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Batool M. Ubed

`pgs.batool.obaid@uobasrah.edu.iq`

University of Basrah

Enas A. Bady

University of Basrah

Ahmed A. A. Alsaad

University of Basrah

Rafeef Amer Abdul-Jabar

University of Basrah

Sabaa Ali Mohammed Al-Fadal

University of Basrah

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Copper Nanoparticles by *Rhodotorula diobovata* as an antimicrobial agent against Antibiotic Resistance

Batool M. Ubed ^{*,1}, Enas A. Bady ¹, Ahmed A. A. Alsaad ², Rafeef Amer Abdul-Jabar ¹ and Sabaa Ali Mohammed Al-Fadal ³

1 Department of Clinical and Laboratory Sciences, College of Pharmacy, University of Basrah, Basra, IRAQ.

2 Department of Pharmaceuticals, College of Pharmacy, University of Basrah, Basra, IRAQ.

3 Department of Pharmacognosy and Medicinal Plants, College of Pharmacy, University of Basrah, Basra, IRAQ.

***Corresponding author**

Abstract

Copper's distinctive physical and chemical characteristics render it a prevalent metallic element. This research aimed to explore the eco-friendly, straightforward, and cost-effective green synthesis of copper nanoparticles (Cu-NPs) using *Rhodotorula diobovata* Strain B2© and copper sulphate as starting material. The influence of the incubation time and the effect of biosynthesis Cu-NPs on the growth of bacteria and yeast were studied in this research. The direct and rapid formation of Cu-NPs occurs when *Rhodotorula diobovata* interacts with Cu⁺² ions, which is visually indicated by a color transition from light blue to dark bluish-green. UV-Vis, FT-IR, DLS and zeta-potential, TEM, and EDX were used to characterize the shape, size, and structure of the produced Cu-NPs. The UV-Vis measurement revealed a surface plasmon resonance (SPR) peak at 606.5 and 622.5 nm due to the capping activity of *Rhodotorula diobovata* biomolecules in the synthesis of Cu-NPs. Energy dispersive spectroscopy (EDX) revealed low-intensity peaks of phosphorus (P), calcium (Ca), and potassium (K) elements and high-intensity metallic peaks of copper (Cu), oxygen (O), and sulfur (S). The synthesis of Cu-NPs is triggered by the presence of functional groups -OH, C=C, and C-H, as proven by FT-IR analysis. The TEM investigations also showed that the particles ranged in size from 33 to 63 nm and had a spherical form. DLS and zeta-potential analysis indicate that the particle sizes are 22.1 nm and 19.5 nm, and the zeta-potentials are -26.8 and -17.3, confirming the stability of the Cu-NPs. The antibacterial activity of Cu-NPs against *Staphylococcus aureus* from diabetic foot infection and *E. coli* and *Candida albicans* from vaginal swabs was demonstrated. The produced Cu-NPs' hemolytic activity was comparable to that of the control Ringer solution.

Keywords: Nanotechnology, antibiotic resistance, copper nanoparticles, green synthesis.

Introduction

Nanotechnology is considered an emerging interdisciplinary research domain that concentrates explicitly on nanoparticles (NPs) exhibiting enhanced size-dependent characteristics, including the durability of the colloidal component, extensive surface area, and heightened bioavailability, among others (1)(2). Nanoparticles (NPs) are defined as ultra-fine particles, characterized by a size ranging from 1 to 100 nm in diameter (3). The utility of nanoparticles extends across various domains within the biomedical and pharmaceutical fields, encompassing diagnostics, bio-markers, bio-imaging, cosmetics, immunological applications, cardiology, genetic engineering, as well as bioremediation for environmental purposes, water purification, and energy generation (4)(5)(6). Moreover, there is a burgeoning interest in creating pharmaceuticals at the nanoscale to address global health challenges. To illustrate, the antibiotic resistance crisis, which has imposed significant clinical and economic strains on healthcare infrastructures, can be mitigated through the antibacterial properties of nanoparticles (7)(8). According to estimates from the World Bank, it is projected that by 2050, antimicrobial resistance (AMR) could be responsible for as many as 10 million deaths annually. This projection serves as a critical warning to nations worldwide, emphasizing the urgent need to implement stringent regulations governing the sale and utilization of antibiotics to mitigate this escalating public health crisis (9)(10). The etiological factors of viral, bacterial, and other microscopic infections operate on a microscopic scale; hence, the most effective approach to combating them is at the nanoscale. Consequently, Nanotechnology serves as the pathway to addressing and detecting various viral, bacterial, and fungal illnesses (11). Among the various transition metals being studied, copper nanoparticles are comparatively more cost-effective than other metals such as platinum, silver, and gold (12). Copper possesses distinctive physical and chemical characteristics, rendering it a prevalent metallic element, ranking as the 26th most abundant element in the Earth's crust (13). These Cu-NPs exhibit notable physical and chemical characteristics (14), including optical (15), catalytic (16), heat transfer (17), and magnetic properties (18), as well as a high surface area to volume ratio and biocidal effects (19).

Traditionally, the production of nanoparticles has been achieved using physical, chemical, and mechanical methods. Nevertheless, the elevated expenses and hazardous nature associated with these techniques have led to a transition in nanoparticle manufacturing towards biological systems (20)(21). The process known as "Green biosynthesis" or "biological fabrication" of nanoparticles involves the utilization of plant and microbial extracts. Biogenic manufacturing stands as a straightforward, cost-effective, and environmentally conscious substitute to physical and chemical methodologies in nanoparticle production, yielding biocompatible and non-toxic nanoparticles. These alternatives steer clear of the utilization of expensive and potentially hazardous chemicals like solvents and stabilizing agents (22). The compounds generated through this method exhibit enhanced stability, are easily reproducible, cost-effective, reliable, sustainable, and generate minimal waste. Green synthesis obviates the requirement for capping moieties by leveraging microbial enzymes that function as both capping and reducing agents, resulting in the stabilization of nanoparticles (23).

Microbes possess the capacity to accumulate and deactivate heavy metals while simultaneously converting metal salts into metal or metal oxide nanoparticles via various enzymes. Bacteria, fungi, and yeast have been employed for the intra- or extracellular biosynthesis of metal and metal oxide nanoparticles (24)(25). The extracellular synthesis technique, specifically, offers advantages over the intracellular method because it eliminates numerous synthesis stages, such as sonication for cell wall degradation, multiple centrifugations, and washing steps for nanoparticle purification, thereby enhancing the feasibility of the approach (24).

As a eukaryotic, unicellular microbe, yeast is a member of the phyla Ascomycota, Basidiomycota, and the kingdom Fungi (26) (27). In the field of nanotechnology, yeasts have drawn the attention of researchers because they are unicellular fungi with similar properties to fungi, but they grow more quickly, making them better organisms for the biofabrication of NPs with high yields in a short amount of time (28)(29). Yeasts have numerous benefits for use in the extracellular and intracellular biosynthesis of metal and metal oxide nanoparticles with high-yield production.

Rhodotorula species are common yeasts that can be found in milk, fruit juice, lakes, soil, air, and ocean water. The red, orange, or pink colonies of *Rhodotorula* species frequently grow on toothbrushes, shower curtains, bathtubs, and toilet bowls (30)(31). The most common infections caused by *Rhodotorula* spp. were found to be related to the bloodstream, central nervous system (CNS), ocular infections, and peritoneal dialysis-associated peritonitis (30)(32). *Rhodotorula* spp. are commensals of human and animal microbiota and colonize the skin, nails, and gastrointestinal, urinary, and respiratory tracts (30)(33).

An oleaginous, marine red yeast called *Rhodotorula diobovata* (Basionym: *Rhodosporidium diobovatum*) can simultaneously produce high-quality single-cell oils for the generation of biodiesel and health-promoting substances called carotenoids that are helpful as food coloring agents in the food industry (34)(35). This study was the first to report on the synthesis of Cu NPs using *Rhodotorula diobovata*.

2. Materials and Methods

2.1. Yeast and Bacterial Isolates

Rhodotorula diobovata strain was used in this study as a source of copper nanoparticle synthesis. It was previously isolated from soil and identified microscopically and genetically by the 16S ribosomal RNA gene. *Staphylococcus aureus*, *E. coli* and *Candida albicans* were previously isolated from diabetic foot infections and vaginal infections respectively and identified microscopically by Gram staining to evaluate the antimicrobial activity of the Cu-NPs. All these isolates were obtained from the Microbiology lab/ Clinical and Laboratory Sciences/ College of Pharmacy/ University of Basrah.

2.2. Biosynthesis of Copper Nanoparticles (Cu-NPs)

In this investigation, *Rhodotorula diobovata* supernatant was used to synthesize Cu-NPs, as described by (36). An isolate of *Rhodotorula diobovata* was activated on nutrient agar and incubated for 24 hours at 37 °C. Then, *Rhodotorula diobovata* was inoculated in two flasks (250 ml) of nutrient broth for 24 hours in an incubating shaker at 150 rpm and 30°C. After that, two flasks (250 ml) of CuSO₄.5H₂O (10 mM) were added drop by drop to the yeast cultures, and the first flask was left in the laboratory without incubation for 1 hour, and the second flask was incubated for 48 hours in an incubating shaker at 150 rpm and 30°C. The light blue color of the solution turned greenish blue. After the yeast cell pellet was separated by centrifuging for 20 minutes at 6,000 rpm, the leftover supernatant was centrifuged for 15 minutes at 10,000 rpm to create the Cu-NPs. After being produced, the Cu-NP was dried in an oven at 80°C and used for additional characterization.

2.3. Characterization of Copper Nanoparticles (Cu-NPs)

2.3.1. UV-Visible Spectroscopy

The UV-vis spectrophotometer (Shimadzu UV-1800, Japan) was used to determine the optical characteristics of the samples, scanning the 200-800 nm range at a resolution of 1 nm (37).

2.3.2. FTIR Spectroscopy

FTIR spectroscopy was used to identify Cu-NPs organic functional groups (38). To achieve translucent circular pellets, 2 mg of biogenic Cu-NPs and 2 g of potassium bromide (KBr) were combined and crushed. The samples were then scanned using FTIR Alpha II (BRUKER Germany) in the 4,000 to 400 cm⁻¹ wavenumber range. Note that the control was a KBr pellet.

2.3.3. DLS and Zeta potential

As previously reported, the average size and stability of Cu-NPs were assessed at room temperature and neutral pH (38). In short, 1 milliliter of each sample was diluted in deionized water to assess the average size distribution. Samples were then analyzed using a zeta potential analyzer (Malvern Zeta-sizer, UK) and DLS.

2.3.4. Transmission Electron Microscopy

TEM was used to examine the topographical and morphological features of Cu-NPs (37). To do this, 1 milliliter of each sample was put onto copper grid thin films after being diluted in deionized water. TEM (Tecnai FEI, United States) was used to obtain the micrographs. Using ImageJ software, 10 particle sizes for each sample were determined to create the particle size distribution (PSD) plots.

2.3.5. Dispersive Energy X-ray Spectroscopy

As described by (39), Dispersive Energy X-ray spectroscopy was employed to conduct qualitative and quantitative measurements, elemental distribution mapping, and investigations into the catalyst's chemical states. A high-energy laser reflects off the surface, generating X-ray emissions. 1 g of dried Cu-NPs powder was used to measure EDX by the device BRUKER.

2.4. Antimicrobial Activity of the Biosynthesized Copper Nanoparticles (Cu-NPs)

The agar well diffusion method (40) was used to assess the produced Cu-NPs' antimicrobial properties against several human infections. Gram-positive *Staphylococcus aureus*, Gram-negative *E. coli* and *Candida albicans* were among the types that were evaluated. Sterile cotton swabs were used to evenly swab the pathogens onto sterile nutrient agar plates after they had been inoculated in nutrient broth and incubated for 24 hours at 37 °C., after that, microbial cultures were adjusted until the turbidity of 0.5 of the McFarland standards was achieved. A sterile stainless-steel cork-borer was used to create agar wells that were 6 mm in diameter. Using a micropipette and aseptic conditions, 50 µl of two mixtures of copper nanoparticles without incubation (1 hr.) and with incubation (48h.), solution of CuSO₄ and *Rhodotorula diobovata* extract, were placed into four wells. Following incubation for 24 hours at 37 °C, the zone of inhibition was measured with a centimeter ruler, and the value for each organism was noted and recorded in millimeters (mm).

2.5. Susceptibility Test for Selected Antibiotics and Antifungals

The Kirby-Bauer disk diffusion method, as described by (41), was used to measure the antibiotic and antifungal susceptibility test. Eighteen antibiotics and five antifungals were assessed against *S. aureus*, *E. coli*, and *Candida albicans*. First, bacterial pathogens were activated onto nutrient agar and incubated for 24 hours at 37°C, then the colonies were taken using sterile loops and suspended in 5 mL of nutrient broth, adjusting their turbidity compared to a 0.5 McFarland standard. The bacterial suspension was inoculated onto nutrient agar using a sterile cotton swab. After that, antibiotic and antifungal disks were placed on the inoculated plates and incubated for 24 hours at 37 °C. The zone of growth inhibition surrounding each disk was measured and classified as sensitive (S) or resistant (R) as compared to reference tables from the Clinical and Laboratory Standards Institute CLSI (42).

2.6. Minimum Inhibitory Concentration (MIC) against *S. aureus*

A stock suspension of copper nanoparticles was prepared using a gravimetric method and diluted using deionized water to obtain a range of nanoparticle concentrations (0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, and 64 mg/ml) (43). Vancomycin antibiotic and deionized water were used as positive and negative controls, gar well technique was

utilized (44). *S. aureus* was cultivated onto a nutrient agar plate and wells (6 mm) were punched on the surface of the agar (45). Each well was filled with a copper nanoparticle suspension of a specific concentration and the plates were then incubated at 37 °C. After 18-24 hours of incubation, the distance across each well was measured and evaluated (45). The lowest concentration at which bacterial growth is inhibited is considered the minimum inhibitory concentration MIC (44).

2.7. Hemolytic Assay of Copper Nanoparticles (Cu-NPs)

Copper Nanoparticles' hemolytic activity (46) was examined in one donor's entire blood. Thirty-eight from each of the five Cu-NPs test samples were incubated individually for one hour at 37 °C in a biological thermostat with an equivalent volume of blood. SDS was added as a positive control (PC) and Ringer solution as a negative control (NC) to the produced blank samples. The samples were centrifuged for 20 minutes at 2000 min⁻¹ following incubation.

The hemolysis coefficient was used to measure hemolytic activity. It was calculated spectrophotometrically (Unico 2802, Unico Sys, USA) using the samples' optical density at a wavelength of 415 nm, which corresponds to the oxyhemoglobin absorption band. A saline solution was used to bring 200 µl of the sample up to 6 mL to assess optical density.

To calculate hemolytic activity, the following formula was used: The formula for HC is $(O - NC) / (PC - NC) \times 100\%$, where O is the sample's determined optical density, NC is the negative control (the blank sample hemolyzes 0%), and PC is the positive control (100% hemolysis).

3. Results and Discussion

3.1. Biosynthesis of Copper Nanoparticles (Cu-NPs)

As seen in Figure (3-1), the solution of CuSO₄·5H₂O changed from light blue to greenish blue, confirming the biosynthesis of Cu NPs (1).

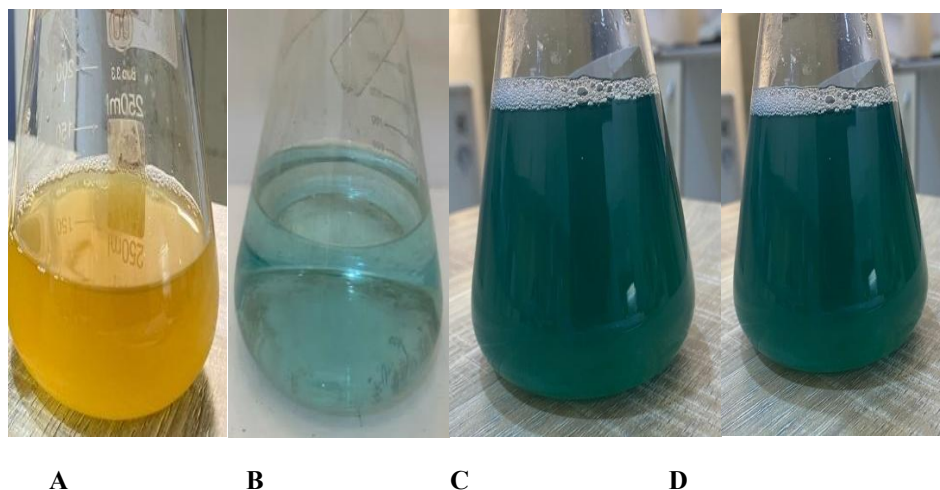


Figure 3-1. Shows the visual observation of the formation of Cu-NPs. A: *Rhodotorula diobovata* extract. B: CuSO₄·5H₂O solution. C: Cu-NPs without incubation (1 hour). D: Cu-NPs with incubation for 48 hours.

3.2. Characterization of Copper Nanoparticles (Cu-NPs)

3.2.1. Optical Properties

To determine the optical characteristics of Cu-NPs, UV-vis spectroscopy was used. Surface plasmon resonance (SPR) peaks were detected at 606.5 and 622.5 nm, as shown in Figure 3-2. These peaks were attributed to the effective bio-reduction of CuSO₄ to Cu-NPs. The distinctive surface plasmonic resonance (SPR) peak of copper nanoparticles is often seen in the 606.5–622.5 nm range; bigger particles may cause this resonance peak to move toward longer wavelengths. Depending on the specific characteristics of each particle, such as its size, shape, capping agent, and chemical makeup, the precise location of the SPR peak may change (47). This largely explains the appearance of the distinctive surface plasmonic resonance peak of the produced Cu-NPs at 622 nm. In the case of the reaction mixture without incubation (1 hour), the wavelength of Cu-NPs was at 622.5 nm, while in the case of the reaction mixture with incubation for 48 hours, the wavelength was at 606.5 nm. The solution of Cu-NPs that was without incubated had a longer wavelength than the solution of Cu-NPs that was incubated for 48 hours, which means the size of Cu-NPs that were incubated for 48 hours is smaller than the size of Cu-NPs without incubated.

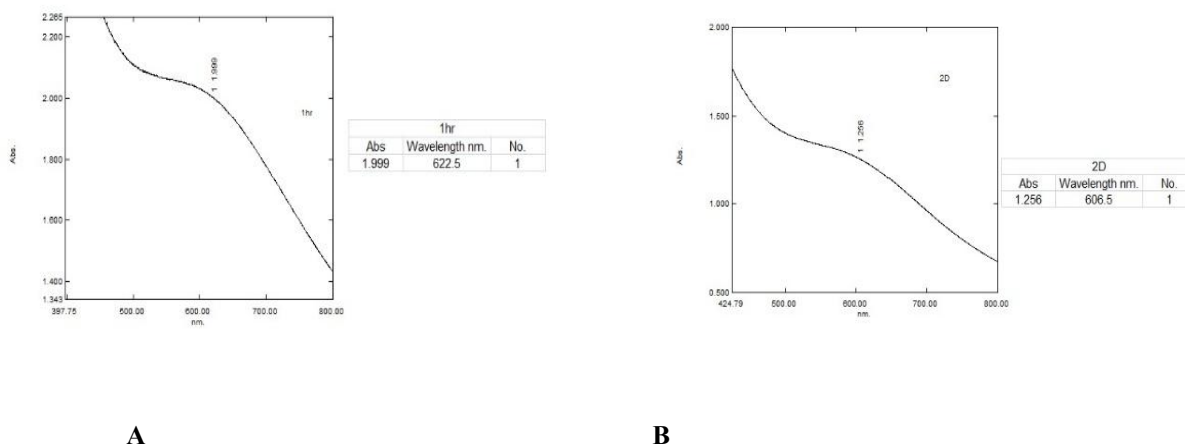


Figure 3-2. The UV-visible spectroscopy of Cu-NPs. A: Without incubation (1 hour). B: With incubation for 48 hours.

3.2.2. FTIR analysis

Similar absorbance bands were observed in the wavenumber ranges of 3,218–3,062 cm⁻¹, 2,958 cm⁻¹, and 1,635–1,335 cm⁻¹, as well as 1095 cm⁻¹ in the FTIR spectra of Cu-NPs without incubated (1 hour) and with incubated for 48 hours (Figure 3-3). A distinct band was observed at 610 cm⁻¹, as can be seen. *Rhodotorula diobovata*, which reduces copper ions to Cu-NPs and then caps them, was examined using FTIR spectrophotometry. According to earlier reports, the organic functional groups encasing Cu-NPs were identified (48)(49)(50). N-H stretching vibrations of amine groups or amide linkages in the protein contents of *Rhodotorula diobovata* plasma membrane were shown by the peak at 3,218. The aldehyde compound's C-H stretching vibration was linked to the peak seen at 2,958 cm⁻¹. The stretching vibration of C=O, which is often present in proteins, was identified by the band at 1,635 cm⁻¹. The stretching vibration of C–N in aliphatic and aromatic amines was further linked to the peaks observed at 1,095 and 1,398 cm⁻¹. According to this study (51), the presence of bands at 610 cm⁻¹ indicated the likelihood that copper or copper oxide nanoparticles (CuO-NPs) were present.

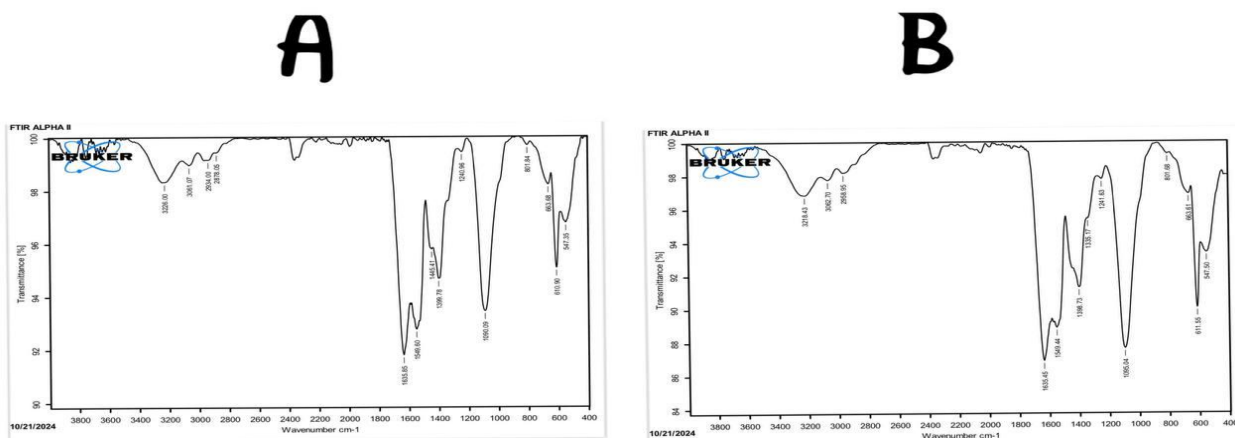


Figure 3-3. A: FTIR of Cu-NPs without incubation (1 hour), B: FTIR of Cu-NPs with incubation for 48 hours

3.2.3. EDX

According to the EDX results, the dried powder of biosynthesized copper nanoparticles contains various components (Figure 3-4). The generated nanoparticles had an optical absorption band peak at about <1 keV, which is typical for copper absorption (52). Cooper's band at 8.00 keV observed in the EDX analysis of the produced copper nanoparticles (Figure 7b) is caused by the copper metallic nanomaterials. In the sample under study, the mass of Cu was 16.6%. Furthermore, the EDX patterns of the biosynthesized Cu-NPs at around 1.00, 2.00, 2.80, and 3.30 keV also showed a few faint indications of Na, P, Cl, and K, respectively. These could be the result of a yeast bio flocculant material utilized in the Cu-NP synthesis (53). S and O components are due to the salt CuSO_4 used in the synthesis of Cu-NPs.

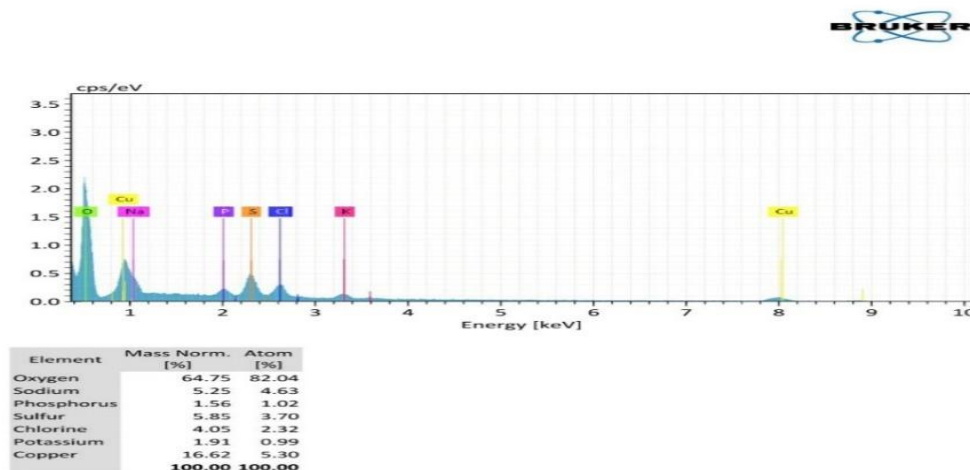


Figure 3-4. EDX for Cu-NPs solution

3.2.4. TEM

TEM analysis of the Cu-NP-containing solution (Fig. 3-5) showed the development of spherical nanoparticles ranging in size from 33 to 64 nm. TEM data show that Cu- NPs cultured for 48 hours were spherical and had an average size of 33–54 nm. This is smaller than Cu- NPs incubated for 1 hour, which were spherical and had a size of 51–64 nm. These results are consistent with those documented by (54)(52), who employed TEM examination to ascertain the spherical, small-diameter shape of the produced copper nanoparticles.

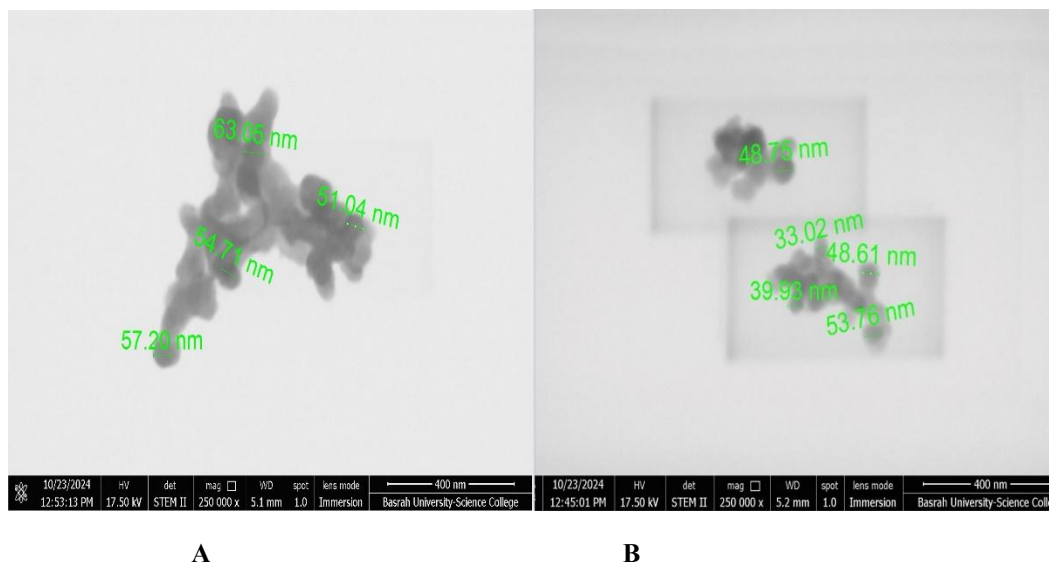


Figure 3-5. TEM. A: Without incubation (1 hour). B: With incubation for 48 hours.

3.2.5. Stability and Particle Size Measurements

Copper nanoparticles without incubated (1h.) and with incubated for 48 hours had average zeta potentials of -17.3 ± 4.36 mV and -26.8 ± 6.06 mV, respectively, demonstrating their great stability (Figure 6 C, D). Nanoparticles with zeta potential values greater than +30 mV or lower than -30 mV often have a high degree of stability, which is of vital importance to avoid their aggregation in colloidal solution (1) The size distribution histograms derived from DLS analysis demonstrated that the Cu-NPs without incubated (1h.) recorded size 19.57 nm, with polydispersity index (PDI) 0.40 nm, whereas those incubated for 48 hours recorded size 22.14 nm with polydispersity index (PDI) 0.2 nm (Figure 6A, B). These results indicate that Cu-NPs that were incubated for 48 hours had a smaller size range than those incubated for 1 hour.

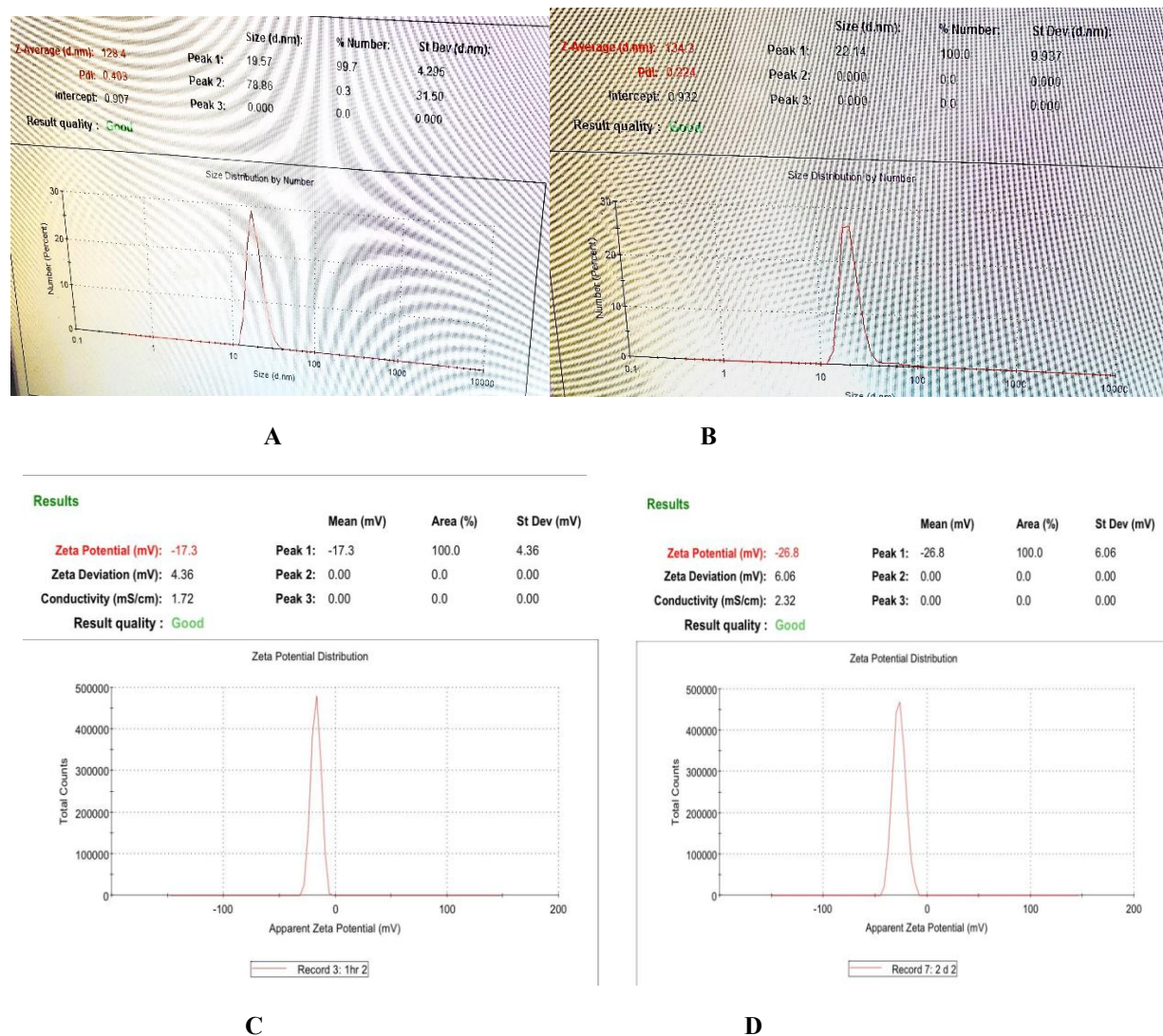


Figure 3-6. DLS. A: Without incubation (1 hour). B: With incubation for 48 hours. Zeta potential. C: Without incubation (1 hour). D: With incubation for 48 hours

3.2. Antimicrobial Activity of Copper Nanoparticles (Cu-NPs) against pathogenic microorganisms by Agar Well Diffusion Method

Since harmful bacteria are rapidly developing resistance to widely used therapeutic drugs, the pharmaceutical industry is finding that screening for novel antibiotics from natural sources is becoming more and more crucial (55). In this work, the antibacterial activity of the produced Cu-NPs against three types of human pathogenic microorganisms was examined. Cu-NPs that were biosynthesized had strong antibacterial properties. The Gram-positive bacterium *Staphylococcus aureus* was more vulnerable to the generated Cu-NPs than Gram-negative bacterium *E. coli* and the yeast *Candida albicans*. The biosynthesized Cu-NPs that were incubated for 48 hours exhibited greater activity against three types of pathogens compared to the biosynthesized Cu-NPs that were without incubated (1h.), yeast supernatant,

and the salt of CuSO₄. The results also indicate that the biosynthesized Cu-NPs without incubated displayed no activity against *E. coli*, while demonstrating more activity against *S. aureus* and *Candida albicans* in comparison to the yeast supernatant and the salt of CuSO₄ (table 3-1), (figure 3-7). These findings contradict those (40), which show that the biosynthesized copper nanoparticles were more active against *Candida albicans* than *E. coli* and *S. aureus*.

Table (3-1) The antimicrobial activity of Cu-NPs against *S. aureus*, *E. coli* and *Candida albicans*.

	Zone of inhibition ZOI (mm)		
	<i>S. aureus</i>	<i>E. coli</i>	<i>Candida albicans</i>
CuSO ₄ .5H ₂ O (10 mM)	15.26±0.73	0.00	10.43±1.02
Yeast supernatant (S)	15.26±0.73	0.00	0
Without incubation 1hr.	17.07±0.15	0.00	11.13±0.21
With incubation 48hrs.	35.03±0.15	14.1±1.55	10.43±1.02

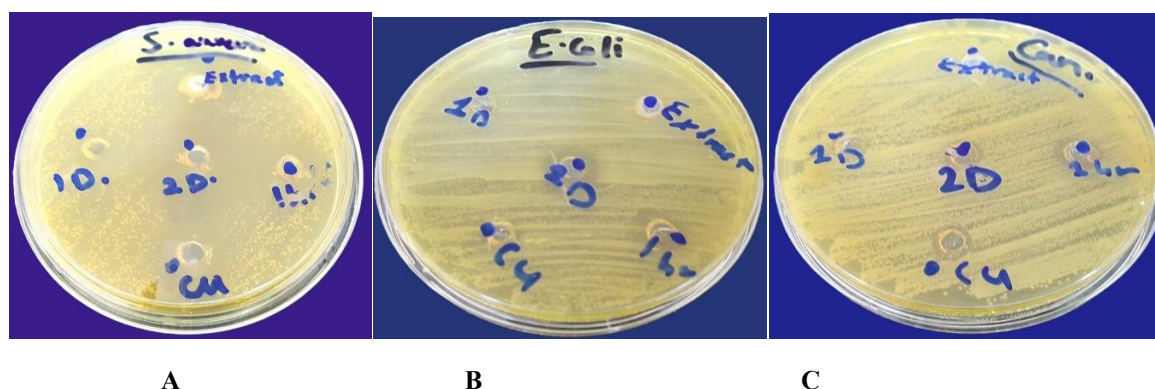


Figure (3-7) The antimicrobial activity of Cu-NPs against A: *S. aureus*, B: *E. coli* and C: *Candida albicans*.

3.3. The Comparison of Antibacterial Activity of Antibiotics, Antifungals and Cu-NPs against Pathogenic Microbes.

The antibacterial activity of copper nanoparticles produced in this study was higher inhibition zones than the antibacterial activity of all antibiotics used against *S. aureus* as shown in table (3-2) and figure (3-8). While against *E. coli*, the copper nanoparticles appeared to have higher antibacterial activity by inhibition zones than some antibiotics used and lower antibacterial activity than other antibiotics as shown in table (3-3) and figure (3-9). The copper nanoparticles appeared to have higher antibacterial activity by inhibition zones than all antifungals against *Candida albicans* except itraconazole as shown in table (3-4) and figure (3-10). Pathogenic microbes such as bacteria and others are affected by antimicrobial copper nanoparticles (56). Nanoparticles can easily interact with bacterial membranes (57). Metal particles' bactericidal effect has been related to their small size and high surface to volume ratio, which permit them to interact closely with microbial membranes (58). Antibacterial activity of copper-based nanoparticles has been reported against different pathogens by in numerous studies (59)(60).

Table (3-2) The Comparative of Antibacterial Activity between Antibiotics and Cu-NPs against *S. aureus*

Antibiotics	IZ (mm)	Cu-NPs IZ (mm)
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1. Ceftriaxone **	0.00	17.07±0.15 35.03±0.15
2. Vancomycin *	20.07 ± 0.61	35.03±0.15
3. Levofloxacin*	30.10 ± 0.36	35.03±0.15
4. Linezolid *	29.00 ± 0.33	35.03±0.15
5. Oxacillin **	0.00	17.07±0.15 35.03±0.15
6. Chloramphenicol*	20.00 ± 0.7	35.03±0.15
7. Gentamicin**	0.00	17.07±0.15 35.03±0.15
8. Azithromycin**	0.00	17.07±0.15 35.03±0.15
9. Ampicillin**	8.03 ± 0.25	17.07±0.15 35.03±0.15
10. Streptomycin*	17.07±0.15	35.03±0.15
11. Gatifloxacin*	20.07 ± 0.61	35.03±0.15
12. Trimethoprim/Sulfamethoxazole*	17.07±0.15	35.03±0.15
13. Erythromycin**	0.00	17.07±0.15 35.03±0.15
14. Polymyxin B**	0.00	17.07±0.15 35.03±0.15
15. Penicillin G**	0.00	17.07±0.15 35.03±0.15
16. Nalidixic acid**	9.00±0.10	17.07±0.15 35.03±0.15
17. Meropenem*	29.10±0.30	35.03±0.15
18. Amoxicillin/Clavulanic acid*	22.07±0.31	35.03±0.15

** Means the antibiotic lower than two types of Cu-NPs,

* Means the antibiotic lower than one types of Cu-NPs



Figure (3-8) The antibacterial activity of Antibiotics against *S. aureus*

Table (3-3) The Comparative of Antibacterial Activity between Antibiotics and

Cu-NPs against *E. coli*

Antibiotics	IZ (mm)	Cu-NPs IZ (mm)
1. Ceftriaxone *	11.13±0.21	14.1±1.55
2. Vancomycin *	0.00	14.1±1.55
3. Levofloxacin	17.07±0.15	14.1±1.55
4. Linezolid *	0.00	14.1±1.55
5. Oxacillin *	0.00	14.1±1.55

6.Chloramphenicol	25	14.1±1.55
7.Gentamicin*	10.43±1.02	14.1±1.55
8.Azithromycin*	10.43±1.02	14.1±1.55
9.Ampicillin*	0.00	14.1±1.55
10. Streptomycin*	11.13±0.21	14.1±1.55
11.Gatifloxacin	18.03±0.21	14.1±1.55
12.Trimethprim/Sulfamethoxazole*	0.00	14.1±1.55
13.Erythromycin*	0.00	14.1±1.55
14.Polymyxin B	15.26±0.73	14.1±1.55
15.Penicillin G*	0.00	14.1±1.55
16.Nalidixic acid*	0.00	14.1±1.55
17.Meropenem	35.03±0.15	14.1±1.55
18.Amoxicillin/Clavulanic acid	15.03±0.30	14.1±1.55

* Means the antibiotic is lower than one type of Cu-NPs



Figure (3-9) The antibacterial activity of Antibiotics against *E. coli*

Table (3-4) The Comparative of Antibacterial Activity between Antibiotics and Cu-NPs against *Candida albicans*

Antifungal	IZ (mm)	Cu-NPS IZ (mm)
Fluconazole*	0	11.13±0.21
Nystatin*	0	11.13±0.21
Ketoconazole*	0	11.13±0.21.
Itraconazole	30.13±0.30	11.13±0.21
Amphotericin B*	0	11.13±0.21

* Means the antifungal lower than one types of Cu-NPs

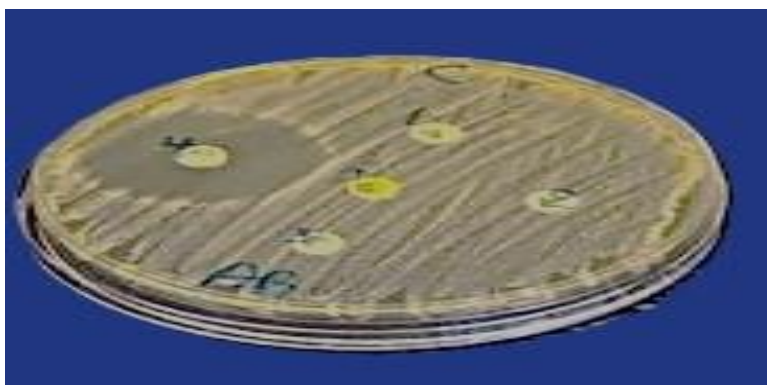


Figure (3-10) The antibacterial activity of Antibiotics against *C. albicans*

3.4. Minimum Inhibitory Concentration (MIC) of Copper Nanoparticles (Cu-NPs) against *S. aureus*

Using the broth microdilution method, the antibacterial activity of Cu-NPs was assessed against Gram-positive bacterium. The results of MIC calculations showed that Cu-NPs suppressed *S. aureus* growth at the lowest inhibitory concentration (64 mg/ml), while the result of the MIC of vancomycin (control) was 0.125 mg/ml shown in Figure (3-11). Although vancomycin had a greater antibacterial potential than Cu-NPs, the evaluation of Cu-NPs antibacterial activity suggested that they had inhibitory effects on Gram-positive infections. Consistent with the present findings, earlier research revealed that metal nanoparticles (NPs), like Cu-NPs, had exceptional antibacterial activity because of their small size and incredibly large surface area, which facilitates improved contact with microbes (1).

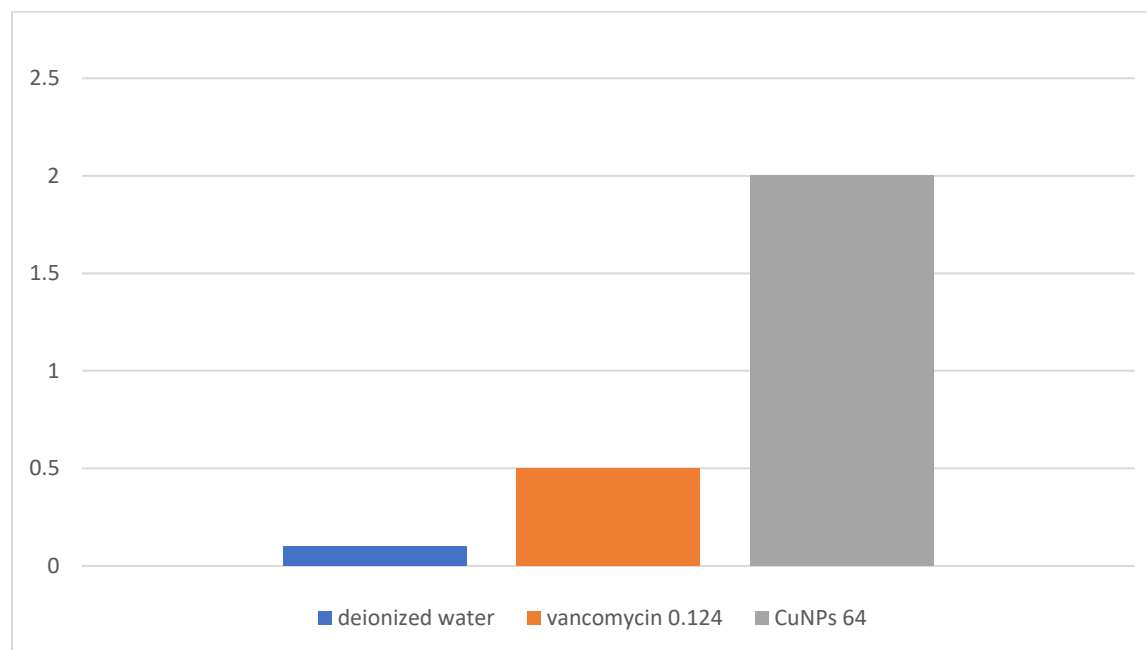


Figure 3-11. The minimum inhibitory concentration (MIC) of copper nanoparticles against *Staphylococcus aureus*.

3.5. Hemolytic assay of Copper Nanoparticles (Cu-NPs)

As shown in Table (3-5) and Figure (3-12), there is no hemolytic effect of Cu-NPs (incubation at 48hrs.) at concentrations of 5 mg/ml and 10 mg/ml, which is similar to the negative control used in the experiment. However, at concentrations of 20 mg/ml, 25 mg/ml, and 50 mg/ml of Cu-NPs, there is a negligible effect on hemolysis, with the hemolytic coefficient being less than 1% (62).

Table (3-5) Hemolytic coefficient of different concentration of Cu-NPs

	Hemolytic coefficient
Ringer solution	0
SDS	100
50 mg/ml	0.066
25 mg/ml	0.034
20 mg/ml	0.033
10 mg/ml	0
5 mg/ml	0

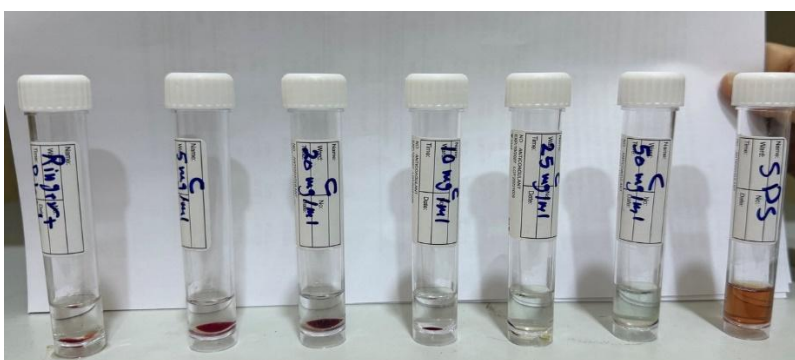


Figure 3-12. Hemolytic coefficient of different concentration of Cu-NPs

Conclusion

The global public health community faces an unsolvable problem with the emergence of antibiotic-resistant bacteria. Using *Rhodotorula diobovata*, we were able to successfully create functional Cu NPs. Cu-NPs' small size, crystalline nature, and spherical shape were shown by TEM, EDX, DLS, and UV vis spectroscopy. Furthermore, functional groups were identified by FTIR spectroscopy, and zeta potential analysis verified the biogenic Cu-NPs' great stability and good dispersion. Cu- NPs demonstrated their antibacterial efficacy against *S. aureus* but had no biological effect against *E. coli* when their biological effects were evaluated. Cu-NPs' hemolytic activity was determined to be insignificant.

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