

Green Synthesis of Copper Oxide Nanoparticles using Protein Extracted from *Thymus Vulgaris* Leaf Extract: Characterisation and Antibacterial Activity Against MDR Strains of Bacteria

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Abstract: The growing resistance to conventional antibiotics has made the use of antimicrobial medicines less effective in recent years. Because nanoparticles interact with bacterial membranes and can be developed into powerful antimicrobial agents, they can help combat antimicrobial resistance, according to recent discoveries. This study examines the impact of sonication-mediated production of copper oxide nanoparticles (Thy-CuO NPs) on multidrug-resistant bacteria (MDR) using protein obtained from *Thymus vulgaris* leaf extract (Thy-Protein). The antibacterial activity of the synthesised Thy-CuO NPs using Thy-Protein was evaluated against Gram-positive Methicillin-Resistant *Staphylococcus aureus* and Gram-negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* by calculating the Zone of Inhibition (ZOI) and Minimum Inhibitory Concentration (MIC). FTIR, XRD, HRTEM-EDS, and a double-beam UV-Vis Spectrophotometer were used to characterise Thy-CuO NPs. XRD confirmed that the copper oxide nanoparticles were crystalline. The nanoparticles ranged from 20–22 nm. The most inhibited bacteria were *Escherichia coli* (23.5 mm for Thy-CuO NPs, 18 mm for Thy-Protein) and *Klebsiella pneumoniae* (20.5 mm for Thy-CuO NPs, 13 mm for Thy-Protein). These results highlight the potential future uses of Thy-CuO NPs using Thy-Protein for a range of drug-delivery applications, as an affordable, biodegradable and environmentally benign way to suppress MDR bacterial strains.

Keywords: Copper Oxide Nanoparticles; Antimicrobial Resistance; *Thymus Vulgaris*; Multidrug-Resistant Bacteria; Vancomycin-Resistant Enterococci; *Pseudomonas Aeruginosa*; Methicillin-Resistant *Staphylococcus Aureus*.

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1. Introduction

Before antibiotics were discovered in the mid-20th century, ancient civilisations used antimicrobial substances to fight illnesses because of their therapeutic qualities [2]. Unfortunately, pathogens have become tolerant of frequently used antibiotics because antibiotics cannot change when a pathogen's resistance increases [1]. Clinical isolates that are known to develop antibiotic resistance and spread quickly in hospital settings include *Pseudomonas aeruginosa*, Methicillin-Resistant *Staphylococcus*

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aureus (MRSA), Enterococci, particularly Vancomycin-Resistant Enterococci (VRE), and members of the Enterobacteriaceae family, including *Klebsiella pneumoniae*, *Escherichia coli*, and *Proteus* sp [6]. Over the past 20 years, public health standards have declined in many parts of the world due to rising infectious diseases, bringing the world back to a pre-antibiotic era [3]. Recent attempts to combat antimicrobial resistance include using nanoparticles as potent antibiotics and investigating antimicrobial nanomaterials that microbial pathogens cannot easily resist [4]. Because of their distinct chemical and physical characteristics, nanomaterials have shown this potential through their high surface area-to-volume ratio, which allows surface modifications to interact directly with microbial membranes and contribute to more effective antibacterial medications [5]. Recently, researchers have investigated metal-based nanoparticles, particularly against pathogens the WHO has designated as priority [9]. Nanomaterials combat bacteria using several techniques [7]. They can interact with biological components, produce reactive oxygen species, dissolve the cell wall and membrane, and transport drugs by fusing with the membrane [10].

When paired, antibiotics and nanoparticles are more stable than free antibiotic molecules and can penetrate bacterial cell membrane barriers to reach specific locations more precisely [8]. Because nanoparticles can adhere to targets inside bacterial cells, preventing bacterial growth and metabolism and ultimately killing them, drugs administered as nanoparticles at the same dose are more effective against bacteria [11]. Because of their modified physicochemical characteristics, nanoparticles exhibit a variety of antibacterial properties. This is because nanoparticles are smaller than their chemical equivalents. This size reduction changes their pharmacokinetics, making it easier for them to pass through bacterial barriers and enter target tissues. This allows nanoparticles to exhibit potent antibacterial properties even without an antibiotic molecule [12]. Copper nanoparticles can be used as bacterial adjuvants to combat MDR bacteria [13]. Certain organisms, such as *Pseudomonas aeruginosa*, are more vulnerable to copper nanoparticles than others, such as *Enterococcus faecalis*, *S. aureus*, and *E. coli* [15]. Researchers can produce copper nanoparticles using physical, chemical, or biological processes. The biological approach uses fungi, bacteria, and even plant extracts. Physical methods for producing nanoparticles include ball milling, pulse wire discharge, and pulse laser ablation [14]. Chemical reduction, sono-chemical, electrochemical, microwave reduction, sono-electrochemical, hydrothermal, microemulsion, sol-gel, photochemical, and thermal breakdown are some of the most varied chemical processes. The synthesis technique determines the size, shape, and particle size distribution of the produced copper nanoparticles [16].

Nanoparticles can be synthesised using various methods. Sonication is an efficient technique for Cu^{2+} reduction in nanoparticle synthesis. It uses high-intensity ultrasonic waves, which offer several advantages, including improved mixing, faster reaction times, and control over particle size [17]. As a result, sonication is a useful technique for creating copper nanoparticles. In this study, Researchers chose a biological approach to synthesise copper oxide nanoparticles because it is eco-friendly. Researchers used protein extracted from plant leaves, as protein-conjugated nanoparticles may have therapeutic uses [18]. Tannins, alkaloids, and flavonoids are only a few of the many secondary metabolites found in plants, many of which exhibit potent antibacterial qualities in vitro [20]. Proteins and peptides with antimicrobial activity, found naturally in the innate defense systems of bacteria, fungi, insects, animals, and plants, have received more attention in recent years [37]. These defensive chemicals can specifically target and inhibit both Gram-positive and Gram-negative bacteria [21]. Compared with metal- or carbohydrate-based nanoparticles, protein-based nanoparticles are often more suitable for use in food, medicine, cosmetics, and other industries [33]. This is because proteins are easy to modify on their surface, allowing drugs or other biomolecules to bind via covalent, ionic, hydrogen, and other bonds [38]. Numerous synthesis methods have been established for creating nanoparticles using both plant- and animal-based proteins [19]. This work aimed to investigate the effects of copper oxide nanoparticles produced using a protein extracted from *Thymus vulgaris* leaves. Researchers used gentamicin as the positive control and compared the antibacterial activity of the extracted protein (Thy-Protein) and the synthesised copper oxide nanoparticles (Thy-CuO NPs) [22].

2. Materials and Methods

All the chemicals were of standard quality. Dried Thyme leaves (*Thymus vulgaris*) were used for the extraction of protein.

2.1. Extraction of Protein

A 10-gram sample of dried thyme leaves was soaked in 200 mL of distilled water and then heated in a water bath at 60°C for 30 minutes. Afterward, the mixture was incubated for 24 hours in an orbital shaker [23]. Following filtration, 150 mL of extract was obtained, to which 50 g of ammonium sulfate was added gradually at intervals. At the same time, the sample was placed on a magnetic stirrer at 4000 rpm to obtain protein using the salting-out method [24]. After letting the protein precipitate for half an hour using a magnetic stirrer, the sample was refrigerated. The sample was agitated for 30 minutes the following day, then centrifuged for 15 minutes at 8000 rpm to extract the precipitated protein. Phosphate-Buffered Saline (PBS) (4 mL) was added to this precipitate and properly mixed before being transferred to the dialysis membrane (Himedia). The dialysis membrane containing the sample was transferred into a reagent bottle filled with 200 mL PBS and was allowed to stir for 1 hour. The sample (Thy-Protein) was then collected in a Falcon tube and refrigerated until needed.

2.2. Synthesis of Copper Oxide Nanoparticles

To synthesise copper oxide nanoparticles, 100mL of 0.1 M aqueous copper sulfate solution was prepared in a 100mL beaker by stirring for 2 minutes. Next, 0.5mL of Thy-Protein was added to the mixture and sonicated in an ultrasonic cleaner for 10min at 27-30°C. Protein denaturation may occur above this optimal temperature. The effect of reaction time was assessed by sonicating for 10, 20, 30, 40, 50 and 60 min. Similarly, several precursor concentrations (0.05 M, 0.1 M, and 0.2 M) were used during synthesis to maximise the efficiency of the resulting copper oxide nanoparticle [25]. The solution's color changed from blue to brownish-green, indicating the production of Thy-CuO NPs. Following a 10-minute centrifugation at 10,000 rpm, the resulting copper oxide nanoparticles (Thy-CuO NPs) were dried for 24 to 48 hours in a glass desiccator. The synthesised Thy-CuO NPs were primarily characterised using a Double-Beam UV-Vis Spectrophotometer (Laboholic LH-2201), and spectra were recorded from 200 to 800nm. Further characterisation was done by Fourier-transform infrared spectroscopy (FTIR, SHIMADZU ITRACER 100), X-ray diffraction (XRD, BRUKER), High-Resolution Transmission Electron Microscopy (HRTEM, JEOL, Japan), and energy-dispersive spectroscopy (EDS). The extracted protein from dried thyme leaves (Thy-Protein) was analysed using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI - TOF MS/MS, Bruker) to obtain the protein sequence.

2.3. Antibacterial Activity

According to Galal et al. [26], the antibacterial activity of the synthesised Thy-CuO NPs was assessed by measuring the Zone of Inhibition (ZOI) using the Agar well diffusion method. Both ZOI and Minimum Inhibitory Concentration (MIC) were determined in Methicillin-Resistant *Staphylococcus aureus* (gram-positive bacteria) as well as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* (gram-negative bacteria). Thy-CuO NPs and gentamicin (positive control) were prepared at a concentration of 1 mg/mL by adding distilled water to the test ZOI. Researchers added 100 μ L of Thy-CuO NPs and 40 μ L of gentamicin stock solution to the corresponding wells for further investigation. Similarly, Researchers immediately filled the corresponding wells with 100 μ L of isolated Thyme-leaf protein (Thy-Protein). This allowed us to compare the effects of the synthesized Thy-CuO NPs and the Thyme-leaf protein (Thy-Protein) on each bacterium. Researchers assessed bacterial inhibition by observing the development of a clear zone around the wells after 24 hours of incubation at 37 °C. Additionally, Researchers computed the MIC at various concentrations (6.25 μ g/mL, 12.5 μ g/mL, 25 μ g/mL, 50 μ g/mL, and 100 μ g/mL) [27].

3. Result and Discussions

3.1. Synthesis and mechanism of Thy-CuO NPs formation

From the extracted Thy protein, 0.5mL was added to 100mL of 0.1 M aqueous copper sulfate solution, and the mixture was sonicated at 27-30°C for 10min to obtain Thy-CuO NPs. The solution color changed from blue to brownish-green, indicating nanoparticle formation. A Double Beam UV-Vis Spectrophotometer (Laboholic LH-2201) was used for the preliminary characterisation of Thy-CuO NPs produced by sonication (Figure 1).

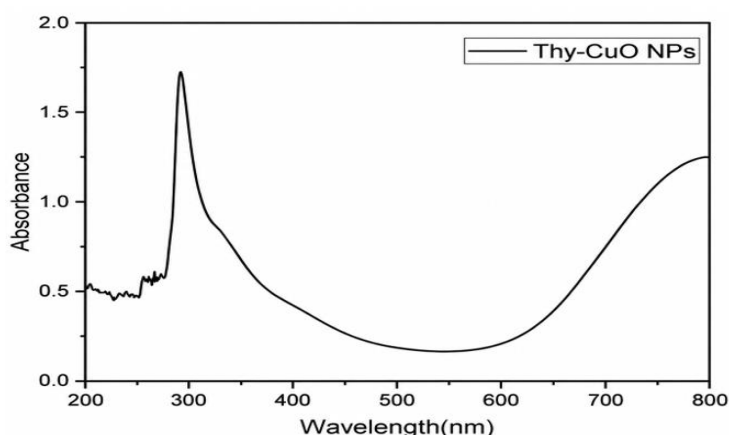


Figure 1: UV spectrum of Thy-CuO NPs synthesised under optimal conditions (temperature range of 27–30°C, reaction period of 10 minutes, and precursor concentration of 0.1 M)

According to Sankar et al. [28], an absorption peak at 292 nm in spectra obtained between 200 and 800 nm confirmed the synthesis of copper oxide nanoparticles (Thy-CuO NPs).

3.2. Protein Analysis of Thy-Protein

Protein estimation was done using Lowry's method, which provided a protein concentration of 3.612mg/mL (Figure 1, Supplementary). Next, Researchers measured the molecular weight of the isolated protein by SDS-PAGE [29]. As illustrated in Figure 2, the molecular weight of the isolated protein (Thy-Protein) was found to be 51kDa. Researchers stained the protein bands on the gel with Coomassie Brilliant Blue R-250 and rinsed the stain after some time to improve protein visibility.

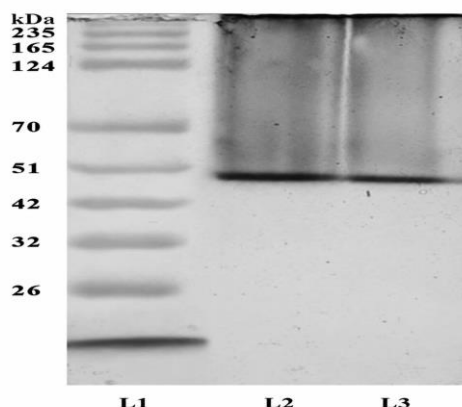


Figure 2: SDS-PAGE electrophoresis of *Thymus vulgaris* leaf protein. the lanes are as follows:(1) L1 is ladder with respective protein masses. (2) L2 and L3 are ammonium sulfate-precipitated protein samples (Thy-Protein)

Researchers excised the bands from the gel and analysed them by matrix-assisted laser desorption/ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF MS/MS; BRUKER). Researchers obtained peptide matches for the experimental m/z dataset using the Mascot search program [30]. Below is the identified protein sequence from the peptide matches.

3.2.1. Fasta Sequence

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1      MTLNGYLSTD FHIKSPAKKF  FQTCIETMDL  PKDDVTVEIE  EVPLEKKKTT
51     FRIEGFQISE  WYKSFKGTIT  PDMATWQNP  DGYKKLEGTM  ITHVEDNDCD
101    RAILTVKYEK  INSDIKDPGT  IMDTFVEFFK  EMDEYLVEDF  N

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3.3. Effect of Parameters

To determine the optimal conditions for producing efficient nanoparticles, Researchers examined the effects of various precursor concentrations and reaction time.

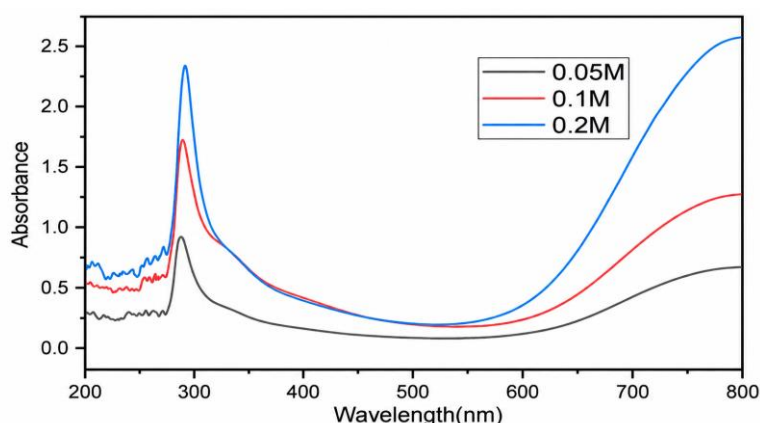


Figure 3: Thy-CuO NPs' UV spectrum at different precursor concentrations of 0.05 M, 0.1 M, and 0.2 M

Color changes were observed at precursor concentrations of 0.05 M, 0.1 M, and 0.2 M. Figure 3 shows the UV spectra of the produced Thy-CuO NPs at different precursor concentrations.

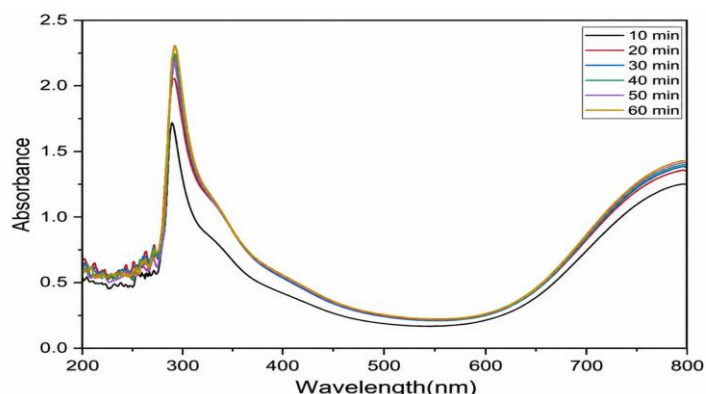


Figure 4: Thy-CuO NPs' UV spectrum at different reaction times of 10, 20, 30, 40, 50, and 60 minutes

Similarly, Researchers used sonication times of 10, 20, 30, 40, 50, and 60 minutes to evaluate the impact of reaction time; as shown in Figure 4, reaction time did not affect Thy-CuO NP formation. Therefore, a 0.1 M precursor concentration and a 10-minute reaction period were considered suitable for synthesising the most effective nanoparticles.

3.4. Characterisation of Thy-CuO NPs by FTIR, XRD, HRTEM and EDS

3.4.1. FTIR

The production of Thy-CuO NPs was verified by FTIR (SHIMADZU, IRTACER 100) analysis [31]. As shown in Figure 5, the resulting FTIR spectra of Thy-Protein and Thy-CuO NPs were recorded and compared by analysis of the FTIR spectra from 400-4000 cm^{-1} which revealed new peaks at 420.48 cm^{-1} indicating metal-oxygen bond (Cu-O), 605.35 cm^{-1} indicating the compounds likely derived from plant proteins which contains sulphur, 864.11 cm^{-1} (C-H bend), 1091.71 cm^{-1} (C-N stretch) indicating the presence of aliphatic amines, 1396.46 cm^{-1} (OH bend), 3034.03 cm^{-1} suggesting ammonium ion in protein, and 3118.9 cm^{-1} indicating (aromatic C-H stretch) in both Thy-CuO NPs and Thy-Protein as seen from Table 1 [32].

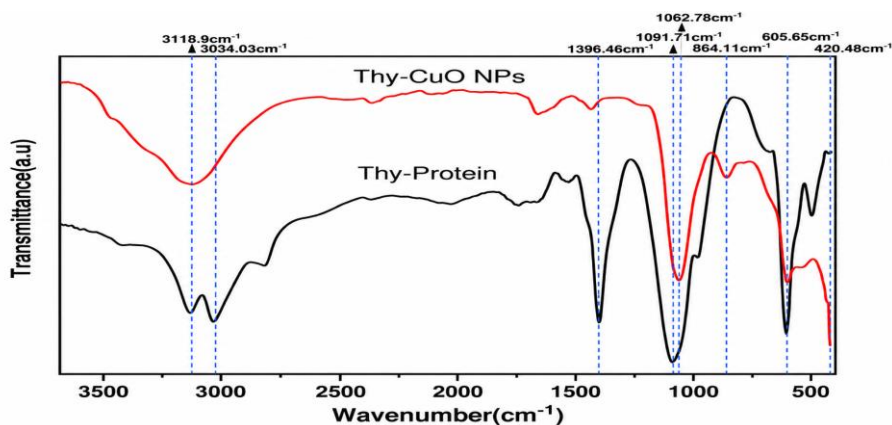


Figure 5: Comparative analysis of the FTIR spectrum of Thy-Protein and Thy-CuO NPs showing shifts in characteristic peaks

Two separate peaks were identified at 1091.71 cm^{-1} and 1062.78 cm^{-1} , both of which are associated with (C-N stretch). However, the peak at 1062.78 cm^{-1} is linked to the (C-N) groups in the synthesised nanoparticle.

Table 1: FTIR characteristic peaks of Thy-Protein and Thy-CuO NPs with their corresponding functional group assignments

Wavenumber (cm^{-1})		Functional groups
Thy-Protein	Thy-CuO NPs	
	420.48	Cu-O
605.35	605.35	S-S stretch
	864.11	C-H bend

1091.71	1062.78	C-N stretch
1396.46		OH bend
3034.03		Ammonium ion
3118.9	3118.9	C-H stretch

In contrast, the peak at 1091.71 cm⁻¹ is associated with C-N stretching in plant extract proteins, indicating their role as stabilising and capping agents during nanoparticle formation.

3.4.2. XRD

The crystalline structure of the synthesized copper oxide nanoparticles (Thy-CuO NPs) was determined using XRD analysis (BRUKER) (Figure 6). The diffraction intensities were measured between 30° and 80° (2θ). Distinct diffraction peaks were observed at 32.00°, 35.58°, 39.18°, 43.78°, and 54.52°, corresponding to Miller indices (110), (-111), (111), (111), and (020), respectively, which match the reference pattern for copper (II) oxide (CuO) (ICDD #089-5899), confirming the monoclinic structure of Thy-CuO NPs.

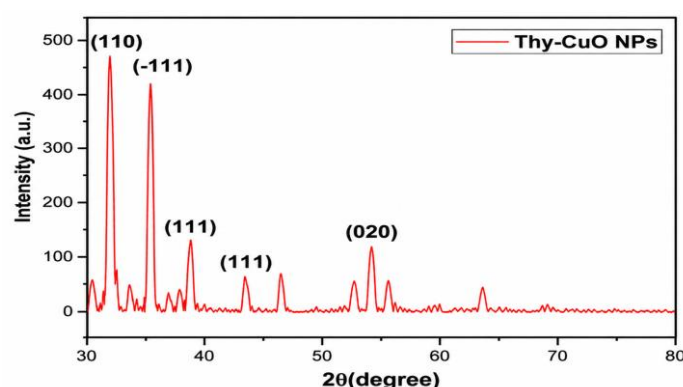


Figure 6: XRD pattern of synthesised Thy-CuO NPs

The strong, distinct diffraction peaks suggest that the synthesised Thy-CuO NPs have a high degree of crystallinity. Similar results supporting the monoclinic phase of CuO have been previously documented for copper oxide nanoparticles made with *E. alata* extract [34].

3.4.3. HRTEM

HRTEM (JEOL, Japan) was used to assess the morphology of Thy-CuO NPs, revealing their shape, size, and size distribution. The resulting nanoparticles tend to aggregate and exhibit an irregular shape (Figure 7). This apparent clustering likely results from the increased concentration of plant material (Thy-Protein) used as a capping agent during synthesis.

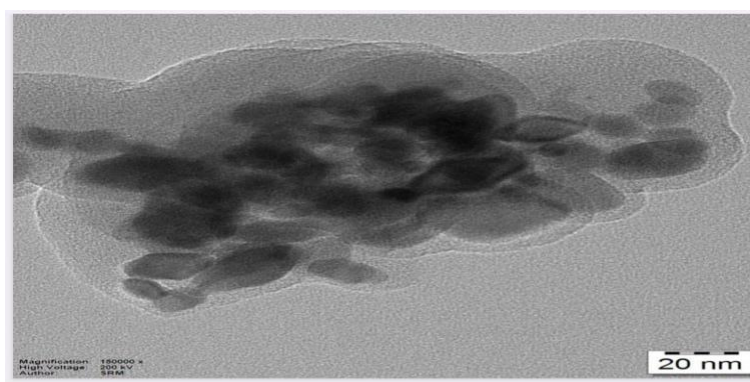


Figure 7: HRTEM image of Thy-CuO NPs showing particle morphology

The interaction between the protein-encased nanoparticles causes copper particles to group together, which is perceived as clusters [35] (Figure 8).

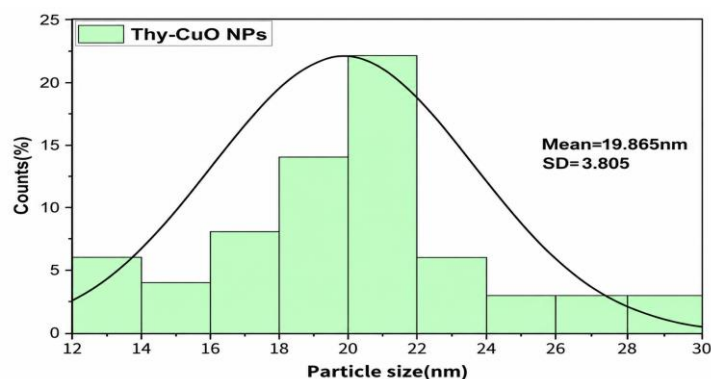


Figure 8: Histogram showing size distribution of Thy-CuO NPs measured from HRTEM images; the average size of Thy-CuO NPs ranges from 20-22nm

The size distribution was evaluated by analyzing the HRTEM images using ImageJ software, and the average size of the produced Thy-CuO NPs ranged from 20 to 22 nm [36]. A comparable histogram is shown.

3.4.4. EDS

The elements in the synthesised copper oxide nanoparticles (Thy-CuO NPs) were analysed both qualitatively and quantitatively using EDS (Figure 9).

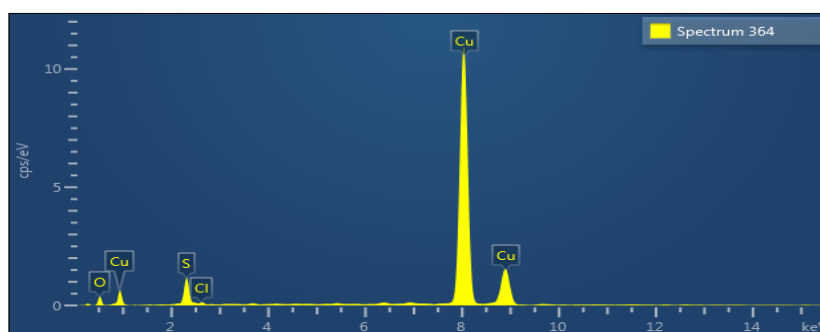


Figure 9: The EDS spectrum of Thy-CuO NPs, where the peaks for copper and oxygen indicate their formation

Since copper (Cu) was present in the greatest quantity, as shown in Table 2, the researchers used EDS to confirm the purity of the Thy-CuO NPs. Cu (92.83%), S (4.71%), O (2.00%), and Cl (0.46%) were the main constituents of Thy-CuO NPs.

Table 2: The elemental composition of Thy-CuO nanoparticles as determined through energy-dispersive X-ray spectroscopy (EDS)

Element	Weight%
O	2.00
S	4.71
Cl	0.46
Cu	92.83
Total:	100.00

The protein recovered from *Thymus vulgaris* leaf extract (Thy-Protein) and the starting material were the sources of all these components.

4. The Antibacterial Activity of Thy-CuO NPs

The antibacterial activity of the synthesized copper oxide nanoparticles (Thy-CuO NPs) against multidrug-resistant strains of bacteria, including Methicillin-Resistant *Staphylococcus aureus* (gram-positive bacteria), *Escherichia coli*, *Pseudomonas*

aeruginosa, and *Klebsiella pneumoniae* (gram-negative bacteria), was evaluated by measuring the ZOI (using the Agar well diffusion method), as indicated in Tables 3 and 4.

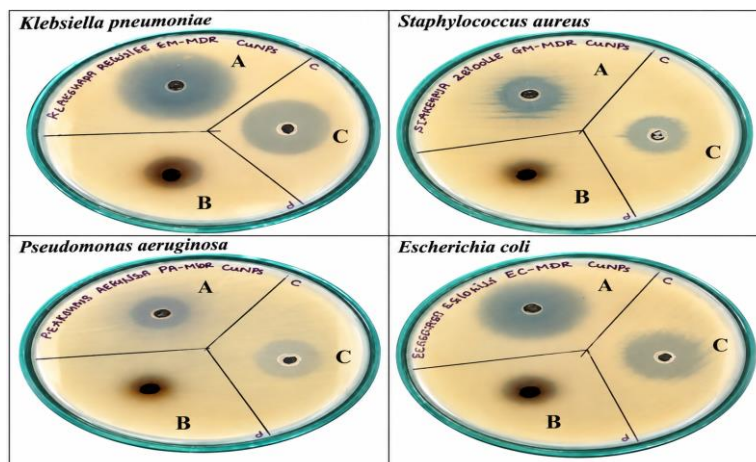


Figure 10: The zone of Inhibition (ZOI) of different bacterial strains observed, where (A) is synthesised Thy-CuO NPs, (B) protein from *Thymus vulgaris* leaf extract (Thy-Protein), and (C) is the positive control(gentamicin). Thy-CuO NPs have better antibacterial activity compared to Thy-Protein, as seen from the clear zones formed

The ZOI was measured between protein extracted from *Thymus vulgaris* leaf extract (Thy-Protein) and synthesised copper oxide nanoparticles (Thy-CuO NPs) (Figure 10).

Table 3: Zone of inhibition(mm) of Thy-CuO NPs, Thy-Protein, and positive control against MDR bacterial strains, highlighting the enhanced antibacterial activity of nanoparticles

Sample	Zone of Inhibition (mm) (Mean $\pm$ Standard Deviation)			
	<i>Klebsiella Pneumoniae</i>	<i>Escherichia Coli</i>	<i>Pseudomonas Aeruginosa</i>	Methicillin-Resistant <i>Staphylococcus Aureus</i>
Thy-CuO NPs	20.5 $\pm$ 0.70	23.5 $\pm$ 0.70	15 $\pm$ 1.41	16.5 $\pm$ 0.70
Thy-Protein	13 $\pm$ 1.41	18 $\pm$ 1.41	8.5 $\pm$ 0.70	10 $\pm$ 1.41
Positive Control(Gentamicin)	25.5 $\pm$ 0.70	23.5 $\pm$ 0.70	20.5 $\pm$ 0.70	19 $\pm$ 0

This was done to compare each bacterium's response to the produced Thy-CuO NPs and extracted Thy-Protein, with gentamicin used as the positive control.

Table 4: Minimum inhibitory concentration(μ g/mL) of Thy-CuO NPs against MDR bacterial strains

Concentration of Thy-CuO NPs (μ g/mL)	<i>Klebsiella Pneumoniae</i>	<i>Escherichia Coli</i>	<i>Pseudomonas Aeruginosa</i>	Methicillin-Resistant <i>Staphylococcus Aureus</i>
6.25	18.814 $\pm$ 0.007	11.219 $\pm$ 0.015	12.330 $\pm$ 0.014	17.146 $\pm$ 0.009
12.5	25.858 $\pm$ 0.013	35.299 $\pm$ 0.014	34.417 $\pm$ 0.014	29.027 $\pm$ 0.012
25	44.271 $\pm$ 0.008	53.747 $\pm$ 0.019	51.084 $\pm$ 0.010	51.260 $\pm$ 0.012
50	56.174 $\pm$ 0.006	73.259 $\pm$ 0.016	66.847 $\pm$ 0.011	77.902 $\pm$ 0.013
100	85.510 $\pm$ 0.004	87.139 $\pm$ 0.014	81.481 $\pm$ 0.011	89.558 $\pm$ 0.015

Escherichia coli showed the greatest inhibition (23.5 mm for Thy-CuO NPs, 18 mm for Thy-Protein), followed by *Klebsiella pneumoniae* (20.5 mm for Thy-CuO NPs, 13 mm for Thy-Protein).

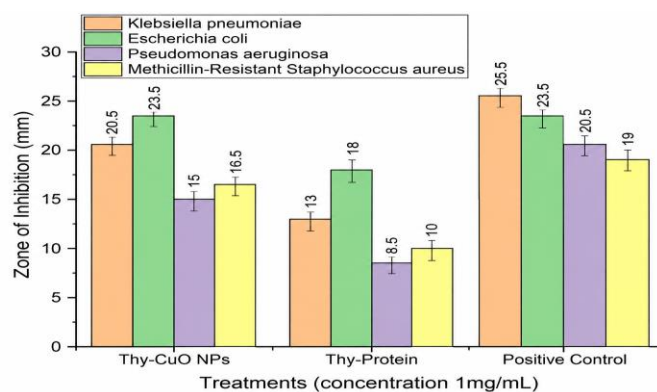


Figure 11: Zone of inhibition(mm) observed during various treatments such as Thy-CuO NPs, Thy-Protein, and positive control against MDR bacterial strains, highlighting the enhanced antibacterial activity of Thy-CuO NPs in comparison to Thy-Protein. Error bars represent deviations from the mean

Significant ZOI was also demonstrated by Methicillin-Resistant *Staphylococcus aureus* (16.5 ± 0.70 for Thy-CuO NPs, 10 ± 1.41 for Thy-Protein), followed by *Pseudomonas aeruginosa* (15 ± 1.41 for Thy-CuO NPs, 8.5 ± 0.70 for Thy-Protein), as seen in Figure 11 and Table 3 (Figure 12).

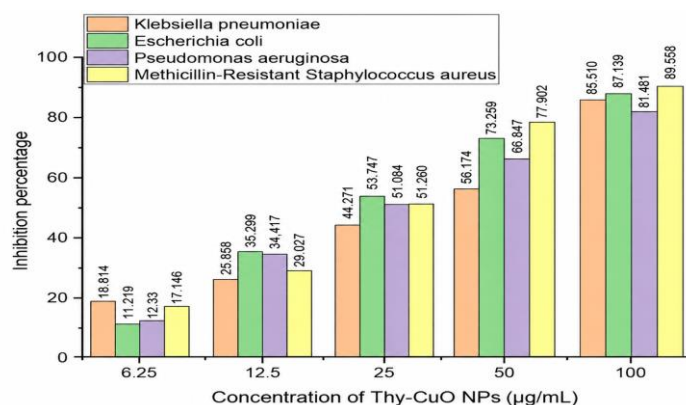


Figure 12: Inhibition percentage obtained for different concentrations of Thy-CuO NPs against MDR bacterial strains. Error bars represent the deviations from the mean

This finding shows that the synthesized Thy-CuO NPs exhibit superior inhibition compared with the plant protein (Thy-Protein). MIC values were also obtained for each bacterium, as shown in Table 4.

5. Conclusion

This study reports the production of copper oxide nanoparticles (Thy-CuO NPs) using protein obtained from the leaf extract of *Thymus vulgaris* (Thy-Protein) as a stabilising agent, via sonication. Various characterization techniques used confirmed the formation of Thy-CuO NPs. A TEM image showing the generated Thy-CuO NPs, with a diameter ranging from 20 to 22 nm, was used to demonstrate their production, along with a distinctive UV absorption peak at 292 nm and FTIR analysis verifying the capping and efficient stability of the metal nanoparticles by Thy-protein. Thy-CuO NPs demonstrated strong antibacterial activity against a variety of multidrug-resistant bacteria, such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and Methicillin-Resistant *Staphylococcus aureus* (a Gram-positive bacterium), by calculating the zone of inhibition (ZOI) and the minimum inhibitory concentration (MIC). The largest Zone of Inhibition was observed in *Escherichia coli* (23.5 mm for Thy-CuO NPs, 18 mm for Thy-Protein), followed by *Klebsiella pneumoniae* (20.5 mm for Thy-CuO NPs, 13 mm for Thy-Protein).

Significant ZOI was also demonstrated by Methicillin-Resistant *Staphylococcus aureus* (16.5 ± 0.70 for Thy-CuO NPs, 10 ± 1.41 for Thy-Protein), followed by *Pseudomonas aeruginosa* (15 ± 1.41 for Thy-CuO NPs, 8.5 ± 0.70 for Thy-Protein). The superior antibacterial activity of Thy-CuO NPs, especially against multidrug-resistant bacteria, establishes the originality of this work. In addition to outperforming the native Thy-Protein, the 23.5 mm Zone of Inhibition in *Escherichia coli* and 20.5

mm in *Klebsiella pneumoniae* also exceed previously reported *Thymus vulgaris*-mediated metal nanoparticles. Thy-CuO NPs were more effective than *T. vulgaris*-stabilized silver nanoparticles, which showed ZOI values of (14.5 ± 0.7) for *K. pneumoniae* and (15.6 ± 0.4) for *E. coli*. Similarly, Thy-CuO NPs function better than iron nanoparticles produced from *T. vulgaris*, which displayed ZOI values of (15 ± 0.20) for *S. aureus* and (15 ± 0.19) for *E. coli*. The study confirms that synthesising nanoparticles from plant protein is an effective, simple, and non-toxic method. This innovative approach contributes to nanotechnology by providing an eco-friendly alternative to existing antimicrobials. Further research can examine specific microbial mechanisms and in vitro toxicity profiles to advance Thy-CuO NPs toward clinical and commercial applications.

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Ethics and Consent Statement: This research adheres to ethical guidelines and obtains informed consent from all participants. Confidentiality measures were implemented to safeguard participant privacy.

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