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Full Length Research Paper

Synthesis of ZnO nanoparticles and their antibacterial effects

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The zinc oxide nanoparticles with the average particle size of about 30 nm were synthesized by the chemical technique and their properties were studied with the help of scanning electron microscope and X-ray diffraction. The aim of this study was to detect the antibacterial properties of 0.01, 0.5 and 1% nano-ZnO against *Escherichia coli. E. coli* was cultured in liquid and agar nutrient medium to evaluate the antibacterial effects of 0.01, 0.05 and 1% of ZnO via the optical density (OD) and log CFU/ml measurements. Non-significant effect was seen for 0.01% of ZnO nano-particles, while 0.05 and 1% of nanoparticles showed considerably decreased bacterial number. A 4.385 and 2.04 times decrease in the OD value was found in the presence of 1 and 0.5% nano-ZnO, respectively ($P<0.001$) as compared to the control. In the second study, 6.3 log CFU/ml of *E. coli* were present in the cultures treated with 1% nano-ZnO at 4°C in water. Control *E. coli* cells survived for 12 days, while complete cell death was seen when 1% nano-ZnO was applied for 24 h. In the third study, *E. coli* was grown in the agar medium with and without nanoparticles and suppressed growth (8.56 times; $P<0.001$) was seen in the presence of 1% nano-ZnO.

Key words: ZnO-nanoparticle, antibacterial, bactericidal, *Escherichia coli.*

INTRODUCTION

Nanomaterials are being used in many branches of science such as harmful microorganisms, recognition and treatment of various diseases (Sun et al., 2003). Nanotechnology has also invaded engineering, biology, chemistry, medicine, physics etc. Metallic nanoparticles have different functions, such as antibacterial characteristics (Te-Hsing et al., 2007). Metallic nanoparticles are continuously being used in the manufacture of manufacture of bactericides, but unfortunately the application in these processes reduces the antibacterial characteristics of nanoparticles. However, in the meantime, inorganic nanoparticles seem to have been good bactericides because of their tolerance to high temperatures (Wu et al., 2003).

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Metal nano particles have various functions that are not observed in bulk phase (Sosa et al., 2003; Sun et al., 2003). Antibacterial agents used in textile industry are divided into two parts: the organic and inorganic matters. The organic antibacterial materials have been used as insecticides and bactericides for many years. Unfortunately, high temperatures in manufacturing process reduce their antibacterial properties. Nonetheless, inorganic antibacterial agents show excellent resistance against the bacterial and thermal stability (Te-Hsing et al., 2007). Over the past few decades, inorganic nanoparticles whose structures exhibit significantly novel and improved physical, chemical and biological properties, as well as functionality due to their nano-scale size, have elicited much interest. Nano-structured materials are attracting a great deal of attention because of their potential for achieving specific processes and selectivity, especially in biological and pharmaceutical applications (Wu et al., 2003; Fortner et al., 2005; Li et al., 2005).

At present, the use of nano-structured materials is becoming more widespread and a major advantage over either organic or inorganic nanoparticles offers many possibilities of applications in the areas of physics, chemistry, pharmacy, surface coating agents, textile sizing, agriculture, biochemistry and so on (Wu et al., 2003; Fortner et al., 2005; Li et al., 2005; Feng et al., 2000; Herrera et al., 2001; Hranisavljevic et al., 2002). It has been demonstrated that specially formulated metal oxide nanoparticles have good antibacterial activity, and antimicrobial formulations comprising nanoparticles could be effective bactericidal materials (Stoimenov et al., 2002). Nano-materials are called “a wonder of modern medicine”. It is stated that antibiotics kill perhaps a half dozen different disease-causing organisms but nano-materials can kill some 650 cells (Sungkaworn et al., 2007). Resistant strains fail to develop if nanoparticle-based formulations are applied in their culture media. In laboratory tests with nanoparticles, the bacteria, viruses and fungi are killed within minutes of contact.

The effect of nanoparticles on bacteria is very important since they constitute the lowest level and hence enter the food chain of the ecosystems (Fortner et al., 2005; Li et al., 2005; Feng et al., 2000; Herrera et al., 2001). Recent studies have demonstrated that specifically formulated nanoparticles demonstrate good antibacterial activity and constitute the antimicrobial formulations (Matsumura et al., 2003; Sondi and Salopek-Sondi, 2004; Lee et al., 2003). Since nano-silver has been used for imparting antibacterial properties (Lee et al., 2003; Dura´n et al., 2007), this study aimed to investigate the potent long-lasting antibacterial activity of nano-ZnO toward the Gram-negative bacterium *Escherichia coli*, which is known as diarrhea-causing organism.

MATERIALS AND METHODS

Chemicals, growth media and bacterial strain

E. coli (ATCC 25922) was used for the present experiment. Nutrient broth (BD234000; Becton Dickinson & Company, MD, USA) was used in growing and maintaining the bacterial cultures as per supplier’s protocol. Zinc nitrate, sodium hydroxide (NaOH), ethanol, potassium nitrate (KNO_3), dihydrogen potassium phosphate (K_2HPO_4), potassium hydrogen phosphate ($KHPO_4$), acetic acid (CH_3COOH) and sodium acetate (CH_3COONa) was purchased from Sigma and Merck Co. The chemicals such as ascorbic acid, sodium citrate tribasic dehydrate, ammonium sulphate, ethanol and cetyltrimethylammonium bromide (CTAB) were purchased from Sigma and were of the highest purity available. These reagents were used as received without further purification. All the solutions used in the study were prepared with double distilled water.

Synthesis of ZnO nanoparticles

To prepare ZnO nanoparticles, in a typical experiment, a 0.45 M aqueous solution of zinc nitrate (Zn $(NO_3)_2.4H_2O$) and 0.9 M aqueous solution of sodium hydroxide (NaOH) were prepared in distilled water. Then, the beaker containing NaOH solution was heated at the temperature of about 55°C. The Zn $(NO_3)_2$ solutions were added drop wise (slowly for 1 h) to the above heated solution under high speed stirring. The beaker was sealed at this condition for 2 h. The precipitated ZnO nanoparticles were cleaned with deionized water and ethanol and then dried in air atmosphere at about 60°C. The scanning electron microscopic image of the synthesized ZnO nanoparticles was taken by a scanning electron microscope Model XL30- Philips Company, operated at 30 KV.

Bacterial susceptibility to nanoparticles

To examine the susceptibility of *E. coli* to nano-ZnO, three different estimation methods were used with three tiles repetition.

Bacterial growth in the presence of nano-ZnO in liquid medium

In the first method, the bacteria were grown in nutrient broth (NB). To start the growth, 2 ml of the overnight-cultured *E. coli* stock was added to 100 ml NB containing 0.12% glucose with and without 0.01, 0.5 and 1% nano-ZnO. The bacteria were aerobically cultured at 30°C for 24 h. Optical density (OD) measurements were taken at 600 nm to monitor the bacterial concentration.

Bacterial killing in the presence of nano-ZnO in liquid medium

In the second method, the culture solution was centrifuged and the cells were washed and re-suspended in distilled water, reaching a final concentration of 6.3 log CFU/ml in each of the sample flasks and incubated at 4°C. The final concentration of the *E. coli* suspensions was made in 100 ml distilled water. Different amounts of nano-ZnO (0.01, 0.5 and 0.1%) were then separately added to the bacterial suspensions to keep in contact with the bacterial cells and shaken at 4°C for 48 h. Optical density (OD) was measured to obtain the results. Aliquots of 0.1 ml of the growth mixtures (water +bacterial cells+ nanoparticles) were sampled every 2 h. The number of resulting bacterial cells was noted after every 2 h of incubation. Bacterial number was determined by measuring the optical density (OD) at 600 nm. The OD values were converted into the *E. coli* concentration as log CFU/ml (Sondi and Salopek-Sondi, 2004).

Bacterial growth in the presence of nano-ZnO in agar medium

In the third method, the same bacteria strain was grown on a solid NB containing 0.12% glucose, 2% agar (control plates) alone or in the presence of 1% nano-ZnO. Bacterial cells were grown at 30°C for 48 h. Afterwards, the plates were visually estimated and bacterial colonies counted. The pictures were taken by an Olympus C2020Z digital camera. The data obtained in all tests were compared with the control. Student’s *t*-test was used to evaluate the significance of experimental results ($P<0.05$).

RESULTS AND DISCUSSION

Size distribution of synthesized nanoparticles by SEM and XRD

The Figure 1 shows the scanning electron microscope (SEM) image of the synthesized ZnO nanoparticles. SEM provided images of the particles by magnification of about

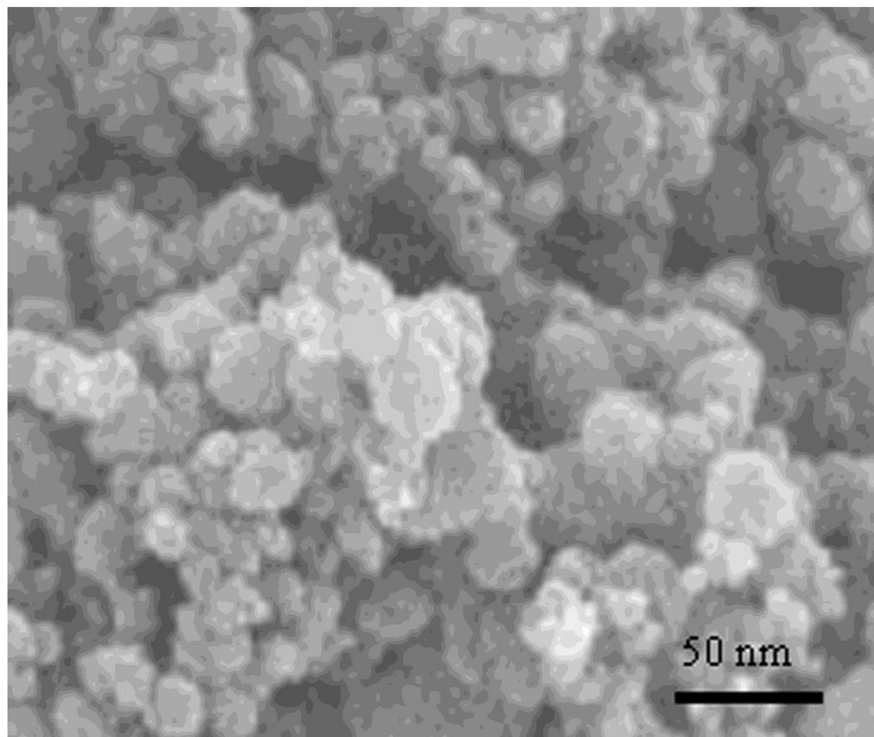

Figure 1. Scanning electron microscope image of synthesized ZnO nanoparticles for corresponding sample 2 h 160 to 2-180°C

one million times greater. The assembly was attached with a computer software programming to analyze the mean size of the particles in sample. It should be noted that the particle diameter is always overestimated due to the distortion of SEM images (Dura´n et al., 2007).

Figure 2a and b demonstrate the X-ray diffraction (XRD) patterns of the synthesized ZnO nanoparticles. The X-ray diffraction data were recorded by using Cu Kα radiation (1.5406 A^o). The intensity data were collected over a 2θ range of 20 to 80°. The average grain size of the samples was estimated with the help of Scherrer Equation using the diffraction intensity of (101) peak. X-ray diffraction studies confirmed that the synthesized materials were ZnO with wurtzite phase and all the diffraction peaks agreed with the reported JCPDS data and no characteristic peaks were observed other than ZnO. The mean grain size (D) of the particles was determined from the XRD line broadening measurement using Scherrer equation (1):

$$D=0.89\lambda / (\beta \cos\theta) \quad (1)$$

Where, λ is the wavelength (Cu Kα), β is the full width at the half- maximum (FWHM) of the ZnO (101) line and θ is the diffraction angle. A definite line broadening of the diffraction peaks is an indication that the synthesized materials are in nanometer range. The lattice parameters calculated were also in agreement with the reported values. The reaction temperature greatly influences the particle morphology of as-prepared ZnO powders. The results of nanoparticle size measurement of samples by XRD and SEM indicate that the size of the ZnO nanoparticles was about 30 nm.

UV-visible absorption spectra of ZnO nanoparticles

The UV-visible absorption spectra of ZnO nanoparticles are shown in Figure 3. Although the wavelength of our spectrometer is limited by the light source, the absorption band of the ZnO nanoparticles shows a blue shift due to the quantum confinement of the excitations present in the sample as compared with the bulk ZnO particles. This optical phenomenon indicates that these nanoparticles have a quantum size effect (Xin et al., 2004).

Effect of nano-ZnO on the growth of *E. coli* in liquid medium

In the first study, we investigated the effect of different concentrations of nanoparticles in liquid culture of *E. coli.* The optical density of the medium was investigated as the number of bacteria after contact with the nanoparticles. Figure 4 shows the effect of different concentration of (0.01, 0.5 and 1%) nano-ZnO on the in growth and killing of *E. coli.* As demonstrated by the Figure, 0.01% nano-ZnO did not have antibacterial

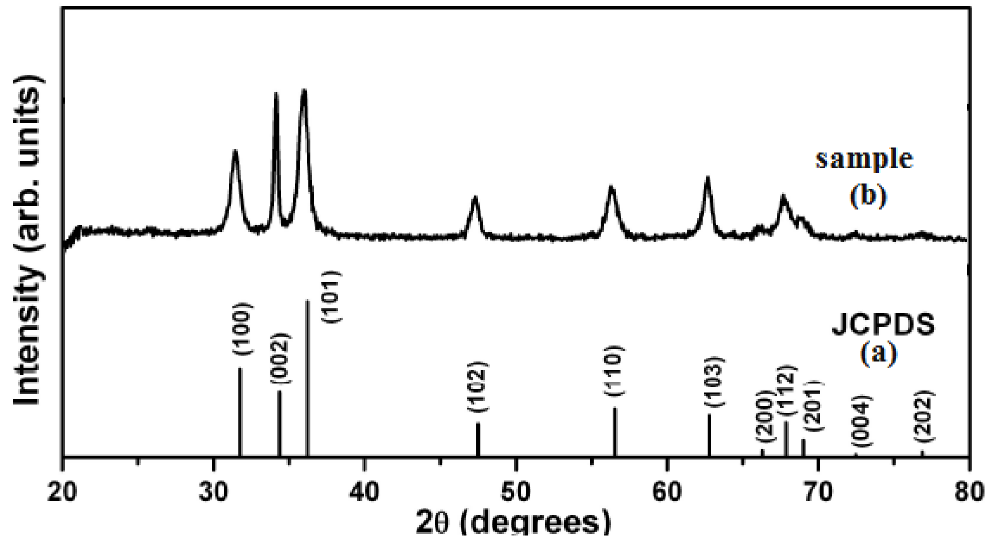

Figure 2. XRD patterns of ZnO nanoparticles. (a) standard XRD pattern and (b) sample XRD pattern.

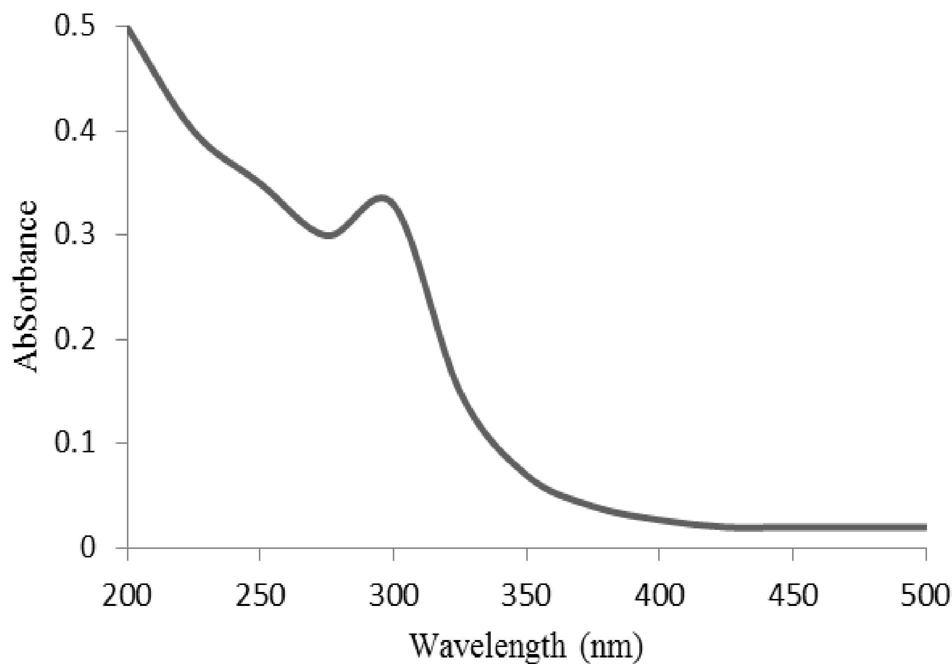

Figure 3. UV-visible absorption spectra for ZnO nanoparticles.

efficiency on *E. coli* but the concentrations of 0.5 and 0.1% nano-ZnO inhibited the bacterial growth. Figure 4 shows that 0.5% nano-ZnO showed 2.04 times decrease in the optical density of bacterial cultures (*P*<0.05) as compared to the control while in the presence of 1% nano- ZnO, the optical density of *E. coli* cultures decreased 4.385 times as compared to the control experiment.

Bactericidal effect of nano-ZnO on *E. coli* in liquid medium

In the second study, estimation of the number of viable *E. coli* cells in contact with 1% nano-ZnO was carried out in water at 4°C for different contact time intervals. Our result show the reduction of *E. coli* cells from 6.3 log CFU/ml to undetectable levels after 12 days (data not shown). The

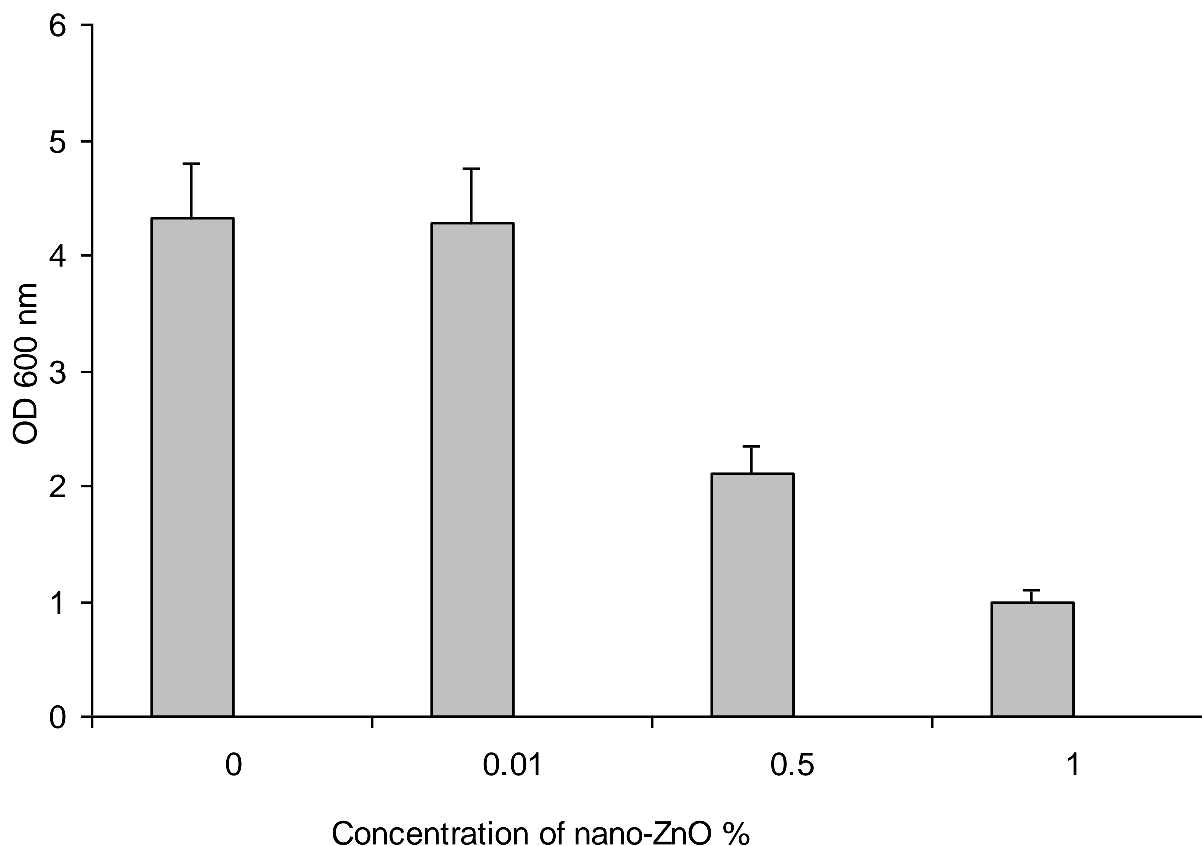

Figure 4. *Escherichia coli* concentration dependence upon different concentrations of nano-ZnO in the culture medium.

addition of these nano-materials to the bacterial culture showed decreased survival rate within 2 days as compared to that of 12-day experiment for control group. Figure 5 represents the number of viable *E. coli* cells in contact with 1% nano-ZnO suspended in water at 4°C for different contact times. From the Figure, it can be clearly observed that nano-ZnO exhibited different antibacterial properties. After the *E. coli* were suspended in water along with ZnO, the number of microbial cells reached zero after 24 h. These results demonstrate that nano-ZnO have a high antibacterial efficiency against *E. coli*.

Effect of nano-ZnO on the *E. coli* growth in agar medium

In the third investigation, *E. coli* was grown on agar medium without (control) or with 1% nano-ZnO. Distinct bacterial colonies were observed in 10^5 times dilution. The visual estimation and bacterial colony counts were performed at this dilution. In Figure 6, we can see smaller number of *E. coli* colonies on the agar medium with nano-ZnO (plate B) as compared to the control group (plate A). In the control plates, 625 ± 42 bacterial colonies were obtained, while in the experimental plates with 1% nano-ZnO, 73 ±14 bacterial colonies were seen. Hence, nano-ZnO suppresses the bacterial growth 8.56 times (P<0.05) in the agar medium.

DISCUSSION

The antibacterial activities of different concentrations of nano-ZnO were investigated during the analysis. *E. coli* (ATCC 25922) was used as the test organism during the experiments. Good growth-inhibition results were observed when the bacterial cells were incubated with nanoparticles during the liquid and solid cultures. The quantitative examination of bacterial activity was estimated by the survival ratio as calculated from the number of viable cells, which formed colonies on the nutrient agar plates (Tsuang et al., 2008). The present data demonstrate that a formulation made with the biologically stabilized ZnO nanoparticles can be useful in the treatment of infectious diseases caused by *E. coli*. A strong binding of nanoparticles to the outer membrane of *E. coli* causes the inhibition of active transport, dehydrogenase and periplasmic enzyme activity and eventually the inhibition of RNA, DNA and protein synthesis, which finally leads to cell lysis as was seen for *E. coli* during the present study. Such effective and less-time consuming formulations can be useful in the clinical

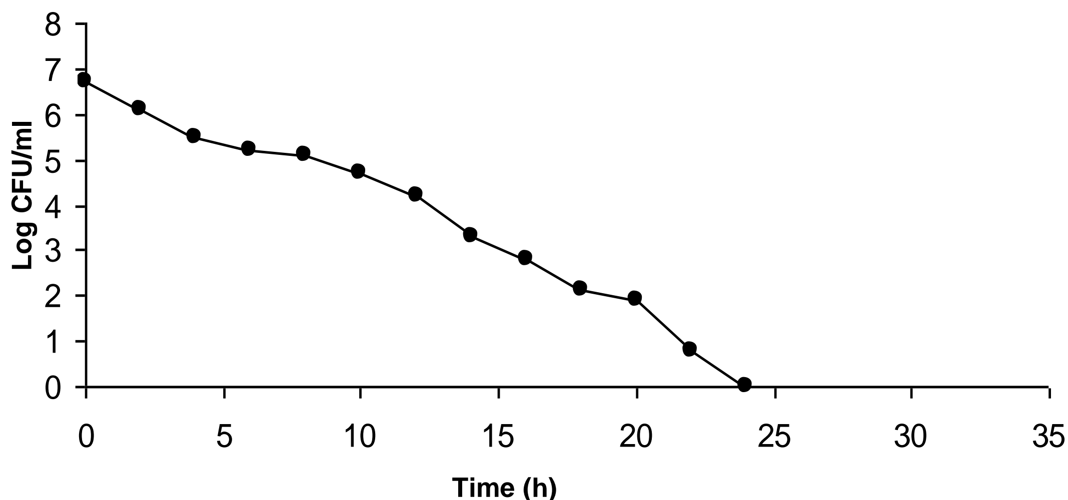

Figure 5. Killing kinetics of 1% nano-ZnO on the *E. coli* cultures.

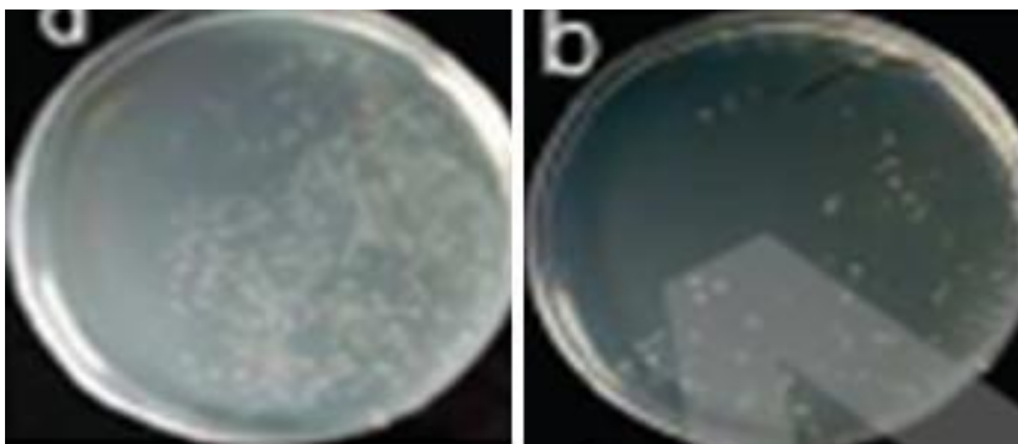

Figure 6. *E. coli* growth on the agar medium without nano-particles (A) and with 1% nano-ZnO (B).

practices where *E. coli* causes urinary tract infections (UTIs). It has been known that nano-materials exhibit strong inhibitory effects towards a broad spectrum of bacterial strains (Russell and Hugo, 1994).

During the present study, different concentrations of nano-scale ZnO were tested to find out the best concentration that can have the most effective antibacterial property against the *E. coli* culture. Our data is in accordance with the previous studies; dealing with the antibacterial effects of nano-materials (Zhang and Chen, 2009; Cook and Costerton, 2000; Jones et al., 2006). Several investigations have suggested the possible mechanisms involving the interaction of nano-materials with the biological macromolecules. It is believed that microorganisms carry a negative charge while metal oxides carry a positive charge. This creates an "electromagnetic" attraction between the microbe and treated surface. Once the contact is made, the microbe is oxidized and dies instantly. Generally, it is believed that nano-materials release ions, which react with the thiol groups (-SH) of the proteins present on the bacterial cell surface. Such proteins protrude through the bacterial cell membrane, allowing the transport of nutrients through the cell wall. Nano-materials inactivate the proteins, decreasing the membrane permeability and eventually causing the cellular death. Nano-materials also retard the bacterial adhesion and bio-film formation (Zhang and Chen, 2009).

Antimicrobial modification to prevent the growth of detrimental microorganisms is a highly desired objective. Microbial cell growth and colonization result in the formation of a compact bio-film matrix, capable of protecting the underlying microbes from antibiotics and host defense mechanisms. Microbial infestation can

result in serious infection (Cook and Costerton, 2000). Such infections are also implicated in food spoilage, spread of food-borne diseases, and bio fouling of materials (Jones et al., 2006). Hence, there is a significant interest in the development of antimicrobial materials and surfaces for applications in the health, biomedical, food and personal-hygiene industry. The nanomaterials based on the metal ions, exhibit broad-spectrum biocides activity towards different bacteria, fungi, and viruses (Greenberg and Steffek, 2005). Nanomaterials are known to deactivate cellular enzymes and DNA by coordinating to electron-donating groups such as thiols, carboxylates, amides, imidazoles, indoles, hydroxyls and so forth. They cause pits in bacterial cell walls, leading to increased permeability and cell death (Holt and Bard, 2005).

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