JOURNAL OF PURE AND APPLIED MICROBIOLOGY, FEBRUARY 2014. Vol. **8**(1), p. 293-300

# Comparative Studies Between Zinc Oxide and Manganese Oxide Nano-Particle for their Antimicrobial Activities

**Enas N. Danial[1,2]* and Jehad M. Yousef[1]**

[1]Biochemistry Department, Sciences Faculty for Girls, King Abdulaziz University, P. O. Box 51459, Jeddah, 21453, Saudi Arabia.
[2]Department of Chemistry of Natural and Microbial Products, National Research Center, Dokki, Cairo, Egypt.

(Received: 12 October 2013; accepted: 09 December 2013)

**The biosynthesis of metal nanoparticles is an expanding research area due to the potential applications for the ecofriendly development of novel technologies. Present study reported the antimicrobial properties of zinc oxide and manganese oxide nano-particles were investigated using : Gram positive bacteria namely (*Bacillus megaterium*, *Bacillus subtilus*, *Sarcina lutea and Staphylococcus aureus*), Gram negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *proteus vulgaris*) and the pathogenic yeast (*Candida albicans* and fungi *Aspergillus niger*). The average sizes of the zinc and manganese nanoparticles were 111nm and 157 nm, respectively, as determined through transmission electron microscopy. The microbial effect of zinc oxide and manganese oxide nano-particles were compared based on diameter of inhibition zone in well diffusion tests and minimum inhibitory concentration (MIC). Bacterial sensitivity to nanoparticles was found to vary depending on the microbial species. Well diffusion studies with *E. coli* and *B.subtilis* revealed greater effectiveness of the zinc nanoparticles compared to the manganese nanoparticles. *P. aeruginosa* depicted the highest sensitivity to nanoparticles compared to the other strains and was more adversely affected by the manganese than zinc nanoparticles. Good correlation was observed between MIC and minimum bactericidal concentration (MBC) measured in liquid cultures. For zinc nanoparticles a good negative correlation was observed between the inhibition zone observed in disk diffusion test and MIC/MBC determined based on liquid cultures with the various strains.**

**Key words:** Nanoparticles, zinc, Manganese, Antimicrobial, MIC, MBC.

In recent years, there has been an increasing interest in developing materials with low dimensional nanostructure such as nanoparticles, nanotubes and nanorods due to their potential technological application in nano scale devices. Also, it has been obvious that their properties depend sensitively on their size and shape. Therefore, the challenges in nanocrystal synthesis are to control not only the crystal size but also the shape and morphology. In order to produce the desired nanostructural materials, various method have been developed, such as electrodeposition[1]

The effect of nanoparticles on bacteria is very important since they constitute the lowest level and hence enter the food chain of the ecosystems [2]. Recent studies have demonstrated that specifically formulated nanoparticles demonstrate good antibacterial activity and constitute the antimicrobial formulations[3]. Antibacterial agents 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

* To whom all correspondence should be addressed.
E-mail: Enas_mahdy@yahoo.com

process reduce their antibacterial properties. However, inorganic antibacterial agents show excellent resistance against the bacterial and thermal stability[4].

Heavy metals such as Co, Cu, Fe, Ni, Mn andZn exist in trace amounts as essential elements in biological systems and play important roles in biochemical reactions of living systems [5]. Such essential trace metals as well as others with no essential biological function (e.g. Al, Cd, Cs, Hg, Pb and Sn) are all increasingly being found at very high concentrations in biological systems of humans, animals and even microbes due to increased industrial use accompanied by improper disposal[6]. Although there have been reports on the activity of drug-metal complexes, most researchers used presynthesized complexes requiring special conditions (e.g high temperatures and reûuxing for long periods) for their studies. Such requirements may however not be met in living systems such as the human body. Also, little or no work has been done on the speciûc eûects of Cd, Cr, Mn, or Zn on the activity of chloramphenicol in solution on the growth of the microorganisms used in this study[7].

Manganese has attracted the attention of several researchers owing to the fascinating physical and biochemical characteristics. The utility of manganese is quite diversified such as an alloying agent for aluminium, in dry cell batteries (carbon-zinc Leclanche type), oxidizing agent etc[8]. The best known complexes of manganese with sulfur ligands are dithiophosphinates the dithiocarbamates[9].

Inorganic materials such as metal and metal oxides have attracted lots of attention over the past decade due to their ability to withstand harsh process conditions[10-11]. Of the inorganic materials, metal oxides such as $TiO_2$, ZnO, MgO and CaO are of particular interest as they are not only stable under harsh process conditions but also generally regarded as safe materials to human beings and animals[12]. The use of nanoparticles of silver and zinc oxide has been seen as a viable solution to stop infectious diseases due to the antimicrobial properties of these nanoparticles. The intrinsic properties of a metal nanoparticle are mainly determined by size, shape, composition, crystallinity and morphology[13].

Reddy[14] report selective toxicity of nano-materials, including metal oxides, to prokaryotes and eukaryotes. Studies on eukaryotes, primarily involving mammalian cells, indicate that nano-particles of ZnO cause higher levels of oxidative stress resulting in inflammation and cytotoxicity. In prokaryotes, oxidative stress can induce cell death due to interactions between reactive oxygen species and proteins, DNA or the cell membrane[15-16].

The objective of this study was to compare the microbial effect of Zinc oxied and manganese nanoparticles using various microbial strains. Such a comparative study would reveal strain specificities and would eventually lead to better utilization of nanoparticles for specific application. Ten representative microbes typically recommended for use in antimicrobial assays. The antimicrobial effect was quantified based on the inhibition zone measured in the agar well diffusion tests conducted in plates and by determining the minimum growth inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC) of nanoparticals in liquid batch cultures.

## MATERIALS AND METHODS

Commercial quality of nano MnO and ZnO nanoparticals, were obtained from local suppliers. Surface morphologies of the specimens were observed with a scanning electron microscope (SEM, PHILIP XL-30). Sample preparation and characterization Semi-quantitative analysis of nanoparticals was carried out by energy-dispersive X-ray spectroscopy (EDS, FEI Quanta 200, Holland). The crystallinity of the nanoparticals was further characterized using a X-ray diractometer (XRD, Philips PW3040/60 X'pert PRO, The Netherlands) employing Cu K a radiation [17].

**Electron spin resonance spectroscopy**

Structural investigation by recording their powder X-ray diffraction patterns transmission electron microscopy (TEM) of ZnO and MnO nanostructures. The patterns were recorded on a Bruker D8 Advance X-ray diffractometer at room temperature (300 K) using monochromatic Cu Ká radiation (ë=0.15406 nm). For this purpose, a fine drop of ZnO nanoparticles and MnO nanoparticals dispersed in hexane were placed on carbon-coated copper grids and the hexane was allowed to evaporate slowly at room temperature. The

elemental concentration of ZnO and MnO nanoparticals in the core– shell nanostructures was determined by inductively coupled plasma atomic emission spectroscopy (ICP AES)[1,18]

**Microorganisms**

The organisms used were: Gram positive bacteria namely *Bacillus megaterium* ATCC 25848, *Bacillus subtilus* NRRL B-543, *Sarcina lutea* ATCC27853 and *Staphylococcus aureus*; NRRL B-313, Gram negative bacteria *Escherichia coli*; NRRL B-210, *Pseudomonas aeruginosa* NRRL B23 27853, *Klebsiella pneumoniae* ATCC 27736 and *proteus vulgaris* NRRL B-123. The pathogenic yeast was *Candida albicans* NRRL Y-477 and fungi *Aspergillus niger* NRRL-3. These microorganisms were obtained from Natural Research center, Department of Chemistry of Natural and Microbial product, Cairo, Egypt.

**Antimicrobial Activity**

ZnO and Mn nanoparticals were tested in vitro for their antimicrobial activities against pathogenic strains by the agar diffusion technique[19]. The tested nanoparticals were dissolved in dimethyl sulfoxide (DMSO) to prepare chemicals of stock solutions of 50 ìg/ml. The pathogenic bacteria and fungi were maintained on nutrient agar and Czapek's-Dox agar media, respectively in Petri dishes with an inner diameter 9 cm to provide thin agar plates after solidification of thickness 3.4-3.5 mm. After solidification, hollows of 10 millimetre diameter wells were cut from the agar using a sterile cork-borer, and 100ìl of each of the tested solutions were poured into the wells. The Petri dishes were incubated at 5-8°C for 2-3 h to permit good diffusion and then incubated at 30°C for 24 h in case of bacteria and at 28°C for 48 h in case of yeast and fungi. After the incubation the diameters of inhibition zone (mm) were measured[20].

**Minimum Inhibitory Concentration (MIC)**

The antimicrobial activities of the samples were evaluated through the determination of the (MIC) by the method of micro dilution in culture broth[21]. For both the antibacterial and the antifungal assays, the particles were dissolved in DMSO (50 mg/ml). Further dilutions were prepared at the required quantities of 50, 45, 40, 35, 30, 25, 20, 15, 10, 5, 1 and 0.5 ìg/ml concentrations. The values of (MIC) values were determined using the method of twofold serial dilutions[22].

Nutrient Broth, which contained tested particles and controls, were inoculated with approximately $5\times10^5$cfu/ml of actively dividing bacterial and fungal cells or spores. The cultures were incubated for 24 h and 48 h at 30°C on a metabolic rotary shaker (220 rev/min), and the growth was monitored visually and spectrophotometerically (at 540 nm)[22]. In order to ensure that the solvent had no effect on bacterial growth, a control test was also performed containing inoculated broth supplemented with only DMSO at the same dilutions used in our experiments and found inactive in culture medium. The MIC was defined as the lowest concentration required to arrest the growth of the bacteria at the end of 24 h of incubation. The MBC was determined by sub culturing a 0.1 ml volume of the medium drawn from the culture tubes after 48 h on Nutrient Agar and incubated further for bacterial growth. The growth was scored for relative numbers of the bacterial colonies. The lowest concentration of the antimicrobial agent causing negative growth (fewer than three colonies) was considered the MBC. Since the MIC and MBC were virtually the same, we generally reported only the MBC in the results[23].

## RESULTS AND DISCUSSION

**Electron spin resonance spectroscopy**

The scanning electron microscopy (SEM) images of ZnO and MnO nanoparticals indicating the homogeneous size and high purity of the product (Fig1 a and b respectively), these fig are contrast with this obtain by Zawrah and Sherein [24]. On the other hand (Fig. 2 a and b) conûrmed that the metal particles of ZnO and MnO nanoparticals are in the nanorange and that they are approximately spherically in shape. Subsequent image analysis revealed that the ZnO nanoparticals are relatively smaller (mean±SD: 111 to 122 nm; size range: 176–214nm for a scan of n=2582 particles) than the MnO nanoparticles (157 to 162 nm; range: 176–214 nm for a scan of n=683 particles). Considerable asymmetry was observed in the particle size distribution profile.

**Antimicrobial Activity**

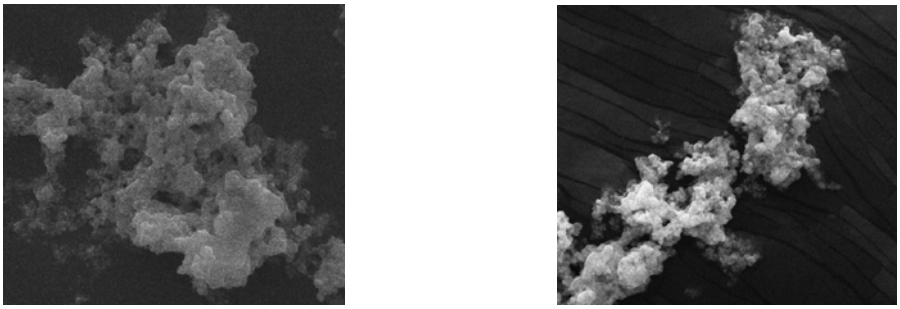
The antibacterial activity of nanoparticles was compared for various microorganisms using the diameter of inhibition zone in well diffusion

test. The diameter of inhibition zone (DIZ) reflects magnitude of susceptibility of the microorganism [25]. The strains susceptible to disinfectants exhibit larger DIZ, whereas resistant strains exhibit smaller DIZ. The well with ZnO nanoparticles were surrounded by a larger DIZ compared to the MnO nanoparticals for all *E. coli* and *B.subtilis* strains selected for this. While the DIZ was affected by the type of microorganisms, it was essentially invariant cross the various strains.

Similarly, for *S. aureus* the ZnO nanoparticals impregnated wells were found to be more effective compared to manganese nanopartical impregnated wells, however the difference in the DIZ was merely 20%. In contrast, using *P. aeruginosa* and *B. megaterium*, the wells impregnated with manganese nanoparticals showed a significantly larger DIZ, almost 10% greater compared to that observed with ZnO nanoparticals. This effect is due to the generation of high rate of oxygen species from the surface of ZnO nanoparticals, which penetrate in the cell membrane and leads to the death of the bacteria. The generation of highly reactive species such as OH, $H_2O_2$, and $O_{2\text{-}2}$ [26].

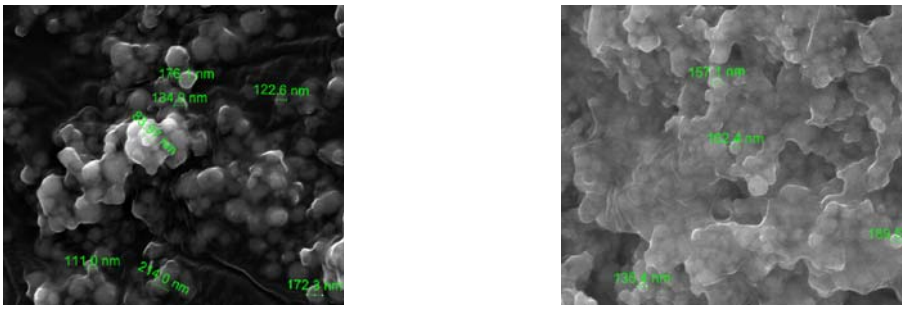
Since DIZ was measured on agar plates using a ruler with 1mm resolution, the possibility of measurement errors exist; however, the method illustrates the potential biocidal effect of nanoparticals to different microbial strains. As for the well diffusion tests, the batch studies also reveal differences in sensitivity to ZnO and MnO nanoparticals for the various microbial strains[27]. These results correspond with the results of Rajendran[28] as ZnO nanoparticals showed antibacterial activity was much higher against *S.aureus*.

The antibacterial data reveals that MnO nanoparticals in solution show prominent inhibition capacities antibacterial properties against the bacterial species under study. The results also confirmed with Tweedy and Loeppky[29], that the antimicrobial activity of the metal chelates show more inhibitory effects than the parent ligand. Furthermore, the MnO nanoparticals enhanced activity, due to chelating the polarity of the metal ion will reduce to a greater extent due to the overlap of the ligand orbital and partial sharing of the positive charge of the metal ion with donor groups.



(a) (b)

**Fig. 1.** (1a and b) Electron spin resonance spectroscopy of ZnO and MnO nanoparticals



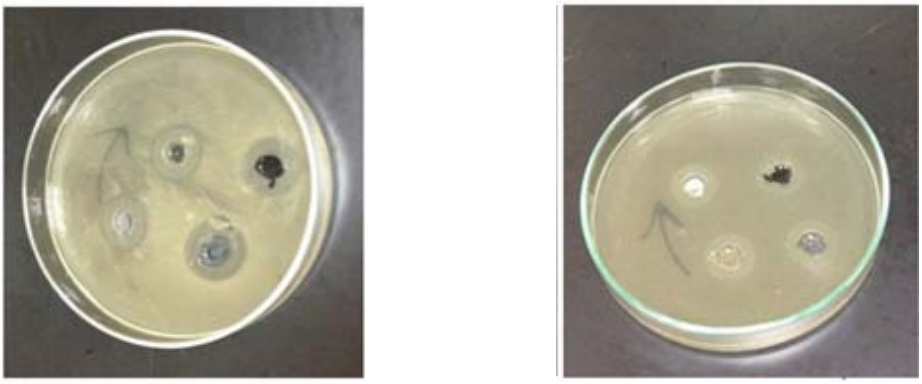

(a) (b)

**Fig. 2.** (2 a and b) TEM images of ZnO and MnO nanoparticals

**Minimum Inhibitory Concentration (MIC)**

MIC and MBC were determined by the standard microbial method. ZnO and MnO nanoparticles suspensions with different concentrations were incubated with *E.coli* and *P. aeruginosa* in aqueous NB as shown in fig (5 a and b) respectively. In batch studies, a greater lag phase and lower maximum absorbance were observed as the concentration of nanoparticles increased. Similar observation was reported by Sondi[25] in their studies on effect of silver nanoparticles on a single strain of *E. coli*. As concentration of nanoparticles increased to MIC of the respective strains, no growth was observed in the flask. The bactericidal effect of nanoparticles is dependent on the concentration of nanoparticles and the initial bacterial concentration[27].

Bacterial growth was studied by using a spectrophotometer inspecting the NB for turbidity. If the material being tested does not kill but instead inhibits the growth of bacteria (bacteriostatic agent), the bacteria will grow when it is removed from the solution containing the material, and colonies will be observed upon plating an aliquot. If the material being tested is bactericidal, the absence of bacterial colonies will be observed upon plating. To establish whether the suspensions were bacteriostatic or bactericidal, 100µl aliquots were taken from the incubated NB, each containing ZnO or MnO nanoparticals and *E. coli*, or *P. aeruginosa*



(a) ZnO nanopartical (b) MnO nanoparticals

**Fig. 3.** Agar plates containing ZnO and MnO nanoparticals impregnated wells and DIZ for (a) *E. coli* and (b) *P. aeruginosa*

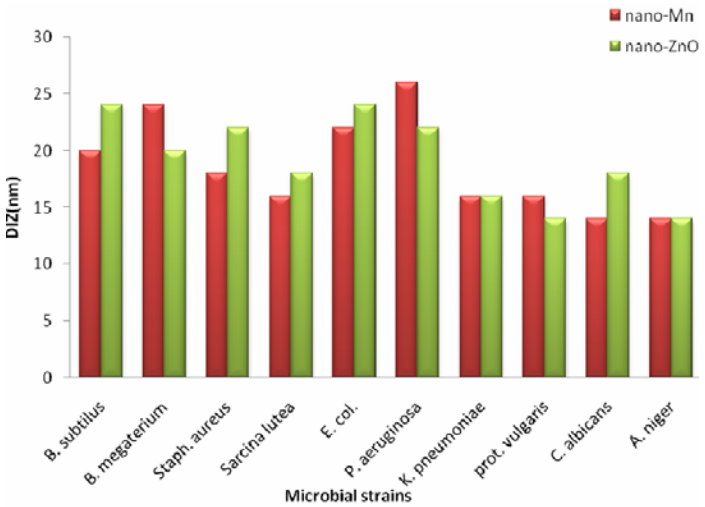



**Fig. 4.** The diameter of inhibition zone (DIZ) surrounding ZnO/MnO nanoparticals impregnated wells in presence of various microorganisms.

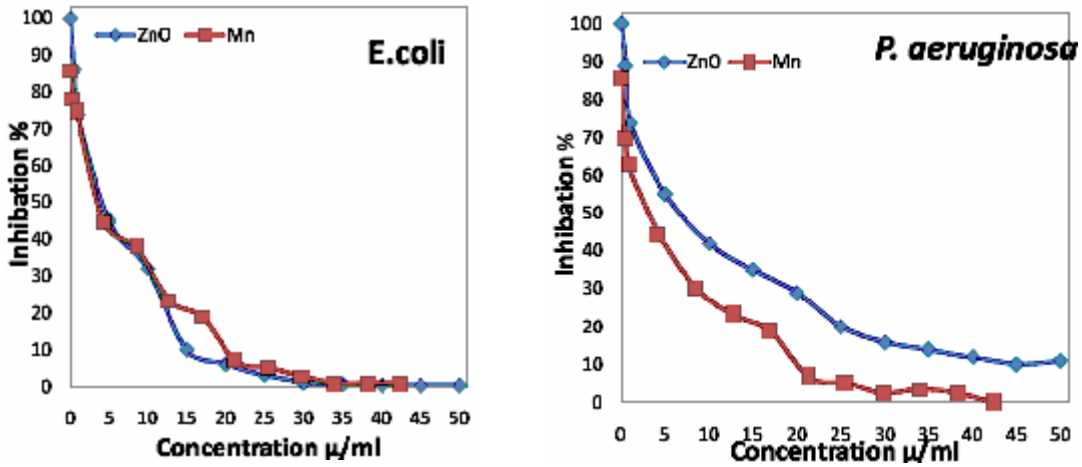



**Fig. 5.** MIC of ZnO and MnO nanoparticals for *E. coli* and *P. aeruginosa*

were placed on nutrient agar plates and incubated for 18–20h. The results are showed that ZnO nanoparticals suspension with a concentration in the range of 0.5 to 50ìg effectively inhibits bacterial growth of *E. coli* and *P. aeruginosa* on using ZnO nano particales. this results are appears because of ZnO nanoparticals strongly resisted to microorganisms[26]. There are some reports on the considerable antibacterial activity of CaO, MgO and ZnO, which is attributed to the generation of reactive oxygen species on the surface of these oxides.

The antibacterial activity was observed at concentrations mor than 5ìM for all the ZnO and MnO nanoparticals on using *P. aeruginosa*. Nanoparticles can act as antibacterial and antifungal agents, due to their ability to interact with microorganisms[30-32]. Exerting their antibacterial properties, nanoparticles attach to the surface of the cell. This interaction causes structural changes and damage, markedly disturbing vital cell functions, such as permeability, causing pits and gaps, depressing the activity of respiratory chain enzymes, and finally leading to cell death[33-34].

Furthermore, Nanoparticles can act as antibacterial and antifungal agents, due to their ability to interact with microorganisms[30-35]. Exerting their antibacterial properties, nanoparticles attach to the surface of the cell. This interaction causes structural changes and damage, markedly disturbing vital cell functions, such as permeability, causing pits and gaps, depressing the activity of respiratory chain enzymes, and finally leading to cell death[33-34].

## REFERENCES

1. Karimian R., Zandi M., Shakour N. and Piri F. Synthesis and characterization of manganese oxide and cobalt oxide nano-structure *journal of nanostructure*; 2012: **1**: 39-43.
2. Herrera M, Carrion P, Baca P, Liebana J, Castillo A. In vitro antibacterial activity of glass-ionomer cements. *Microbios*; 2001; **104**(404): 141-48.
3. Matsumura Y, Yoshikata K, Kunisaki S, Tsuchido T. Mode of bactericidal action of silver zeolite and its comparison with that of silver nitrate. *Appl Environ Microbiol*, 2003; **69**(7): 4278–81.
4. Te-Hsing W, Yi-Der T. and Lie-Hang S. The novel methods for preparing antibacterial fabric composites containing .nano-material. *Solid State Phenomena*; 2007; **124**: 1241-44.
5. Obaleye, J.A. and Lawal, A. Synthesis, characterization and antifungal studies of some metronidazole complexes. *Jour. Applied Sci. Environ. Management*; 2007; **11**(4): 15–18.
6. Adarsh V.K., Madhusmita M. and Sanhita C. Studies on metal microbe interaction of three bacterial isolates from east Calcutta wetland. *Online Jour. Biol. Sci.;* 2007; **7**(2): 80–88.
7. Ogunniran, K.O., Ajanaku K.O.and James O.O. Synthesis, physical properties, antimicrobial potentials of some mixed antibiotics complexed with transition metals and their effects on alkaline phosphatase activities of selected rat tissues. *Sci. Res. Essay.;* 2008; **3**(8): 348–354.
8. Denger C., Keck H., Kuchen W., Mathow J. and Wunderlich H. Synthesis, properties and structure of bis (dialkyldithiophosphinato) manganese (II) complexes. *Inorg. Chim. Acta.*1987; **132**: 213-215.

9. Holah D. G. and Murphy C. N. Reactions of Sodium N,N-Diethyldithiocarbamate and Potassium Ethyl Xanthate with some 3d Transition Metal Halides in the presence of 2,2'-Bipyridyl and 1,10-Phenanthroline. *Can. J. Chem*. 1971; **49**: 2726-2732.
10. Fu L., Liu Z., Liu Y., Han B., Hu P., Cao L. and Zhu D., Beaded Cobalt oxide nanoparticles along carbon nanotubes: towards more highly integrated electronic devices, *Advanced Materials*; 2005; **17**: 217-221.
11. Makhluf S., Dror R., Nitzan Y., Abramovich Y., Jelnek R. and Gedanken A. Microwave-assisted synthesis of nanocrystalline MgO and its use as a bacteriocide, *Advanced Functional Materials*; 2005;**15**: 1708-1715.
12. Stoimenov P.K., Klinger R.L., Marchin G.L. and Klabunde K.J. Metal oxide nanoparticles as bactericidal agents, *Langmuir*; 2002; **18**: 6679–6686.
13. Dickson R.M. and Lyon L.A., Unidirectional plasmon propagation in metallic nanowires, *Journal of Physical Chemistry*; 2000; **104**: 6095 -6098.
14. Reddy K.M., Feris K., Bell J., Wingett D.G., Hanley C. and Punnoose A. Selective toxicity of zinc oxide nanoparticles to prokaryotic and eukaryotic systems. *Appl Phys Lett*, 2007; **90**: 2139021-2139023.
15. Desnues B., Cuny C., Grégori G., Dukan S., Aguilaniu H. and Nyström T. Differential oxidative damage and expression of stress defense regulons in culturable and non-culturable *Escherichia coli* cells. *EMBO Rep*, 2003; **4**: 400-404.
16. Aertsen A. and Michiels C.A. Stress and how bacteria cope with death and survival. *Crit Rev Microbiol*, 2004; **30**: 263-273.
17. Kim J. S., Kuk E., Yu K. N., Kim J., Park S. J., Lee H.J., Kim S. H., Park Y.K., Park Y. H., Hwang C.Y., Kim Y.K., Lee Y.S., Jeong D. H. and Cho M.H., *Antimicrobial effects of silver nanoparticles. Nanotechnology, Biology, and Medicine*; 2007; **3**: 95–101.
18. Yanagi H., Ohno S.and Kamiya T. Magnetic and carrier transport properties of Mn-doped p-type semiconductor LaCuOSe: an investigation of the origin of ferromagnetism. *J Appl Phys*; 2006; **100**: 033717.
19. Perez C., Pauli M. and Bazevque P. An antibiotic assay by the agar well diffusion method. *Acta Biologiae et Medicine Experimentalis*; 1990; **15**: 113-115.
20. Li W.R., Xie X.B., Shi Q.S., Zeng H.Y., OuYang Y.S. and Chen Y.B. Antibacterial activity and mechanism of silver nanoparticles on *Escherichia coli*. *Appl Microbiol. Biotechnol*.; 2010; **85**: 1115–1122.
21. Hamouda T., Myc A., Donovan B., Shih A., Reuter J.D.and Baker J. J. A. A novel surfactant nanoemulsion with a unique non-irritant topical antimicrobial activity against bacteria, enveloped viruses and fungi.*Microbiol Res*; 2000; **156**: 1-7.
22. Dragieva I., Stoeva S., Stoimenov P., Pavlikianov E. and Klabunde K. Complex formation in solutions for chemical synthesis of nanoscaled particles prepared by borohydride reduction process. *Nanostruct Mater*; 1999; **12**: 267-70.
23. Dibrov P., Dzioba J.and Gosink K.K. Chemiosmotic mechanism of antimicrobial activity of Ag(+) in Vibrio cholerae. *Antimicrob Agents Chemother*; 2002; **46**: 2668-70.
24. Zawrah M. F. and Sherein A. E. Antimicrobial Activities of Gold Nanoparticles against Major Foodborne Pathogens. *Life Science Journal*. 2011; **8**(4): 37-44.
25. Sondi I. and Salopek-Sondi B. Silver nanoparticles as antimicrobial agent: a case study on *E. coli* as a model for gram-negative bacteria. *J Colloid Interf Sci*; 2004; **275**: 177–82.
26. Nawaz H. R., Solangi B. A., Zehra B. and Nadeem U. Preparation of Nano Zinc Oxide and its Application in Leather as a Retanning and Antibacterial Agent. *Canadian Journal on Scientific and Industrial Research*; 2011; **2**(4): 164-170.
27. Pal S., Tak Y.K. and Song J.M. Does the antimicrobial activity of silver nano particles depend on the shape of the nanoparticle? A study of the gram-negative bacterium *Escherichia coli*. *Appl Environ Microbiol*; 2007; **73**: 1712–20.
28. Rajendran R., Balakumar C., Hasabo A. M., Jayakumar S., Vaideki K. and Rajesh E.M. Use of zinc oxide nano particles for production of antimicrobial textiles. *International Journal of Engineering, Science and Technology*; 2010; **2**(1): 202-208.
29. Tweedy B.G. and Loeppky C., The use of 14C-labeled glucose, glucuronate, and acetate to study the effect of atrazine, simazine, and fluometuron on glucose catabolism in selected plant pathogenic fungi, *Phytopathology*; 1968; **58**: 1522-1531.
30. Hernandez-Sierra J.F., Ruiz F., Pena D.C. The antimicrobial sensitivity of *Streptococcus mutans* to nanoparticles of silver, zinc oxide and gold. *Nanomedicine NBM*; 2008; **4**: 237–240.
31. Dror-Ehre A., Mamane, H., Belenkova, T., Markovich, G. and Adin, A. Silver nanoparticle – *E. coli* colloidal interaction in water and effect

on E. coli survival. *J Colloid Interface Sci.*; 2009; **339**: 521–526.

32. Eby D.M., Shaeublin, N.M., Farrington K.E., Hussain S.M. and Johnson G.R. Lysozyme catalyzes the formation of antimicrobial silver nanoparticles. *ACS Nano*.; 2009; **3**: 984–994.
33. Rai M., Yadav A. and Gade A. Silver nanoparticles as a new generation of antimicrobials, *Biotechnology Advances;* 2009; **27**: 7683.
34. Sharma V.K., Yngard R.A. and Lin Y. Silver nanoparticles: Green synthesis and their antimicrobial activities. *Adv. Colloid Interface Sci*.; 2009; **145**: 83–96
35. Panacek A., Kolar M. and Vecerova R. Antifungal activity of silver nanoparticles against *Candida* spp. *Biomaterials*; 2009; **30**: 6333–6340.