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Synergic Effect of Biosynthesized ZnO- Nanoparticles with Some Antibiotic on Multi-Drug Resistance Bacteria

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Abstract
In this study, we extant an ecofriendly, harmless, biosynthesized zinc oxide nanoparticle by *Escherichia coli*bacteria. Biosynthesized ZnO NPs was characterized by different techniques,and results of UV-vis showed peak at 266 nm,while X-Ray Diffractionproved the hexagonal phase structures of ZnO NPs, andFourier Transform Infrared spectra exhibited the active groups of molecules and ZnO NPswere the lower energy at 432.05. Flower shape morphology with particle size 23.87was observed in Scanning Electron Microscopeanalysis.The highest diameter of inhibition zones for *P.aeroginosa*, *E.coli*, *K.pneumoniae*, and *A.baumannii*as follows (14, 24,16, 17) mm for Ciprofloxacin and ZnO- NPs respectively. While, the diameter of highest inhibition zone for *S.aureus* 14mm for Tetracycline and ZnO- NPs.The effect of ZnO NPs have antibiofilm activity against MDR isolates in the presence of antibiotics (Ciprofloxacin for Gram negative and Tetracycline for Gram positive bacteria).In addition, anti-biofilm effect of ZnO NPs against *A. baumannii* when coated tracheal tube was studied and the result exhibit maximum inhibition of 86.7٪ at concentration 50mg/ml. The effect of ZnO-NPs on WRL 68 normal cells did not show significant effect of viability rate at concentration (400, 200, 100, 50, 25). These outcomes of combined biosynthesized ZnO NPs with antibioticshowed antibacterial and antibiofilm activities against Multi-drug resistant bacteria, and when coating the medical materials. So, these nanoparticles can be further used in biomedical, pharmaceutical and other applications as an effective antimicrobial and antibiofilm agent.
Key words: ZnO- NPs and antibiotic, Antibacterial, antibiofilm, medical materials.

Introduction

Nanoparticles (NPs) are small particles with a size range of 1- 100 nm, which play an important role in our everyday lives, and are of significant importance in the biotechnological fields such as food, medical and pharmaceutical industries (Menon *et al.,* 2017). In microbiology, nano-sized antimicrobial agents are extremely reacting and favored over original-sized antimicrobials, primarily due to their high surface area, researchers have accepted that NPS has antimicrobial and anti-biofilm effects against various fungal, bacterial and other microbial species (Qayyum and Khan, 2016).

Microbes like bacteria, fungi and yeast play a significant role in the biological synthesis of NPs of metal and metal oxides. In general, not all microbes are capable of synthesizing NPs since each microbe has various metabolic processes and enzymes. Thus, the selection of suitable microbes (regardless of their enzyme activity and biochemical pathway) is crucial to the formation of NPs.

The NPs developed are characterized physio-chemically to determine their properties, including scale, shape, surface load, functional group and purity (Król *et al.,* 2017). The synthesis of NPs of metal and metal oxide depends on the ability of microbes to withstand heavy metals. In addition,

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it is well known that high metal stress can have an impact on various microbial activities (Giller *et al.,* 2009).

Zinc is an essential nutrient in living organisms (Swain *et al.,* 2016). Zinc is also an essential trace mineral that plays an important role in several physiological functions in the body (Sahoo *et al*., 2014). Evidence has shown that ZnO NPs have a high potential in biological applications, especially as antimicrobial agents (Jamdagni *et al.,* 2018). In addition, numerous studies have been published on the efficacy of ZnO NPs in inhibiting the growth of a wide spectrum of pathogens (Moghaddam *et al.,* 2017) that could potentially replace traditional antibiotics.

Biofilm are aggregates of cells (Bacteria or Fungi) from one microbial or polymicrobial that are packed together to make up a biofilm (Hall-Stoodley *et al*., 2004). Biofilm-related issues are that the bacteria become antibiotic resistant and more resistant to disinfectant chemicals or to the immune system's reaction. (Hoiby *et al*., 2017).High percentage of biofilm are related with medical devices, and about 60٪from 1 million cases estimated medical devices are because of biofilms that formed on medical devices (Darouiche, 2004). Establishment of these devices may occur quickly within 24 hours and numerous studies have examined the of several types of antimicrobial treatment in regulatory biofilm formation on these devises(Namasivayam*et al*., 2012)

This study aimed to study the assessment of the combined impact of antibiotics and biosynthesized ZnO- NPs against Multi-Drug Resistant bacteria as antibacterial and antibiofilm agents, and determine antibiofilm activity on medical device.

MATERIALS AND METHODS

Chemicals

Zinc sulphate and Sodium hydroxide (BDH- England), complicated in the biosynthesis of ZnO-NPs were purchased from local medical shop, and type of human liver cell line WRL 68 display morphology identical to that of hepatocytes also liver primary cultures (Malaysia), while antibiotics from (Bioanalyse company- Turkey).

Bacterialisolates

E.coli bacteria (65 sample) was collected from stool samples of non-infectious people, and the test bacterial isolates *P.aeroginosa, A.baumannii, K.pneumoniae, E.coli,* and *S.aureus* were collected from 40 clinical specimens from different sources (urine, blood, wound, chest infection) from different hospitals in Baghdad city- Iraq. Nutrient agar and MacConkey agar used for the cultivation of Gram negative bacteria,whileMannitolsalt agar for *S.aureus* were obtained from HI media company- India.

Biosynthesis of Zinc oxide (ZnO_2) Nanoparticles

Sterilized 50 ml of Nutrient broth was inoculated with 18 hr. of *Escherichia coli* and incubated at 37 C° for 24 hr. (Hasan, 2016). About 50ml of 0.1 M Zinc sulphate ($ZnSO_4$) and 50ml of 0.4 M sodium hydroxide (NaOH) were mixed in 250ml flask, after adding the mixture to the bacterial broth, shake vigorously and then heating in the water bath at 40°C for 15 minutes, the flask was placed in microwave for 1-2 minutes, after this, cooled for 1 hr.to allowed the nanoparticles to settle down. The formation of a white precipitate at the bottom of the flask is evidence of the formation of nanoparticles.The nanoparticle was collected by centrifugation at 3000 rpm for 10 minutes and this process was repeated three times, after any centrifugation the supernatant were washed properly with deionized water. Finally, the supernatant was collected in a small glass petri

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dish and kept in oven to drying at 40˚C for 8 hr. Then ZnO nanoparticle was obtained in powdered form, and the dry weight was estimated (Mishra *etal*., 2013).

Characterization of biosynthesized ZnO nanoparticles

UV-Vis spectroscopy

The maximum optical absorption of the sample was characterized using UV-Vis spectroscopy (100CONC plus, NIR) in the wavelength range of (200-900) nm.

X- ray diffraction (XRD)

The crystalline structure of ZnO NPs was determined by using X- ray diffraction analyzer (XRD 6100 diffraction- Shimadzo). The average crystallite size (D) was calculated using Debye Scherer's formula: $D=\frac{K\lambda}{\beta cos\theta}$

Were K□ is the shape factor (0.9), λ□ is the wavelength of X−ray (1.5406 Å) Cu Ka radiation, β□ is the full width half maximum (FWHM), θ□ is the diffraction angle(Manyasree*et al.*, 2018).

Fourier Transform Infrared (FTIR)

The functional groups involved in the formation of ZnO NPs was determined by using Fourier Transform Infrared (FTIR) spectra (Perkin-Elmer model) at the wave number range of 3900- 400 cm^{-1}.

Scanning electron microscope (SEM)

Morphological study of ZnO NPs was determined using scanning electron microscopy(Carl Zeiss Ultra55 model).

Determination antibacterial effect of combined antibiotics and biosynthesized ZnO-NPs

To determine the combined effects of antibiotics (Tetracycline, Methicillin, Oxacillin, Erythromycin, Ampicillin, Ciprofloxacin, Gentamicin, Cefepime, and Meropenem) with biosynthesized ZnO nanoparticles followed the described method by (Chauhan *et al*. 2015) with some modification.Each standard paper of antibiotics disc was further impregnated with sub- MIC of the biosynthesized ZnO nanoparticles and adding filter paper disc saturated with sub- MIC concentration of ZnO- NPs

A single colony of MDR bacteria was suspended in the test tube containing 4ml of distilled water, then turbidity was adjusted to obtain 0.5 McFarland, and by a sterile cotton swab, a portion of bacterial suspension was spread on Mueller - Hinton agar plates along, then with a sterile forceps the discs with sub –MIC of biosynthesized ZnO nanoparticles were placed. After incubation at 37°C for 24 h, the zones of inhibition were measured.

Effect of combined ZnO-Nps with antibiotic on biofilm formation

The antibiofilm activity of synergicbiosynthesized ZnO Nps and antibiotics against Multi drug resistance (MDR) bacteria were determined by (Ashajyothi *et al*, 2016) with modification. Each of the bacterial suspension in the brain heart infusion broth with 2% sucrose (100µl) was added to 96 well flat-bottomed microtiter plate together with (100µl) sub-MIC of ZnO nanoparticles or antibiotic or equally(50µl of ZnO NPs together with 50µl Antibiotic).

Control wells contained 180 µl of brain heart infusion broth with 2% sucrose and 20 µl of bacterial suspension.The covered microtiter plate was sealed with parafilm during incubation at 37°C for (24,48) h. Un-attached bacterial cells were removed by washing the wells three times with PBS(PH 7.2).After drying at room temperature, 200 µl of crystal violet (0.1%) was added to the wells for 20 min. The stained attached bacterial cells were rinsed three times with PBS(PH

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7.2), and allowed to dry at room temperature then extracted twice with 200 µl of 95% ethanol and the absorbance of each well was measured at 630 nm using ELISA Reader. The inhibition of biofilm formation of each pathogenic bacterium was calculated as equation described by (Namasivayan *et al*., 2013).

% Inhibition of biofilm formation = $\frac{ODcontrol - DOtratment}{ODcontrol} \times 100$

The microtiter plate antibiofilm assay estimates the percentage of bacterial biofilm reduction in relation to the control wells, which were set at 100% to indicate the absence of ZnO nanoparticles. In contrast, negative percentage results indicate no inhibition activity of ZnO nanoparticles on biofilm formation.

Antibiofilm of biosynthesized ZnO NPs coated medical device (Tracheal tube)

Tracheal tube was obtained from local medical shop (Baghdad- Iraq), the tube was cut in to 1×2 cm^2 surface and the pieces were put in a tube containing 10 ml of ZnO NPs suspension with (25 and 50) µg/ml concentration and kept for 24 hrs. at room temperature. Coating of nanoparticles was proven by color change of the tracheal tube surface. The cut pieces were put in a test tube containing (5ml) brain hart infusion broth and inoculated with fresh bacterial colony, the inoculated tubes were incubated in 37C° for 3 days.

The hole contents were removed after the incubation period and 3ml of 1٪ crystal violet was added and kept at room temperature for 10 min. Crystal violet was removed and washed using sterile phosphate buffer saline to removed unbound or free planktonic cells, then (5 ml) of ethanol added and kept at room temperature for 15 min. the reaction mixture was transferred into microtiter plate and read at 590 nm by ELISA redder, the biofilm inhibition (٪) was calculated by the same formula above (Namasivayan *et al*., 2013).

MTT Assay for cytotoxicity

To determine the cytotoxic effect of ZnO-NPs onWRL 68 cells, First the cell viability assay is done using 96-well plates. Cell lines were seeded at 1×104 cells/well. After 24 hrs. Or a confluent monolayer was achieved, cells are treated with ZnO-NPs at different concentration (400-25) µg/ml. Cell viability is measured after 24 hrs of treatment by removing the medium and adding 28 µL of 2 mg/mL solution of MTT stain and incubating the cells for 2.5 h at 37°C. After removing the MTT solution, the crystals remaining in the wells are solubilized by the addition of 130 µL of DMSO (Dimethyl Sulphoxide) followed by 37°C incubation for 15 min with shaking (Ali *et al.*, 2019). The absorbency is determined on a microplate reader at 492 nm; the assay was performed in triplicate. The inhibition rate of cell growth (the percentage of cytotoxicity) is calculated as the following equation (Alsaedi *et al*., 2019). Cytotoxicity = A-B/A *100

Where A and B are the optical density of control and the optical density of test respectively.

Results and Discussion

In this study, 49 isolates of *E. coli* were identified (75 ٪)from 65 stool sample, and the process of identification was performed by using the traditional methods. While, 6 isolates of *Acinetobacter baumannii* (20٪), 5 isolates of *pseudomonasaeruginosa* (16.66٪), 8 isolates for each *Klebsiella pneumoniae* and *Escherichiacoli* (26.66) and 3 isolates of *Staphylococcusaureus* (10٪)from different clinical sourcesas shown in Table(1).

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Table (1): Number of pathogenic bacterial isolates

Bacterial isolates	No. of isolates	%	Source
A.baumannii	6	20	Sputum
P.aeroginosa	5	16.66	2 wounds
			3 urine
K.pneumoniae	8	26.66	3 wounds
			5 urine
E.coli	8	26.66	2 wounds
			6 urine
S.aureus	3	10	1 wound
			2 Nasal swabs
Total	30	100%	30

Biosynthesis of ZnO Nanoparticles

A white cluster deposited appeared at the bottom of the flasks of *Escherichiacoli*indicates the biosynthesis process. Biosynthesized ZnO nanoparticles were obtained in powdered form, and the dry weight was estimated (Figure 1).

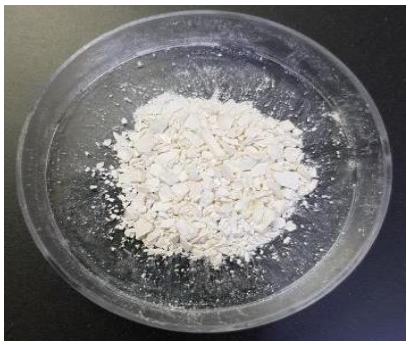

Figure (1): White cluster of ZnO NPs synthesized by *E.coli*

The change in color is due to surface Plasmon resonance (SPR), which is a distinguishing property of the nanoparticles (Thamer, 2016). The biosynthesized of NPs by microorganisms are classified into extracellular and intracellular synthesis (Hamida*etal*., 2020). The biosynthesized of NPs controlled by general steps such as metal ions are trapped on the microbial surface or in the microbial cells in the presence of enzymes, finally reduced to form NPs (Yin *etal*., 2016).

Characterization of ZnO-Nps

UV- visible spectroscopy analysis

Figure-2 presence an absorption peak observed at 266 nm indicates the successful biosynthesis of ZnO NPs. UV –Visible spectroscopy is highly used technique to distinguish the optical properties of the nanoparticles.

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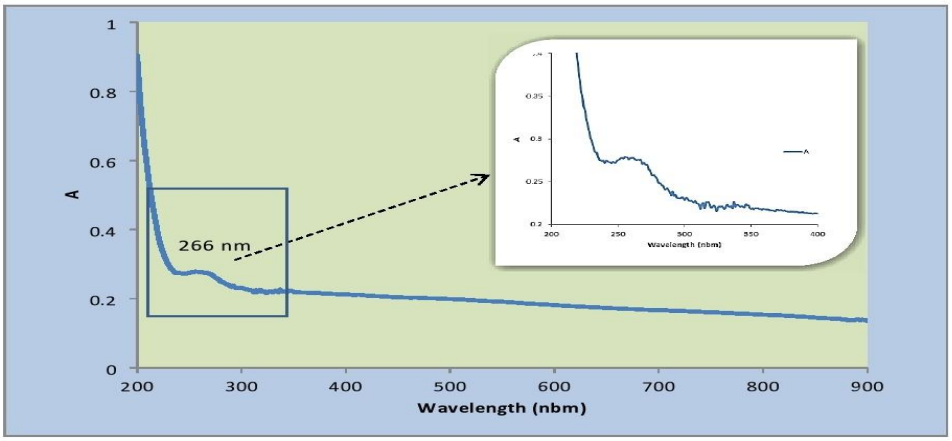

Figure (2) □ UV−visible absorption spectrumof ZnO NPs synthesized by *E.coli*

Ifeanyichukwu *et al*., (2020) reported that's indicate combined vibration of electrons of the biosynthesized nanoparticles with the light wave. This result is agreement with result which reported by Balogun *et al*., (2020), where absorption peak at 270 nm was obtained. Huda (2018) reported a corresponding absorption peak of ZnO NPs at 207 nm due to the transmission of electron between the ranges from the deep level of the valance range.

Fourier Transform Infrared Spectra Measurement

FTIR spectrum of the biosynthesized ZnO NPs are illustrated in Figure (3), showing the broad peak at (3684.04 and 3263.56) cm^{-1} which correspond to O–H stretching vibrations.FTIR spectral band exhibits weak absorption near 2315.44 cm^{-1} which is indicated free C−H group. The peak at 1396.46 cm^{-1} showed C−N vibration stretch in protein amide linkages. The sharp absorption peak observed at 904.61cm^{-1} assigned to the N–H bending vibration of amine. The lower energy region at 432.05 is assigned to the ZnO stretching vibration.

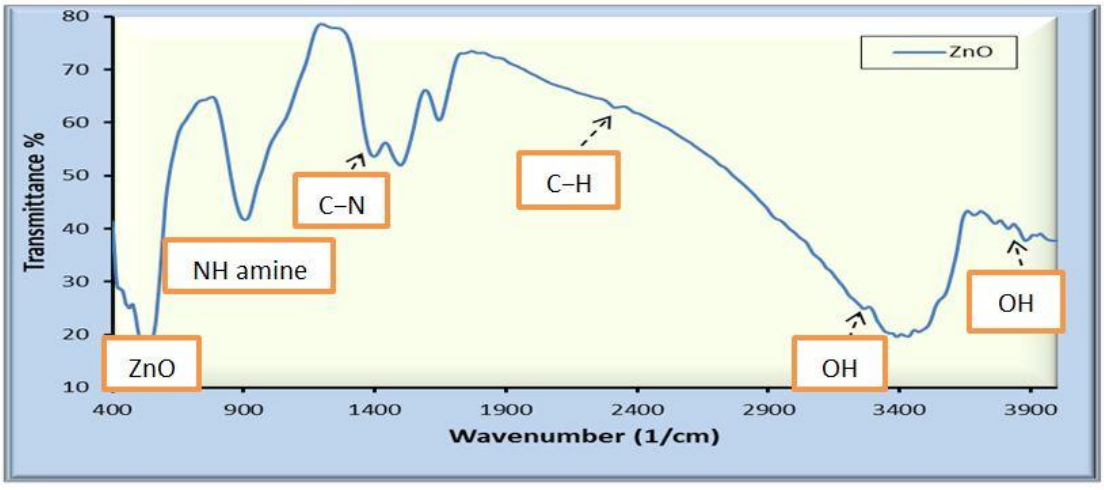

Figure (3) □ FTIR result of ZnO NPs synthesized by*E.coli*

Steffy *et al*., (2018**)** showed in his study to biosynthesized ZnO NPs the broad peak at 3428 and 3399.89 cm^{-1} which corresponds to O−H stretching vibration. Sana *et al*, (2020**)** reported 2353 cm^{-1} in ZnO NPs is corresponding to C−Hstretching vibration which attributed to the presence of alkaline group. The peak was found at 450 cm^{-1} corroborative Zn–O bond bending

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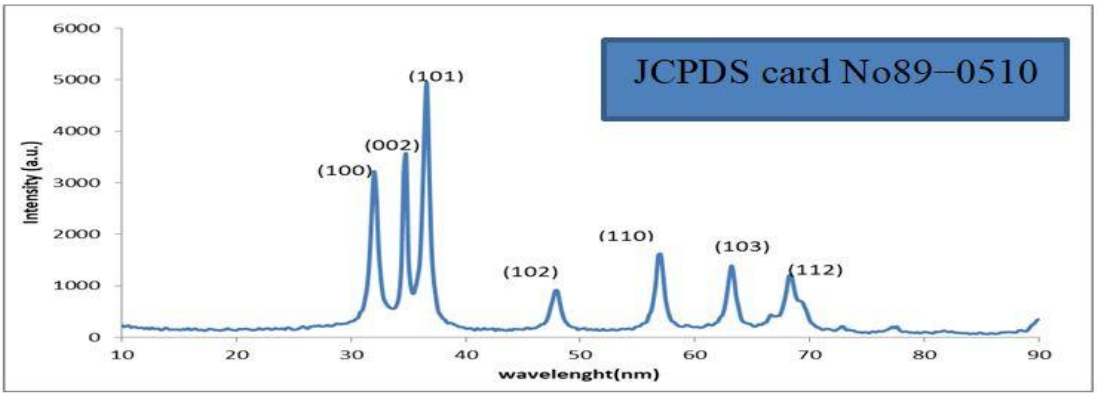
vibration as metal-oxygen is reported in the range of 400 to 600 nm (Nithya and Kalyanasundharam refers, 2019).

X –ray Diffraction

The crystal phase and crystallinity of the biosynthesized ZnO NPs were analyzed using XRD pattern, and the calculated average particle diameter is found to be 10 nm as shown in Figure (4), the observed lattice planes of (100), (002), (101), (102), (110), (103)and (112) corresponded to the 2θ values of 32.48°, 34.73°, 36.57°, 47.84°, 56.90°, 62.22°and 63.15° respectively, and the diffraction peaks are well steady with the hexagonal phase of ZnO according to (JCPDS card No89−0510). Line broadening of the diffraction peak is an indication that the synthesized material in the nanometer range (Osuntokun et al., 2019). The average crystalline size was obtained from the XRD peak using Scherer's equation (Rajeshkumar*et al.*, 2018).

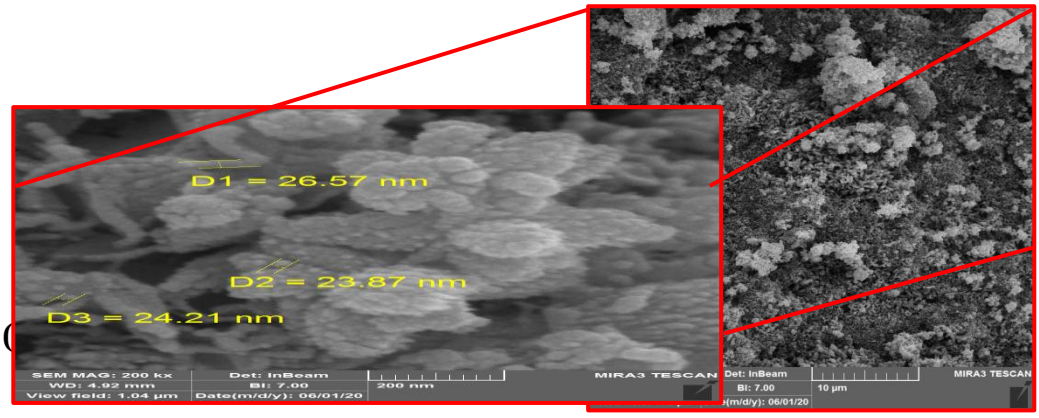


(Figure 4) □ XRD pattern of ZnO NP synthesized by*E.coli*

Scanning Electron Microscope (SEM) analysis

Figure (5) represents the SEM image to studying morphological properties of the ZnO NPs. The dimensions of the particle range between (23.87−26.57) nm are aggregated as flower shape. The morphology of NPs is important sides that participate to the physiochemical properties of the substances (Kuruppu*etel*.,2020). A research suggests that the annealing temperature have important effect on the shape of NPs were in some process, the morphology of the NPs seemed to change after calcination at high temperatures (Gopal and Kamila 2017).

Figure (



Antibacterial of combined Antibiotics and ZnO NPs

The results showed the diameter of inhibition zone for *P.aeroginosa* as follows 14, 9, 9, 8, and 5 mm for Ciprofloxacin, Gentamicin, Cefepime, Meropenem and ZnO NPs alone respectively as

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shown in figure (6- A). For *A.baumannii* the results as follows 17, 13, 16, 14, and 2mm for the same disks respectively as shown in figure 7(6-,B). While the result for *E.coli* as follows 24, 13, 0, 22, and 2mm for Ciprofloxacin, Gentamicin, Cefepime, Ampicillin and ZnO NPs alone respectively as shown in figure (6- C). The diameter of inhibition zone of *K.pneumoniae* as follows 16, 6, 14, 0, and 2mm for the same disks respectively as shown in figure (6- D). Finally the diameter of inhibition zone for *S.aureus* as follows 14, 8, 7, 5, and 4mm for Tetracycline, Methicillin, Erythromycin, Oxacillin and ZnO NPs respectively as shown in figure (6- E).

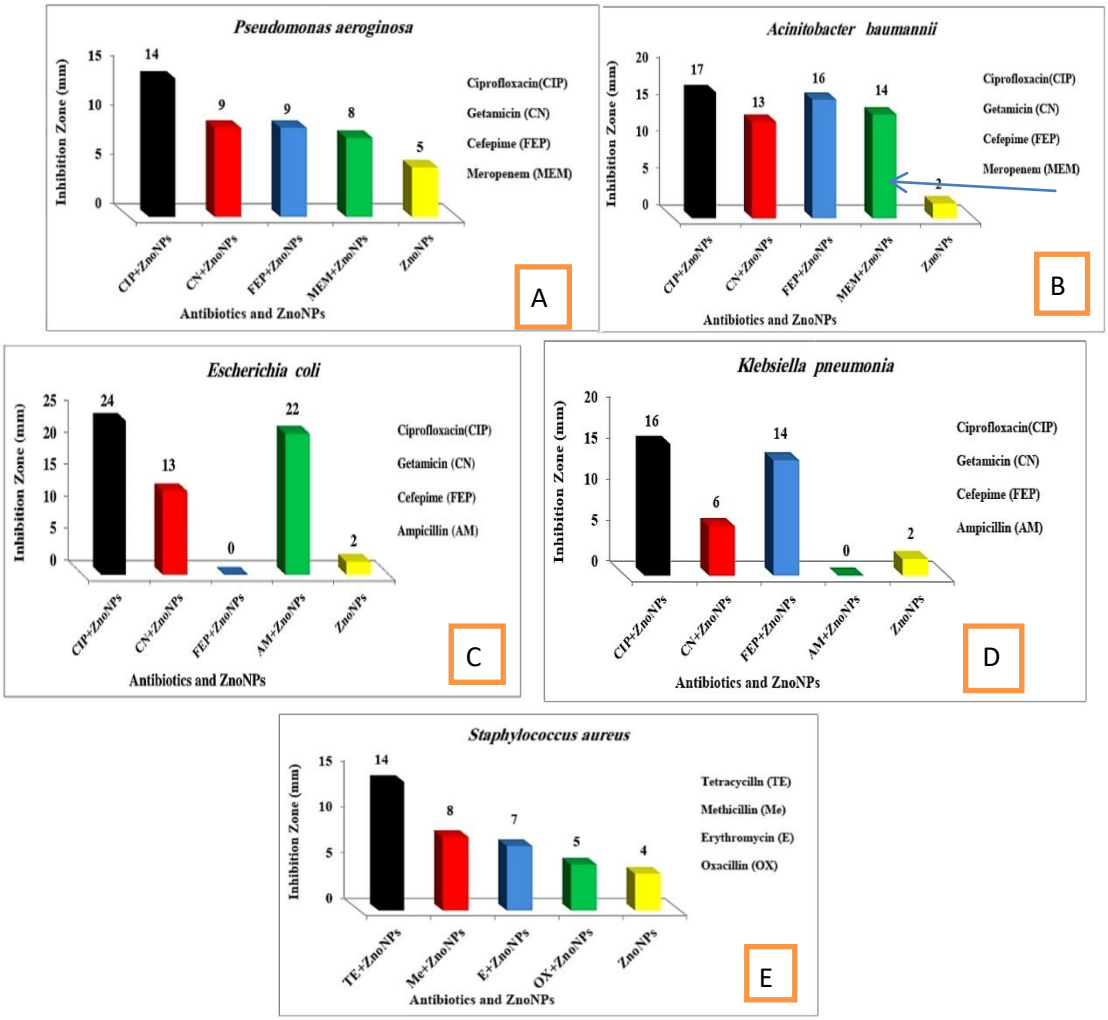

Figure 6 Antibacterial effect of ZnO NPs and Antibiotics for A- *P.aeroginosa; B- A. baumannii; C- E. coli; D- K. pneumoniae; E- S. aureus*

The highest antibacterial activities of antibiotics combined with biosynthesized ZnO nanoparticles by nonpathogenic *E.coli* were observed with Ciprofloxacin and showed high inhibition zone (24 mm) for *E.coli* while the highest inhibition zone of antibiotic combined with ZnO NPs were observed with Tetracycline for *S.aureus*. The combined effect of ZnO NPs synthesized by nonpathgenic *E.coli* and antibiotics was promising against pathognic *E.coli* followed by *A.baumannii ,P. aeruginosa, S. aureus, and K. pneumoniae* respectively. This result good agreement with TULI*etal*, (2020) reported zinc oxide nanoparticles are able to enhance the efficiency of antibiotics. Among five tested bacterial strains (*Staphylococcus*, *Escherichiacoli*, *Klebsiella*, *Shigella*, and *Pseudomonas*) had been show resistant against tested drugs. Zinc oxide

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nanoparticles which have therapeutic roles in different diseases have been recognized (Sharma *etal*.,2016).

Antibiofilm of combined Antibiotics and ZnO NPs

Results showed that ZnO NPs was able to inhibit biofilm formation of MDR bacterial isolates. Moreover, this study also observed the synergistic effect of ZnO NPs of antibiofilm activity against MDR isolates in the presence of antibiotics (Ciprofloxacin for Gram negative and Tetracycline for Gram positive bacteria) samples treated with antibiotic alone showed negligible activity on biofilm inhibition (Table-2).

Table (2) Antibiofilm effect of ZnO NPs synthesized by *E.coli* against MDR bacterial isolates after 24 hr. incubation time

Bacterial isolates	Biofilm inhibition %					
	Incubation time (24 hours)			Incubation time (48 hours)		
	ZnO NPs	Antibiotic	Antibiotic +ZnO NP	ZnO NPs	Antibiotic	Antibiotic +ZnO NPs
S.aureus	12.18	4.06	40.3	19.6	11.4	48.2
P.aeroginosa	12.6	9.4	16.2	29.8	20.8	37.9
A.baumannii	20.65	15.56	30.2	37.3	26.6	50.7
K.pneumoniae	18.68	8	23	20.3	14.1	35.5
E.coli	18	10	30	38.6	19.47	40.19

In addition, the large surface area and presence of functional groups, like amino group, hydroxyl group, etc. lead nanoparticles to interact with antibiotics by chelating reactions (Kazemnia, 2019). Because of the resistant properties of biofilm, eradication of biofilm related disease is challenging (Miyaue*etal*., 2018) ZnO nanoparticles have bioactivity properties such as control of biofilms formation according to the concentration of this nanoparticles, for this reason it has been as promising antibacterial agents than conventional antibiotics (shakerimogliaddam *et al.*, 2017).

Antibiofilm of biosynthesized ZnO- NPsand antibiotic coated medical device

Coating of biogenic Zinc oxide nanoparticles combined to antibiotic was easily recognized by color change of tracheal tube (White color) as shown in figure (7).

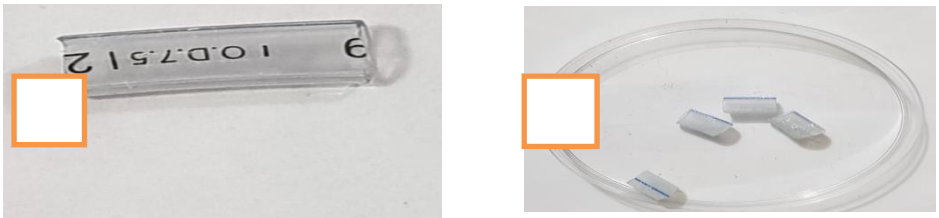

Figure (7) Tracheal tube A−without coated by ZnO NPs, B− ZnO NPs+ antibiotic coated tracheal tube

Biofilm inhibition study clearly observed at the tested concentration inhibited biofilm of *Acinetobacter baumannii*. Results were identified as inhibition percentage of biofilm development in microtiter plate assay, the best inhibition percentage is 86.7% at the concentration 50 mg/ml, whereas the inhibition zone for a concentration of 25 mg/ml was 58.8 as shown in Table (3).

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Table (3) Percentage of biofilm inhibition by nanoparticles and antibiotic

Bacterial isolate	Concentration (ml)	Biofilm inhibition (٪)
Acinetobacterbaumannii	25	58.8
Acinetobacterbaumannii	50	86.7

Namasivayam *et al*., (2012) shown in his study when coated catheter with Ag NPs in the concentration 100mg/ml different percentage inhibition biofilm was recorded at 6 hr. of incubation with 61.5% of inhibition as well as 78.5% inhibition at 12 hr. of incubation and complete inhibition (100%) was obseved in 18 hr. of incubation. Dose dependent manner of ZnO NPs for inhibit biofilm production by clinical isolate of *S. aureus* after coated medical devices with biogenic Silver nanoparticles because of the effect of Ag NPs on biochemical composition of biofilm matrix such as protein and carbohydrate produce by S. aureus isolates (Namasivayam *et al*., 2013).

Cytotoxicity of ZnO-NPs

The WRL 68 cells did not show significant effect of viability rate ranged between (62.6 ± 4.4, 71.3 ± 7.1, 88.3 ± 6.2, 87.6 ± 5.2 and 94.2 ± 1.7) at concentration (400, 200, 100, 50, 25) respectively, as shown in (Table 4).

Table 4 Flow cytometric analysis of WRL68 cells after treated with ZnO-NPs.

Concentration of ZnO NPs(mg/ml)	Viability (%)
	WRL
400	62.6± 4.4
200	71.3± 7.1
100	88.3± 6.2
50	87.6± 5.2
25	94.2±1.7
Values are expressed as mean ±SD of three experiments.	

Namvar *etal*, (2015) revealed that no toxicity effect against normal mouse fibroblast (3T3) cell line, and this correspond with these results.

Conclusions

The results in this study of combined ZnO NPs biosynthesized using *E.coli* with antibioticshowed antibacterial and antibiofilm activities against Multi-drug resistant bacteria, and when coating the medical materials. This synergic effect is useful to use as a bactericidal agent in many

applications, and consider as an affective biofilm disinfectant using in medicine and pharmacology as medical device coating.

References

1. Ali, S. H., G. M. Sulaiman, M. M. F. Al-Halbosiy, M. S. Jabir and A. H. Hameed, (2019). Fabrication of hesperidin nanoparticles loaded by poly lactic co-Glycolic acid for improved therapeutic efficiency and cytotoxicity. Artificial Cells, Nanomedicine, and Biotechnology, 47, 378-394.
2. Alsaedi, I. I. J., Z. J. Taqi, A. M. Abdul Hussien, G. M. Sulaiman and M. S. Jabir, (2019). Graphene nanoparticles induces apoptosis in MCF-7 cells through mitochondrial damage and NF-KB pathway. *Materials Research Express,* **6,** 095413
3. Ashajyothi, C.□ K. Handral Harish□ Nileshkumar Dubey, and R. Kelmani Chandrakanth.(2016). "Antibiofilm activity of biogenic copper and zinc oxide nanoparticles-antimicrobials collegiate against multiple drug resistant bacteria: a nanoscale approach." *Journal of Nanostructure in Chemistry* 6, no. 4: 329-341
4. Balogun, S. W., James, O. O., Sanusi, Y. K., & Olayinka, O. H. (2020). Green synthesis and characterization of zinc oxide nanoparticles using bashful (Mimosa pudica), leaf extract: a precursor for organic electronics applications. Sn Applied Sciences, 2(3), 1-8.
5. Chauhan, R.□ Reddy, A.□ & Abraham, J. (2015). Biosynthesis of silver and zinc oxide nanoparticles using Pichia fermentans JA2 and their antimicrobial property. *Applied nanoscience*, 5(1), 63-71.
6. Darouiche, R. O. (2004). Treatment of infections associated with surgical implants. *New England Journal of Medicine*, *350*(14), 1422-1429.
7. Giller, K. E., Witter, E., &mcgrath, S. P. (2009). Heavy metals and soil microbes. *Soil Biology and Biochemistry*, *41*(10), 2031-2037.
8. Gopal, V. V., & Kamila, S. (2017). Effect of temperature on the morphology of zno nanoparticles: a comparative study. Applied Nanoscience, 7(3-4), 75-82.
9. Hall-Stoodley, L., Costerton, J. W., & Stoodley, P. (2004). Bacterial biofilms: from the natural environment to infectious diseases. *Nature reviews microbiology*, *2*(2), 95-108.
10. Hamida, R. S., Ali, M. A., Redhwan, A., & Bin-Meferij, M. M. (2020). Cyanobacteria–a promising platform in green nanotechnology: a review on nanoparticles fabrication and their prospective applications. International Journal of Nanomedicine, 15, 6033.
11. Hasan, Z.H.□ (2016). Inhibition of Biofilm formation by silver nanoparticles biosynthesized by pathogenic *Escherichia coli*. MSC Thesis.Collage of Science, Mustansiriyah University.
12. Hoiby, N. (2017). A short history of microbial biofilms and biofilm infections. *Apmis*, *125*(4), 272-275.
13. Huda M. Jawad, Ahmed N. Abd and Wasna'a M. Abdulridha, 2018. Characterization of a Zinc Oxidethin Film Used Simple Chemical Method. Journal of Engineering and Applied Sciences 13(18) □ 7537
14. Ifeanyichukwu, U. L., Fayemi, O. E., & Ateba, C. N. (2020). Green synthesis of zinc oxide nanoparticles from pomegranate (Punica granatum) extracts and characterization of their antibacterial activity. Molecules, 25(19), 4521.

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 3, 2021, Pages. 2293 - 2305
Received 16 February 2021; Accepted 08 March 2021.

15. Jamdagni, P., Khatri, P., & Rana, J. S. (2018). Green synthesis of zinc oxide nanoparticles using flower extract of Nyctanthes arbor-tristis and their antifungal activity. *Journal of King Saud University-Science*, *30*(2), 168-175.
16. Kazemnia, A. (2019). Effect of silver nanoparticles combined with antibiotics on different phylogenetic groups of Escherichia coli. Journal of the Hellenic Veterinary Medical Society, 70(3).
17. Król, A., Pomastowski, P., Rafińska, K., Railean-Plugaru, V., & Buszewski, B. (2017). Zinc oxide nanoparticles: Synthesis, antiseptic activity and toxicity mechanism. *Advances in colloid and interface science*, *249*, 37-52.
18. Kuruppu, K. A. S. S., Perera, K. M. K. G., Chamara, A. M. R., & Thiripuranathar, G. (2020). Flower shaped zno—nps; phytofabrication, photocatalytic, fluorescence quenching, and photoluminescence activities. Nano Express, 1(2), 020020.
19. Manyasree, D., Kiranmayi, P., & Venkata, R. K. (2018). Characterization and antibacterial activity of zno nanoparticles synthesized by co-precipitation method. *Int J App Pharm*, *10*(6), 224-228.
20. Menon, S., Rajeshkumar, S., & Kumar, V. (2017). A review on biogenic synthesis of gold nanoparticles, characterization, and its applications. *Resource-Efficient Technologies*, *3*(4), 516-527.
21. Mishra, M.□ Paliwal, J. S.□ Singh, S. K.□ Selvarajan, E.□ Subathradevi, C.□ & Mohanasrinivasan, V. (2013). Studies on the inhibitory activity of biologically synthesized and characterized zinc oxide nanoparticles using lactobacillus sporogens against Staphylococcus aureus. *J Pure Appl Microbiol*, *7*(2), 1-6.
22. Miyaue, S., Suzuki, E., Komiyama, Y., Kondo, Y., Morikawa, M., & Maeda, S. (2018). Bacterial memory of persisters: bacterial persister cells can retain their phenotype for days or weeks after withdrawal from colony–biofilm culture. Frontiers in microbiology, 9, 1396.
23. Moghaddam, A. B., Moniri, M., Azizi, S., Rahim, R. A., Ariff, A. B., Saad, W. Z., ... & Mohamad, R. (2017). Biosynthesis of zno nanoparticles by a new Pichia kudriavzevii yeast strain and evaluation of their antimicrobial and antioxidant activities. Molecules, 22(6), 872.
24. Namasivayam, S. K. R., Preethi, M., Bharani, A., Robin, G., & Latha, B. (2012). Biofilm inhibitory effect of silver nanoparticles coated catheter against Staphylococcus aureus and evaluation of its synergistic effects with antibiotics. *Int J Biol Pharm Res*, 3(2), 259-265.
25. Namasivayam, S. K. R.□ Christo, B. B.□ Arasu, S. K.□ Kumar, K. A. M.□ & Deepak, K. (2013). Anti-biofilm effect of biogenic silver nanoparticles coated medical devices against biofilm of clinical isolate of Staphylococcus aureus. *Global Journal of Medical Research*, *13*(3), 1-7.
26. Namvar, F., Rahman, H. S., Mohamad, R., Azizi, S., Tahir, P. M., Chartrand, M. S., & Yeap, S. K. (2015). Cytotoxic effects of biosynthesized zinc oxide nanoparticles on murine cell lines. *Evidence-Based Complementary and Alternative Medicine*, *2015*.
27. Nithya, K., & Kalyanasundharam, S. (2019). Effect of chemically synthesis compared to biosynthesized zno nanoparticles using aqueous extract of C. Halicacabum and their antibacterial activity. *Opennano*, *4*, 100024.

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 3, 2021, Pages. 2293 - 2305
Received 16 February 2021; Accepted 08 March 2021.

28. Osuntokun, J.; Onwudiwe, D.C. and Ebenso, E. (2019). Green synthesis of zno nanoparticles using aqueous Brassica oleracea L. Var. Italica and the photocatalytic activity. *Green chemistry letters and reviews*,Vol. 12, No. 4, 444–457.
29. Qayyum, S., &Khan, A. U. (2016). Nanoparticles vs. Biofilms: a battle against another paradigm of antibiotic restsiance. Medchemcomm, 7(8), 1479-1498.
30. Rajeshkumar, S., Kumar, S. V., Ramaiah, A., Agarwal, H., Lakshmi, T., & Roopan, S. M. (2018). Biosynthesis of zinc oxide nanoparticles usingmangifera indica leaves and evaluation of their antioxidant and cytotoxic properties in lung cancer (A549) cells. Enzyme and microbial technology, 117, 91-95.
31. Sahoo, A. Swain, R. K. and Mishra, S. K. (2014). Effect of inorganic, organic and nano zinc supplemented diets on bioavailability and immunity status of broilers. *International Journal Advance Resource*. 2; 66-99.
32. Sana, S. S., Kumbhakar, D. V., Pasha, A., Pawar, S. C., Grace, A. N., Singh, R. P., ... & Peng, W. (2020). Crotalaria verrucosa Leaf Extract Mediated Synthesis of Zinc Oxide Nanoparticles: Assessment of Antimicrobial and Anticancer Activity. Molecules, 25(21), 4896.
33. Shakerimoghaddam, A. Ghaemi, E. A. & Jam alii, A. (2017). Zinc oxide nanoparticle reduced biofilm fonnation and antigen 43 expressions in uropathogenic Escherichia colt. Iranian Journal of Basic Medical Sciences, 20(4),451-456.
34. Sharma, N., Jandaik, S., & Kumar, S. (2016). Synergistic activity of doped zinc oxide nanoparticles with antibiotics: ciprofloxacin, ampicillin, fluconazole and amphotericin B against pathogenic microorganisms. Anais da Academia Brasileira de Ciências, 88(3), 1689-1698.
35. Steffy, K., Shanthi, G., Maroky, A. S., & Selvakumar, S. (2018). Synthesis and characterization of zno phytonanocomposite using Strychnos nux-vomica L.(Loganiaceae) and antimicrobial activity against multidrug-resistant bacterial strains from diabetic foot ulcer. *Journal of advanced research*, *9*, 69-77.
36. Swain, P. S., Rao, S. B., Rajendran, D., Dominic, G., & Selvaraju, S. (2016). Nano zinc, an alternative to conventional zinc as animal feed supplement: A review. *Animal Nutrition*, *2*(3), 134-141.
37. Thamer, N. A. (2016). Green Synthesis of Silver Nanoparticles Using *Crocus Sativus* L. Extracts and Evaluation of some Biological Effects in Induced Pre- Hepatocellular Carcinoma in Male Rats, thesis. Chemistry department. College of Science. Babylon University.
38. Tuli, H. S., Kaur, G., Sood, S., Sharma, D., Kaur, P., Kumar, P., & Kumar, M. (2020). Zinc oxide nanoparticles and antibiotics mediated combinatorial approach to enhance antibacterial potential. International Journal of Pharmaceutical Research, 12(3).
39. Yin, Y., Yang, X., Hu, L., Tan, Z., Zhao, L., Zhang, Z., ... & Jiang, G. (2016). Superoxide-mediated extracellular biosynthesis of silver nanoparticles by the fungus *Fusarium oxysporum*. Environmental Science & Technology Letters, 3(4), 160-165.