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Green synthesis of copper nanoparticles using a hydroalcoholic extract of *Moringa oleifera* leaves and assessment of their antioxidant and anti-microbial activities

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Abstract

The synthesis of metal nanoparticles using plant extracts is a very promising method in green synthesis. The medicinal value of *Moringa oleifera* leaves and the anti-microbial activity of metallic copper were combined in the present study to synthesize copper nanoparticles having a desirable added-value inorganic material. The use of a hydroalcoholic extract of *M. oleifera* leaves for the green synthesis of copper nanoparticles is an attractive method as it leads to the production of harmless chemicals and reduces waste. The total phenolic content in the *M. oleifera* leaves extract was 23.0 ± 0.3 mg gallic acid equivalent/g of dried *M. oleifera* leaves powder. The *M. oleifera* leaves extract was treated with a copper sulphate solution. A color change from brown to black indicates the formation of copper nanoparticles. Characterization of the synthesized copper nanoparticles was performed using UV-Vis spectrophotometer, FT-IR spectrometer, TEM, SEM, and XRD. The synthesized copper nanoparticles have an amorphous nature and particle size of 35.8-49.2 nm. We demonstrate that the *M. oleifera* leaves extract and the synthesized copper nanoparticles display considerable antioxidant activity. Moreover, the *M. oleifera* leaves extract and the synthesized copper nanoparticles exert potent anti-bacterial activity against *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Enterococcus faecalis* (MIC values for the extract: 500, 250, 250, and 250 µg/mL; MIC values for the cooper nanoparticles: 500, 500, 500, and 250 µg/mL, respectively). Similarly, the *M. oleifera* leaves extract and the synthesized copper nanoparticles exert relatively more potent anti-fungal activity against *Aspergillus niger*, *Aspergillus flavus*, *Candida albicans*, and *Candida glabrata* (MIC values for the extract: 62.5, 62.5, 125, and 250 µg/mL; MIC values for the cooper nanoparticles: 125, 125, 62.5, and 31.2 µg/mL, respectively). Our study reveals that the green synthesis of copper nanoparticles using a hydroalcoholic extract of *M. oleifera* leaves was successful. In addition, the

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synthesized copper nanoparticles can be potentially employed in the treatment of various microbial infections due to their potent antioxidant, anti-bacterial, and anti-fungal activities.

Keywords: *Moringa oleifera*; copper nanoparticles; polyphenolics; anti-bacterial; anti-fungal; antioxidant

1. Introduction

Moringa oleifera (family Moringaceae) is known as the "tree of life". For thousands of years, it has been widely cultivated for its industrial and medicinal value. It is cultivated for its leaves and fruits, which are used in common man's kitchen. Almost all parts of the plant have been utilized in home remedies and traditional medicine [1]. *M. oleifera* leaves are edible and their vitamin and amino acid content make them a well-balanced diet [2]. The phenolic compounds present in *M. oleifera* leaves possess antioxidant properties and they are used in various medical applications [3]. It has been envisaged that plant compounds and their derivatives may be applied in several fields and industries such as textiles, fabrics, polymers for food and non-food applications, biomaterials for oral hygiene and prevention of dental caries, prevention of biofilm formation, and wrapping foil polymers for food packaging and food safety [4-10]. New materials based on the antimicrobial properties of plant compounds conjugated to silver nanoparticles and other nanoparticles-related technologies have been produced and studied [11-15].

Previous studies reported the successful use of active components of plant extracts (e.g. proteins, flavonoids and carboxylic groups of arabinose and galactose, reducing sugars, tannins, aliphatic amines, aliphatic alkenes of alkaloids, polysaccharides, aromatic amines, secondary alcohols, water-soluble heterocyclic components and saponins) in the synthesis of silver nanoparticles [16]. Typically, plant extracts possess intrinsic biological activities, which may further manifest in the biological activities of silver nanoparticles as a result of combining the two materials. Therefore, plant extracts can potentially be developed into novel nanomaterials with diverse biological activities [17]. Gold and silver nanoparticles were synthesized using leaves extracts of *Erythrina suberosa (Roxb.)*, *Paederia foetida*, *Acalypha indica*, *Cassia auriculata*, *Sorbus aucuparia*, and *Azadirachta indica*, and their antimicrobial efficacy was evaluated [18-23]. Moreover, studies evaluated the *in vitro*

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assessment of both the antioxidant and antimicrobial activities of silver nanoparticles synthesized using various plant extracts [24-28]. In many cultures, people used to, and some still, drink water stored in copper vessels. Such water has a copper content of 177 ± 16 ppb, which is well within the permissible limits according to the World Health Organization (WHO) [29]. Storing water in copper vessels purifies water by killing some species and strains of bacteria like *Escherichia coli*, as metallic copper surfaces rapidly and efficiently destroy bacteria by a contact-killing mode [30].

Our study attempts to bring together the properties of *M. oleifera* leaves' phytochemical constituents and the anti-microbial activity of copper. We aimed at the green synthesis and characterization of copper nanoparticles using a hydroalcoholic extract of *M. oleifera* leaves. Using different species of bacteria and fungi, we evaluated the potential anti-bacterial and anti-fungal activities of the *M. oleifera* leaves extract and the synthesized copper nanoparticles.

2. Results and Discussion

2.1.Phytochemical Analysis of the *M. oleifera* Leaves Extract

The qualitative evaluation of different chemical constituents in the *M. oleifera* leaves extract was performed using the test methods indicated in Table1. The presence is indicated with (+).

Table 1. Phytochemical analysis of the *M. oleifera* leaves extract.

Functional group	Test method	*M. oleifera* leaves extract
Alkaloids	Dragendroff's test	+
Tannins	Ferric chloride	+
Flavonoids	Shinoda test	+
Steroids	Salkowski reaction	+
Saponins	Foam test	+
Polyphenols	Puncal-D	+
Glycosides	Conc. H_2SO_4 and heat	+
Carbohydrates	Anthrone test	+
Proteins	Ninhydrin test	+
Amino acids	Millon's test	+

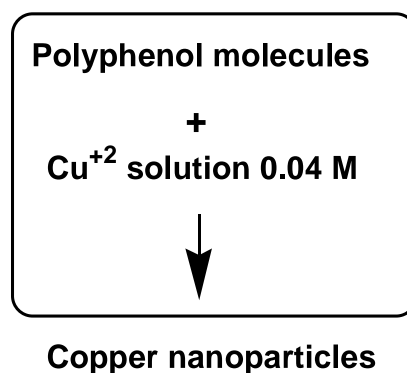
The protein content in the *M. oleifera* leaves extract was estimated to be 0.1% of the dry leaves powder. The total phenolic content in *M. oleifera* leaves extract was 23% of the dry leaves powder. An earlier study demonstrated that alanine, tyrosine, lysine, and threonine are among the major amino acids present in the *M. oleifera* leaves extract [31]. The concoction of *M. oleifera* leaves extract contains isomers of ceffeoylquinic acid, isomers of feruloylquinic acid, tannins, gallic acid, and several flavonoids like quercetin, kaempferol (Suppl. Fig. 1) and their glycoside derivatives [32]. Collectively, these findings suggest that the *M. oleifera* leaves extract could serve as a nutritional supplement and a possible stabilizing agent for the formed copper nanoparticles.

From the total phenolic content estimation of the *M. oleifera* leaves extract before and after the reaction (Table 2), Scheme 1 is proposed. The total phenolic content of 60 mg gallic acid equivalent from 10g of dried *M. oleifera* leaves powder was used in the synthesis of 250 mg

of copper nanoparticles from 0.04 M of copper (II) ion solution. The other reducing and binding chemical entities present in the concoction aid in the formation and stabilization of the synthesized copper nanoparticles.

Table 2. Estimation of total phenolic content before and after the synthesis of copper nanoparticles.

Sample	**Total phenolic content (mg/g of dried leaves)**
M. oleifera leaves extract (before synthesis)	23.0 ± 0.3
M. oleifera leaves extract (after synthesis)	17.0 ± 0.4



Scheme 1. Schematic representation of the synthesis of copper nanoparticles.

2.2.Characterization

2.2.1.Size and Morphology of the Synthesized Copper Nanoparticles

The synthesized copper nanoparticles were imaged using JEOL-TEM (JEM) (Figure 1) and EDS (Figure 2).

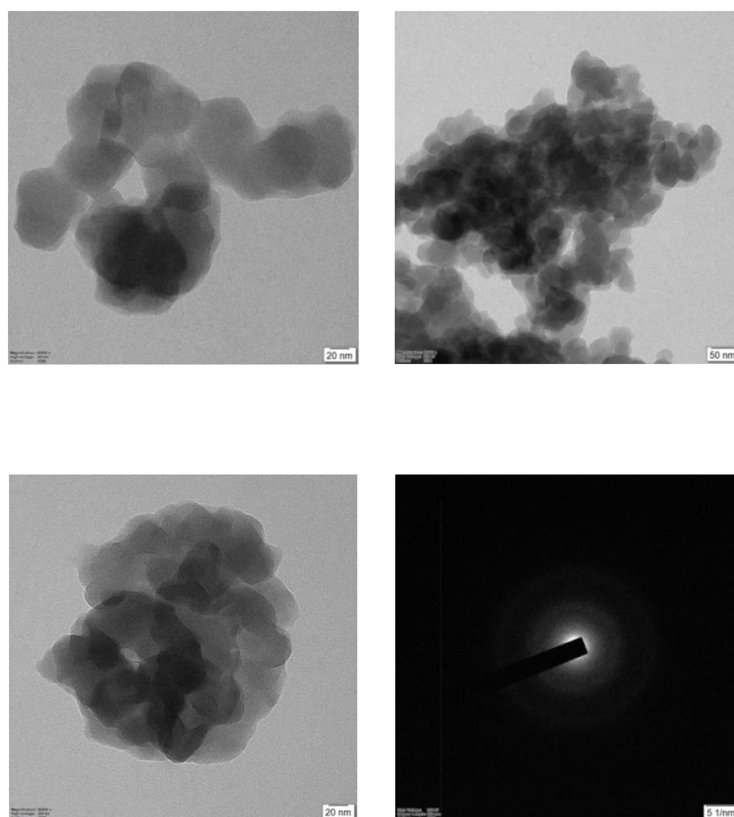


Figure1. JEOL-TEM (JEM) images of the synthesized copper nanoparticles.

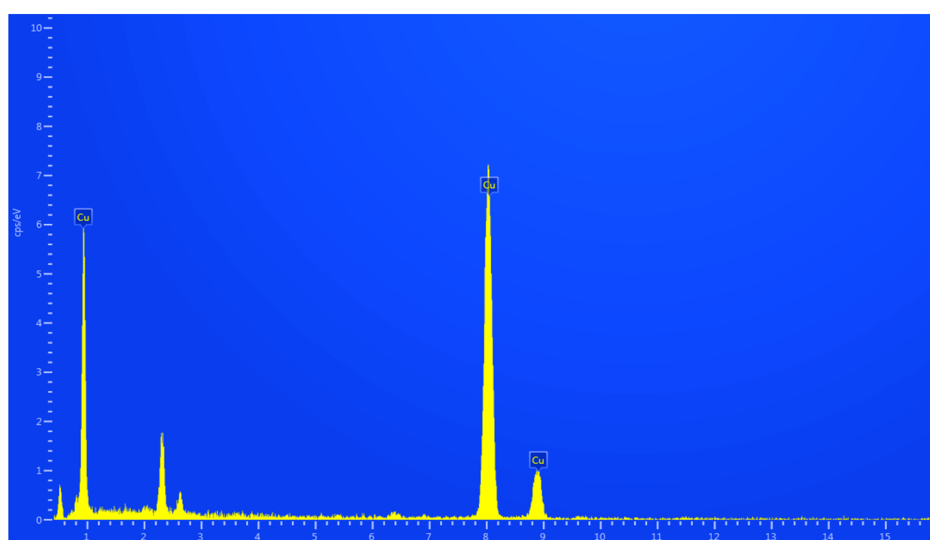


Figure 2.EDS spectrum with peaks corresponding to copper.

The synthesized copper nanoparticles were obtained in ethanol to give a colloidal solution. The synthesized copper nanoparticles are amorphous in nature and they agglomerate upon

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storage. The energy dispersive X-ray spectroscopy analysis confirmed the presence of the elemental copper nanoparticles. The absence of other elemental peaks reflects the purity of the sample. The SEM images (Figure 3) indicate that the size of the synthesized copper particles is 35.8-49.2 nm.

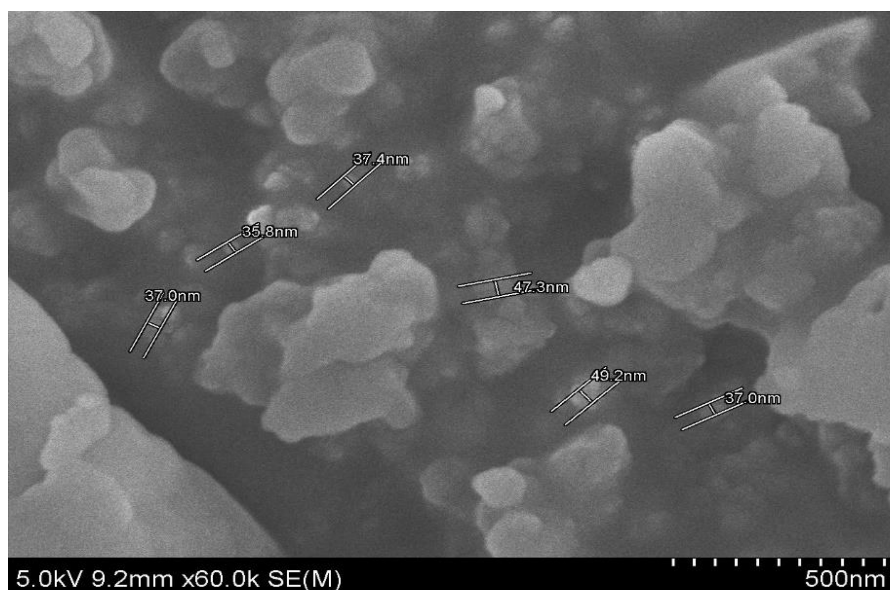

Figure 3.SEM image of the synthesized copper nanoparticles.

X-ray diffraction analysis confirms the amorphous nature of the synthesized copper nanoparticles (Figure 4). This substantiates the diffraction pattern observed in the JEOL-TEM (JEM) images (Figure 1), suggesting that the synthesized copper nanoparticles are amorphous in morphology.

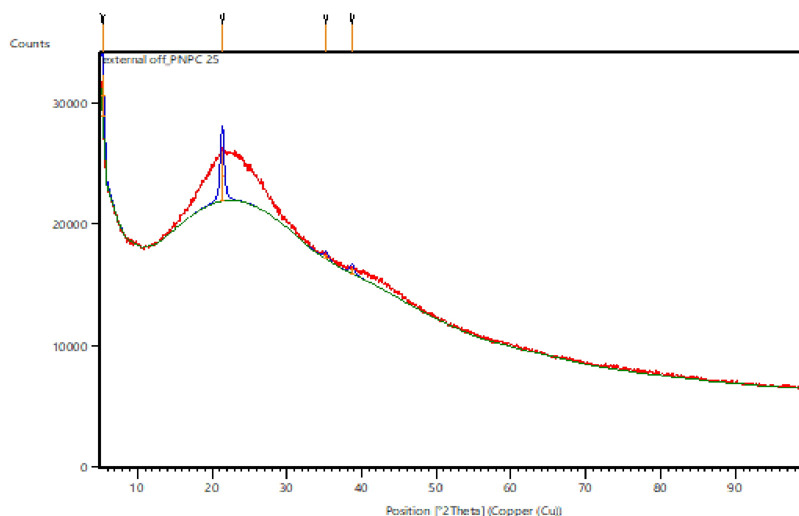

Figure 4. XRD spectrum of the synthesized copper nanoparticles.

2.2.2. Fourier Transform Infrared Spectroscopy (FT-IR) of the *M. oleifera* Leaves Extract and the Synthesized Copper Nanoparticles

The FT-IR spectra of the *M. oleifera* leaves extract and the synthesized copper nanoparticles are shown in Figure 5 and Figure 6, respectively. This vibration spectroscopy data can be used to understand the biomolecules involved in the synthesis of copper nanoparticles. The bands around 3400 cm^{-1} and 1630 cm^{-1} are broad in the *M. oleifera* leaves extract (Figure 5), corresponding to the vibration mode of hydroxyl group, mostly found in polyphenolic molecules such as tannins, flavonoids, and glycoside derivatives. The FT-IR spectrum of the synthesized copper nanoparticles (Figure 6) depicts a sharp band at 3431 cm^{-1}, corresponding to the N-H vibration mode. This comparison shows the participation of the hydroxyl group in the synthesis of copper nanoparticles. Moreover, the binding characteristics of amino groups in the *M. oleifera* leaves extract are observed in Figure 5 and Figure 6. This could be the reason for agglomeration of the synthesized copper nanoparticles to give an amorphous morphology.

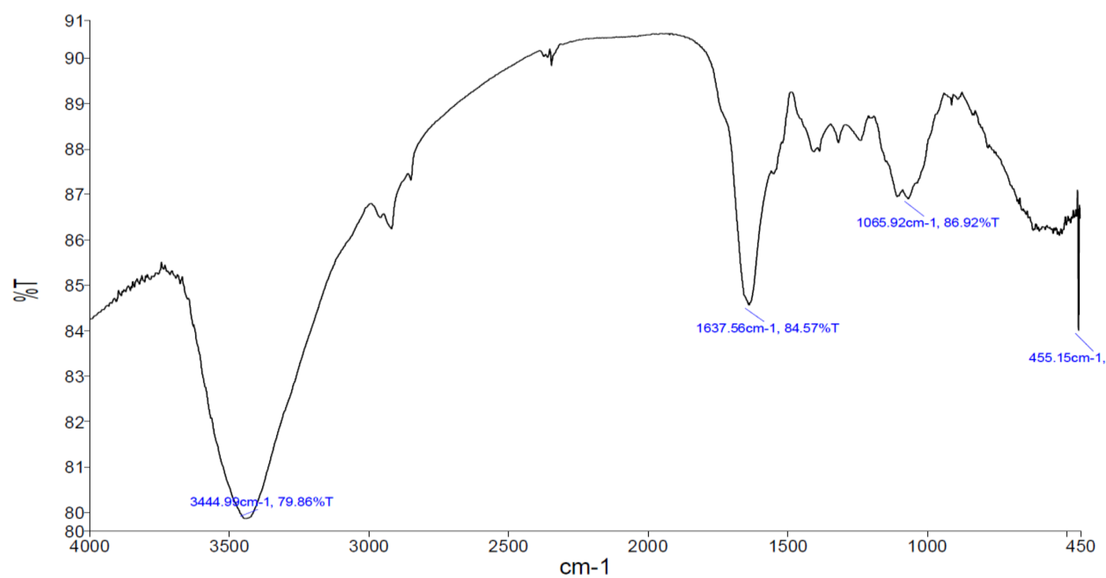

Figure 5. FT-IR spectrum of the *M. oleifera* leaves extract.

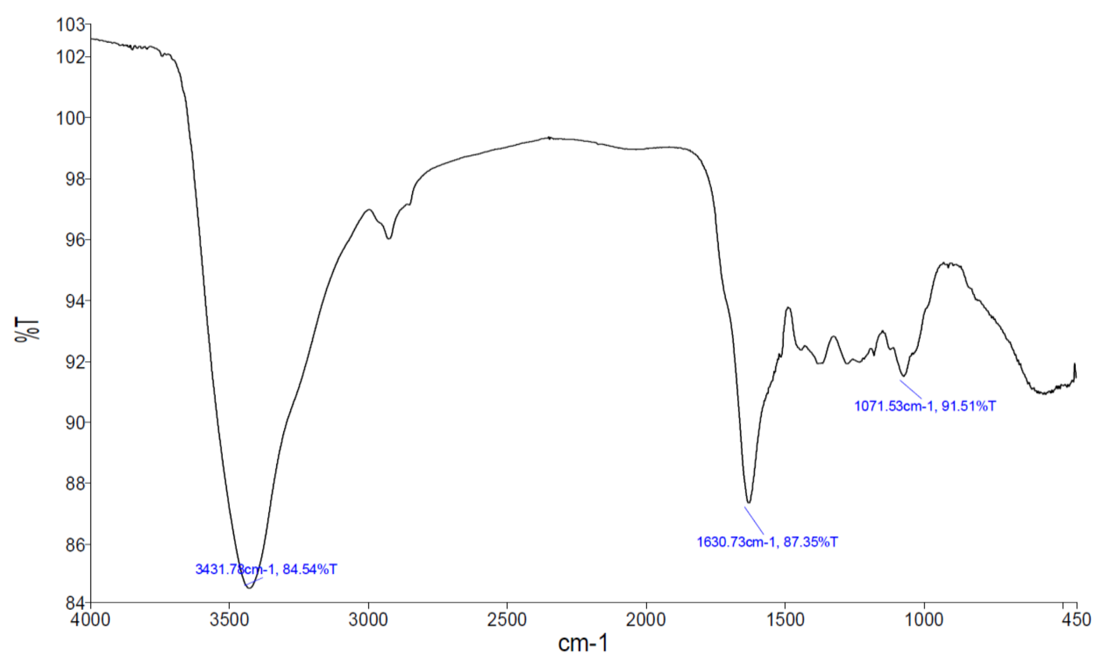

Figure 6. FT-IR spectrum of the synthesized copper nanoparticles.

2.2.3. UV-Vis Spectroscopy of the *M. oleifera* Leaves Extract and the Synthesized Copper Nanoparticles

The UV-Vis absorption spectrum of the *M. oleifera* leaves extract is shown in Figure 7, λ_{max} at 390 nm. The UV-Vis spectrum of the synthesized copper nanoparticles reconstituted in dimethyl sulfoxide (DMSO) solvent is shown in Figure 8, λ_{max} at 260 nm.

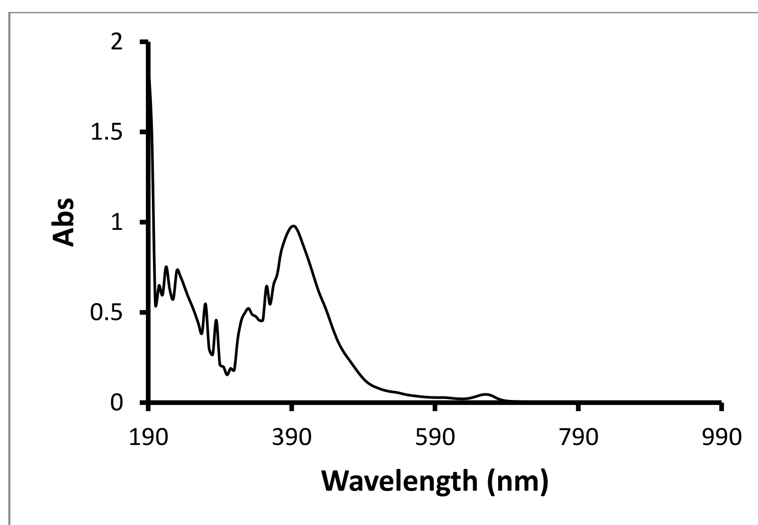

Figure 7. UV-Vis spectrum of the *M. oleifera* leaves extract.

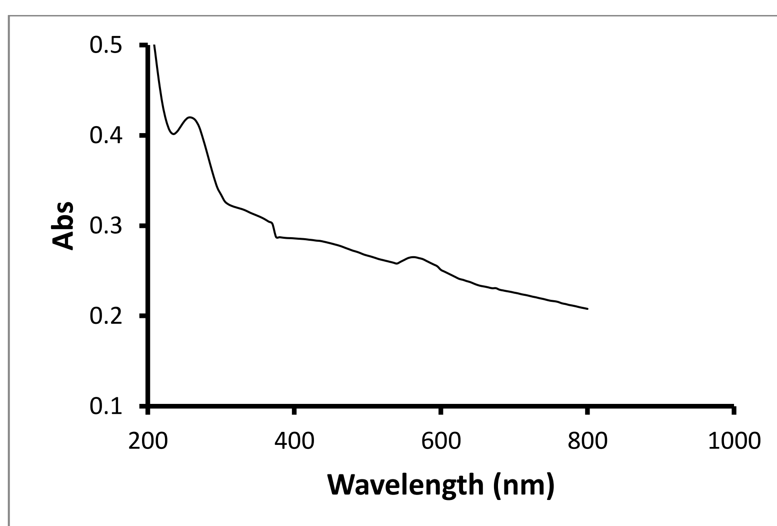

Figure 8. UV-Vis spectrum of the synthesized copper nanoparticles.

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2.3. Antioxidant Activity of the *M. oleifera* Leaves Extract and the Synthesized Copper Nanoparticles

Using DPPH assay against ascorbic acid as a standard, the percentage of antioxidant activity of the *M. oleifera* leaves extract and the synthesized copper nanoparticles was assessed. As shown in Table 3, the *M. oleifera* leaves extract exerted considerable antioxidant activity, while the synthesized copper nanoparticles displayed a lower activity. These results were supported with total antioxidant capacity measured by phosphomolybdate assay (Table 4).

Table 3. Antioxidant activity percentage (AA%) using DPPH assay.

Sample	Amount (µg)				
	100	200	300	400	500
Ascorbic acid (standard)	34.4	55.1	67.2	75.8	84.4
M. oleifera leaves extract	55.1	58.6	63.7	65.5	65.5
Copper nanoparticles	12.0	13.7	17.2	20.6	29.3

Table 4. Total antioxidant capacity(TAC) using phosphomolybdate assay.

Sample	Concentration(µg/mL) Ascorbic acid equivalent)				
	50	100	150	200	250
M. oleifera leaves extract	32.5	65.0	102.5	132.5	172.5
Copper nanoparticles	12.5	12.5	17.5	25.0	47.5

2.4.Anti-Bacterial Activity of the *M. oleifera* Leaves Extract and the Synthesized Copper Nanoparticles

The potential anti-bacterial activity of synthesized copper nanoparticles was evaluated against *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *and Enterococcus faecalis*. Streptomycin (10 µg/500 µL) was used as a positive control, while water-ethanol (1:1) solution and DMSO solvent were used as negative controls for the *M. oleifera* leaves extract

and the copper nanoparticles, respectively. The nutrient broth was also used as a negative control. The classification of these species of bacteria and their localization in the human body is given in Table 5. The minimum inhibitory concentration (MIC) values measured in presence of the *M. oleifera* leaves extract and the copper nanoparticles are given in Table 6. The growth of the indicated species of bacteria in presence of difference concentrations (7.8-1000 µg/mL) of *M. oleifera* leaves extract and the copper nanoparticles is shown in Table 7. The resazurin microtiter assay plates for the *M. oleifera* leaves extract and the copper nanoparticles are shown in Figure 9. In presence of the *M. oleifera* leaves extract, the MIC values against *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Enterococcus faecalis* were found to be in the range of 250-500 µg/mL (Table 6). Very similar MIC values (250-500 µg/mL) were observed in presence of the synthesized copper nanoparticles (Table 6), indicating that the anti-bacterial activity was not lost in the process of green synthesis of the copper nanoparticles (Table 7 and Figure 9).

Table 5. Species of bacteria used for the anti-bacterial study.

No.	Bacterial species	Classification	Localization in the human body
1	*Escherichia coli*	Gram-negative	Lower intestine
2	*Klebsiella pneumoniae*	Gram-negative	Flora in the mouth, skin, and intestines
3	*Staphylococcus aureus*	Gram-positive	Upper respiratory tract and skin
4	*Enterococcus faecalis*	Gram-positive	Gastrointestinal tract

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Table 6. Anti-bacterial activity (MIC values).

Bacterial species	MIC (µg/mL)
***M. oleifera* leaves extract**	
Escherichia coli	500
Klebsiella pneunomiae	250
Staphylococcus aureus	250
Enterococcus faecalis	250
Copper nanoparticles	
Escherichia coli	500
Klebsiella pneunomiae	500
Staphylococcus aureus	500
Enterococcus faecalis	250

Table 7. Anti-bacterial activity data (growth (+) and no growth (-)).

No.	Bacterial species	Growth of bacteria										
	Concentration (µg/mL)	1000(1)	500 (2)	250 (3)	125 (4)	62.5 (5)	31.2 (6)	15.6 (7)	7.8 (8)	Streptomycin (10 µg/500µL)	Negative control	Nutrient broth
	***M. oleifera* leaves extract**											
1	*Escherichia coli*	-	-	+	+	+	+	+	+	-	+	+
2	*Klebsiella pneumoniae*	-	-	-	+	+	+	+	+	-	+	+
3	*Staphylococcus*	-	-	-	+	+	+	+	+	-	+	+

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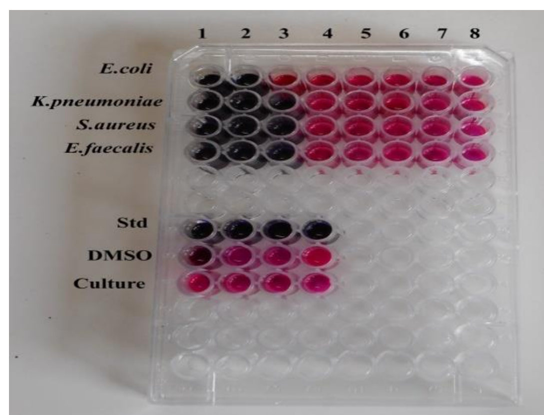
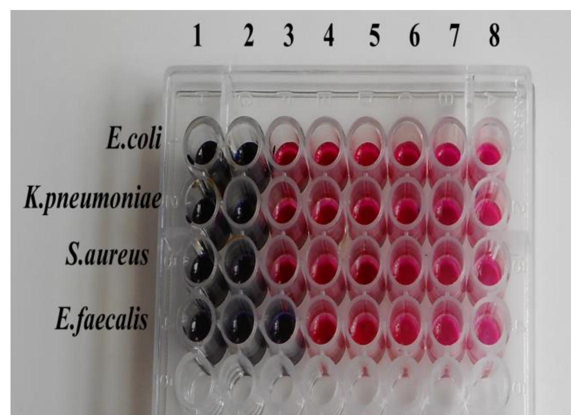
	aureus											
4	*Enterococcus faecalis*	-	-	-	+	+	+	+	+	-	+	+
	Copper Nanoparticles											
1	*Escherichia coli*	-	-	+	+	+	+	+	+	-	+	+
2	*Klebsiella pneumoniae*	-	-	+	+	+	+	+	+	-	+	+
3	*Staphylococcus aureus*	-	-	+	+	+	+	+	+	-	+	+
4	*Enterococcus faecalis*	-	-	-	+	+	+	+	+	-	+	+



M. oleifera leaves extract



Copper nanoparticles

Figure 9. Resazurin microtiter assay plates for the *M. oleifera* leaves extract and the synthesized copper nanoparticles.

2.5. Anti-Fungal Activity of the *M. oleifera* Leaves Extract and the Synthesized Copper Nanoparticles

The potential anti-fungal activity of synthesized copper nanoparticles was evaluated against *Aspergillus niger*, *Aspergillus flavus*, *Candida albicans*, and *Candida glabrata*. Ketoconazole (10 µg/500 µL) was used as a positive control, while water-ethanol (1:1) solution and DMSO solvent were used as negative controls for the *M. oleifera* leaves extract and the copper nanoparticles, respectively. The nutrient broth was also used as a negative control. The classification of these species of fungi and their localization in the human body is given in Table 8. The minimum inhibitory concentration (MIC) values measured in presence of the *M. oleifera* leaves extract and the copper nanoparticles are given in Table 9. The growth of the indicated species of fungi in presence of difference concentrations (7.8-1000 µg/mL) of *M. oleifera* leaves extract and the copper nanoparticles is shown in Table 10. The resazurin microtiter assay plates for the *M. oleifera* leaves extract and the copper nanoparticles are shown in Figure 10. The copper nanoparticles displayed more effective anti-fungal activity against *Candida albicans* and *Candida glabrata* than the *M. oleifera* leaves extract (Table 9, Table 10, and Figure 10). In presence of the *M. oleifera* leaves extract, the MIC values against *Aspergillus niger*, *Aspergillus flavus*, *Candida albicans*, and *Candida glabrata* were found to be 62.5, 62.5, 125, and 250 µg/mL, respectively (Table 9). In presence of the synthesized copper nanoparticles, the MIC values against *Aspergillus niger*, *Aspergillus flavus*, *Candida albicans*, and *Candida glabrata* were found to be 125, 125, 62.5, and 31.2 µg/mL, respectively (Table 9). These findings indicate that the copper nanoparticles displayed more effective anti-fungal activity against *Candida albicans* and *Candida glabrata* compared to the *M. oleifera* leaves extract (Table 9, Table 10, and Figure 10). Moreover, it is concluded that the anti-fungal activity was not lost in the process of green synthesis of the copper nanoparticles.

Table 8. Species of fungi used for the anti-fungal study.

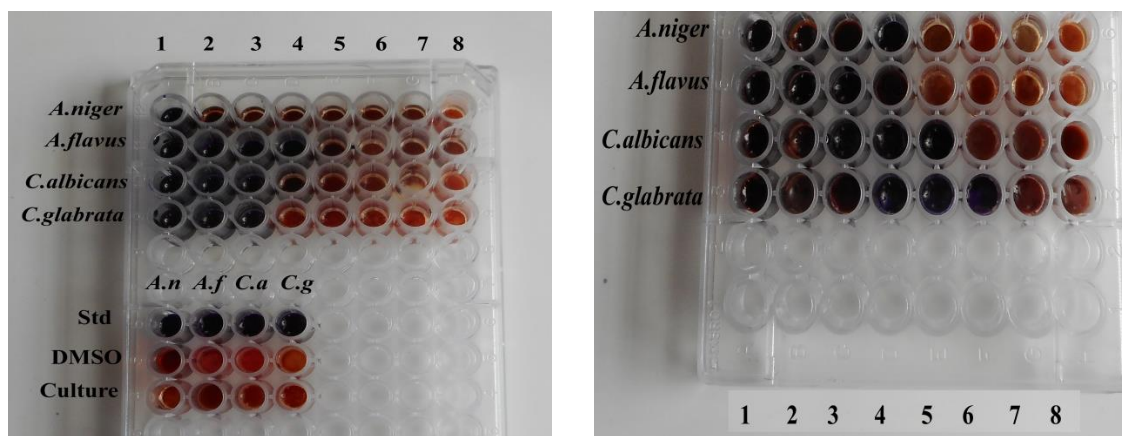
No.	Fungal species	Localization in the human body
1	*Aspergillus niger*	Lungs
2	*Aspergillus flavus*	Lungs, eyes, and ears
3	*Candida albicans*	Flora in gastrointestinal tract
4	*Candida glabrata*	Mucosal tissues

Table 9. Anti-fungal activity (MIC values).

Fungal species	MIC (µg/mL)
***M. oleifera* leaves extract**	
Aspergillus niger	62.5
Aspergillus flavus	62.5
Candida albicans	125
Candida glabrata	250
Copper nanoparticles	
Aspergillus niger	125
Aspergillus flavus	125
Candida albicans	62.5
Candida glabrata	31.2

Table 10. Anti-fungal activity data (growth (+) and no growth (-)).

No.	Fungal species	Growth of fungi										
	Concentration (μg/mL)	**1000 (1)**	**500 (2)**	**250 (3)**	**125 (4)**	**62.5 (5)**	**31.2 (6)**	**15.6 (7)**	**7.8 (8)**	**Ketoconazole (10 μg/500 μL)**	**Negative control**	**Nutrient broth**
	***M. oleifera* leaves extract**											
1	*Aspergillus niger*	-	-	-	-	-	+	+	+	-	+	+
2	*Aspergillus flavus*	-	-	-	-	-	+	+	+	-	+	+
3	*Candida albicans*	-	-	-	-	+	+	+	+	-	+	+
4	*Candida glabrata*	-	-	-	+	+	+	+	+	-	+	+
	Copper Nanoparticles											
1	*Aspergillus niger*	-	-	-	-	+	+	+	+	-	+	+
2	*Aspergillus flavus*	-	-	-	-	+	+	+	+	-	+	+
3	*Candida albicans*	-	-	-	-	-	+	+	+	-	+	+
4	*Candida glabrata*	-	-	-	-	-	-	+	+	-	+	+



M. oleifera leaves extract Copper nanoparticles

Figure 10. Resazurin microtiter assay plates for the *M. oleifera* leaves extract and the synthesized copper nanoparticles.

3. Materials and Methods

3.1. Preparation of the *M. oleifera* Leaves Extract

Fresh *M. oleifera* leaves were collected and shade-dried. The dried leaves were smoothly crushed. The *M. oleifera* leaves powder was stored at room temperature. 10 g of *M. oleifera* leaves powder was taken and soaked in 100 mL water-ethanol (1:1)solution. The mixture was macerated for 1 h and *M. oleifera* leaves extract was filtered through Whatman filter paper 1. The *M. oleifera* leaves extract was used immediately for the preparation of copper nanoparticles and other experimental analyses.

3.2. Synthesis of the Copper Nanoparticles

Copper sulfate pentahydrate (1g) was dissolved in 20 mL de-mineralized water and added to 80 mL of *M. oleifera* leaves extract. The reaction mixture was stirred for 3 h at 60°C. The synthesized copper nanoparticles were collected by centrifugation and repeatedly washed with de-mineralized water. The synthesized copper nanoparticles were dried at 105°C for 1 h, and subsequently reconstituted in DMSO solvent.

3.3. Characterization of the Size and Morphology of the Synthesized Copper Nanoparticles

The completion of synthesis was characterized by UV-Vis spectroscopy. The formation of copper nanoparticles and the role of biomolecules in this synthesis were confirmed by BRUKER ALPHA-E FT-IR spectrometry. The crystalline nature of the synthesized copper nanoparticles was ascertained using Shimadzu XRD-6000 diffractometer. The size of the synthesized copper nanoparticles was measured by SEM using Quanta 200 FEG scanning electron microscope. The morphology assessment of the synthesized nanoparticles was done by JEOLJEM-2100 plus transmission electron microscopy. The sample was dispersed in ethanol, coated on the grid, and dried for TEM analysis, along with energy dispersive X-ray analysis.

3.4. Phytochemical Analysis of the *M. oleifera* Leaves Extract

The qualitative analysis of biomolecules present in the *M. oleifera* leaves extract was carried out for the presence of alkaloids, tannins, flavonoids, steroids, saponins, polyphenols, glycosides, carbohydrates, proteins, and amino acids. The total phenolic content in the *M. oleifera* leaves extract was estimated as gallic acid equivalent by Folin-Ciocalteu polyphenol assay [33].The protein content in the *M. oleifera* leaves extract was estimated by Lowry's method [34].

3.5. Antioxidant Activity

3.5.1. DPPH Assay (Antioxidant Activity Percentage - AA%)

The antioxidant activity percentage (AA%) (scavenging activity) of the *M. oleifera* leaves extract and the synthesized copper nanoparticles was assessed by DPPH free radical scavenging assay. 1 mg of ascorbic acid (standard) was dissolved in 1 mL methanol.

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Different aliquots (serial dilution) of the ascorbic acid solution (0.1-0.5 mL), corresponding to 100-500 µg, were used for calibration. To each tube containing ascorbic acid solution, 1 mL of 0.1 mM DPPH radical solution in ethanol was added, and the final volume was adjusted to 4mL using ethanol. The stock solutions for the *M. oleifera* leaves extract and the synthesized copper nanoparticles were prepared by dissolving 1 mg of each sample in 1 mL of an appropriate solvent (methanol for the *M. oleifera* leaves extract; DMSO for the synthesized copper nanoparticles). Different aliquots from the stock solutions (0.1-0.5 mL), corresponding to 100-500 µg, were added to all tubes except the blank tube (control). The volume in each tube was adjusted to 3 mL using ethanol. To each tube, 1 mL of 0.1 mM DPPH radical solution in ethanol was added. The blank tube (control) was prepared by mixing 3 mL of ethanol and 1 mL of DPPH radical solution in ethanol. All tubes were incubated for 30 min at room temperature, and absorbance at 517 nm was recorded. AA% was determined using the following formula:

AA% = {(absorbance of blank) – (absorbance of sample) / (absorbance of blank)} X 100

3.5.2.Phosphomolybdenum Assay (Total Antioxidant Capacity - TAC)

The total antioxidant capacity (TAC) of the *M. oleifera* leaves extract and the synthesized copper nanoparticles was assessed by phosphomolybdenum assay as ascorbic acid equivalent. 1 mg of ascorbic acid (standard) was dissolved in 1 mL methanol. Different aliquots (serial dilution) of the ascorbic acid solution (0.1-0.5 mL), corresponding to 100-500 µg, were prepared. The volume in each tube was adjusted to 4 mL using distilled water. The stock solutions for the *M. oleifera* leaves extract and the synthesized copper nanoparticles were prepared by dissolving 1 mg of each sample in 1 mL of an appropriate solvent (methanol for the *M. oleifera* leaves extract; DMSO for the synthesized copper nanoparticles). Different aliquots from the stock solutions (0.1-0.5 mL), corresponding to 100-500 µg, were added to

all tubes except the blank tube (control). The volume in each tube was adjusted to 3 mL using distilled water. To each tube, 1 mL of phosphomolybdenum reagent (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) was added. The volume in each tube was adjusted to 4 mL using distilled water. After incubation for 90 min at 95°C, absorbance at 695 nm was recorded. The calibration curve for the ascorbic acid solution (standard) was plotted for the absorbance at 695 nm against known amounts of ascorbic acid with the phosphomolybdate reagent, so as to express the TAC values as ppm equivalent of ascorbic acid. Using the ascorbic acid calibration curve, the TAC values for the *M. oleifera* leaves extract and the synthesized copper nanoparticles were calculated and expressed as ppm equivalent of ascorbic acid.

3.6.Anti-Bacterial Activity using Resazurin Microtiter Assay

The most rapid and inexpensive way to screen several microorganism isolates at the same time, with better correlation in comparison to other techniques, is the resazurin microtiter assay [35-37]. The resazurin solution was prepared by dissolving a 270mg tablet of resazurin in 40 mL of sterile distilled water. The test was carried out in 96-well plates under aseptic conditions. A volume of 100 µl of sample containing 10 mg/mL was pipetted into the first well of the plate. To all other wells, 50 µL of nutrient broth was added, and the tested sample was serially diluted. Subsequently, 10 µL of resazurin solution and 10 µL of bacterial suspension were added to each well. Each plate was wrapped loosely with cling film to prevent dehydration. The plates were incubated at 37°C for 18-24 h. The color change was then assessed visually. A color change from purple to pink (or colorless) was recorded as positive, indicating cell growth (i.e. (+) means growth and (-) means no growth). The lowest concentration at which color change occurred was considered to be MIC. Streptomycin (10µg/500 µL) was used as a positive control, while water-ethanol (1:1) solution and DMSO

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solvent were used as negative controls for the *M. oleifera* leaves extract and the copper nanoparticles, respectively. The nutrient broth was also used as a negative control.

3.7.Anti-Fungal Activity using Resazurin Microtiter Assay

The resazurin solution was prepared by dissolving a 270 mg tablet of resazurin in 40 mL of sterile distilled water. The test was carried out in 96-well plates under aseptic conditions. A volume of 100 µl of sample containing 10 mg/mL was pipetted into the first well of the plate. To all other wells, 50 µL of nutrient broth was added, and the tested sample was serially diluted. Subsequently, 10 µL of resazurin solution and 10 µL of fungal suspension were added to each well. Each plate was wrapped loosely with cling film to prevent dehydration. The plates were incubated at 37°C for 18-24 h. The color change was then assessed visually. A color change from purple to pink (or colorless) was recorded as positive, indicating cell growth (i.e. (+) means growth and (-) means no growth). The lowest concentration at which color change occurred was considered to be MIC. Ketoconazole (10µg/500 µL) was used as a positive control, while water-ethanol (1:1) solution and DMSO solvent were used as negative controls for the *M. oleifera* leaves extract and the copper nanoparticles, respectively. The nutrient broth was also used as a negative control.

4. Conclusion

The green synthesis of copper nanoparticles using a hydroalcoholic extract of *M. oleifera* leaveswas successful. The formation of copper nanoparticles and the role of biomolecules in this synthesis were confirmed by UV-Vis absorption and FT-IR spectrometry. The energy dispersive X-ray spectroscopy analysis confirmed the presence of the elemental copper nanoparticles. The crystalline nature of the synthesized copper nanoparticles was ascertained by XRD. Using SEM, the particle size of the synthesized copper nanoparticles was measured

to be 35.8-49.2 nm. Using TEM with EDS, the synthesized copper nanoparticles were shown to have an amorphous morphology.Our study reveals that the *M. oleifera* leaves extract and the synthesized copper nanoparticles display considerable antioxidant activity, although the latter was relatively less influential. We also demonstrate that the *M. oleifera* leaves extract and the synthesized copper nanoparticles exert potent anti-bacterial activity against *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Enterococcus faecalis*, with MIC values in the range of 250-500 µg/mL. Similarly, the *M. oleifera* leaves extract and the synthesized copper nanoparticles exert relatively more potent anti-fungal activity against *Aspergillus niger*, *Aspergillus flavus*, *Candida albicans*, and *Candida glabrata*, with MIC values in the range of 62.5-250 µg/mL and 31.2-125 µg/mL, respectively. Indeed, the anti-fungal activity of the synthesized copper nanoparticles is more effective than that of the *M. oleifera* leaves extract against *Candida albicans* and *Candida glabrata*. We also conclude that the anti-bacterial and anti-fungal activities were not lost in the process of green synthesis of the copper nanoparticles. These findings suggest that the synthesized copper nanoparticles can be a promising therapeutic candidate for the treatment of various bacterial, and particularly, fungal infections such as candidiasis.

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Author Contributions

N.S., I.A.A.Y, A.F.M., and P.P. designed the experiments and supervised their execution. P.E.D. performed the preparation of the *M. oleifera* leaves extract and the green synthesis of the copper nanoparticles. P.E.D. performed the phytochemical and biological analyses. P.E.D., N.S., I.A.A.Y, A.F.M., and P.P. performed data analyses of the experimental assays. All authors contributed to manuscript writing and preparation.

Conflicts of Interest

The authors declare no conflicts of interest.

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