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Antibacterial Activity of Green Synthesized Copper Oxide Nanoparticles

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Abstract

This study was conducted to investigate the antibacterial activity of green synthesized copper oxide nanoparticles (CuO NPs) using *Aloe vera*. Initially, bacteria were collected from clinical samples of patients having otitis media infection and the isolates were identified at the species level following biochemical tests. Copper oxide nanoparticles were prepared by green synthesis using *Aloe vera* leaves and characterized using UV- visible spectroscopy at 260 nm peak. The shape and size were determined by using transmission electron microscopy (TEM) and the dimensions of the particles were more precisely determined by using scanning electron microscopy (SEM) and x-ray diffraction (XRD). Different concentrations of nanoparticles (25-50-75-100 µg/ml) were tested for antibacterial activity by using the well diffusion method. The results showed that the shape of CuO NPs was spherical with a size range of 40-10 0nm. The TEM images revealed average of dimensions of 32.34, 35.63, 51.85, 74.71 and 100 nm. The antibacterial activity results of the nanoparticles showed the following growth zone inhibition values for the different bacterial species used: *Staphylococci aureus* 17.1 mm, *Pseudomonas aeruginosa* 17 mm, *Escherichia coli* 16.8mm, *Staphylococci epidermidis* 16.4mm, *Pseudomonas oryzihabitans* 15.3mm, *Klebsiella pneumonia* 13.5mm, *Citrobacter freundii* 12.7mm, *Enterobacter Cloacae* 12.2 mm, *Proteus vulgaris* 8mm, Concerning the virulence factor production, the nanoparticle inhibited the production of biofilm and urease more than other virulence factors, such as gelatinase, hemolysin, protease and lecithinase, by some Gram negative and positive bacterial isolates.

Keywords: CuO NPs, *Aloe vera* leaves, Otitis media infection, Antibacterial activity**,** Virulence factors

الفعالية الضد بكتيرية لدقائق اوكسيد النحاس النانوية المصنعة حيويا من النبات

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الخلاصة

أجريت هذه الدراسة للتحري عن الفعالية الضد بكتيرية لدقائق أوكسيد النحاس النانوية المصنعة حيويا باستخدام نبات الصبار. في البدء، تم جمع عزلات البكتيريا سريريا من المرضى المصابين بعدوى التهاب الأذن

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الوسطى ، وتم تحديد هذه العزلات وتشخيصها على مستوى النوع اعتمادا على الفحوصات الكيموحيوية. تم تحضير جزيئات أكسيد النحاس النانوية (CuO NPs) عن طريق التوليف الأخضر باستخدام أوراق الصبار بعدها تم تمييز CuO NPs باستخدام التحليل الطيفي المرئي للأشعة فوق البنفسجية عند ذروة طول موجي 260 نانومتر. كما تم تحديد الشكل والحجم باستخدام المجهر الإلكتروني النافذ وتم تحديد أبعاد الجزيء بدقة أكبر بواسطة اجهزة المجهر الإلكتروني الماسح وانحراف الأشعة السينية. تم استخدام تراكيز مختلفة من الجسيمات النانوية (CuO NPs) بنسب 25−50−75−100مايكروغرام\مل بطرق الانتشار بالحفر في وسط الأكار. أظهرت نتائج هذا العمل أن شكل CuO NP كان كرويًا بحجم بلورات يتراوح ما بين 40−100 نانومتر وبين المجهر الماسح ان معدل أبعاد هذه الدقائق كان كالتالي 32.34, 35.63, 51.85, 74.71 و100 نانومتر. كانت نتائج الجسيمات النانوية المضادة للبكتيريا كما يلي: *Staphylococcus aureus* 17.1، *Pseudomonas aeruginosa* 17، *Klebsiella pneumonia* 13.5 *Pseudomonas oryzihabitans* 15,3، *Staphylococcus epidermidis* 16.4، *Eschereshia coli* 16.8 *Citrobacter freundii* 12.7, *Enterobacter Cloacae*12.2، و12.8 ملم لبكتيريا المتقلبات الاعتيادية *Proteous vulgaris*، فيما يتعلق بإنتاج عوامل الفوعة اتضح بأن الجسيمات النانوية ثبطت إنتاج الأغشية الحيوية وانزيم اليوريا بشكل أكبر من بقية العوامل الاخرى مثل protease ,hemolysin ,gelatinase, و lecithinase بالنسبة لبعض العزلات السالبة والموجبة لصبغة غرام.

Introduction

Copper oxide nanoparticles are interesting for studying because they possess unique physical and chemical properties. Interest has especially increased in the case of such metal oxide nanoparticles because these particles are widely used in medical applications, e.g. as antimicrobials in disinfection, along with their other applications as fillers, opacifiers, catalysts, and semiconductors. They are also useful in the development of cosmetics and microelectronics [1, 2].

Copper oxide nanoparticles also attracted further attention because they are easy to synthesize. They can be obtained by using copper salts such as sulphate, chloride, and others, in a low-cost process. Depending on the properties they show, these nanoparticles can be applied in various fields of biomedicine, especially for their anti-cancer and antimicrobial activities [1]. A recent study showed that there is a sensitive and selective way to detect a virus, which depends on the principle of marking the antibody using the design of a sandwich complex made of CuO NPs linked to the antigen of H1N1 virus [2]**.** Copper oxide nanoparticles can act against Gram-negative and Gram-positive bacteria, following several mechanisms; In the first mechanism, copper ions can bind to DNA molecules. This association causes a spiral structure disturbance by crosslinking the nucleic acid strands and thus disrupting the cell structure. In the second mechanism, copper disrupts the cell membrane and enters the cell, disturbing the functioning of enzymes that are important to the various cellular activities. In the third mechanism, the nanoparticles work on the membrane of the cell directly through the binding of the positively charged copper particles with the negatively charged membrane molecules, leading to defects in the membrane and lysis of the cell [3]**.** This study aimed to synthesize copper oxide nanoparticles using plant extract and reveal their activities on bacteria isolated from clinical sources.

Materials and methods

Bacterial culture condition

Clinical samples were collected from patients having chronic otitis media using cotton swabs under the supervision of a physician. The samples were cultured and diagnosed based on microscopic examination and cultural characteristics, including colonies growth on various media (nutrient agar, blood agar, MacConkey agar, eosine methylene blue agar (EMB), and mannitol salt agar). The growing bacteria were characterized in terms of shape, size, color, edge, blood hemolysis pattern, and lactose fermentation. Biochemical tests were also conducted to investigate the properties of isolated bacteria. These tests included the indole test to investigate the production of indole, the methyl red test to investigate sugar fermentation and acid production, the Vogus-Proscauer test for acetone compound detection, the citrate utilization test to investigate the consumption of citrate as the sole source of carbon and the formation of sodium carbonate, the urease test to indicates the hydrolysis of urea and the formation of ammonium, the oxidase test to investigate the production of cytochrome, the catalase and fermentation tests for sugars, the motion screening test, and the coagulase test in its two

ways based on a previously described method [4, 5]**.** The bacteria identified at the species level were preserved on agar slants until further tests [6].

Plant material extraction

The *Aloe vera* aqueous extraction was prepared by weighting 25 g of the plant leaves, which were then washed, dried and chopped. Then, 100 ml of deionized water was added and the samples were placed on a heat source at 80 C˚ for 5 minutes. After being left to cool at room temperature, the preparations were filtered using filter paper. The extract was stored in refrigerator at 4 C˚ until being used [7]**.**

Synthesis of CuO NPs

Copper oxide nanoparticles were synthesized according to previously published methods [8 ,9]. However, slight modifications were applied (raising temperature till 170C˚ instead of 120 C˚ and decreasing duration time from 24 to 7 hours only). Copper sulphatepentahydrate ($CuSO_4.5H_2O$, 10 mmol/L) was added to 100 ml of distilled water and mixed until melted. Then, 5 ml of *Aloe vera* extract was added to 50 ml of copper sulphate solution with continuous stirring by magnetic stirrer for 7 hours at 170° C, when the color was turned from light green to dark green. The mixture was left to cool at room temperature and centrifuged at 3500 rpm for 20 minutes. The process was repeated twice, then the deposit was taken and dried at 130C˚ for 6 hours. The formed black precipitate was stored in closed tubes for the purpose of testing. All the steps of CuO NPs synthesis are shown in Figure-1.

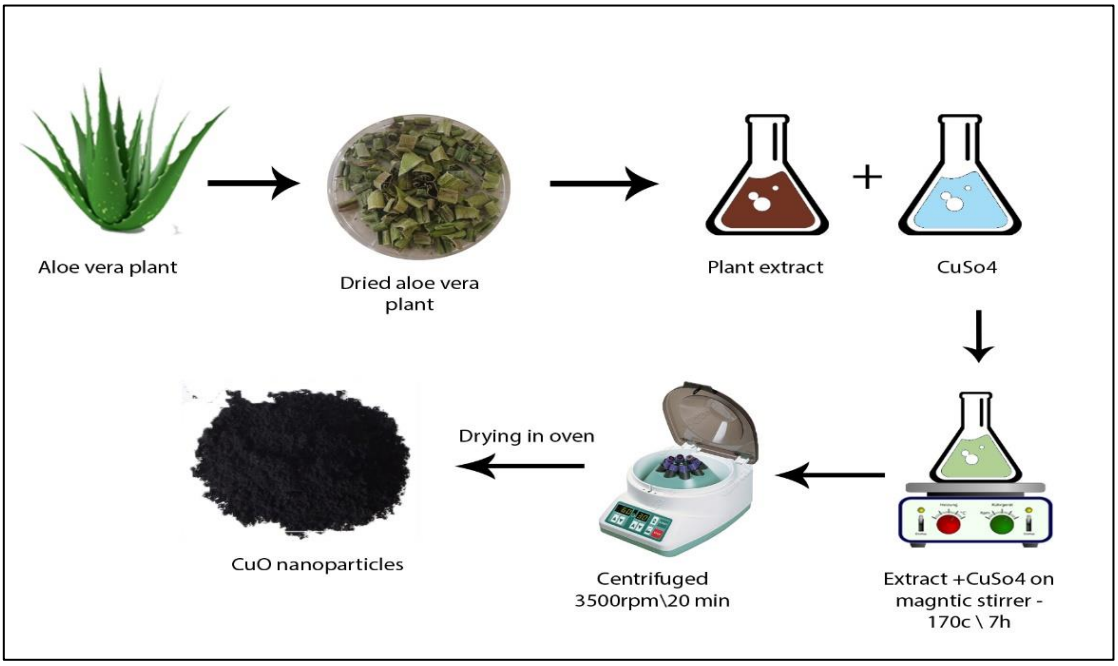

Figure 1- Steps of Biosynthesis of Copper Oxide Nanoparticles by Using *Aloe vera* Leaf Extract

Characterizations of CuO NPs

The copper oxide nanoparticles were extensively investigated using UV-Vis spectroscopy, X-ray diffraction, Fourier transform infrared spectroscopy (FT-IR), and X-ray photoelectron spectroscopy, while electro-kinetic properties were assessed by using transmission electron microscopy and atomic force microscopy.

Antibacterial tests of CuO NPs

The inhibitory activity of CuO NPs against the isolated bacterial species was measured following the procedure presented by Balouiri *et al*. [4]. The agar diffusion method was performed via making wells in Mueller Hinton agar petri dishes at concentrations of 100, 75, 50, and 25 %. The bacterial samples were compared with 0.5 McFarland solution ($1.5^8\times10$). Then, the bacterial suspension was spread on the media. A volume of 100 microliters from the different concentrations of nanoparticles was then added to the wells (6 mm diameter), then the cultures were incubated at 37 °C for 24 hours. The inhibition zone diameter was measured in mm using zone reader. The experiments were conducted with three replicates

Virulence factors detection tests

Biofilm production test was conducted using Congo red agar. This medium was prepared by dissolving brain heart infusion broth (37 g/l), sucrose (50 g/l), and agar No 1 (10 g/l) in 900 ml distilled water. This medium was added to Congo red aqueous solution (0.8 g/l) dissolved in 100 ml distilled water. The media and solution were sterilized separately in an autoclave (121°C for 15 minutes) and poured into bacteria-containing Petri dishes at 55°C [10].

Gelatinase test was conducted using gelatin medium prepared by adding 15g agar, 1g yeast extract, 4g peptone, and 15g gelatin to 1000 ml of distilled water. The components were dissolved by heating then the medium was distributed into test tubes. The test tubes were sterilized by autoclaving at 121°C for 15 minutes [5].

Protease production test was prepared by dissolving 87.5 ml of nutrient agar, which was left to cool down to 50 ° C. Then 12.5 mL of skimmed milk were added in sterile places and mixed well, then poured into bacteria-containing Petri dishes [6].

Lecithinase and lipase production were detected by using egg yolk agar, prepared with 85 ml of nutrient agar, which was left to cool down to 55 ° C. Then, 15 mL of egg yolk was added to the nutrient in sterile tube and mixed, then pour into bacteria-containing Petri dishes.[10]

Hemolysin production was tested by using blood base agar prepared routinely using AB+ human blood added to the medium at 5-10% [11]. The preparation was then poured into bacteria-containing Petri dishes.

Urease production was detected by using urea agar base prepared by adding 24 g of urea to 900 ml of distilled water. The mixture was dissolved by a heat source, sterilized by autoclave, and left to cool at 50°C. Then, 100 mL of the filtrated urea solution was added and distributed to sterile test tubes for exposure to the nanoparticles. Tests were conducted according to Malarkoi *et al.* [4].

The bacteria under study were transferred to 1.2 ml of nutrient broth in sterile test tubes. A volume of 0.8 ml of a concentration of μg/mL of nanoparticles was added. The mixture was incubated in a shaker incubator (250 rpm) at 37° C for 24 hrs. Then, the same tests of virulence factors detection were performed for all the above mentioned tests.

Statistical analysis

The data were statistically analyzed depending on one-way ANOVA test and average means were compared using Duncan multiple range with a confidence levels of 95 and 1% [10].

Results and Discussion

The current study involved collecting samples from patients with chronic suppurative otitis media admitted to Samarra Hospital and outpatient clinics. The patients were of both genders with different age groups, all tested under the supervision of a specialized physician. From a total of eighty-four specimens, 74 (88%) samples had recovered bacterial growth while 10 (12%) samples had no bacterial growth. The common isolated bacterial species collected from otitis media of the current study were *Staphylococcus aureus*, followed, respectively, by *Pseudomonas aeruginosa*, *Klebsiella pneumonia, Staphylococcus epidermidis* and *Escherichia coli, Proteus vulgaris, Citrobacter freundii. Enterococcus cloacae*. *Hemophilus influenza*, and *Pseudomonas oryzihabitans*.

We have chosen chronic otitis media as an infection site since it is characterized by recurrent infections and that the nanoparticles are known to be able to eliminate the microorganisms with high efficacy during the ear therapy, overcoming all the disadvantages of antibiotic treatments [5].

Structural characterization of biosynthesized CuO NPs

In this work, a traditional medicinal plant (*Aloe vera* leaves extract) was used as a stabilizing and reducing agent for the green synthesis of CuO NPs. To confirm the presence of these particles and study their properties, several microscopic techniques were conducted as described below.

UV-Vis Spectroscopy Analysis

The presence of copper oxide nanoparticles was confirmed by UV-visible spectroscopy. It is one of the important and widely used techniques for characterization of metal nanoparticles in aqueous media. The absorption peak appeared 260 nm, as illustrated in Figure- 2, which indicates the presence of CuO NPs. The λmax value of CuO NPs ranges from 250 to 395, depending on the plant extract used. Also, the absorption on the plasmon surface reflects the shape and size of nanoparticles [6], as shown in Figure- 2.

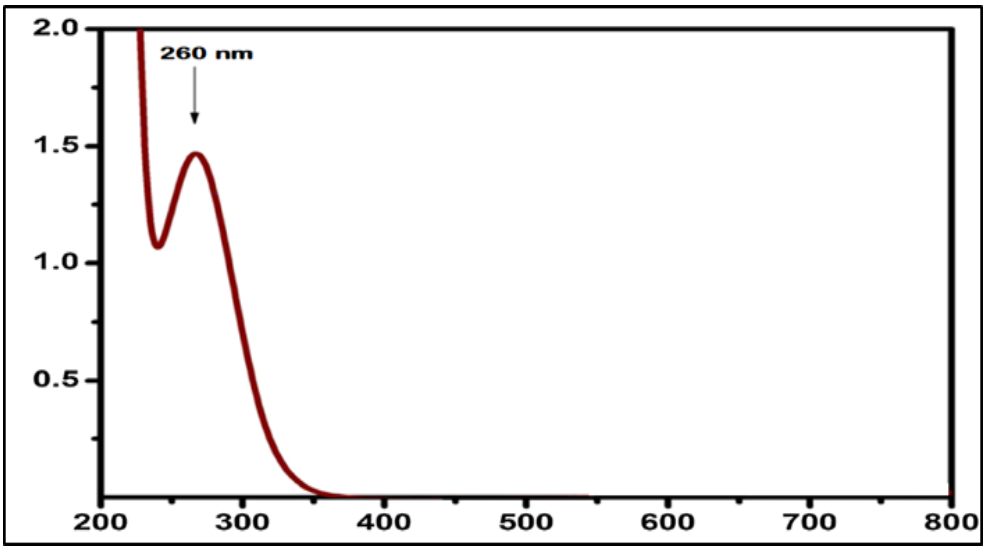

Figure 2 - UV-Vis Absorption Spectrum of CuO NPs

FTIR Chemical Analysis

The FTIR analysis was applied to identify the biomolecules and determine the functional groups present in the synthesized CuO NPs. The FTIR spectrum of CuO NPs synthesized using *Aloe vera* extract showed various peaks at 3415.93, 2131.34, 1625.99, 1573.91, 1425.40, 1120.64 and 607.58 cm^{-1}, as shown in Figure- 3. The broad and strong peak were observed at around 3415.93 cm^{-1} (O-H alcohol groups) 2131.34 cm^{-1} (C=C, alkanes groups), 1652.99 and 1576.91 cm^{-1} (C-C alkenes groups), and 1425 cm^{-1} (O-H, bending, carboxylic group). The peaks at 1120.64 and 607.58 cm^{-1} (Cu-O) indicate the metal-O, which indicates the formation of CuO NPs in a pure form.

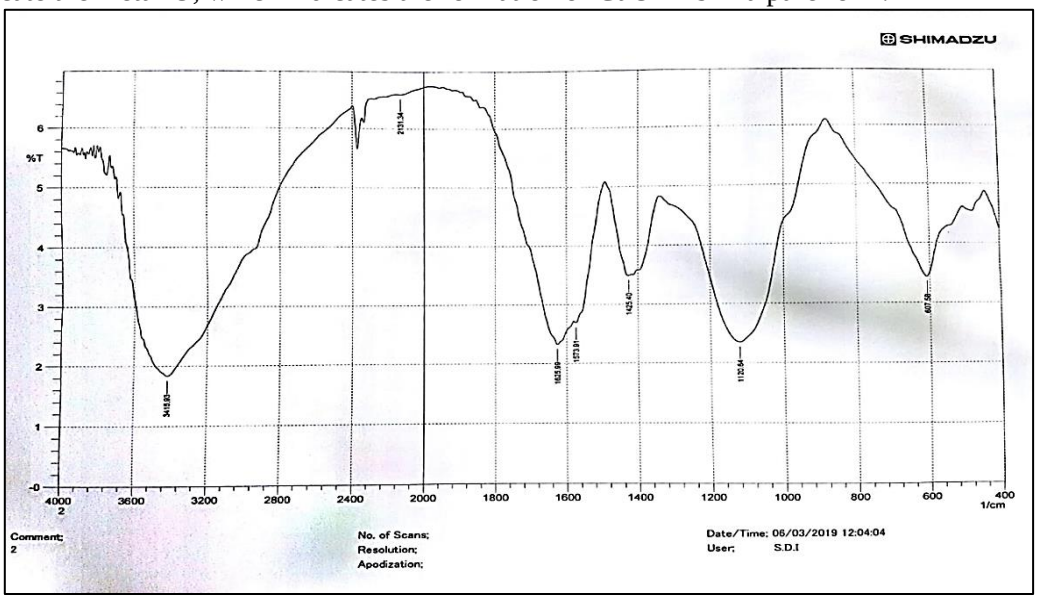

Figure 3-FTIR Spectra of Green Synthesized CuO NPs Using *Aloe vera* Leaf Extract

X-Ray Diffraction Analysis

Figure- 4 shows the XRD pattern of the synthesized copper oxide nanoparticles. This technique was used to confirm the formation of crystals and determine their size, The results showed diffraction peaks at 29.29 and 31.74. The average size of the crystals was calculated by Debye – Scherrer's and the related formula is expressed as follows:

$$\boldsymbol{D = \frac{k\,\lambda}{\beta\,cos\,\theta}}$$

where D is the particles size in nm, *k* is a constant of 0.94, and λ represents the full-width at half maximum (FWHM) of the peak. The average size of crystals was found to range between 3.36 and 12.1 nm.

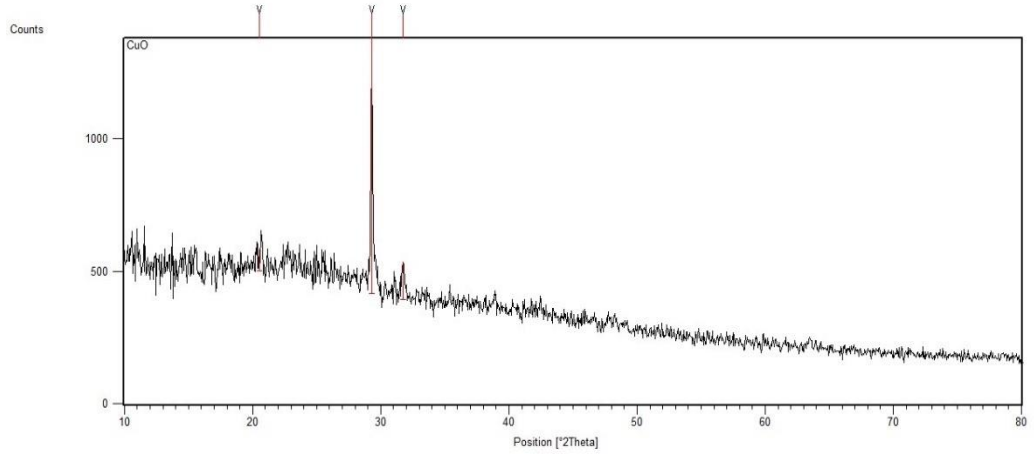

Figure 4- XRD Pattern of Green Synthesized CuO NPs Using *Aloe vera* Leaf Extract

Energy Dispersive X-ray Spectroscopy (EDS)

The EDS was employed to determine the chemical composition of CuO NPs. The EDS spectra showed peaks related to the catalysts of O and Cu, as showen in Figure-5.

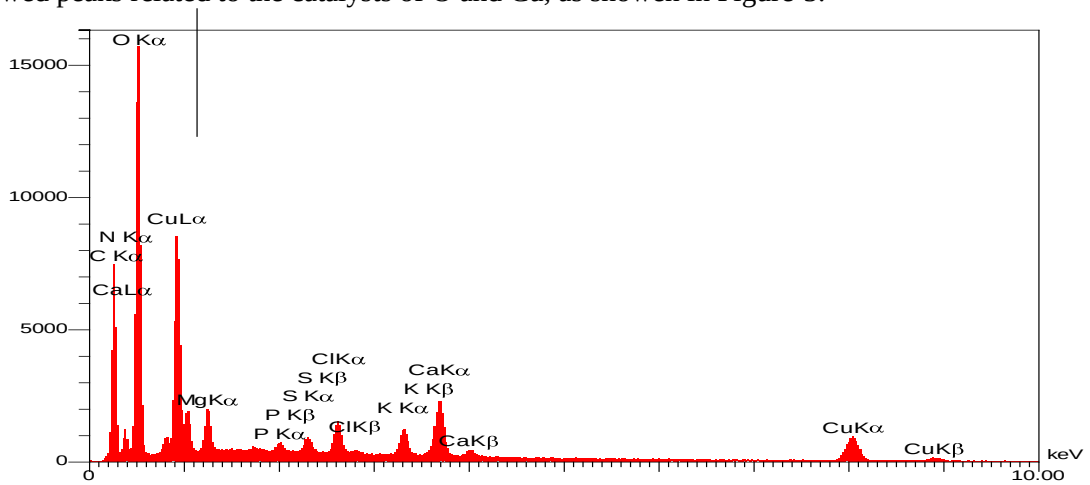

Figure 5 - Energy Dispersive X-ray Spectroscopy of Green Synthesized CuO NPs Using *Aloe vera* Leaf Extract

Transmission Electron Microscopy

The TEM test was carried out to determine the crystalline characteristics of the CuO NPs. The particles are observed to be spherical in shape and the size ranges between 40 and 100 nm (Figure- 6).

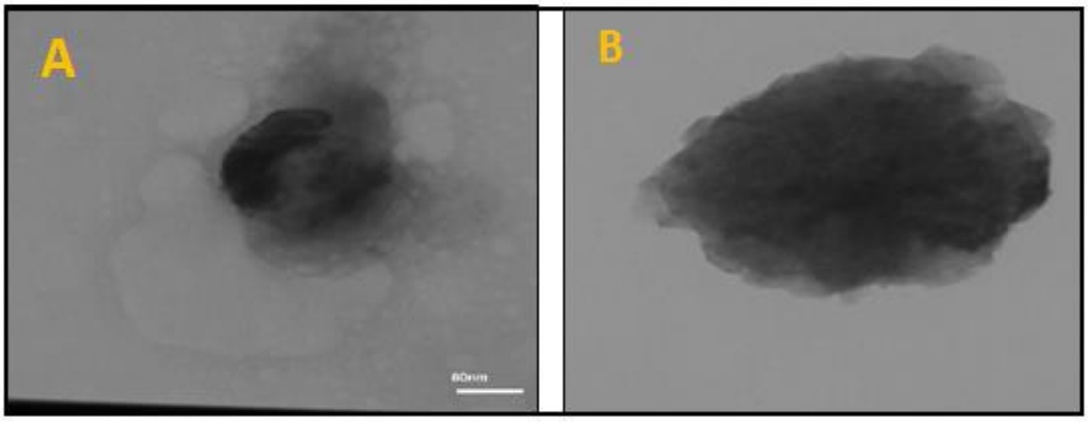

Figure 6- Transmission Electron Microscopic (TEM) Images of the Copper Oxide Nanoparticles Synthesized Using *Aloe vera* Leaf Extract

Scanning Electron Microscopy

The SEM image in Figure-7 shows that the size of nanoparticles had the values of 32.34, 35.63, 51.85, 74.71 and 100 nm. The particles are almost spherical in nature and free from agglomeration, with random size distribution.

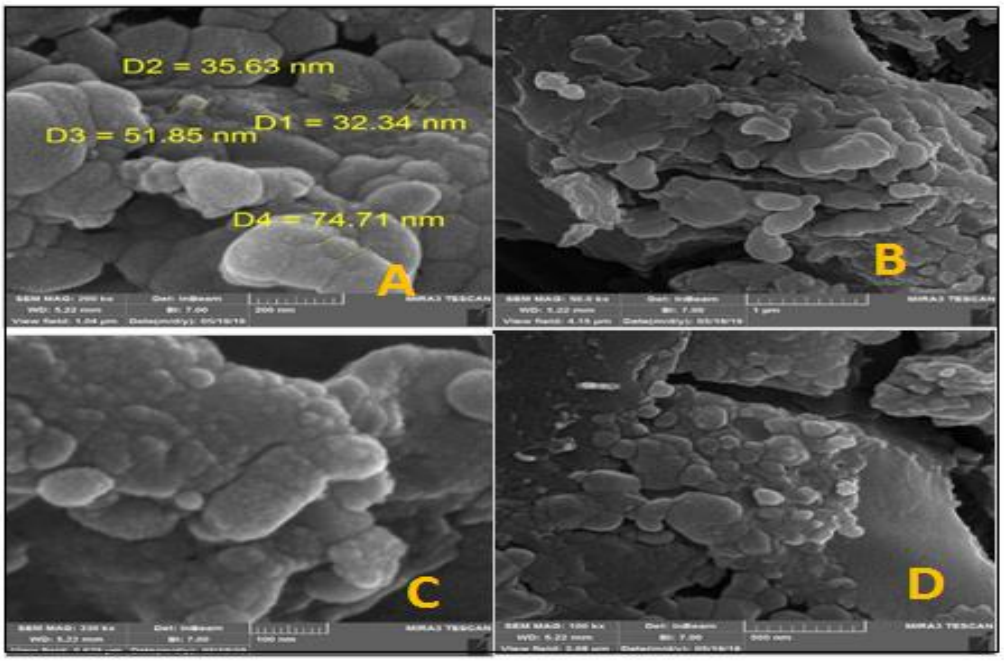

Figure 7-Scanning Electron Microscopic (SEM) Images of the Copper Oxide Nanoparticles Synthesized Using *Aloe vera* Leaf Extract

Antibacterial Activity of CuO NPs

The antibacterial activity of the copper oxide nanoparticles was tested on several species of Gram negative bacteria (*Pseudomonas aeruginosa, Klebsiella pneumonia, Escherichia coli, Proteus vulgaris, Citrobacter freundii, Enterobacter Cloacae, HemophilusI influenzae* and *Pseudomonas oryzihabitans*) and Gram positive bacteria (*Staphylococci aureus* and *Staphylococcus epidermidis*) using the agar well diffusion method. The data were analyzed using variation analysis and table 1 Illustrates detailed results.

Table 1- Statistical analysis of the results of antibacterial activity of CuO NPs in relation to NPs concentrations and bacterial isolates

CONC. / **Bacterial Isolates**	**25** (µg/mL)	**50** (µg/mL)	**75** (µg/mL)	**100** (µg/mL)	**Mean of Isolates**
P. aeruginosa	13.26	15.2	16.4	17	15.465±1.65 a
K. pneumonia	10.1	13.4	12.4	12.7	12.15±1.429 bcd
E. coli	9	16.8	16.2	13.5	13.875±3.550 abc
P. vulgaris	11	12.2	11.8	12.8	11.95±0.755 cd
C. freundii	8.5	12.7	11.1	12.5	11.2±1.936 d
H. influenzae	13.5	13.6	16.7	13.5	14.325±1.584 a
P. oryzihabitans	13.8	14.3	14.9	15.3	14.575±0.660 a

E. cloacae	0	12.2	11.3	11.8	8.825±1.890 e
S. aureus	14.3	14.6	14.5	17.1	15.125±1.323 a
S. epidermidis	11.6	13.5	15.2	16.4	14.175±2.090 ab
Mean of Conc.	10.506 ±2.220 b	13.85 ±1.432 a	14.05 ±2.198 A	14.26 ±2.003 a	

Lower-case letters indicate significant differences at p< 0.001 we got: a>b, different (mean according to concentration). Different letters in a column mean significant differences of mean values according to the bacterial isolate.

The nanoparticles showed the highest inhibition effect on *S. aureus* bacteria with 100 µg/mL concentration, having 17.1mm diameter of inhibition zone. The inhibition zone diameters were 14.5, 14.6, and 14.3 mm when the concentrations of CuO NPs were 75,50 and 25 µg/mL, respectively. These results are compatible with those reported by Nabila [7], who found that the concentrations of 100, 75, 50, and 25 of CuO NPs caused inhibition zone values of 19.6, 17.6, 16.6 and 15.6 mm, respectively. However, our results differed from those described by Merah and others, who found that the diameter of the inhibition zone was lower than 6 mm [8].

The results of *S. epidermidis* showed that the inhibition zone diameter values were 16.4 mm at a concentration of 100 µg/mL, 15.2 mm at 75 µg/mL, and 13.5 mm at 50 µg/mL, while at the concentration of 25 µg/mL, the diameter recorded was 11.6 mm. The results of the present study agree with those of Alishah *et al*., where CuO NPs were found to have an inhibitory effect on *S. epidermidis* [9].

As for *P. aeruginosa*, the diameter of the zone of inhibition showed values of 17 mm at 100 µg/mL, 16.4mm at 75 µg/mL, 15.2 mm at 50 µg/mL, and 13.26 mm at 25 µg/mL.

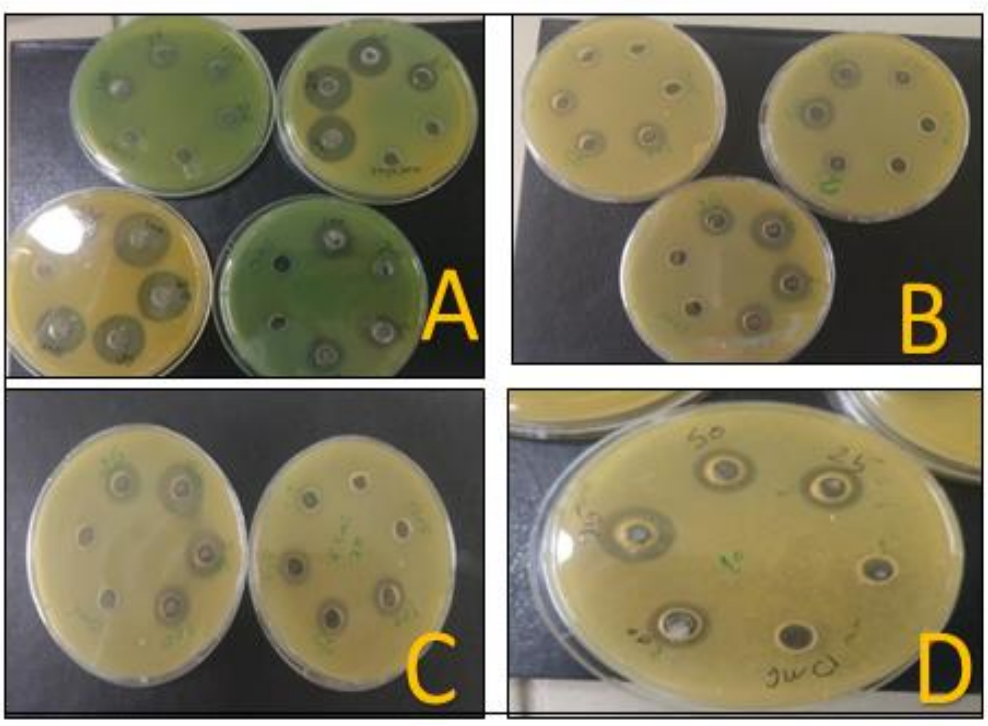

Figure 8-Zone of inhibition of A: *P. aeruginosa, B : K. pneumonia C: E. coli , D: H. influenzae* in response to treatment with the synthesized CuO NPs.

In addition, the inhibitory effects of CuO NPs against *K. pneumonia* were reflected by growth zone diameter values of 12.8, 12.4, 13.4 and 10.1 mm at 100, 75, 50 and 25 µg/mL concentrations, respectively. The results are in agreement with those of Rajgovind and his groups [10], but differed

from those published by Sutradhar and others who reported lower inhibition values by using similar NPs concentrations [22]. The effects of CuO NPs on *E. coli* when using the concentration of 100 µg/mL nanoparticles were reflected by a growth zone diameter of 13.5 mm, whereas the values were 16.2 mm at 75 µg/mL, 16.8 mm at 50 µg/mL, and 9 mm at 25 µg/mL. The inhibition values of *P. vulgaris* were 12.8, 11.8, 12.2 and 11 mm at the same gradient concentrations, respectively. These results agree with those of Rajgovind and his group [17]**.** They are also in agreement with the results of Laha *et al.*, who found that the CuO NPs showed growth inhibition of *P. vulgaris* [18]**.** Furthermore, inhibitory effects of CuO NPs against *P. oryzihabitans* were demonstrated by values of 15.3, 14.9, 14.3 and 13.8 mm, respectively, using the abovementioned NPs concentrations. Lastly, the concentrations of nanoparticles used recorded inhibition values of 11.8, 11.3, 12.2 and 0.0 mm, respectively, in the growth of *E. Cloacae.*

Effects of CuO NPs on Virulence Factors Production

The present study also tested the effects of CuO NPs on the production of virulence factors by the isolated bacteria. It was found that the effect was more obvious on Gram-negative bacteria. Among the tested virulence factors, urease and biofilm showed the highest increase in production, being clearly affected by exposure to nanoparticles, since these particles can reach the active sites of the enzymes.

These results are in agreement with those reported by Murthy *et al.* and Ali *et al.* [19 ,20], who found that CuO NPs were able to inhibit the formation of biofilms of Gram negative bacteria. The study is also in agreement with that of Pugazhendhi *et al.* [21]**.** Biofilm production was recently reported to occur in the middle ear of patients having chronic otitis media all over the world [16]. On the other hand, *S. aureus* and *S. epidermidis* which represent Gram positive bacteria were found to be not affected by CuO NPs. This is due to the strong structure of the thick and highly cross-linked peptidoglycan which prevents the nanoparticles from reaching the target site. As for the production of the other virulence factors, less differences were observed since not all isolates were able to produce these factors before exposure. In general, *P. aeruginosa* was the isolate that showed the most prominent changes in virulence factors production.

The differences among bacterial isolates concerning the inhibition of virulence factors production might be due to the increase or decrease of their cell wall hydrophobicity after exposure to copper oxide nanoparticles.

As was observed previously [22]**,** CuO nanoparticles may penetrate and interact with bacterial membranes. These nanoparticles can generate reactive oxygen species that induce oxidative stress by releasing Cu^{2+} ions, which was found to be a key determinant of toxicity.

Table 2- Production of virulence factors by bacterial isolates before and after exposure to CuO NPs

Bacteria	Biofilm		Urease		Gelatinase		Hemolysin		Protease		Lecithinase	
	A	B	A	B	A	B	A	B	A	B	A	B
S. aureus	+	+	+	+	+	-	+	+	-	-	+	+
P. aeruginosa	+	-	+	-	+	+	+	-	+	-	+	-
K. pneumonia	+	-	+	-	-	-	+	-	-	-	-	-
S. epidermidis	+	-	+	-	-	-	-	-	-	-	-	-
E. coli	+	-	-	-	-	-	+	-	-	-	-	-
P. vulgaris	-	-	+	-	-	-	+	+	-	-	-	-
C. freundii	+	-	+	-	+	-	+	+	-	-	-	-
H. influenzae	-	-	-	-	-	-	-	-	-	-	-	-
P. oryzihabitan	-	-	-	-	-	-	-	-	+	-	-	-
E. cloacae	-	-	-	-	+	-	-	-	-	-	-	-

+: Positive - : Negative; NT: Not tested; B: Before exposure to nanoparticles; A : After exposure to nanoparticles.

Figure 9-Biofilm Detection by Congo Red Agar. Black Colonies Show Biofilm Formation, Red Colonies Show Non Biofilm Formation.

Figure 10-Urease Test. Pink Color indicated Positive Result; Yellow indicates Negative Result.

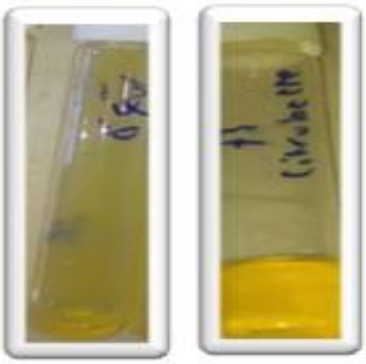

Figure 11-Gelatin test. A: Positive Result B: Negative Result

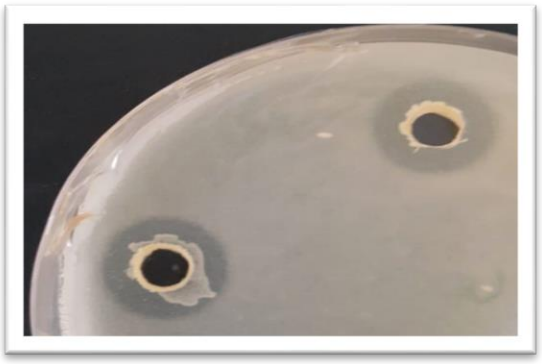

Figure 12- Protease test. Clear zone represents positive result.

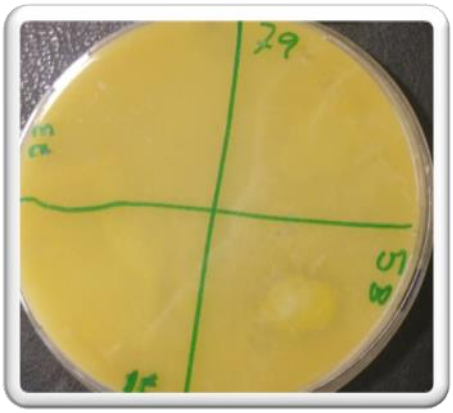

Figure 13-Lecithinase test. Opaque area represents positive result.

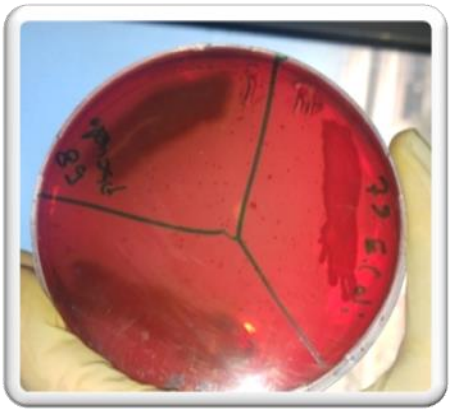

Figure 14- Hemolysin Test: Clear zone around colonies represents positive result.

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