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Evaluation of Antibacterial Activity of ZnO and TiO_2 Nanoparticles on Planktonic and Biofilm Cells of *Pseudomonas aeruginosa*

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In this study antimicrobial activity of ZnO nanoparticles and TiO_2 nanoparticles on the most common pathogen bacterium *Pseudomonas aeruginosa* was evaluated. *P. aeruginosa* is one of the prevalent pathogen that caused nosocomial infection. It is generally multi-drug resistant. *Pseudomonas aeruginosa* (ATCC 27853) were cultured on nutrient agar medium (NA) for 24h at 37°C. TiO_2 and ZnO nanoparticles was synthesized from $TiCl_4$ and : Zinc acetate dehydrate after several steps, and the type and size of these particles were characterized by scanning electron microscopy (SEM) and X-Ray-: Diffraction (XRD). The microbial suspension with concentration of 1×10^6 cells/ml was prepared. Minimum Inhibitory Concentration (MIC) for TiO_2 and ZnO was measured.This present study showed that synthesized ZnO and TiO_2 nanoparticles can limit and prevent the growth of *Pseudomonas aeruginosa.*

Key words: *Pseudomonas aeruginosa*, ZnO, TiO_2nanoparticles.

Pseudomonas aeruginosa is one of the most perilous pathogenic agents which are generally found in water, soil, plants and animals[1] it is one of the important pathogen that caused nosocomial infection which is responsible for significant morbidity in the hospitals[2, 3]. It has the capability of forming biofilms. Biofilms are well-organized and complex aggregate of microorganisms, surrounded by a protective matrix of exopolysaccharides and can adhere to each other on various surfaces[4]. Because of increase the drug resistance and the side effect of over use of antibiotic, it is necessary to find a suitable agent to reduce the growth of microorganism.

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Nanoparticlesare is a special group of materials with unique features[5] which attract many researchers to study these particular features. In compare to bulk size counterparts, nanoparticles illustrate amazing properties[6].

Metal oxide nanoparticles have some special characteristic such as less toxicity and heat resistance and greater effectiveness on resistant strains of microbial pathogens which make them the selective candidates for eradicating bacteria[7, 8].

However, to obtain the maximum effects of metal oxide nanoparticles we should select the best way to prepare it.

There are different methods to produce nanoparticles. Vapor phase deposition methods such as physical vapor deposition (PVD), chemical vapor deposition (CVD) and liquid phase based methods such as the sol – gel way , solid phase

methods, thermal methods, chemical and is a mechanochemical method[9].

In recent study, we utilized sol gel way. Obtaining the homogeneous particle, the particle size distribution to achieve the higher density and lower cost of raw materials are the advantages of the sol - gel method[10].

Among metal oxide nanoparticles, ZnO and TiO_2appeal many attentions for their properties.

ZnO nanoparticles as one of the multifunctional inorganic nanoparticles has many significant features such as chemical and physical stability, high catalysis activity, effective antibacterial activity as well as intensive ultraviolet and infrared adsorption with broad range of applications as semiconductors, sensors, transparent electrodes, solar cells, etc.[1, 11]. Also in recent years ZnO has received considerable attention because of its unique optical, piezoelectric, and magnetic properties[11]. In addition ZnO nanoparticles has the potential to impact many aspects of food and agricultural systems because of its antimicrobial efficacy especially with the growing need to find alternative methods for formulating new type of safe and cost-effective antibiotics in controlling the spread of resisted pathogens in food processing environment[12]. Some data suggest the selective toxicity of the ZnO nanoparticles toward cancer cells[11,12]. The anticancer effects of ZnO nanostructures on human brain tumor U87 and cervical cancer Hela were obtained and indicate promising activity that varies with the changes in the structure and the size[11, 13].

Unique properties of these nanocrystals depend strongly on their dimensions[14]. Controlling the particle size in the nanometer range by varying the synthesis condition is always a difficult task.

In the last decade, TiO_2 has been widely utilized as a self-cleaning and self-sterilizing material for coating many clinical tools including sanitaryware, food tableware and cookingware, and items for use in hospitals[15] When exposed to sunlight or ultraviolet light, TiO_2 exhibits antimicrobial activity due to its strong oxidizing property. TiO_2 particles come into contact with microbes; the microbial surface was the primary target of the initial oxidative attack. Reactive oxygen species (ROS) (such as hydroxyl radical, superoxide anions and hydrogen peroxide) generated on the irradiated TiO_2 surface initially promote peroxidation of the polyunsaturated phospholipids component of the lipid of the microorganism described that light and scanning electron microscopic examinations suggest that the microbial destruction achieved takes place through direct damage to cell walls caused by hydroxyl radical[16].

There are many studies which investigated the effect of nanoparticle. But to our knowledge there is not study which investigates the effect of ZnO and TiO_2 nanoparticles synthetized by sol-gel method on planktonic and biofilm cells of *Pseudomonas aeruginosa*.

Objectives

The present investigation was aimed to evaluate the antibacterial activity of ZnO and TiO_2 nanoparticles which synthesized by sol gel method towards planktonic and biofilm cells of *Pseudomonas aeruginosa*.

MATERIAL AND METHODS

Preparation TiO_2 nanoparticles in sol-gel way

In this study TiO_2 nanoparticles were synthesized through the hydrolysis of $TiCl_4$ (99% TiO_2 merck). In the first step, 1 mL of $TiCl_4$ was added to 58 ml twice distilled deionized water (as a nanoparticle solvent). Then it was mixed using a mixer without heating for 5 hours. After 5 hours colloidal TiO_2 (sol) is obtained. The resulting solution was subjected to laboratory temperature for 24 hours. The solution was in a drying oven at temperatures from 80 to 100 °C for 12 hours. Then it was heated in the oven for 2 hours in 550°C as a white powder is obtained.

In this study,the testafter confirming the test by the XRD method it has been shown that Two-thirds of the structure of the synthesized nanoparticles TiO_2 from titanium tetrachloride precursor in the oven 550 ° C, had anatase phase and one- third had rutile phase . ZnO nanoparticles were hexagonal phases also.

Preparation of TiO_2 nanoparticles

For Micro dilutionTest, first prepared serial dilutions of 200, 20, 10, 5, 3 and 1 micro litter of a colloidal solution of TiO_2 (sol) in distilled water and then was sterile with 0.22 micrometer filter.

Preparation ZnO nanoparticles

In our experiments, Zinc acetate dehydrate $[Zn(CH_3COOH)_2.2H_2O]$ has been used as precursors, and ethylene glycol (EG) has been used as capping . Water was also used as a solvent. For synthesis of ZnO nanoparticles, $Zn(CH_3COO)_2.2H_2O$, and EG were dissolved in 50 ml DI water with 10/1 ratio (Zn/EG). The solution was put on a hot plate and stirred at 80°C until a small quantity of gel containing zinc acetate and EG was obtained. Then, the obtained gel was dried in the oven for 12h. The dried gel was eventually calcined at 300 °C for 24h in the furnace without any special atmosphere. The achieved samples were characterized by means of field emission scanning electron microscopy (FESEM-S4160 Hitachi).

Dilution ZnO nanoparticle

First the serial dilutions of 1,3,5,10, 20, 30, 40, 50,60,70,80,90,100microliters of the ZnO colloidal solution prepared and then was sterile with 0.22 micrometer filter.

SEM and XRD test

To survey the morphology and the properties of synthesized nanoparticles we use the SEM (Poland, Philips, XL30) and XRD (XPERT-95) test with) with k± radiation, wavelength (»=1.54178 Å),

In order to confirm the crystalline structure of the nanoparticles X-ray diffraction apparatus was used(Figure 4).X-ray diffraction is a method by which we can examine elements that are crystalline forms.So, the beam of X-rays with wavelengths between 2 to 7 Å were spun onsamples.To determine the crystalline phases and measurement of structural properties we used Bragg's Law,

$$d = \lambda/(2\sin\theta)$$

In this equation,d is the atomic spacing in the crystalline phaseandèis theangle ofthebeam withthe sample surface. λ is a function of scattering angle 2θ.

Microbial tests

Preparation of microbial suspension:

Pseudomonas aeruginosa strain (ATCC 27853) were cultured on nutrient agar medium (NA) for 24h at 37°C. Then the microbial Suspension with concentration 1×10^6 cells/ml was prepared.

Preparation of different dilutions of imipenem antibiotic

Since, imipenem antibiotic is a good soluble in sterile distilled water, its aqueous solution in differentconcentrations was used for different experiments of the study. The produced dilutions included 0.025, 0.016,0.0125, 0.010 and 0.008 (g/mL).

Determination of Minimum Inhibitory Concentration (MIC)

After preparation of different concentrations of the agents, the micro dilution test was done according to clinical and laboratory standard institute performance (CLSI). 10 ml microbial suspension was inoculated on micro titer plate and was added 100µl Mueller Hinton Broth (MHB) medium to it then the different concentration of agents were added. Microtiter plate was incubated overnight at 35°C then the colony formation on nutrient agar medium was calculated.

Biofilm formation

Preparation of microbial suspension

Pseudomonas aeruginosa strain (ATCC 27853) were cultured on nutrient agar medium (NA) for 24h at 37°C. Single colonies grown on the TSA +0.2% glucose was inoculated in TSB +0.2 glucose medium.After mixing the contents of the tube,optical 0.1 for each sample was set at a wavelength of 650. If the absorption rate is less than 0.1, the colonies of bacteria inoculated TSB tubes and then re-read its absorb. Then the microbial Suspension with concentration 1×10^6 cells/ml was prepared 200 µl suspension was added to each well of the 96 microtiter plate, and the incubation temperature was 37 ° C for 24 h. The contents of the wells were emptied slowly. To separate the not connected bacteria initially were added 200 µl sterile PBS buffer to the wells and then were emptied. Concentration equivalent to 2 times the MIC concentrations were added separately to each of the wells containing 100 µl of medium glucose 0.2% + TSB. The tests were repeated three times, once again the plate incubated at 37 ° C for 24 h in incubation. After the time wells were evacuated and was repeated. For washing the wells 20µl of sterile PBS buffer aspirated and plates were poured and the operation was performed two times.

XTT assay

XTT [2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5- (phenylamino) carbonyl)-2H-tetrazolium hydroxide] as colorimetric assay was carried out to evaluate antifungal effect of nanoparticles. This method is based on determining the viability of collected cell. 0.25 mg XTT salt was solute in one ml PBS buffer and was sterile with 0/22 micrometerfilter. Then 100µl XTT sterile solution and 50 µl coenzyme Q was added to the 96 wells of the plate containing a biofilm and was incubated for 2-3 hours at was 37 ° C. The absorbance of each well at 492 nm was read by using the Elisa reader (Model Adolf Fenz Germany).

Statistical analyses

Statistical analyses were conducted using one-way analysis of variance (ANOVA). A significance level of 0.05 was used.

RESULTS

Evaluation of Morphology of the ZnO and TiO_2 nanoparticles with SEM showed that these nanoparticles were spherical. ZnO nanoparticles were 30-90 nm and TiO_2 nanoparticles were 40-65nm (Fig. 2 and 3). The XRD test showed that Synthesized TiO_2 nanoparticles were 2/3 in anatase and 1/3 in rutile phases and ZnO nanoparticles were in hexagonal phases (Fig. 4). Minimum inhibitory

Table 1. Determination of Minimum Inhibitory Concentration (MIC) of on agents *Pseudomonas aeruginosa*

Agents	MIC (µg/ml)
TiO_2	2.2
ZnO	0.0003
imipenem	0.43

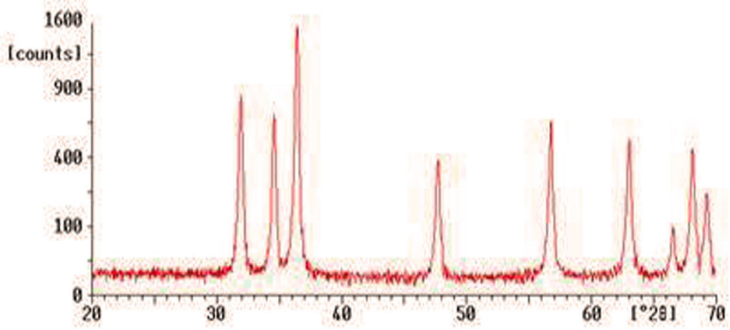

Fig. 1. The graph of crystal structure of nanoparticles by x-ray diffraction

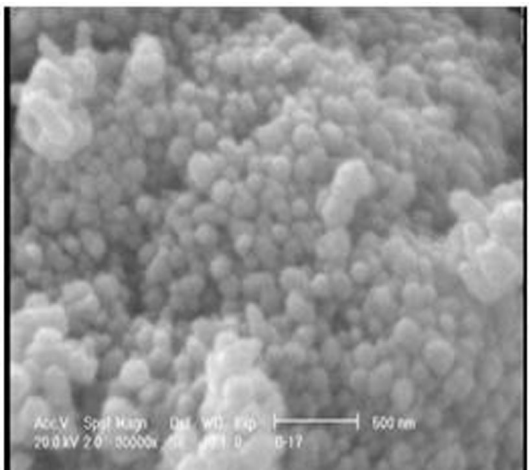

Fig. 2. SEM of ZnO nanoparticles

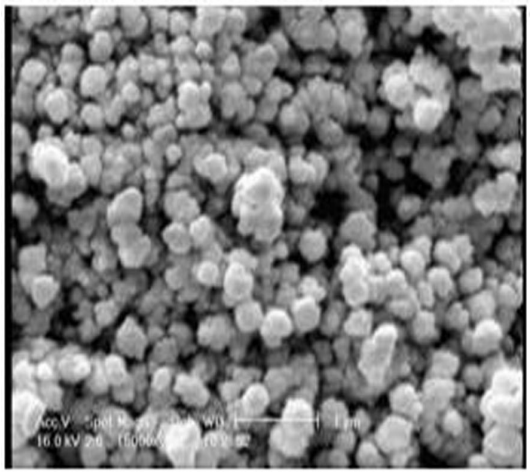

Fig. 3. SEM of TiO_2 nanoparticles

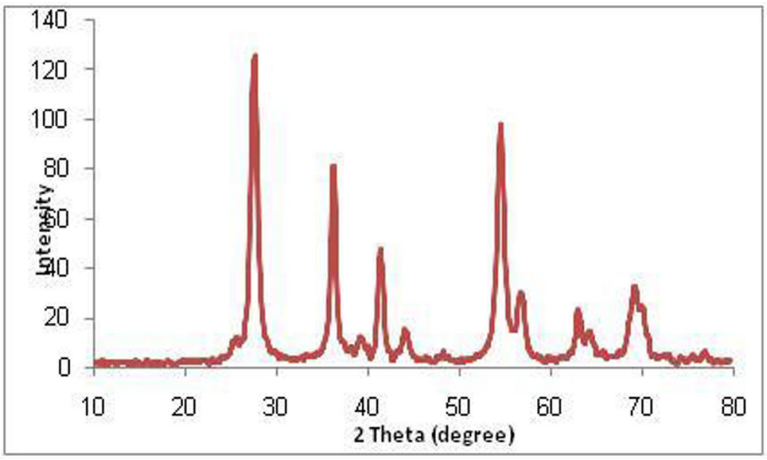

Fig. 4. XRD pattern of TiO_2 nanoparticles

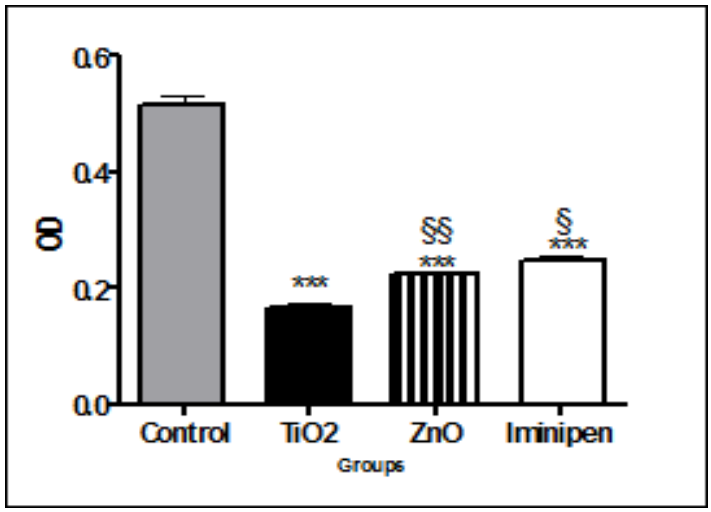

Fig. 5. Results of the MTT assay for evaluation of antimicrobial effects of ZnO, TiO_2 and imipenem on the pseudomonas biofilm. *** denotes $p<0.01$, compared to the control group. §§ denotes $p<0.01$, compared to the TiO_2. § denotes $p<0.05$, compared to the TiO_2 group. Values are presented as mean ± SEM

concentrations (MIC) of TiO_2 on *Pseudomonas aeruginosa* ATCC27853 was 2.20 µg/ml andM IC of ZnO was 0.0003 µg/ml,respectively. And MIC of imipenem as a control group was 0.43µg/ml (Table 1).

According to statistical analysis of the acquired data from the XTT technique, it was indicated that there was a significant difference between the wells containing ZnO , TiO_2, imipenem and the control group ($P< 0.001$). Furthermore, results of this experiment showed a significant difference between the wells containing ZnO($p<0.01$), and imipenem($p<0.05$) to TiO_2. (Fig.5)

DISCUSSION

TiO_2 nanoparticel is inexpensive and stable. It has great electrical and optical properties[17]. Chen *et al*., in 2009 showed that TiO_2 nanoparticles reduced the growth of *E.coli* and *Aspergillus niger*[18]. TiO_2 nanoparticles synthesized in different ways for example Yang J *et al*., in 2001 Synthesized TiO_2 Nanopowders from Tetra alkyl ammonium

Hydroxide in hydrothermal method and Colón G *et al*, in 2002 Synthesized TiO_2 Nanopowders from Alkoxide Precursor and Using Active Carbon as Additive[14, 19]. In this study we synthesized TiO_2 and ZnO nanoparticles in sol-gel method.

Zinc oxide is usually appears as a white powder, nearly insoluble in water. The powder is widely used as an additive into numerous materials and products including plastics, ceramics, glass, cement, rubber, lubricants, paints, ointments, adhesives, sealants, pigments, foods (source of Zn nutrient), batteries, ferrites, fire retardants, first aid tapes, etc[13]. Atmaca *et al*., in 1997 showed that ZnO nanoparticle could inhibit the growth of *Staphylococcus aureus* and *Staphylococcus* epidermidis[12]. Steven *et al*., in 2005 had prohibited the growth of Candida albicans with photocatalic ZnO nanoparticles in 2 hours[20]. The antimicrobial property of nanoparticles depends on the synthesized method, concentration and size of them. The small particles have greater antimicrobial activity[11, 21]. In this study the result of SEM and XRD test showed that ZnO and TiO_2 are spherical and 30-90 and 40-65 diameters. Based on the importance of this size of particles on its properties, there are some studies which was investigated the size. For instance Jiang *et al*.[21] compared three difference sizes of TiO_2. Although we did not investigated the difference sizes, but it seems that 30-90 and 40-65 diameters for ZnO and TiO_2, respectively is completely appropriate to show antibacterial effect.

In addition, we have selected *P. aeruginosa* for investigating the antibacterial effects of ZnO and TiO_2 and we have seen the promising effects meanwhile in Toolabi et al, study they have shown that TiO_2 was not so effective in treatments of *P. aeruginosa* in compare to other bacteria like *S. aureus*. This difference in our study to Toolabi may be due to the physical properties of TiO_2 utilized in the studies. As we have mentioned before the physical characteristics of the NP play the critical role in its effectiveness. In accordance to our study, Vijayalakshmi & Rajendra (2012) prepared TiO_2 via hydrothermal and sol gel methods There results showed that the nanoparticles prepared via sol-gel route were highly crystalline and had smaller crystallite size.

The results of Determination of Minimum Inhibitory Concentration (MIC) of the agants on *Pseudomonas aeruginosa* showed that the nanoparticles have intensive antibacterial property; hence, we can say the suitable methods were applied for synthesis of these nanoparticles. We use imipenem as control. Imipenem, a broad-spectrum β-lactam antibiotic, is one of the most effective drugs against *P. aeruginusa*. But imipenem has side effects and toxicity including CNS toxicity and hypersensitivity[22]. In this study we showed that ZnO could inhibit the growth of *Pseudomonas aeruginosa* in low concentration compared with TiO_2 nanoparticles and imipenem.

Our finding showed that both ZnO and TiO_2 were effective in the treatment of biofilm infections. In addition, analyses showed that TiO_2 was more effective than ZnO. Toolabi *et al*, also showed that LC50 of TiO_2 was more than ZnO which is completely in accordance to our research. The only difference is that they did not examine mentioned NPs on biofilm likewise our study.

The mechanism of nanoparticles on biofilms is unknown, however it may chelates two-capacity cations which are necessary for the stability of biofilm matrix and causes the separation of cells from biofilm and subsequently death of biofilm cells.

Because of the great ability of nanoparticles in coating, and regarding to the antibacterial activity of these nanoparticles, we recommend using these nanoparticles to coat solid surfaces, limitingthe growth of *Pseudomonas aeruginosa* and blocking the attachment of this bacterium,thereby reducing the prevalent of *Pseudomonas* infections.

CONCLUSION

The anti-bacterial effects of ZnO and $TiO_2$was investigated on the *P. aeruginosa* . It has been shown that the chemically synthesized nanoparticles of ZnO and TiO_2 have great ability to limit the growth of the important medical pathogen, *P. aeruginosa*. Therefore, we may conclude that suitable methods were applied for the synthesis of these nanoparticles. The effectiveness of ZnO and TiO_2 made in sol-gel way on other bacteria will be suggested.

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