Journal Homepage: -www.journalijar.com
INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)
Article DOI:10.21474/IJAR01/7115
DOI URL: http://dx.doi.org/10.21474/IJAR01/7115

RESEARCH ARTICLE

ANTIBACTERIAL ACTIVITY OF ZNO NANOPARTICLES ON *S.AUREUS* AND *E.COLI.*

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Manuscript Info

Manuscript History

Received: 16 March 2018
Final Accepted: 18 April 2018
Published: May 2018

Keywords:-
ZnO nanoparticle, Escherichia coli, Staphylococcus aureus, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC).

Abstract

In this study, the effects of zinc oxide (ZnO) nanoparticles (NPs) on, Gram–ve bacteria (***E.coli***) and Gram+ve bacteria (***S.aureus***) were investigated. The bacterial strains were inoculated into media containing different concentrations of ZnO (16, 8, 4, 2 m M) and 1% of NP-free solution and incubated at 37 °C for 24 h. The presence and characterization of ZnO nanoparticles on bacterial cells were investigated by scanning electron microscopy (SEM)The bacteria, for up to 12 h of incubation, the numbers of treated cells were within 1 log CFU/mL less than that of the control.Morphological changes of bacterial cells were observed, but many cells remained in normal shapes. Results indicate that ZnO nanoparticles has very inhibitory effects on bacteria.

Introduction:-

Nanotechnology offers possibilities of great advancements in a variety of industries by manipulating materials on the atomic or molecular level and thus obtain novel characteristics and functions of smaller constructed materials; these smaller materials are referred to as nanomaterials and defined as a particle less than 100 nm in at least one direction (**Meyer and Kuusi 2002).**

Medicinal sciences are investigating the use of nanotechnology to improve medical diagnosis and treatments (**Andersen *et al.*, 2009).**

The considerable antimicrobial activities of inorganic metal oxide nanoparticles such as ZnO and its selective toxicity to biological systems suggest their potential application as therapeutics, diagnostics, surgical devices and nanomedicine based antimicrobial agents**(Reddy *et al.*, 2007).**

Among the metal-based NPs, ZnO NPs is one of the most studied showed that as the concentration of ZnO NPs increased, the inhibition of ***E.coli*** bacterial growth also increased. The results showed that concentrations of 1.3 mM or lower did not significantly affect the growth of ***E. coli***. At concentrations of 3.0 to 10 mM ,ZnO NPs showed 100% inhibition of bacterial growth. (**Brayner *et al.*, 2006)**

Another study showed that ZnO NPs have inhibitory effect on various bacteria, including, ***S. aureus, S. epidermidis, S. pyogenes***, and ***B. subtilis***.(**Jones *et al.*, 2008).**

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The antibacterial activity of ZnO NPs was demonsterated by two concentrations, 5 and 10 mM, of ZnO NPs were inoculated with ***E. coli*** and ***S .aureus***. As a result, after 8 h of incubation, the growth number of ***S. aureus*** treated with ZnO NPs was 2 log CFU/mL lower than its control, while the number of ***E. coli*** was less than 1 log lower than the control. The antibacterial effect of ZnO NPs on ***S .aureus*** was stronger than ***E. coli***. These findings imply that antibacterial efficacy of ZnO NPs(**Mirhosseini and Firouzabadi ,2012)**.

Different sizes of NPs influence their effectiveness against bacterial growth. The study showed that the smaller size of ZnO NPs, the stronger the antibacterial effect. An increasingly positive surface charge (which results in the NPs being drawn to the negatively charged surface of the bacteria, is another characteristic that generally enhances the antibacterial effects of ZnO NPs. (**Seil and Webster , 2012).**

Material and Methods:-

Preparation of Zinc oxide nanoparticles by chemical aquous preciptation method:-

5gm. of Zinc acetate Zn $(CH_3COO)_2$ was dissolved in 500 ml of boiling ethanol under vigorous stirring for 15 minutes (the boiling time is about 2 minutes). The solution was cooled in an ice bath for 15 minutes. During that time; 2.90gm of lithium hydroxide ($LiOH.H_2O$) was dissolved in 600 ml ethanol under gentle stirring. The two solutions were mixed together for 30 minutes. The new solution was covered and left for 24 hours to get a white precipitate in the gel form. This precipitated gel was washed 5 times by using distilled water under stirring for 15 minutes each time. The solution was covered and left for 24 hours to get the desired zinc hydroxide gel which can be used for making nanoparticles ZnO powder after releasing of the distilled water.

The previously prepared Zinc hydroxide gel and FTO glass were used for preparing nanoparticles ZnO powder using the following ; the Zinc hydroxide gel was placed in the furnace at a temperature 70˚C for 6 hours.

The temperature was gradually increased to 450˚C (sintering temperature) with a heating rate of 10˚C/min and was kept in 450˚C for 30 minutes. Finally, the furnace was turned off and samples were removed when the temperature reached 80˚C.Finally, the obtained ZnO powder was used.

ZnO Suspension Preparation:-

ZnO nanoparticles powder nanoparticles were initially sterilized at 160°C for 3 h, and then dispersed in ultrapure water , vigorously vortexed for 10 min and additionally sonicated for 30 min to avoid aggregation and deposition of particles. The resulting suspensions (100 mL with concentration of 1 M) were considered as stock solution to be diluted and used for bacterial susceptibility evaluation.

Bacterial Strains:-

The bacterial strains (***S.aureus and E.coli***) obtained from bacteriology,immunity and mycology department ,Banha univerisity and were examined for their susceptibility and sensitivity toward the treatment with ZnO nanoparticles using different concentrations.

Antimicrobial Testing Assay:-

Two different assays (qualitative and quantitative) were carried out to evaluate the antimicrobial activity of ZnO nanoprticles against examined bacterial strains. Bacterial cultures were kept in dark throughout the assays to avoid the possible effect of light on the antibacterial activity.

Paper Disc Diffusion Assay:-

Sterile paper discs (Whatman No.1, 6 mm in diameter) were placed on the surface of suitable media plates, freshly inoculated with bacterial cells, then 10 mL from ZnO stock solution was dispensed onto the surface of each disc. Plates were then incubated for 24 h at the optimum temperature for each strain, and diameters of the growth inhibition zones were recorded in mm. Each experiment was made in triplicate and the inhibition zones are given as the mean ± standard deviation.

Determination of the Minimal Inhibitory Concentrations:-

The modified microdilution method of Eloff (1998) as described by **Tayel *et al*. (2010)** was applied for the determination of minimal inhibitory concentrations (MIC) of ZnO suspension against examined bacteria. Briefly, 20 mL of 24-hour-old bacterial culture (~108 cfu/mL) was poured in microplate 96-wells as followed by 100 mL of a ZnO suspension along with an equal volume of broth media. The ZnO suspensions were produced by serial dilutions

to give a final ZnO concentration in the range of 1–200 mM. ZnO-free solution was used as a control. The microplates were then incubated overnight. *p*-iodonitro-tetrazolium violet aqueous solution (20 mL; INT, Sigma-Aldrich), with a concentration of 4% w/v, was added to all wells as an indicator of bacterial growth by the formation of red-colored formazan produced by biologically active cells.MIC was defined as the lowest ZnO concentration that completely inhibited bacterial growth, i.e., a colorless well.As a confirmation test, after microplate incubation for 24 h at the optimum temperature for each strain,50 mL from each well were spread on solidified growth media plates and incubated for further 24 h.Growth-free plates validated that the used concentration inhibited bacterial growth.

Morphological Test of the Bacterial Cells:-

Scanning electron microscope (SEM; S-500, Hitachi, Tokyo,Japan) was used to examine morphological characters/ changes of bacterial cells before and after treatmentwith ZnO NP **(Marrie and Costerton 1984).** First, cells were primarily fixed with a fixative buffer (2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M Na-Cacodylate buffer, pH 7.35) for 30 min. The samples were then rinsed thrice with ultrapurewater, followed by dehydration with a series of ethanol solutions (10, 30, 50, 70, 90 and 99%). The dehydrated samples were dried immediately by critical point dryer (Auto-Samdri-815 Automatic Critical Point Dryer; Tousimis, Rockville,MD), followed by mounting onto SEM stubs and sputter-coated with gold/palladium using a cool-sputter coater (E5100 II,Polaron Instruments Inc.,Hatfield,PA). Sections were then observed under SEM at 8 kV each hour after treatment. Captured areas were selected according to the alteration in the morphology of treated cells.

Results:-

Two bacterial strains (one Gram positive and the other Gram negative)were examined for their susceptibility to ZnO nanoparticles (Table 1). Generally, the Gram positive strain were more susceptible to ZnO Nps using either qualitative or quantitative assay. The diameter of growth inhibition zone increased with the decrement of required MIC of ZnO nanoparticles for each strain.

The antibacterial activity of ZnO nanoparticles was tested by the disc and well diffusion agar methods (Tables 1 and 2).

Table 1:-Zone of inhibition (ZOI) for ***S. aureus.***

ZnO concentration in wells (mg / ml)	ZOI(mm)	ZnO concentration in discs(mg / ml)	ZOI(mm)
10	**30± 1.1**	**5**	**23± 1.3**
5	**29± 1.4**	**2.5**	**22± 1.1**
2.5	**27± 1.1**	**1.25**	**19± 1.2**
1.25	**25± 1.3**	**0.625**	**16± 1.2**
0.625	**2.1± 1.2**	**0.312**	**14±1.1**
0.312	**17± 1.2**	**0.156**	**12±1.3**
0.156	**14± 1**	**0.078**	**9± 1**
0.078	***14± 1**	**0.039**	***9± 1**
0.039	**0**	**0.0195**	**0**
0.0195	**0**	**0.00975**	**0**
Control	**0**	**Control**	**0**

Minimum concentrations of ZnO nanoparticles at which zone of inhibition started to appear.

Table 2:-Zone of inhibition (ZOI) for ***E-coli***.

ZnO concentration in wells (mg / ml)	ZOI(mm)	ZnO concentration in discs(mg / ml)	ZOI(mm)
10	**20± 1.2**	**5**	**27± 1.3**
5	**19± 1.1**	**2.5**	**25± 1.2**
2.5	**16± 1.3**	**1.25**	**25± 1.2**
1.25	**12± 1.2**	**0.625**	**20± 1.1**
0.625	***10±1**	**0.312**	***14± 1**
0.312	**0**	**0.156**	**0**
0.156	**0**	**0.078**	**0**

0.078	**0**	**0.039**	**0**
0.039	**0**	**0.0195**	**0**
Control	**0**	**Control**	**0**

* Minimum concentrations of ZnO nanoparticles at which zone of inhibition started to appear.

Number of colony forming unit (cfu) of ***E. coli*** and ***S.aureus*** after overnight incubation at the presence of different concentrations of ZnO nanoparticles was showing in (Figure 1). The minimum concentration of ZnO nanoparticles which inhibited the growth of bacteria was3.1 mg/ml for ***E. coli*** and 1.5 mg/ml for ***S.aureus***.

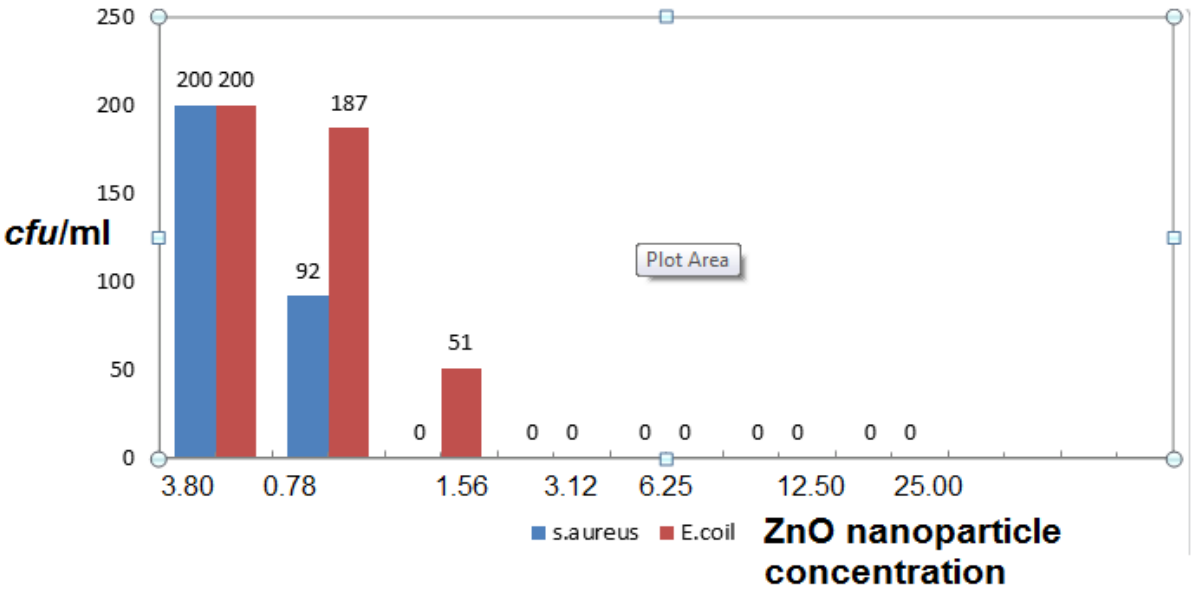

Fig 1:-Number of colony forming units (cfu) of ***E. coli*** and ***S. aureus*** after overnight incubation at the presence of different concentrations of ZnO nanoparticles.

The results of MIC and MBC for ***E. coli*** and ***S. aureus*** were summarized (Tables 3 and 4). Based on the results obtained from MIC, MBC, disc and well agar diffusion methods, it can be suggested that in comparison with Gram-positive bacteria, the growth of gram-negative bacteria is inhibited at higher concentrations of ZnO nanoparticles .

Table 3:-Determination of MIC and MBC for E. coli.

Mode of effect	**Concentration (mg/ml)**
Growth	0.125
Growth	0.25
Growth	0.5
Bacteriostatic	1 (MIC)
Bacteriostatic	2
Bacteriostatic	4
Bacteriostatic	8
Bactericidal	16(MBC)

Table 4:-Determination of MIC and MBC for ***S. aureus***.

Mode of effect	**Concentration (mg/ml)**
Growth	0.125
Growth	0.25
Bacteriostatic	0.5(MIC)
Bacteriostatic	1

Bacteriostatic	2
Bacteriostatic	4
Bactericidal	8(MBC)
Bactericidal	16

Morphological test of bacteria cells Treated with ZnO NPs:-

The SEM images of ***E. coli*** and ***S. aureus*** incubated in respective broth medium for 10 h, with and without the presence of 20 mM of ZnO were analyzed.

As shown in Figures (2)and(4), no significant changes in bacterial morphology (e.g. size, shape, appearance, etc) were observed after ZnO NPs treatment for 10 h. Also, ZnO NPs were observed to adhere to the ***E. coli*** cells and ***S.aureus*** Figure (3) and(5).

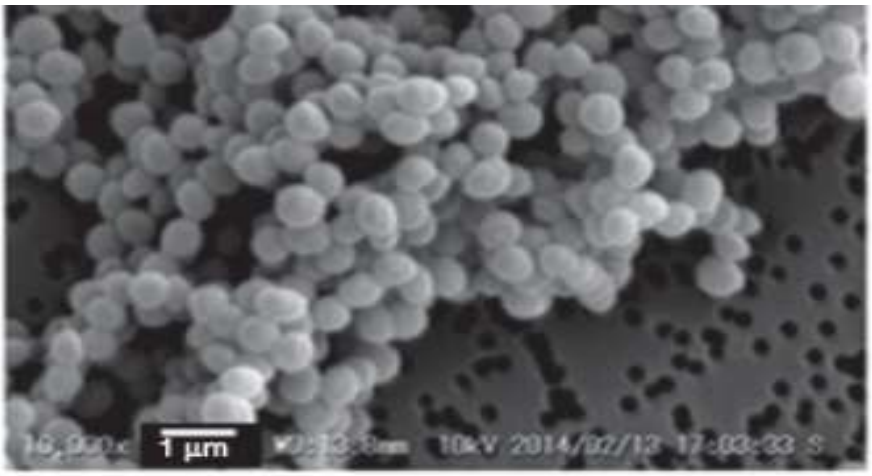

Fig 2:-*S.aureus* (control sample) untreated with ZnO nanoparticles under scan electron microscope.

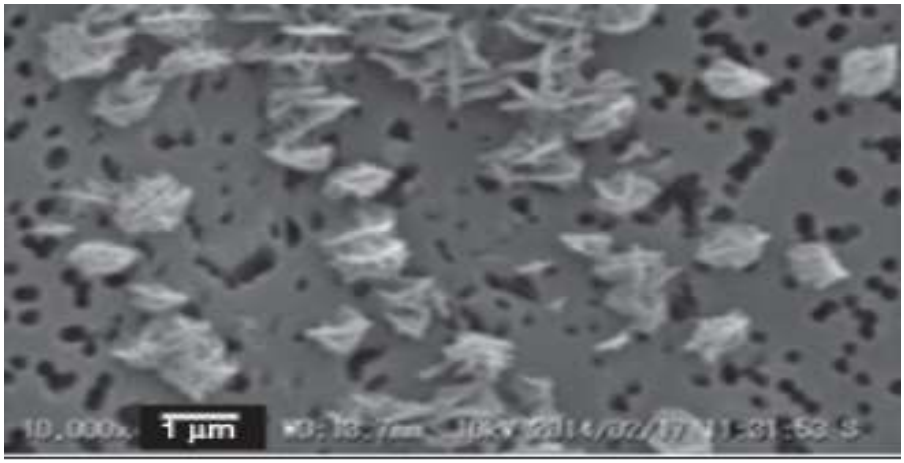

Fig 3:-*S.aureus* treated with ZnO nanoparticles under scan electron microscope.

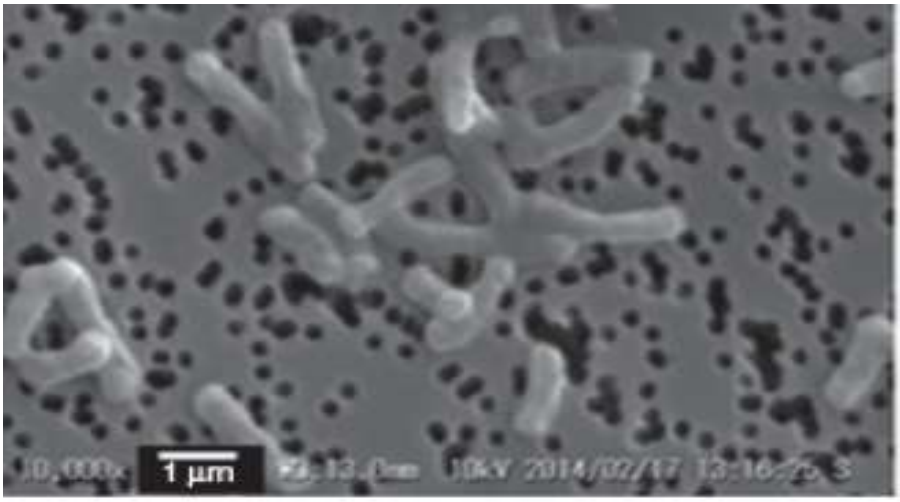

Fig 4:-*E. coli* (control sample) under scan electron microscope.

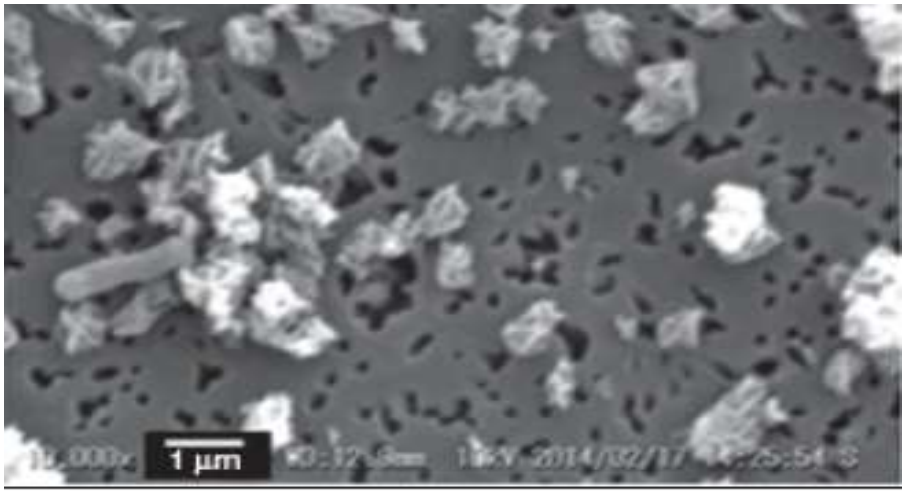

Fig 5:-*E.coli* treated with ZnO nanoparticles under scan electron microscope.

Discussion:-

For the investigation of ZnO antimicrobial capability,two bacterial strains, from both Gram positive and negative were used in this study.

Metal oxide nanomaterials increased cell death with increasing concentrations, affected mitochondrial function, induced lactate dehydrogenase leakage and generated abnormal cell morphology at concentrations as low as 50–100 mg/L **(Chen *et al*., 2007).**

The antibacterial activity of ZnO NP was much stronger than that of ZnO powder. This could be simply explained as smaller particles normally have a larger surface to volume ratio which provides a more efficient mean for antibacterial activity **(Baker *et al*., 2005).** The generation of hydrogen peroxide (H_2O_2) presents another elucidation of the antibacterial activity of ZnO;H_2O_2 that generates from the surface of ZnO is considered as an effective mean for the inhibition of bacterial growth **Yamamoto(2001).** It can be assumed that the concentration of H_2O_2 generated from the surface increases with decreasing particle size, because the number of ZnO powder particles per unit volume of powder slurry increases with particle size decreasing.

The obtained data, regarding the bacterial sensitivity to ZnO, indicated that Gram-positive bacteria were more sensitive than Gram-negative bacteria to ZnO NPs; this could be explained as the antibacterial action of ZnO is suggested to occur through its interaction with specific cell compounds; these compounds may be found or increased

in Gram-positive rather than in Gram-negative bacteria. Potential contenders of these compounds are the outer thick peptidoglycan layer and its amino acid constituent, surface proteins (e.g., adhesions) and teichoic acids plus lipoids (forming lipoteichoic acids), which act as chelating agents and also execute certain types of adherence.

The minimum concentration of ZnO nanoparticles which inhibited the growth of bacteria was 3.1 mg/ml for ***E. coli*** and 1.5 mg/ml for ***S.aureus***. This is in agreement with previously published reports on the antibacterial properties of ZnO nanoparticles which showed that the minimum concentration at which the growth of ***E. coli*** and ***S. aureus*** was inhibited was 3.4 and 1 mM, respectively **(Reddy *et al.*, 2007).**

In this study, the growth of gram-negative bacteria is inhibited at higher concentrations of ZnO nanoparticles Fig (2**).(Reddy et al. 2007)** have reported the same results, emphasizing on the higher susceptibility of Gram-positive bacteria in comparison with Gram-negative bacteria. Inthe study done by **(Selahattin et al., 1998)**, it has been proposed that the higher susceptibility of Gram-positive bacteria could be related to differences in cell wall structure, cell physiology, metabolism or degree of contact.

According to the results, it can be concluded that ZnO nanoparticles are effective antibacterial agents both on Gram-positive and Gram-negative bacteria. The same results were confirmed in the study of (**Zhongbing et al., 2008)** in which Gram-negative membrane and Gram positive membrane disorganization was approved by transmission electron microscopy of bacteria ultrathin sections. In order to use ZnO nanoparticles in *in vivo* condition, further studies should be performed investigating the toxic effect of ZnO nanoparticles on eukaryotic cells.

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