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Original Research

Antibacterial activity of CuO and MgO nanoparticles in combination with levofloxacin against multidrug resistant *Escherichia coli* causing urinary tract infections

Authors:
Baidaa R. Mohammad[1], Zainab M. Alzubaidy[1] and Ammar Algburi[2]

Institution:
1. Biology Department, College of Science, Diyala University, Iraq.

2. Biotechnology Department, College of Science, Diyala University, Iraq.

Corresponding author:
Zainab M. Alzubaidy

ABSTRACT:

Metal nanoparticles (NPs), including oxides of magnesium and copper have been reported with antimicrobial properties. This study speaks about the antibacterial activity of the synthesized magnesium oxide (MgO) and copper oxide nanoparticles (CuO-NPs) against pathogenic *Escherichia coli* strain isolated from Urinary Tract Infection (UTI) patients. In the present study, 200 urine samples were collected and inoculated in sorbitol MacConkey medium to isolate a pure culture of *E. coli* O157:H7. Our results showed that *E. coli* was isolated from 60 (30%) of the total samples; 15 (7.5%) from male and 45 (22.5%) from female, which were inoculated in the MacConkey, eosin methyl blue and blood agar medium. The antibiotic sensitivity test showed that 75% of isolated *E. coli* showed multiple resistance to the tested antibiotics while 25% were sensitive to all antibiotics. The average crystallite size of tested MgO and CuO nanoparticles found to be 18 nm and 57.5 nm respectively using Debye Scherrer equation. MgO and CuO nanoparticles showed remarkable antibacterial activity against all isolated *E. coli*. MgO nanoparticles produced an inhibition zone ranged from (12.2±0.09 - 12±0.1) mm at 400 µg/mL concentration, while CuO nanoparticles produced an inhibition zone ranged from (10.2±0.5 - 9.5±0.21) mm in diameters at 220 µg/mL concentration. The antibacterial activity of levofloxacin combined with MgO at 400 µg/mL was significantly difference at $P<0.001$ in comparison to each of the antimicrobial compounds tested. Also, the Minimum Inhibitory Concentration MIC of CuO nanoparticles was 200 µg/mL. The bacterial inhibition zones were bigger when levofloxacin at 40,20,10 µg/mL were used in combination with CuO nanoparticle compared with using CuO nanoparticles or levofloxacin, separately.

Keywords:
Urinary tract infection, *E. coli*, Levofloxacin, MgO, CuO, Nanoparticles.

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INTRODUCTION

Escherichia coli has been the most frequently isolated pathogen from Urinary Tract Infection (UTI), followed by *Proteus mirabilis*, *Klebsiella pneumoniae*, *Staphylococcus saprophyticus* and *Pseudomonas* sp (Chen *et al*., 2012). Some strains of *E. coli* are found in the intestinal tract where the latter is a, natural habitat and are considered as a normal flora of human beings and animals, providing nutrients (such as vitamins) and regulating the immune response to the invading pathogens (Yan and Polk, 2004). The other strains, are pathogenic associated with the severe infections inside and outside the intestinal tract including; Diarrheagenic *E. coli* (DEC), and Extra intestinal Pathogenic *E. coli* (ExPEC) (Marrs *et al*., 2005). Outside of gastrointestinal tract, *E. coli* can spread and colonize cardiovascular, central nervous system and urinary tract, causing a number of life-threatening infections.

With regard to the UroPathogenic *E. coli* (UPEC), they are associated with community-acquired UTI (90-80%) (Foxman 2014). Many virulence factors were reported producing UPEC including fimbria, biofilm formation, capsule, bacteriocin production and iron chelates to facilitate the cellular penetration during the bacterial pathogenesis (Cheesbrough, 2012). Therefore, an effective strategy is urgent for the control of such infection-associated pathogens. The discovery of antimicrobials plays a key role in significant reduction of UTI incidence but not for a long time. The abuse of the antibiotics has accelerated the emergence of single and multi-drug resistant microbial strains. Also, some antibiotics are unable to penetrate the cellular membrane and fail to attack the intracellular pathogens leading to the failure of the treatment. An alternative and safe antimicrobial substances are publicly required to encounter antibiotic resistance of uropathogenic *E. coli* (Japoni *et al*., 2009).

Nanoparticles (NPs) have been emerged as promising broad-spectrum antimicrobial agents due to their physical and chemical properties. The nanomaterials can be used as alternative antimicrobial agents with a broad spectrum against pathogenic microbes. The small size enables NPs to easily penetrate the membrane barriers and inhibit the growth of intracellular pathogens (Yah and Simate, 2015). This study aimed to evaluate the antimicrobial activity of testing MgO and CuO-NPs,

Table 1. Response *E. coli* isolates to antibiotics in percentage

S. No	Antibiotics	Resistance	Intermediate	Sensitive
1	Ampicillin	60%	20%	20%
2	Pipracillin	15%	0	85%
3	Cefazolin	75%	0	25%
4	Ceftazidime	75%	0	25%
5	Cefepime	75%	0	25%
6	Ceftriaxone	70%	5%	25%
7	Azetreonam	75%	0%	25%
8	Ertapenem	0	0	100%
9	Imipenem	0	0	100%
10	Meropenem	0	0	100%
11	Amikacin	0	0	100%
12	Gentamycin	15%	5%	80%
13	Tobramycin	15%	0	85%
14	Ciprofloxacin	30%	0	70%
15	Levofloxacin	30%	0	70%
16	Tigecycline	0	0	100%

Table 2. Multiple resistance against the antibiotics of *E. coli* isolates

S. No	Number of antibiotics that isolates resist	Isolates	Percentage
1	12	1	6.25%
2	11	1	6.25%
3	10	1	6.25%
4	9	1	6.25%
5	8	1	6.25%
6	4	1	6.25%
7	7	3	18.75%
9	5	3	18.75%
10	6	4	25%

against uropathogenic *E. coli* isolated clinically from patients with UTI.

MATERIALS AND METHODS

Sample collection

Two-hundred urine samples were collected from patients have UTI at the dialysis center of Baqubah teaching hospital and Al-Batool hospital for children and maternity in Diyala, Iraq. Three culture media, were initially used for identification of *E. coli,* viz: (i) blood agar to enhance the bacterial growth, (i) MacConkey agar for identification of lactose fermenter bacteria and (iii) Eosin Methylene Blue (EMB) agar. The agar plates were incubated aerobically at 37°C for 24 h. Then, the pure cultures were transferred to tryptone soy broth and incubated aerobically at 37°C for 24 h.

Bacterial identification

The bacterial species were identified primarily using some biochemical tests including; lactose fermentation, CO_2 and H_2S production on Kligler's iron agar and indole production, also, urease, catalase and oxidase assays. VITEK®2 COMPACT system was utilized to confirm the bacterial diagnosis and to detect bacterial sensitivity to antibiotics (Pincus, 2011).

Synthesis of copper oxide nanoparticles

The CuO-NPs were prepared using sol-gel methods according to Phiwdang *et al.* (2013). Briefly, the copper nitrate (CuO $(NO_3)_2$. $3H_2O$) was dissolved in 100 mL of deionized water to obtain 0.1 M, then a few drops of 0.1 M NaOH solution was added slowly. The mixture was stirred until the black particles participate at the bottom of the beaker. The precipitate was filtered using 0.45 micron filter paper and washed several times with deionized water, then with absolute alcohol until it reaches pH 7. The precipitate was dried at 80°C for 16 h, and then burned at 500°C for four hours. The X-ray Diffractrometry (XRD) was used to confirm the production of CuO-NPs (Abboud *et al.*, 2014).

Synthesis of magnesium oxide nano-particles

The MgO-NPs were prepared by sol-gel method according to Wahab *et al*. (2007). An aqueous 0.2 M of magnesium nitrate ($MgNO_3.6H_2O$) was dissolved in 100 mL of deionized water. A drop of sodium hydroxide (NaOH) solution 0.5 M was slowly added to $MgNO_3.6H_2O$ till a white precipitate with pH 12.5 is produced at the bottom of the beaker which indicated $Mg(OH)_2$ formation:

$$Mg(NO_3)_2 + 2NaOH \rightarrow Mg(OH)_2 + 2NaNO_3$$

The precipitates were filtered and washed several times with methanol to remove the ionic impurities; the product was left at room temperature to obtain a

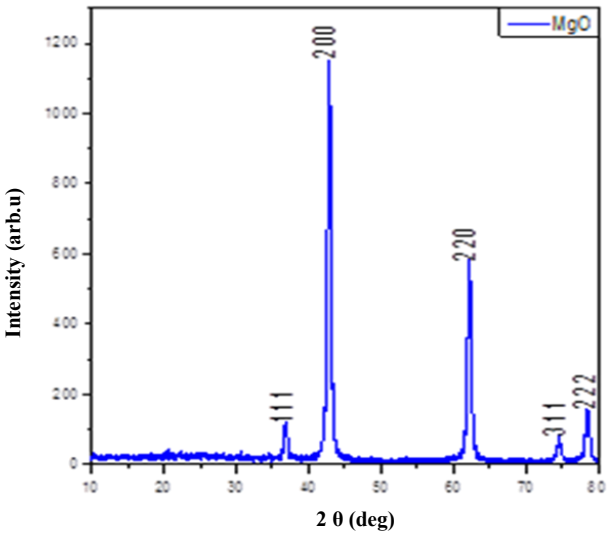


Figure 1. XRD patterns of MgO-NPs

white powder. The precipitate was dried at 80°C for 16 h and then burned at 500°C for 4 h. The X-ray Diffractometry (XRD) was used to confirm the production of MgO-NPs (Abdel-Aziz *et al*., 2020). The average size of NPs was calculated using the of Debye Scherrer equation according to Prabhu *et al*. (2014) method as follow: Dav = 0.9 λ/β cos θ**.** When λ is the wavelength of X-ray falling on the target and it is equal to 154 Å0, β is the Full Width of half Maximum (FWHM), θ: (Prague angle) the fall angle X-ray.

Antimicrobial activity of nanoparticles

CuO and MgO-NPs powder 20 and 40 mg, respectively were dissolved separately into 10 mL of sterile deionized water ddH_2O. Each antimicrobial NPs were tenfold-diluted using tube dilution method ranging from 2000 to 0.2 µg/mL for CuO-NPs and from 4000 to 0.4 µg/mL for MgO-NPs. Agar well diffusion method was used to detect the inhibitory effects of nanoparticles against *E. coli* isolates. The bacterial inoculums, 0.1 mL, which corresponded to No 0.5 McFarland tube was streaked onto Mueller Hinton agar. Wells with 5 mm in diameter were made using crock borer on the surface of the agar plates. Each well was filled with 100 µL of nanoparticle dilutions at three replicates per dilution, then incubated at 37°C for 24 h. The diameters of the inhibition zones were measured using digital caliper.

Determination of Minimum Inhibitory Concentration (MIC) for MgO and CuO nanoparticles

According to Wikler (2006), the Broth macro-dilution method was used to determine the MIC of MgO and CuO nanoparticles. Nutrient broth was used to prepare several dilutions of MgO and CuO. *E. coli* inoculum was prepared by diluting their overnight growth to a density of 10^6 CFU/mL and compared to the 0.5 McFarland standard solutions. Then, 1 mL of the bacterial inoculum was added to each of the diluted tubes containing 1 mL of the previously determined concentrations of NPs. All tubes are incubated at 37°C for 24 h. The MIC was determined as the lowest concentration of antimicrobial which prevents visible bacterial growth.

Determination of MIC of levofloxacin

As mentioned above, broth macro-dilution method was also used to estimate the MIC of levofloxacin with several dilutions ranging from 4000 to 0.4 µg/mL. (Balouiri *et al*., 2016).

Determination of synergistic activity of nanoparticles combined with levofloxacin

Agar well diffusion method was used to estimate the inhibitory effect of antimicrobial mixtures; MgO, CuO and Levofloxacin, against isolated *E. coli*. Briefly, 0.1 mL corresponding to 0.5 McFarland standard solution of *E. coli* was streaked onto Meuller Hinton

Table 3. The effect of Levofloxacin in combination with MgO-NPs against *E. coli*

E. coli isolates	Levofloxacin and MgO nanoparticles (400 µg/mL)						
	Lev 1 (10 mg/mL)	**Lev 2 (20 mg/mL)**	**Lev 3 (40 mg/mL)**	**MgO (400 µg/mL)**	**Lev 2+ MgO (µg/mL)**	**Lev 3 +MgO (µg/mL)**	**Lev 1+ MgO (µg/mL)**
	Diameter of inhibition zones at millimeter (mean ± SEM)						
E_1	0	6.1±0.01	8.1±0.1	12.1±0.9	18.2±0.05	20.1±0.1	11.2±0.1
E_2	0	6.1±0.01	8.1±0.1	12.1±0.09	18±0.09	20.2±0.1	11.2±0.05
E_3	0	6±0.05	7±0.05	12±0.1	18±0.1	20.5±0.09	10.2±0.1
E_4	0	6.2±0.05	7.1±0.1	12.2±0.09	18.1±0.1	20±0.1	10.3±0.05
E_5	0	6.1±0.01	7.1±0.2	12.1±0.1	18.1±0.04	20±0.1	10.2±0.1
P-value		0.01	0.01	0.01	0.001	0.001	0.001

Table 4. The effect of Levofloxacin in combination with CuO-NPs against *E. coli*

E. coli	Levofloxacin and CuO nanoparticles (200µg/mL)						
	Lev 1 (10 mg/ mL)	Lev 2 (20 mg/ mL)	Lev 3 (40 mg/mL)	CuO-NPs (200 µg/mL)	Lev 2+ CuO-NPs (µg/mL)	Lev 3+ CuO-NPs (µg/mL)	Lev 1+ CuO-NPs (µg/mL)
	Diameter of inhibition zones at millimeter (mean ± SEM)						
E_1	0	6.1±0.01	8.1±0.2	10±0.23	14.6±0.03	19.3±0.03	10.3±0.08
E_2	0	6±0.02	8.1±0.3	9.5±0.21	13.9±0.04	18.8±0.02	10.4±0.09
E_3	0	6±0.02	7±0.25	10.1±0.26	14.2±0.05	19.1±0.04	10.1±0.07
E_4	0	6.2±0.03	7.1±0.3	10±0.3	14±0.04	20.1±0.05	10.2±0.08
E_5	0	6.1±0.01	7.1±0.4	10.2±0.5	14±0.06	20.1±0.04	10.1±0.07
P-value		0.01	0.01	0.01	0.01	0.001	0.001

Agar. Four wells with 5 mm were made in each plate. In the first well, 100 µL (MIC of MgO) was added followed by 50 µL into the next wells in addition to 50 µL of the (MIC) Levofloxacin. The process continue using lower concentrations of both antimicrobials. The plates were incubated at 37°C for 24 h, then the measurements of the inhibition zone diameters were recorded. All experiments were performed with three replications (Kareem, 2015).

Statistical analysis

Statistical analysis was used in accordance to chi-square test and student t-test. Data were found to be significantly different when 'P' value was less than 0.05 (Levesque, 2007).

RESULTS AND DISCUSSION

Isolation and identification of *E. coli*

The results showed that 105 (52.5%) of the samples showed positive growth, while 95 (47.5%) of the samples did not show any growth. The samples were distributed as 125 samples from females and 75 samples from males of different ages, diagnostic results showed that the samples were 60 (30%), distributed as 15 (7.5%) isolated from males and 45 (22.5%) from females. The results of our study were comparable with the results of the local study conducted by Al-Hamawandi, (2014) in which, *E. coli* infection rate was 33.3%.

Antibiotics sensitivity test of *E. coli*

The sensitivity test of *E. coli* isolated for antibiotics was carried out using Vitek®2 compact device using 16 different antibiotics to determine the sensitivity and resistance of the isolates to antibiotics. The results indicated that 80 % of the isolates were resistant to a number of tested antibiotics and only 20 % were sensitive to all tested antibiotics, as shown in Table 1. The high resistance to antibiotics was due to many mechanism including their ability to form a biofilm and have outer membrane channels called porins which change permeability and cause the entry of antibiotics into the bacterial cell as well as the bacteria may have the potential to

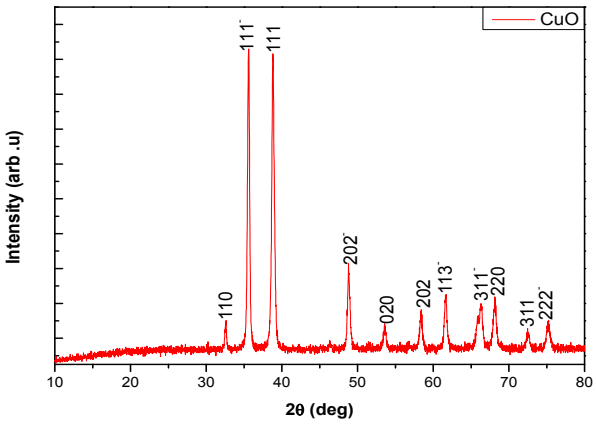


Figure 2. XRD patterns of CuO-NPs

produce (beta-lactamase) enzymes (Manoharan *et al*., 2011). The antibiotic sensitivity test showed that 75% of *E. coli* isolates showed multiple resistance to the antibiotics included in this study while 25% were sensitive to all antibiotics (Table 2).

Inhibitory effects of levofloxacin

Levofloxacin interferes with the basic metabolic processes of the bacterial cell such as DNA replication, cloning and repair and re-association by inhibition of type II enzymes such as topoisomerases and gyrases (Kuntaman *et al*., 2005). The MIC of the antibiotic levofloxacin showed that 40 µg/mL is the lowest concentration of the antibiotic, which prevented the visible growth of *E. coli* and there was a significant difference in the treatment of the bacteria with the antibiotic levofloxacin compared with the control treatment P<0.001. Our results were similar to the results that were recorded by Drago *et al*. (2001) and Li *et al*. (2017) where the MIC value of levofloxacin for *E. coli* isolates was 32 µg/mL.

Characterization of MgO and CuO nanoparticles with X-ray diffraction

The results confirmed the presence of MgO nanoparticles as in Figure 1. The intensities and position of the peaks are in good agreement with the reported values (ICDD card no; (00-045-0946). On the other hand the results as showed in the Figure 2 confirmed the information of CuO nanoparticles. The intensities and position of peaks are in agreement with the reported values (ICDD card no. (00-048-1548). The average crystallite size of MgO and CuO nanoparticles is found to be 18 nm and 57.5 nm respectively, using Debye Scherrer equation,

$$D_{av} = k \lambda / \beta \cos \theta.$$

MIC determination of MgO and CuO nanoparticles

It is found that the MIC of MgO particles against *E. coli* isolates was at 400 µg/mL which inhibited the visible growth of *E. coli* bacteria as illustrated in Figure 3. The results indicated a significant difference at P<0.001when the isolates treated with magnesium oxide vs. control. The MIC of CuO-NPs against *E. coli* was 200 µg/mL which prevented visible growth of *E. coli* (Figure 4) and there were a significant difference (P<0.001) in bacterial growth when treated with CuO-NPs compared to the control group. The study agreed with the results of Krishnamoorthy *et al*. (2012) who showed that the MIC of MgO against *E. coli* was 500 µg/mL. It is suggested that the antimicrobial activity of MgO-NPs related to the presence of defects on the surfaces of these particles lead to the case of lipid peroxidation and the formation of Reactive Oxygen Species (ROS). A study by Nguyen *et al.* (2018) showed that the MIC values of MgO-NPs ranged from 500 to 1200 µg/mL While the Minimum Lethal Concentration (MLC) of these NPs, killed 90% of the cells from 700-1400 µg/mL against different types of pathogenic bacteria and yeasts. Padil and Černík (2013) referred to the enormous inhibitory action of CuO-NPs against both gram-

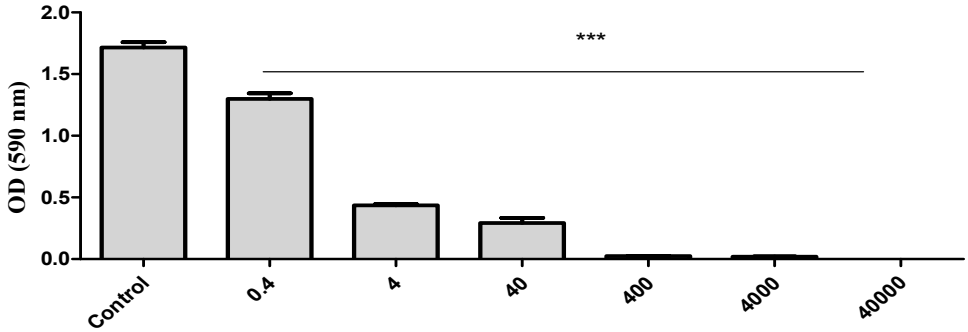


Figure 3. Minimum inhibitor concentration (MIC) of MgO nanoparticles

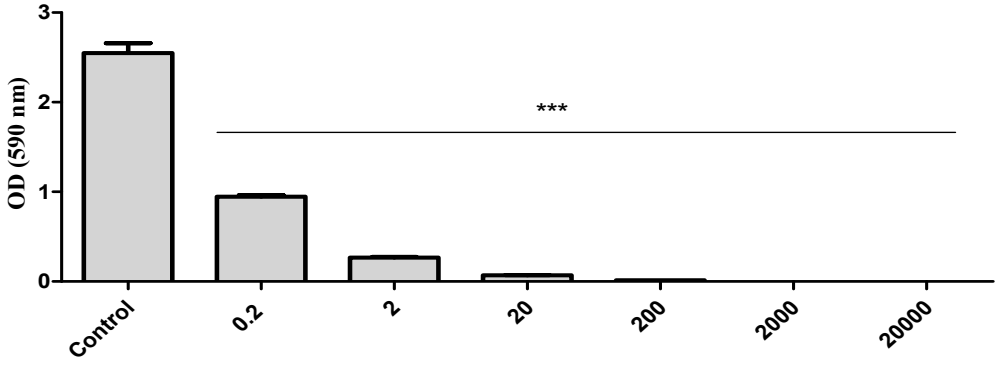
positive and gram-negative bacteria, they found that the lowest inhibitory concentration of CuO-NPs towards both the gram-positive bacteria *S. aureus* and the gram-negative *E. coli* was 120±8.1, 103±4.7 µg/mL, respectively. The authors also showed that the difference in the effectiveness of NPs against both the types of bacteria is due to the differences in cell structure and cell membrane topography (Heinlaan *et al*., 2008).

Antibacterial activity of MgO and CuO-NPs

MgO and CuO-NPs showed remarkable antibacterial activity against all isolates of antibiotic-resistant *E. coli* bacteria. MgO-NPs were recorded at a concentration of 400 µg/mL inhibition diameters ranging from (12.2±0.09 - 12±0.1) mm, (Table 3), While CuO-NPs with a concentration of 200 µg/mL. The inhibition diameters were ranged from 10.2±0.5 - 9.5±0.21 mm around the wells as shown in Table 4. The study conducted by AL-Hazmi *et al*. (2012) confirmed the inhibitory activity of MgO-NPs at a concentration of 700 µg/mL where the diameters of the inhibition areas were 13.8±0.34 mm and confirmed that MgO-NPs are effective against gram negative bacteria compared to gram positive bacteria with an increasing concentration of MgO. According to Azzam *et al*. (2016) the inhibition zone of *E. coli* was 21±2.3 mm and 11±1.1 mm using CuO at 500 µg/mL and 100 µg/mL, respectively. One of the supposed mechanisms of NP action is the accumulation and melting of NPs in the bacterial membrane that alters its permeability, causing the dissolution and release of LPS, proteins and cellular biomolecules and thus dissipating the proton pumping force through the plasma membrane (Azam *et al*., 2012) and the generation of Reactive Oxygen Species (ROS) (Applerot *et al*., 2012).

Inhibitory effect of MgO and CuO-NPs in combination with levofloxacin

Agar-well diffusion method was used to estimate the inhibitory effects of levofloxacin antibiotic at the concentrations of 40, 20, 10 µg/mL. The results showed that the antibiotic at 10 and 20 µg/mL showed no inhibition zone around the wells against *E .coli* isolates, while 40 µg/mL showed that the inhibition zone was between 7.1±0.3 - 8.1±0.3 mm. The inhibition zones were 12±0.1 - 12.2±0.09 and 9.5±0.21 - 10.2±0.50 mm when using MgO-NPs at 400 µg/mL and CuO-NPs at 200 µg/mL respectively, (Tables 3 and 4). The results are consistent with the Ahmad *et al*. (2014) when they found the diameter of the inhibition zone of the CuO-NPs against *E. coli* bacteria 10 mm. The antibacterial activity of the levofloxacin antibiotic was evaluated at different concentrations with MgO at 400 µg/mL as follows 10+400, 20+400 and 40+400 and the inhibition zones diameters were 10.2±0.1 to 11.2±0.05, 18±0.1 to18.2±0.5 and 20±0.1 to 20.5±0.09, respectively and there was a significant difference P<0.001 when treating bacterial isolates with levofloxacin and MgO-



Minimum inhibition concentration of CuO nanoparticles against *E. coli*

Figure 4. Minimum inhibitor concentration (MIC) of CuO nanoparticles

NPs (Table 3).

The results also showed inhibitory effect of the antibiotic at the concentrations of 40, 20 and 10 µg/mL with CuO-NPs at MIC (200 µg/mL) or mixing CuO-NPs with levofloxacin as follows: (10+200), (20+200) and (40+200) and their diameters of bacterial inhibition zones were 10.1±0.07 to 10.4±0.09, 13.9±0.04 to 14.6±0.03 and 18.8±0.02 to 20.1±0.05 mm, respectively. As given in Table 4, statistically there were significant difference $P<0.001$ when bacteria were treated with Levofloxacin and CuO-NPs.

CONCLUSION

Recent studies have shown that the combination of nanoparticles and antibiotics reducing the need for high doses, also enhances their antibacterial properties. Combining antibiotics with nanoparticles restores their ability to destroy bacteria that have gained resistance. In addition, antibiotic-treated nanoparticles have been shown to increase the concentration of antibiotics at the site of interaction between bacteria and antibiotics, and to facilitate the binding of antibiotics to bacteria.

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