispersive X-ray spectroscopy analysis (SEM-EDX)

Figure 7 shows the EDS spectrum of the prepared copper nanoparticles (Cu-NPs1 and Cu-NPs2) that measuring the prepared copper nanoparticles (Cu-NPs1 and Cu-NPs2) by SEM-EDS shows the main peaks of the coppers at the positions including 0.9-1, 8.0-8.1, and 8.7-8.9 keV which belong to the standard position of copper nanoparticles (Cu-NPs1 and Cu-NPs2). The presence of oxygen alongside copper indicates that at least part of the Cu nanoparticles was oxidized by air.

Field emission scanning electron microscopy (FESEM)

Figure 8 shows the FESEM for the investigation the topography of the prepared copper nanoparticles (Cu-NPs1 and Cu-NPs2). A is the image of Cu-Nps1 which has observed at magnification of 20000x while B is the image of Cu-NPs2 was observed at 50000x magnification. The images showed the presence of spheroid like shape particles ranging from about 35 to 320 nanometer (8-A) with few agglomerated random and spheroidal shapes at a scale ranging from 400 to 640 nanometer (8-A and 8-B). The observation of such structures because the deposition has been bypassed the exact time during the synthesis since it took 45 min which is in our consideration has exceeded about 4-5 min a time at which the spherical structure tends mostly to extend itself leading to the agglomeration [49, 50].

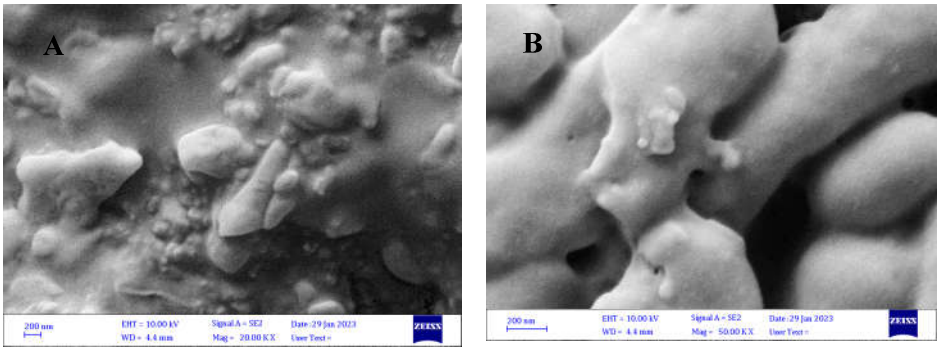

Figure 8. FESEM of (A) Cu-Nps1 (B) Cu-NPs2 at 130 ºC with magnification of 100k.

In vitro antimicrobial activity

Minimum inhibitory activity (MIC)

In vitro biological activity of the copper nanoparticles (Cu-NPs1 and Cu-NPs2) and extract (Ext. 2) were investigated as shown in Table 1. Table 1 shows the MIC values of both copper nanoparticles (Cu-NPs1 and Cu-NPs2) and extract (Ext. 2). The activity was tested at 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, and 0.05 µg/L concentrations. The majority of the synthesized copper nanoparticles (Cu-NPs1 and Cu-NPs2) exhibit moderate to potent inhibitory activity against the whole tested bacteria (*S. aureus*, *Escherichia faecalis*, *Pneumocystis pneumoniae*, *E. coli*, *P. mirabilis* and *K. pneumoniae*) demonstrating our design strategy. Copper nanoparticles (Cu-NPs2) were effective against three bacterial strains including *S. aureus*, *E. coli* and *K. pneumoniae* with MIC values from 8-16 µg/L compared to the positive control of amoxicillin (MIC = 30 µg/L). On the other hand, copper nanoparticles (Cu-NPs2) and the extract (Ext. 2) exhibit moderate inhibitory activity in comparison to the positive control of amoxicillin. Copper nanoparticles (Cu-NPs1) were effective against the whole bacterial strains with MIC values from 8-16 µg/L in comparison to the positive control of amoxicillin (MIC = 30 µg/L) as shown in Table 1.

Table 1. MIC and zone of inhibition for the nanoparticles and long pepper extract against bacterial strains.

Entry	MIC (µg/mL)					
	Gram positive bacteria			Gram negative bacteria		
	S. aureus	*E. faecalis*	*S. pneumoniae*	*P. mirabilis*	*K. pneumoniae*	*E. coli*
Cu-NPs1	8	16	8	8	8	16
Cu-NPs2	16	32	32	32	8	16
Ext2	32	64	32	64	32	128
Amoxicillin	30	30	30	-	-	30
	Inhibition zone					
Cu-NPs1	11	12	13.5	14	13	14
Cu-NPs2	9	8	7	6	9	8
Ext2	5	6	4.5	5	5.5	5
Amoxicillin	8	9	10	-	-	9

Zone inhibition

According to the Table 1, the results show that prepared copper nanoparticles (Cu-NPs) were significantly effective inhibitory activity against the whole tested bacterial strains than the positive control of amoxicillin as shown in Figure 9. The zones of inhibition (11-14 mm) were confirmed the potency of the copper nanoparticles (Cu-NPs1) in comparison to the positive control of amoxicillin (8-9 mm).

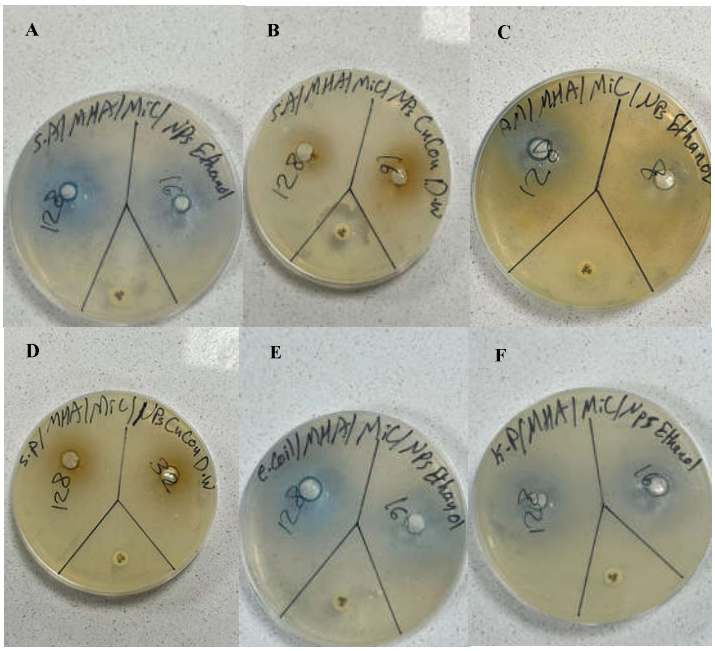

Figure 9. Zone of inhibition of A) CuNPs1, B) CuNPs2, C) CuNPs1, D) CuNPs2, E) CuNPs1, and F) CuNPs1 against the bacterial strains.

CONCLUSION

Copper nanoparticles (CuNPs1 and CuNPs2) were prepared via a green method involving the use of the ethanolic and water extracts of long pepper fruit. The reaction was performed at ambient temperature and for a short time. The copper nanoparticles have been identified using UV-VIS and FTIR to prove the change of absorbance peak compared to the long paper extracts. The techniques including XRD, HR-TEM, and EDX were used to investigate and confirm the crystalline, elemental composition and morphology of copper nanoparticles (CuNPs1 and CuNPs2). In vitro antibacterial activity was tested and showed potent inhibitory activity of both copper nanoparticles and water extract against all bacterial strains.

ACKNOWLEDGEMENTS

Many thanks to Department of Biology at Cihan University-Erbil for full support during this study. The authors are grateful to their respective affiliation institutions for providing infrastructure and administrative support. This work was supported by Princess Nourah bint Abdulrahman University researchers supporting project number (PNURSP2024R205), Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia.

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