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Antibacterial activity of modified zinc oxide nanoparticles against *Pseudomonas aeruginosa* isolates of burn infections

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ABSTRACT

In this research antimicrobial activity of nanoparticles ZnO on perilous bacteria such as *Pseudomonas aeruginosa* was evaluated. *P. aeruginosa* is important pathogen that caused burn wound infections as it is multi-drug resistant and has several virulence factors. Fifteen samples of *P. aeruginosa* were collected from patients who suffering from Burn infections in Al-Hilla teaching hospital burn unit with the age range between (7-80) years old for both genders. After collecting burn samples, the diagnosis and characterization were performed by culturing and biochemical tests. ZnO NPs were synthesized by chemical method, Zinc oxide nanoparticles are well-known to be one of the multifunctional inorganic compounds which are widely used in medical applications. This study aims to prepare ZnO nanoparticles with particle size ranging from 23-29 nm. In the present study, surface modification of ZnO nanoparticles was performed, and influence of modification of the structure and morphological properties was investigated. The resulting nanoparticles were characterized by X-ray diffraction (XRD), scanning electron microscopy (SEM) and atomic force (AFM). Zinc oxide nanoparticles with the average diameter of about 29 nm were modified with an oleic acid to exert more compatibility. From the results obtained it is suggested that modified ZnO-nanoparticles could be used effectively in safety environmental and medical applications. Antibacterial activity for nanoparticle ZnO against *P. aeruginosa* isolates was measured by: Agar Diffusion Technique and Minimum Inhibitory Concentration (MIC)/Minimum bactericidal Concentration (MBC) with microdilution. The best zone of inhibition was (35.5mm) at a concentration of 40 μg/ml of nano-ZnO in one strain of *P. aeruginosa* while the lowest inhibition zone was (16 mm) at a concentration of 20

μg/ml of nano ZnO in one strain also. In addition, all *P. aeruginosa* isolates were completely inhibited at the concentration of 3.7 μg/ml of nano-ZnO (MIC) but no significant antibacterial activity was observed at concentrations less than 1.8 μg/ml of nano-ZnO and the (MBC) was same as MIC (3.7 μg/ml) for all *P.aeruginosa* isolates.

Keywords: ZnO; nanoparticles; surface modification; burn infection; Pseudomonas aeruginosa; Antimicrobial activity

1. INTRODUCTION

Despite the increased knowledge of microbial pathogenesis and applications of modern therapeutics, the morbidity and mortality associated with the microbial infections still remain high; Therefore, there is an increasing in the infectious diseases and in the drug resistance in the pathogenic bacteria at an alarming rate is a matter of serious concern [1]. From that, there is a needing to discover novel strategies and identify new antimicrobial agents from natural and inorganic substances to develop the next generation of drugs or agents to control microbial infections. In the recent times, the advances in the field of nano sciences and nanotechnology has brought to the fore the nano sized inorganic and organic particles leading to increasing applications in industry, medicine and therapeutics, synthetic textiles and food packaging products [2].

Greater effectiveness on resistant strains of microbial pathogens, less toxicity and heat resistance are the characteristic of metal oxide nanoparticles, which make them the selective candidates for eradicating bacteria [3,4]. The small size of nano-ZnO referred which is 250 times smaller than a bacterium the might be giving it the antimicrobial ability, this makes them easier to adhere with the cell wall of the microorganisms causing its destruction and leads to the death of the cell, in addition to that metal nano-particles are harmful to bacteria [5]. Nano ZnO can disrupt the bacterial cell membrane integrity (the particles interact with the building elements of the outer membrane and might cause structural changes), reduce cell surface hydrophobicity and down-regulate the transcription of oxidative stress-resistance genes in bacteria, then degradation and finally cell death [6]. ZnO has recently achieved a special attention regarding potential electronic application due to its unique optical, electrical and chemical properties [7].

One of the most frequent and major complications in patients with burn injuries is the infection and is the main cause for prolonged in-hospital stay and death in cases of wide-spread burns despite marked progress in the development of treatment methods for these patients. The development of multi-resistant organisms complicate burn infections, besides the infected wound may be a potential source of spreading of antibiotic-resistant microorganisms. The colonization and infection of these wounds are a dual clinical problem. The infected wound is a cause of pain and discomfort for patients, as well as life-threatening septic conditions. As a result, the treatment cost and the medical care increase, respectively [8-10].

P. aeruginosa is a Gram-negative, rod-shaped bacterium, facultative anaerobe, it belongs to the group of γ-Proteobacteria. This bacteria is an opportunistic pathogen and a ubiquitous organism that present in soil and water and can be isolated from plants, animals and humans [11,12]. *P. aeruginosa* able to tolerate a variety of physical conditions and

survive on minimal nutritional requirements [13,14]. *P. aeruginosa* is one of the important pathogen that caused nosocomial infection, especially in immune suppressed patients like severe burns, and in patients suffered from cancer [15,16]. The mortality rate of *P. aeruginosa* infections were reported from 18% to 61% in hospital-acquired infections [17-19]. Selective antibiotic pressure led to emerging of acquired multidrug resistance in several countries in the past; and some multidrug resistant *P. aeruginosa* infections have been untreatable [20]. The antibacterial activity of zinc oxide nano particles were probed by many researchers. Zinc oxide nano particles are better antibacterial agent [21-22]. Therefore, we aimed in this study to test the antibacterial activity of ZnO nanoparticles on important and perilous bacteria such as *P. aeruginosa* isolated from burn infections.

2. MATERIALS AND METHODS

2. 1.Patients

Fifteen (15) samples are collected from patients who suffering from burn infections with the age range between (7-80) years old for both genders. The period extended from (February to April-2015) and the test isolates were obtained from samples taken from patients submitted to Al-Hilla teaching hospital in the burn unit.

2. 2. Collection of specimens and bacterial identification:

Sterile swap samples collected from patients suffering from burn infections and did not receive any antibiotic treatment before swabbing, moisten swaps with sterile saline were passed over the infected area in a zigzag motion while twisting, swapped firmly from the center of infection site outward to the edge (this may be painful for the patient). The samples were transported as quickly as possible to the laboratory [23].

Then the swab samples had been inoculated on the culture media *P. aeruginosa* and were identified by biochemical differentiation tests, including growth on cetrimide agar, oxidase and catalase tests, motility, growth at 42 °C, growth in oxidation fermentation (OF) medium, TSI agar and Simon's citrate Then the confirmed *P. aeruginosa* samples were cloned three successive times on nutrient agar and stored on a nutrient agar slant at 4 ºC [24,25]. Also, bacterial samples were maintained in the brain, heart infusion broth containing 15% glycerol at -75 °C during the research period.

2. 3. Preparation of ZnO Nanoparticles

Dissolve acetate, zinc in a mixture of methanol and Mono ethanol secretary at room temperature and then mixed with a magnetic mixer for one hour until a homogenous solution occur left for 24 hours and then the solution is heated for 3 hours at 200 Celsius the black material precipitate calcined at 500 Celsius and then we get a white powder (nano zinc oxide).

2. 4. Preparation of Nano ZnO concentration for Agar Diffusion Method:

Nano zinc oxide was weighed as 10 mg of then dissolved in 10 ml dimethyl sulfoxide (DMSO) yielding stock solutions of (1 mg/ml) concentration after that (1 ml) of this solution was diluted to (10 ml) with DMSO again giving a solution of 100 µg/ml concentration, then from this solution, the required concentration which include: 20-30-40 µg/ml had been

prepared for agar diffusion method and dilutions: 30-15-7.5-3.7-1.8 had been prepared for the determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) [26].

2. 5. Preparation of bacterial samples

From each bacterium sample of the 15 *P. aeruginosa* isolates, small portion by using sterilized loop was transferred to 3 ml of nutrient broth media previously prepared and sterilized then incubated at 37 °C for 24 hrs. After that 0.1 ml from these cultures of each 15 *P. aeruginosa* isolates were transferred to fifteen sterilized test tubes containing 0.9% NaCl solution.

2. 6. Determination of activity by agar diffusion method

Antimicrobial activities of nano-ZnO were tested in vitro against all *P. aeruginosa* strains by the agar diffusion technique, seeded with a 24 hr- old culture of the microrganism strains (by sterile cotton swab dipped into the broth of these microorganisms). After solidification of 25 ml nutrient agar in Petri plates, hollows of three wells (5 millimetr diameter) were cut into the agar by cork borer and the pathogenic bacterial strains of *P. aeruginosa* were tested on this agar, 0.1 ml of nano ZnO solutions dissolved in (DMSO) prepared earlier in different concentrations which include: 20- 30-40 µg/ml was applied in these wells. The inoculums size was adjusted so as to deliver final inoculums of approximately 108 colony forming unit (CFU)/ml, compared with the turbidity of a sample of the 0.5 McFarland standards. The Petri dishes were incubated at 5-8 °C for 2-3 h to permit good diffusion and then incubated for 24 h at 37 °C. The assessment of antibacterial was based on measuring the diameter of the inhibition zone (mm) formed around the well. In addition to that the activity of (DMSO) alone without nano ZnO was tested on *P. aeruginosa* and it had been found there was no any effect of it on this bacteria [26,27].

2. 7. Determination of Minimum inhibitory concentration and Minimum Bactericidal concentration (MIC/ MBC) as antimicrobial activity Nano-ZnO:

The antimicrobial activities of nano-sized zinc oxide were evaluated, showing antimicrobial against all *P. aeruginosa* strains (15 strains) by serial dilution method through the determination of the minimum inhibitory concentration (MIC and MBC) in culture broth. The method of twofold serial dilutions (28) was used in this study for determination of the minimum inhibitory concentration (MIC) values, 1 ml of media was taken in a test tube, to which, 1ml of test solution (100 µg/ml) was added, thereafter, 0.1ml of the bacterial strains (*P.aeruginosa*) prepared in 0.9% NaCl was added to the test tube containing media and test solution. Serial dilution was done five times giving concentrations of 30-15-7.5-3.7-1.8 µg/ml. The Nutrient Broth, which contained tested samples and controls were incubated for 24 h at 37 °C. Also control test was performed to ensure that the solvent had no effect on bacterial growth, this control test was containing inoculated broth supplemented with only DMSO at the same dilutions used in this research and found inactive in culture medium. The MIC values were taken as the lowest concentration required to arrest the growth of the bacteria in the test tube after incubation (showed no turbidity) while the minimum bactericidal concentration (MBC) was determined by sub culturing 50 µl from each test tube showing no apparent growth (clear), if there was no growth this concentration was taken as MBC.

3. RESULT AND DISCUSSION

3. 1. Analysis Results of ZnO Nanoparticles

3. 1. 1. Scanning Electron Microscopy (SEM)

Figure (1) shows different magnifications of ZnO nanoparticles. The results of this analysis showed the highly homogeneity distribution of ZnO nanoparticles and this agrees with the results of [29].

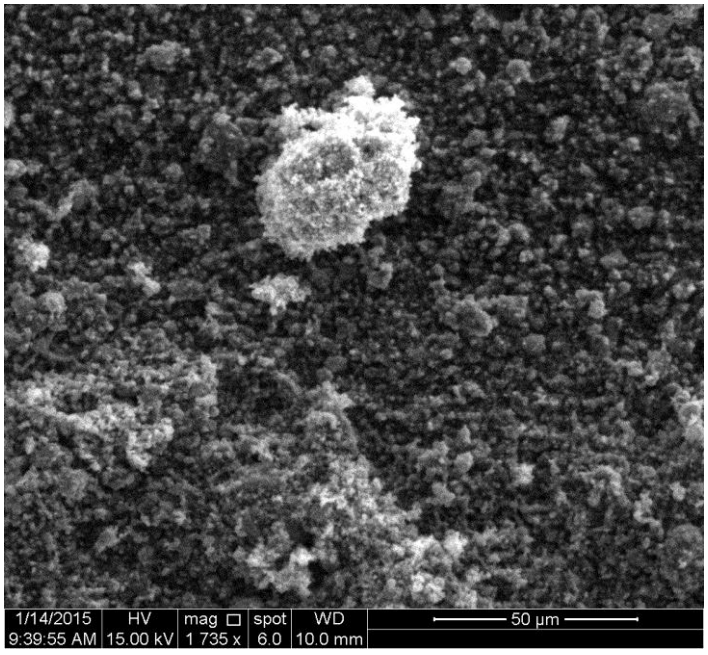

Figure 1. SEM images of different magnification of nano-ZnO.

3. 1. 2. SEM/Energy Dispersive X-Ray Spectroscopy (EDS)

Figure (2) shows that spectrum of crystalline ZnO nanoparticles. One can conclude from the Figure (2) that the purity of ZnO is 100% since there is no elements appears in the spectrum and element analysis agrees with the research [30].

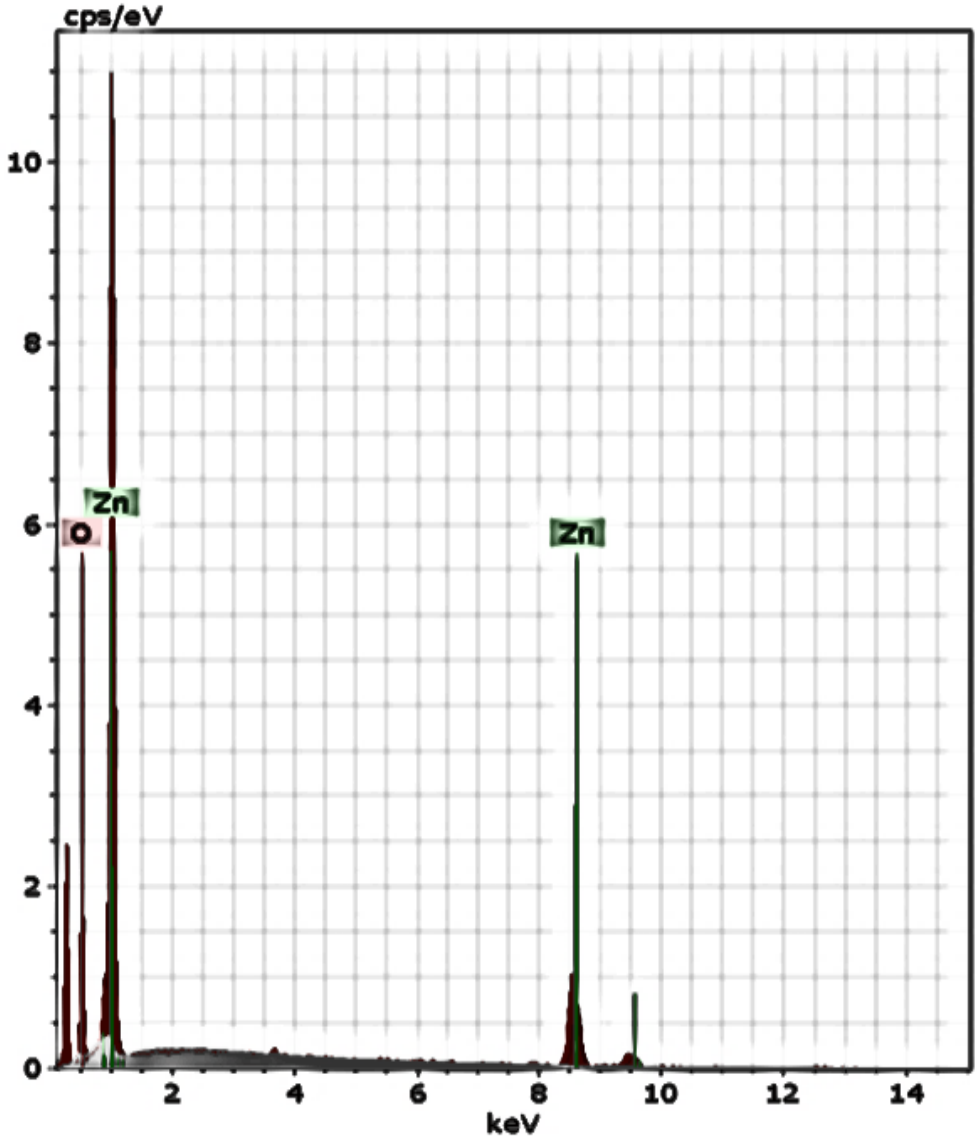

Figure 2. EDS spectrum of ZnO modified nanoparticles.

3. 1. 3. Atomic Force Microscopy

Figure (3) shows the AFM (3-D) images of ZnO nanoparticles. AFM images prove that the grains are distributed homogeneously within the scanning area (1518x1514) nm. The average diameter of synthesized ZnO is measured from AFM analysis using software and is found to be around (47.69) nm.

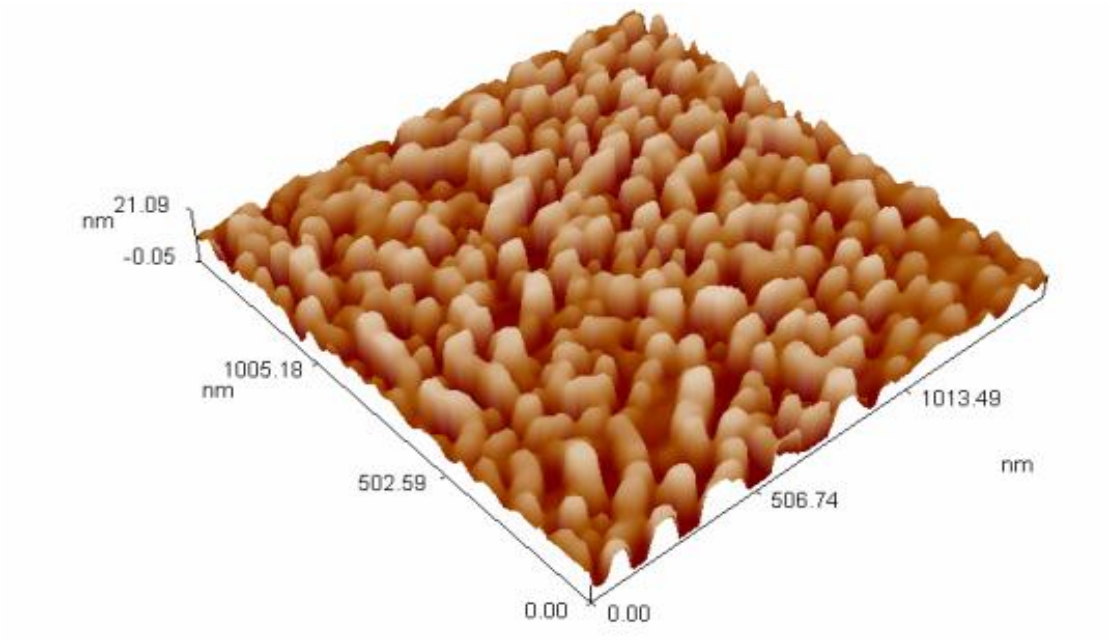

Figure 3. AFM image of the ZnO nanoparticles.

The surface morphology of the ZnO unmodified nanoparticles obtained from the AFM analysis in Figure (3) shows the surface is very smooth, the average roughness of modified ZnO is 1.33 nm. This result agrees with [31].

3. 1. 4. X-ray Diffraction Analysis (XRD)

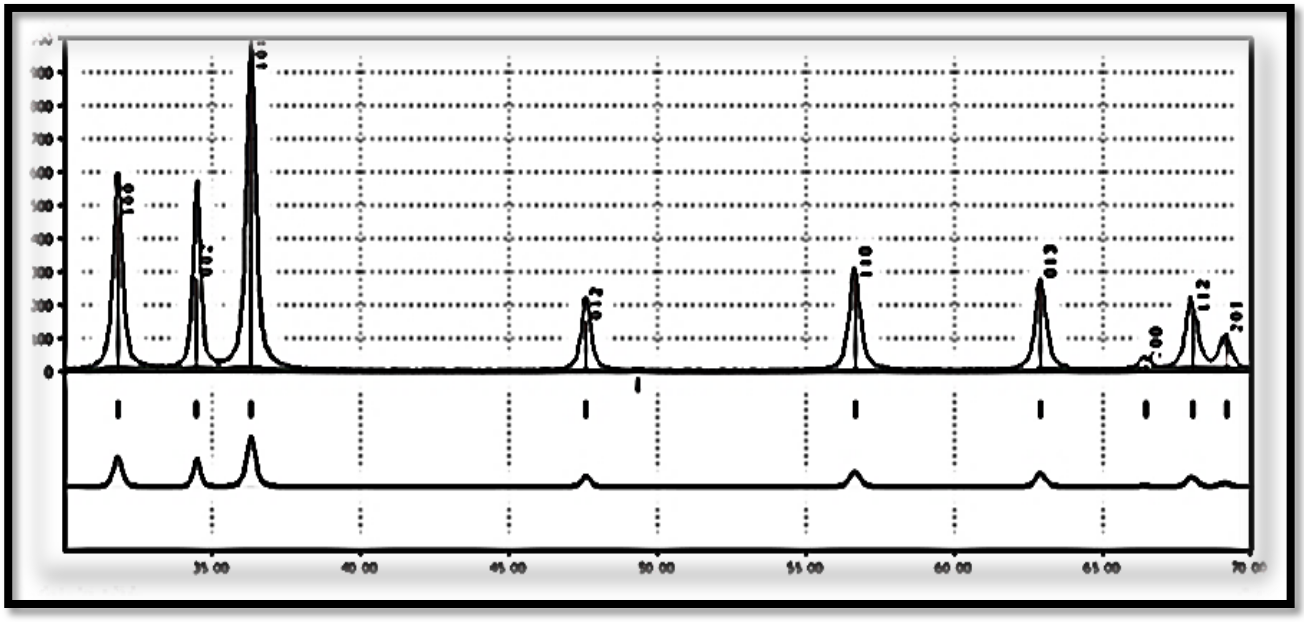

Figure 4. Analysis of X-ray diffraction for ZnO nanoparticles.

From the X-ray test Figure (4A) of ZnO nanoparticles at a diffracted angle (30° to 70°), a crystalline peak appeared which indicate crystalline structure at (2θ = 34). This indicates that crystalline material is prepared and were still in accordance with the standards ZnO XRD which agrees with the results of [32].

3. 2. Agar Diffusion Technique

Researchers find new compounds that have anti-bacterial effect may provide strategies for the control of infections and problems related to *P. aeruginosa* especially burn infections caused by it. In our research, we have tested the activity of nano ZnO on the identified *P. aeruginosa* bacteria isolated from hospitalized patients.

The uses of nano ZnO as antibacterial agent against (*P. aeruginosa*) isolated from burn infections in this study. The results of antimicrobial activity of nano-ZnO tested using agar diffusion technique against all 15 strains of *P. aeruginosa* represented in (Table-1) showed that the best zone of inhibition was (35.5mm) at a concentration of 40 µg/ml of nano-ZnO in one strain of *P. aeruginosa* while the lowest inhibition zone was (16 mm) at a concentration of 20 µg/ml of nano ZnO in one strain also.

Table 1. Measured inhibition zone diameters (mm) of nano-ZnO against Pseudomonas aeruginosa samples.

Nano ZnO Conc.	***Pseudomonas aeruginosa* Samples**														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
20 µg/ml	18	16	16.5	20	20	21	19	20	20.5	22	22	18.5	19.5	21	22
30 µg/ml	23	25	23.5	25	24	24.5	24	24	24	25	23.5	22	23.5	25	25
40 µg/ml	30	30.5	35	35	35.5	34	34	34.5	33	30.5	30	33	35	35	33

From agar diffusion method results of our study, it is obvious that the relationship between the concentration of nano ZnO agent and the inhibition zones of *P. aeruginosa* is exponentially proportional as shown in (Figure 5). The concentration of ZnO (50, 60) µg/ml in our knowledge, it is first applied, by us and gave different results about inhibition zone, so it's not included in the results that is there is poor search related with it to compare with.

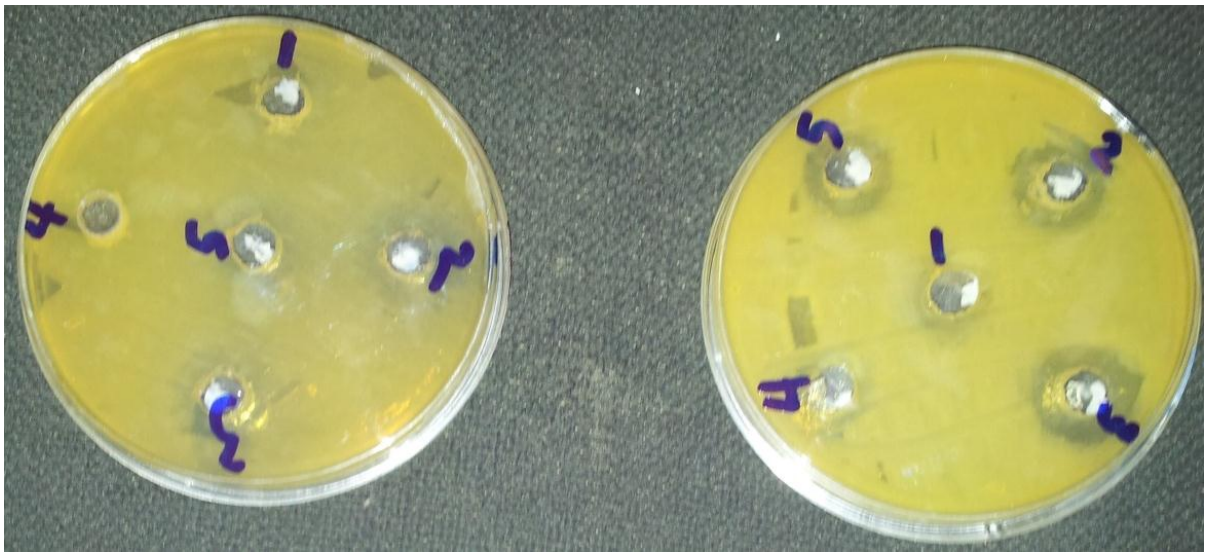

Figure 5. Agar diffusion method.

As the increasing in nano-ZnO concentration lead to increasing in the inhibition zones and this finding is in accordance with the results of (Sangani MH, et al) [33] because they found that the effect of ZnO nanoparticles against *P. aeruginosa* isolates was gradually increased. On the other hand our results were near to the results of Yousef, et al. [34] as they found that the inhibition zone of nano ZnO at a concentration of 20 μg/ml (they use only one concentration of ZnO) against *Pseudomonas aeruginosa* isolates was (22 mm), while the average of inhibition zone of nano ZnO at a concentration of 20 μg/ml in our research was (19.7 mm), they use only one sample of *P. aeruginosa* but in our research we tested fifteen strain of the mentioned bacteria and three of them gave the inhibition zone of (22 mm).

In other study done by (Chauhan, et al) [35] they found that the antibacterial activity of extracellular biosynthesized ZnO nanoparticles against *P. aeruginosa* estimated about 10.33 mm and 12.66 mm as inhibition zone at a concentration of 25 μl 50 μl respectively, while in our result the average of inhibition zone at a concentration of 30 μg/ml and at 40 μg/ml were (22.4 mm) and (33.2 mm) respectively and in other study done by (Voicu, et, al.) [36] used nano ZnO with average particle size of 19 nm, the inhibition zone of ZnO nanoparticles against *P. aeruginosa* at a concentration of 40 μg/ml was 2 mm, these results not in agreement with our results and that may be due to using of completely different preparation method of nano ZnO and different size of nanoparticles, it has been demonstrated that the size, shape, surface area, solubility, chemical composition and dispersion factor of nanoparticles play exceptional roles in determining their biological responses (Oberdorster et al.) [37].

There are some variations in the results of inhibition zones between strains of *P. aeruginosa* such as 16 mm in one strain while the other was 22mm at a concentration of 20 μg/ml of nano-ZnO and 30 mm in one strain, but it was 35 in another strain at a concentration of 40 μg/ml of nano-ZnO, these differences may be due to constitutional variation between these strains such as genetic composition and enzymes possession, antibiotic resistance mechanisms acquisition especially when isolated from hospital.

3. 3. Determination of Minimum inhibitory concentration and Minimum Bactericidal concentration (MIC/ MBC) as antimicrobial activity Nano-ZnO

In our study, the relative antimicrobial activity of nano-ZnO suspensions against pathogenic *P. aeruginosa* fifteen isolates were studied in nutrient broth quantitatively by determination of the MIC and MBC.

Here, five nano-ZnO suspensions with different concentrations were tested (of 30-15-7.5-3.7-1.8 μg/ml) and the results are given in Table 2. The data showed that all *P. aeruginosa* isolates were completely inhibited at the concentration of 3.7 μg/ml of nano-ZnO (Minimum inhibitory concentration -MIC) but no significant antibacterial activity was observed at concentrations less than 1.8 μg/ml of nano-ZnO. The data also showed that the minimum Bactericidal concentration (MBC) was same as MIC (3.7 μg/ml) for all *P. aeruginosa* isolates. Our results identical to the results of (Nagarajan P. and Rajagopalan V.) [38] in that ZnO nanoparticles have bactericidal activity in addition they interpreted in their study that once hydrogen peroxide is generated by ZnO nanoparticles, the nanoparticles remains in contact with the deadly bacteria to prevent further bacterial action and continue to generate and discharge hydrogen peroxide to the medium.

In our study, the MIC of nano ZnO on *P. aeruginosa* isolates was 3.7_μg/ml for all isolates and that differ from (Saadat, et al) [39] who found that MIC of nano-ZnO *on P. aeruginosa* isolates was 300 μg/ml, in other study done by **(**Yousef, et al.) [34] they found that MIC was 0.5 μg/ml and that near to our result if we compare it with the result of (Saadat, et al) [39], these differences from our result may be due to use of preserved bacterial strain of *P. aeruginosa* that not isolated from burn or isolation of clinical isolates that were able to form an effective biofilm, other factors that may lead to different results may be the differences in the preparation methods of nano ZnO, other probable cause may refer to the size of ZnO nanoparticles such as (30-90) nm in (Saadat, et al.) study [39]. While we used ZnO nanoparticles of 40-nm. The antimicrobial property of nanoparticles depends on the synthesized method, concentration and size of them [40-41], the activity was affected by particle size, which is controlled by processing parameters [38].

Table 2. Antimicrobial activity MIC/MBC (μg/ mL) of nano-ZnO against *Pseudomonas aeruginosa* amples.

Antimicrobial activity of nano-ZnO	***Pseudomonas aeruginosa* Samples**														
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
MIC	≥ 3.7 μg/ml														
MBC	≥ 3.7 μg/ml														

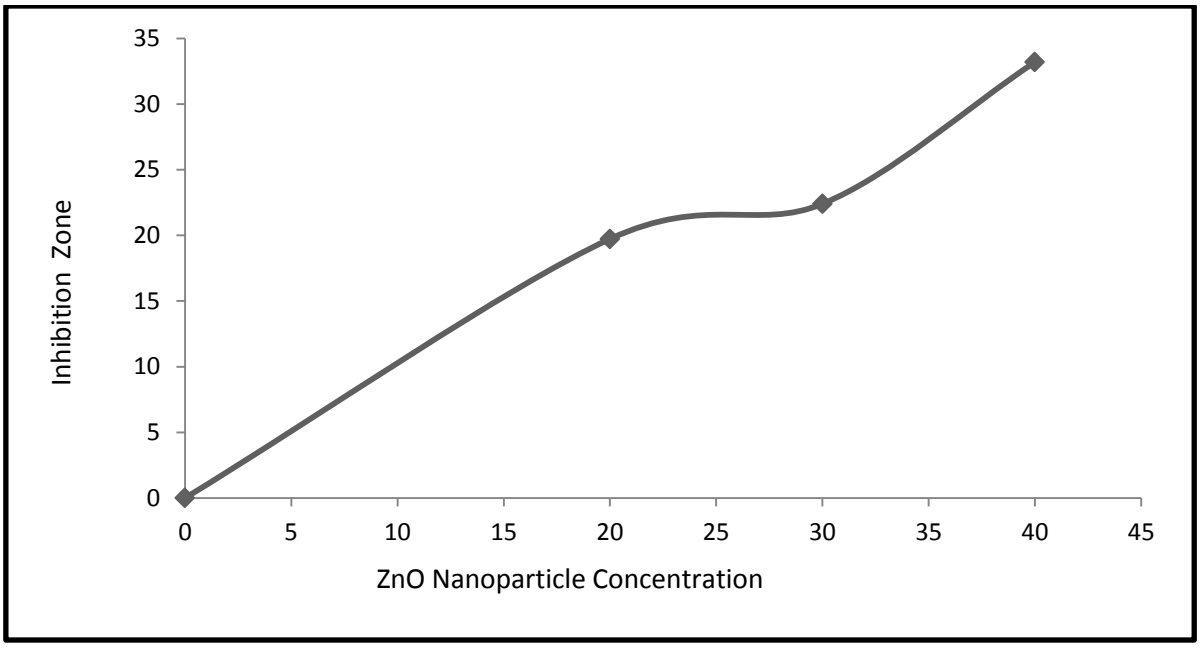

Figure 6. Relationship between the Inhibition zone diameters (mm) and the ZnO Nanoparticle Concentration against *Pseudomonas aeruginosa* samples.

4. CONCLUSION

Our study showed that zinc oxide nanoparticles had strong antibacterial activity and could inhibit one of the most important pathogenic bacteria (*P. aeruginosa*) at the concentrations mentioned.

Recommendation

We recommend more studies using nano-ZnO against *P. aeruginosa* in order to prepare some nano antibacterial remedies that effective and having less resistance from bacteria, especially for burn infections because these infections are critical and life threatening diseases, in addition to that we suggest testing more and higher concentrations of nano-ZnO against *P. aeruginosa*.

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