Preparation, diagnosis and study of the inhibitory effect of copper nanoparticles before and after Erythromycin loading on Pseudomonas aeruginosa

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Abstract: Pseudomonas aeruginosa is one of the opportunistic nurses, which possesses many virulence factors which makes it a high ability to resist antibiotics multidrug-resistant (MDR). leading to make Antimicrobial resistance is one of the main threats to human health because it leads to increased infection rates and mortality. The present study aimed to evaluate the mechanism of inhibitory effect of the free CuO nanocomposite and its synergistic effect with the anti-bacterial erythromycin ER after it was loaded on the CuO / ER nanoparticle on Pseudomonas aeruginosa isolated from Al-Hindiya General Hospital. The CuO nanoparticles were manufactured by using precipitation method. Diagnosis free CUO and nanoparticles after they were loaded onto nanocomposites by using the AFM atomic force microscope. The inhibition zone diameter measured by Disc Diffusion method the results was indicated the highly synergistic effect on bacteria, while the free nanoparticles are less effective, whereas the antibiotic alone gave the lowest inhibition diameter (21.25 ±0.854, 11.75 ±0.750, 8.50 ±1.040) respectively. moreover this study investigated that The release of the antibiotic ER from the CuO / ER that the second pseudo order model is more applicable to the interpretation of the behavior of ER release from CuO / ER (K = 2.60min⁻¹ , r² = 0.997) which indicates that the forces controlling the release of ER are flexible without dissociation and being affected by the metabolism, and the percentage of ER release was significant due to the intensity of the surface ion exchange between CuO / ER and the anion (CO₃)⁻² coming from CaCO₃, which makes the ER release rate to reach more than 98% in the medium of sodium carbonate at a concentration of 0.05 M within 250 minutes of the start time of the reaction.

Key words: Free Copper Oxide Nanoparticals , P. aeruginosa, Erythromycin, Synergistic effect.
1-Introduction

Pseudomonas aeruginosa is one of the opportunistic nurses, which possesses many virulence factors, which makes it an attention to what may cause harm to human health. (1) Such as urinary tract infection, pneumonia, keratitis, external otitis and folliculitis, and it is one of the causes of acquired infection, especially for people who suffer from immune deficiency, and because of the multiple mechanisms that the bacteria as virulence factors, the bacteria have adapted to stay in environments Low nutrition and resistance to different antibiotic classes like biofilm on biotic and non-biotic surfaces (2, 3). These bacteria have a high ability to resist antibiotics multidrug-resistant (MDR). leading to make Antimicrobial resistance is one of the main threats to human health because it leads to increased infection rates and mortality. The first step in combating the development of resistance and limiting the use of antibiotics without benefit is to resort to alternative solutions, including the use of nanocomposites (4).

Nanotechnology is one of the fields of science that deals with dealing with matter at the scale of 1 billion meters (i.e. 10-9 meters = 1 nanometer). Silver nanoparticles are defined as nanoparticles of silver metal whose size ranges between 1 to 100 nanometers, and are used in many fields such as medical, engineering, and magnetic fields, in energy and environmental treatment, as well as in cosmetics (5). Nanotechnology has been adopted as a way to develop new non-traditional antimicrobial agents called nanoparticles that are an effective treatment for infectious diseases and that have many Advantages compared to conventional antibiotics, including the absence of harmful, their low toxicity (6) and their ability to overcome bacterial resistance to antibiotics by interfering with multiple biological pathways, may cause mechanical disruption of the bacterial membrane.( 7).

The present study aimed to evaluate the mechanism of inhibitory effect of the free CuO nanocomposite and its synergistic effect with the anti-bacterial erythromycin ER after it was loaded on the CuO / ER nanoparticle on Pseudomonas aeruginosa isolated from Al-Hindiya General Hospital.

2-Materials and Methods

2-1. Preparation of spherical copper nanoparticle oxide: The CuO nanoparticles were manufactured by precipitation method using aqueous copper nitrate (Cu (NO3) 2.3H2O) according to (8) as follows: Prepare a 0.1 molar solution of dissolving 14 mg (Cu (NO3) 2.3H2O) in 100 ml of deionized water to form (solution No. 1). Prepare 100 ml of solution 10. Mg NaOH)) was added to the solution in the form of drops / second drops on the walls of the vessel and the acidic function (PH) of the solution is measured until it reaches 14 the reaction is stopped. Leave the solution for two hours. after it stagnates and precipitates by centrifuging 10,000 cycles for 5 minutes. Leave the solution for 24 hours. Wash the precipitate that is black in distilled water and absolute ethanol several times until the pH reaches 7 and leaves 16 hours. The compound is dried at 80 ° C. 7. The product is burned in an oven temperature of 500 ° C for 4 hours, and the result is preserved after cooling it for later use.

2-2. Diagnosis of a hybrid nanoparticle: Methods for diagnosing hybrid nanoparticles include Fourier transform.
infrared spectroscopy (FT-IR) and X-ray diffraction (XRD) spectrum, as well as the use of Atomic Force Microscope (AFM) and electronic scanning Election Microscope (SEM).

2-3. Diagnosis using the AFM atomic force microscope: The atomic force microscope was used to examine free and nanoparticles after they were loaded onto nanocomposites, and to measure the diameters, sizes, and assemblies of nanoparticles. The Crystallinity Index was extracted using the following formula

\[ \text{Crystallinity Index} = \frac{D_p}{L} \]

As:

\[ D_p = \text{the partial volume to be measured by the AFM} \]
\[ L = \text{the average crystal size, which is calculated using the Scherrer equation.} \]

2-4. Study of the release of therapeutic substance:
The method described by (9) was followed in a study that edits the therapeutic substance into a number of aqueous solutions.

Solutions used:
1- Solution No. (1): Na2CO3 sodium carbonate solution
This solution was prepared by dissolving 2.65 g of sodium carbonate in a quantity of distilled water removed from the ions and after completing the dissolution process, complete the volume to 50 ml with distilled water removed from the ions also, and from this concentration the concentration was prepared 0.05M .

2- Solution No. (2): Hydrochloric acid (2M) HCl solution
Prepare this solution by mixing 4.2 ml of concentrated hydrochloric acid with a quantity of distilled water removed from the ions and then complete the volume to 50 ml with distilled water removed from the ions as well.-

2-5. Determination of Calibration Curve
The titration curve, which represents the relationship between absorption and concentration, was determined by preparing four consecutive concentrations within the range (5–20 ppm) of the antibiotic A solution used in the study. Absorption of these concentrations was measured at the maximum wavelength (max 213nm) of the antagonist. The standard between absorption and concentration was shown in Figures (3-1, 3-2). From the following straight-line equation \( Y = mx + b \), the concentration value is found over time.

2-6. Method for estimating the inhibitory efficacy of nanocomposites and antibiotics against bacteria:
The inhibitory effect of the free CuO nanocomposite and the Erythromycin was tested by measuring the inhibition zone diameter around bacterial colony by using the disc diffusion method (10) as follows: A series of decimal dilutions have been made for each bacterial isolation. 0.1 ml of appropriate dilution for each bacterial isolation was well spread on the surface of the dish by the L-Shape diffuser and the dishes were left for an hour in the refrigerator. Dilute the nanocomposites with the use of the dimethyl sulfoxide (DMSO) to obtain concentrations of macrograms / ml, as well as prepare antibiotics loaded with the nanocomposites with both concentrations. Dishes have been made saturated with free nanoparticles, antibiotic and nanocompounds with antibiotic, to be placed on the surface of the agar with a diameter of 5 mm, as the distance was equal between one disk and another. Place the saturated tablets from the free nanocomposite concentrations and loaded with the antibiotic under study in the dish containing the bacterial culture, incubate for an hour in
the refrigerator, and then incubated in the incubator at 37 °C for 24 hours, and the antibiotic Erythromycin was used as a positive control. Measures of inhibition of bacterial growth (mm) were measured using a ruler after the completion of the incubation period.

3-Result and Discussion:
Diagnostic of CuO nanoparticles by atomic force microscope images

Atomic Force Microscopic (AFM) The results (3-1) of the AFM images indicate that the CuO nanoparticle has a surface roughness coefficient of 8.43 nm but with respect to the compound's molecular size ratios, it is 10% for molecular sizes less than 55.00 nm, and 50% was for molecular volumes less than 75.00 nm, the sizes in general were 90% less than 100.00 nm.

Figure (3-1) the atomic force microscope images of the free CuO nano-compound: a / three-dimensional image, b / two-dimensional image, c / two-dimensional image showing all the details of the particles.

Table (3-1) the total average particle size for free CuO nanoparticles and the different ratios for these sizes
Avg. Diameter: 80.55 nm
<=10% Diameter: 55.00 nm
<=50% Diameter: 75.00 nm
<=90% Diameter: 100.00 nm

| Diameter (nm) | Volume (e%) | Cumulative (n%) | Diameter (nm) | Volume (e%) | Cumulative (n%) | Diameter (nm) | Volume (e%) | Cumulative (n%) |
|---------------|-------------|-----------------|---------------|-------------|-----------------|---------------|-------------|----------------|
| 55.00         | 1.68        | 1.68            | 80.00         | 10.61       | 50.28           | 105.00        | 7.82        | 93.30          |
| 60.00         | 10.61       | 12.29           | 85.00         | 8.94        | 59.22           | 110.00        | 4.47        | 97.77          |
| 65.00         | 8.94        | 21.23           | 90.00         | 10.61       | 69.83           | 115.00        | 2.23        | 100.00         |
| 70.00         | 8.94        | 30.17           | 95.00         | 8.94        | 78.77           |               |             |                |
| 75.00         | 9.50        | 39.66           | 100.00        | 6.70        | 85.47           |               |             |                |

Figure (3-2) Distribution of the different ratios of free CuO particle size

: The study of the kinetics of Erythromycin antibiotic (ER) loaded with CuO: 3-2

Ion exchange kinetics have been studied by applying the false-zero and first-order and second-order equations to the kinematics of each of the studied nanocomposites after loading it with the ER counterpart as the zero-order was founded according to the following equation:

\[ C_t = k_0 t \] (zero order)

To find the first false rank, we apply the following equation

\[ \log (1-\frac{C_t}{C_0}) = k_1 t \] (first order)

the following equation to find the value of second false rank

\[ \frac{t}{C_t} = k_2 C_t^2 + \frac{t}{C_t} \] (Second order)
Depending on the Zero Order equation indicated above, the values of \( C_t \) are plotted against the time and the correlation coefficient values are known by applying the straight-line equation.

According to the zero-order equation for the anti-ER spread of the nanocomposite and CuO / ER Figures (3-3), Results indicate that there is a deviation of these values from the straight line indicating the non-compliance of the anti-liberation process ER) for the zero-rank model, as slow progressive liberalization begins until time 20 A minute, and then the acceleration begins until it reaches the highest release at a time of 150 minutes.

\[ r^2 = 0.950 \text{ min}^{-1} \quad \text{And constant of speed} \ K = 0.0050. \]

It is noted through the results indicated in Figure (3-3) regarding the release of anti-ER from the nanocomposition CuO / ER, that these release values are not identical to the straight line, which indicates the non-compliance with the release process of anti-ER treatment for the first-order false model as it happened when applying Zero rank model. Therefore, the false second order equation is applied, since ER release began with a time of approximately 20 minutes to reach above at time 140 minutes.

\[ r^2 = 0.916 \text{ min}^{-1} 0.0079 \]

while, when applying the second false rank model for anti-ER release of CuO / ER nanocomposites, Figure (3-4) indicate that this model is more applicable to the interpretation of ER release behavior of these nanocomposites, as we found that

\[ r^2 = 0.997 \text{ min}^{-1} 2.60 \]

Figure (3-3) the zero rank of antibiotic releasing (ER) from CuO nanocomposite.
Figure (3-4) False First Rank for Antibiotic Editing (ER) of CuO Nanomode

Figure (3-5) the second pseudoscopy for atibiotic releasing (ER) from CuO nanocomposite
Table (3-2) Correlation coefficient and velocity constant for the zero, 1st and 2nd false ranks of the anti-ER release from CuO / ER nanocomposite

|                    | Zeroorder | 1st order | 2nd order |
|--------------------|-----------|-----------|-----------|
|                    | r²        | 1K min⁻¹  | r²        | 1K min⁻¹  | r²        | 1K min⁻¹  |
| ER from CuO        | 0.950     | 0.0050    | 0.916     | 0.0079    | 0.997     | 2.60      |

our current study are in agreement with (11), as he showed that the paracetamol release process from the layers of the zergel (magnesium / aluminum) compound is applied to the second-order mathematical model of the lying to explain the behavior of the release process. The results are also consistent with what (12) found, as it showed that the second-order math model is the most appropriate to explain the release of Ellagic acid from the surface of zinc nanocomposite. While it does not agree with (13), they showed that the mathematical model of false first order is the most appropriate to explain the release of calcic acid from the surface of the zinc nanoparticle that approached 1 depending on the value of r2.
3-3. Study of antibiotic release percentage (Cu) / ER nanocomposite

Figure (3-7) shows that the intensity of the surface ion exchange between CuO / ER and the anion-2 (CO3) coming from carbonates is large, which makes the release of ER treatment up to more than 98% in the medium of sodium carbonate with a concentration of 0.05 molar within two and a half hours of Reaction start time. Our finding of the process of launching the anti-ER from the nanocomposite CuO to the medium of sodium carbonate is slow at the beginning of the experiment, and then it was return to be quickly. After approximately (120) minutes of the start time of the experiment are relatively constant, it can be attributed to the high concentration of carbonate ion-2 (CO3), which causes the release of increased amounts of treatment to the surrounding medium, and that the process of release is progressive, which indicates the process of ion exchange of carbonates is to be gradual, and maintains the gradual release ratio of ER and the formation of a new compound with the mutual carbon ion to equal the ion released from treatment (13).
(12) observed the process of release of ellagic acid ((Ellagic acid from the surface of the zinc compound to the solution of sodium carbonate and triple sodium phosphate during a time period of 38 hours, and found that the release process is fast during the first 5 hours of the start of the experiment, as the rate of release reached (44 and 85)% to the medium of sodium carbonate And triple sodium phosphate, respectively, and then the release velocity gradually decelerates.

3.3. Inhibitory effect of CuO before and after loading Erythromycin on pseudomonas aeruginosa

The inhibitory effect of CuO nanoparticles against the bacteria P. aeruginosa was studied. the results compared with Erythromycin (ER) with its free state and after loading it into the nanocomposite and study the difference in the inhibitory effect of the free form and the synergistic form between the compound and the antibiotic. The results of Table (3-3) indicate that there is a clear variation in the effect of the nanocomposite and the antibiotic before and after the loading on the studied bacteria in the inhibition zone (21.25 ± 0.854) mm compared to the inhibition zone for both the free compound and the antibiotic who recorded the inhibition area (8.50 ± 1.040, 11.75 ± 0.750) mm T1 and T2 respectively . (14) suggested that silver nanoparticles work to prevent bacterial growth by their direct effect protein synthesis by correlating silver atoms with the Thiol group (SH) of bacterial enzymes thus disrupting the work of these enzymes, and the AgNP works to Changing the action of the components that make up the plasma membrane and the task in generating energy and ionic transport through this membrane by forming a S-Ag frame with the Thiol group

The nanocomposite works on the formation of the two sulfur bonds in the interaction of the oxygen and hydroxine atoms in the cell with thiol group (R-S-S-R) groups in S.aureus, P.aeruginosa, E.coli, Helicobacterbiylori, Bacillus subtilis and Shigella flexnri.

The Agnihotri ( 15) study indicated that some silver nanoparticles compounds produce ROS, causing bacterial cell death. Moreover, this study suggests that there are several factors that can synergize in generating the inhibitory action of antibacterial substances more quickly by loading onto the nanocomposite. Because it has electrical properties that help in contacting bacteria, which facilitates the arrival of the antibiotic loaded on these molecules, just as the
action of ions that are soon released when they reach the invading bacterial cell cannot be ignored in the extermination of bacteria.

Antibacterial activity refers to a number of activities such as the toxicological activity of nanoparticles that are still unclear and still controversial, and antibacterial activity requires a deep explanation of the mechanisms that antibacterials use to eliminate germs.

Table (3-3) the Inhibition Zone Diameter (IZD) for P. aeruginosa when treated with CuO nanocomposites before and after loading Erythromycin (ER):

| Treatment      | (IZD ± standard error (mm)) |
|----------------|-----------------------------|
|                | pseudomonas aeruginosa      |
| T1 (CuO)       | 1.040 ± 8.50 a              |
| T2 (ER)        | 0.750 ± 11.75 a             |
| T3 (ER / CuO)  | 0.854 ± 21.25 b             |

Figure (3-7) shows the inhibitory effect of CuO nanocomposite and ER antibiotic in its free and synergistic forms before and after loading. A(T1), b(T2), c(T3)

References
1-Deep, A.; Chaudhary, U.; and Gupta, V. (2011). Quorum sensing and Bacteria Pathogenicity: From Molecules to Disease. Journal of Laboratory Physicians, 3 (1): 4–11.
2- Moradali, M.F.; Ghods, S. and Rehm, B.H.A. (2017). *Pseudomonas aeruginosa* Lifestyle: A Paradigm for Adaptation, Survival, and Persistence. J. Front. Cell. Infect. Microbiol., 7: 39.

3- Babapour, E.; Haddadi, A.; Mirnejad, R.; Angaji, S-A. and Amirmozafari, N. (2016). Biofilm formation in clinical isolates of nosocomial *Acinetobacter baumannii* and its relationship with multidrug resistance. Asian Pac. J. Trop. Biomed., 6(6): 528–533.

4- Chaudhuri, R. G. and Paria, S. (2012). Core/shell nanoparticles: classes, properties, synthesis, mechanisms, characterization, and applications. In Chem. Rev., 112(4): 2373-2433.

5- Sahayaraj, K. and Rajesh, S. (2011). Bionanoparticles: Synthesis and antimicrobial applications. Science against microbial pathogens. Commun. Curr. Res. Technol. Adv., 23: 228-244.

6- Huh AJ, Kwon YJ. (2011)."Nanoantibiotics": a new paradigm for treating infectious diseases using nanomaterials in the antibiotics resistant era. *J Control Release*.;156(2):128-145.

7- Guzman, M.; Dille, J. and Godet, S. (2012). Synthesis and antibacterial activity of silver nanoparticles against gram-positive and gram negative bacteria. Nanomed. Nanotechnol. Biol. Med., 8: 37-45.

8- Phiwdanga, K.; Suphankija, S.; Mekprasarta, W. and Pecharapaa, W.(2013). Synthesis of CuO Nanoparticles by Precipitation Method Using Different Precursors. Energy Procedia, 34 : 740 – 745.

9- Wang , L . ; Liu , Y. ; Zhang , W. ; Chen , X. ; Yang , T. ; Ma , G.( 2013). Microspheres and microcapsules for protein delivery: strategies of drug activity retention. *Curr. Pharm. Des.*, 19 : 6340-6352.

10- Egorove , N.S. (1985) : Antibiotics Scientific approach. mirpublishers, Moscow.

11-Kovanda, F.; Maryskova, Z. & Kvar, P. (2011). Intercalation of paracetomol into the hydrotalcite-like host. Journal of solid state chemistry,184: 3329-3335.

12-Hussein, M. Z. ; Al Ali, S. H.; Zainal Z. & Hakim, M. N. (2011). Development of antiproliferative nanohybrid compound with controlled release property using ellagic acid as the active agent. *International Journal of Nanomedicine*, 6: 1373–1383.

13-Ghotbi,M. Y. & Hussein, M. Z. (2010). Gallate–Zn–Al-layered double hydroxide as an intercalated compound with new controlled release formulation of anticarcinogenic agent. Journal of Physics and Chemistry of Solids, 71(11):1565-1570.

14- Gurunathan, S. ; Han, J.W. ; Kwon, D. ; and Kim, J. (2014). Enhanced antibacterial and anti-biofilm activities of silver nanoparticles against Gram-negative and Gram-positive bacteria. *J Nanoscale Res Lett.*; 9 (1) :373-384.

15- Agnihotri S.; Mukherjiabc, S. & Mukherji, S. (2014). Size-controlled silver nanoparticles synthesized over the range 5–100 nm using the same protocol and their antibacterial efficacy. *RSC Adv.*; 4: 3974-3983.