Comparative and Synergistic Studies of Antibacterial Effect of ZnO Nanoparticles and Antibiotics for Pathogens in Drinking Water

F. Rubab, M. F. Chaudhary and N. M. Butt*

Preston Institute of Nano Science & Technology (PINSAT), Preston University, Sector H-8/4, Islamabad, 44000 – Pakistan
E-mail: nmbutt36@gmail.com
* Fellow Pakistan Academy of Sciences, Islamabd, Pakistan
* Corresponding author

ABSTRACT

The goal of this study was to determine the antibacterial efficiency of zinc oxide nanoparticles against various Gram negative bacteria isolated from local sources of drinking water. We have been able to isolate and identify *Escherichia coli, Serratia marcescens, Enterobacter cloacae, Cronobacter sakazakii, Pseudomonas aeruginosa, klebsiella pneumoniae and Aeromonas hydrophila.* A variety of microbiological tests were carried out using different concentrations and working volumes of zinc oxide nanoparticles with average size of 35 nm. Earlier results have already been reported (TechConnect Briefs 2015, Vol. 1). Current Result showed the antibacterial activity of different concentrations (0, 100, 200, 300 & 500 mg/ml) of zinc oxide nanoparticles against *P. aeruginosa* and *k. pneumoniae*. Highest zone of inhibition i.e. 25 mm was observed at 500 mg/ml against *P. aeruginosa* while *K. pneumoniae* showed a 20 mm zone of inhibition at same concentration. When different volumes (0, 10, 20, 30, 40, 50, 60 µl) of ZnO NPs were tested the highest zone of inhibition i.e. 23 mm was observed at 60 µl against *P. aeruginosa* while *K. pneumoniae* showed 22 mm zone of inhibition. Antibiotic susceptibility of various antibiotics was also tested in combination with ZnO nanoparticles against these water borne bacteria. Results revealed that antibiotic efficacy of Erythromycin (ERYC) was enhanced by the addition of ZnO NPs (5 µl) of 500 mg/ml. Erythromycin showed only DZI of 7 mm against *E. cloacae* but the same antibiotic along with ZnO NPs showed zone of inhibition of 12 mm against *E. cloacae* showing a substantial increase. Therefore ZnO NPs have the potential for their use as a very effective antibacterial agent for antibiotic resistant bacteria and eventually for water purification.

Key words: DZI, Antibiotic Resistance, ZnO nanoparticles, Antibacterial Agent

1. INTRODUCTION

One of the major problems around the globe is inadequate access to clean water and sanitation. Most of the natural resources of drinking water are found to be contaminated with various toxic materials and pathogenic microorganisms (Baruah & Dutta, 2009). The spread of disease; the emergence of antibiotic resistant microorganisms and its propagation are the leading cause of infections from these water borne microorganisms. Despite significant hard work, resistance among bacteria is still thought to be a major hitch in chemotherapy of many infection diseases (Bennet, 2008). One of the recent efforts in addressing this challenge lies in exploring antimicrobial nanomaterials, to which microbial pathogens may not be able to develop resistance. Antibacterial properties of silver (Sharma *et al.,* 2009; Rai *et al.,* 2009; Lok *et al.,* 2007; Fabera, 2009) and copper nanoparticles have been reported in literature (Ren, 2009). ZnO nanoparticles have also been reported to have antibacterial effect (Xin *et al.,* 2004; Vigneshwaran *et al.,* 2006; Fei *et al.,* 2006). ZnO nanoparticles have shown extensive antibacterial activity against variety of microbes including both Gram-negative and Gram-positive bacteria, such as *Staphylococcus aureus*, *Salmonella*, *Listeria monocytogenes* and *Escherichia coli*, O157:H7 (Jones *et al.,* 2008; Liu *et al.,* 2009).

The antibacterial efficacy of antibiotics i.e. erythromycin, penicillin G, clindamycin, amoxicillin, and vancomycin in combination with silver nanoparticles showed significant increase in antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* (Shahverdi *et al.,* 2007). The

antibacterial activity of ciprofloxacin was enhanced in the presence of ZnO nanoparticles against various clinical strains of *E. coli* and *S. aureus* (Maryam *et al.,* 2010). Enhanced synergism of β -lactam antibiotics with ZnO nanoparticles has also been reported (Rashmi *et al.,* 2013).

Clean drinking water is dire need of people particularly in developing countries like Pakistan. In the previous study the antibacterial effect of ZnO nanoparticles and their comparison with certain antibiotics was investigated (Rubab *et al.,* 2015). Therefore the aim of present study is to analyze the antibacterial and synergistic effect of ZnO nanoparticles with various antibiotics against human pathogens isolated from drinking water supply of Islamabad, the capital of Pakistan under laboratory conditions. This study explores new avenues for provision of cost effective and good quality drinking water using nanomaterials particularly in combination with antibiotics.

2. MATERIALS & METHOD

2.1 Provision of zinc oxide (ZnO) Nanoparticles

Zinc Oxide, dispersion Cat # 721077 nanoparticles of size<100 nm particle size (DLS), <35 nm average particle size (APS) and 50 weight percent in H_2O were purchased from M/S Sigma Aldrich.

2.2 Provision of Antibiotics

Antibiotics used in this study include ciprofloxacin, ceftriaxone, erythromycin and trimethoprim-sulfamethoxazole which were purchased from M/S Oxoid and Liofilchem. Details are given in Table-1.

Table 1: List of Antibiotics used

Antibiotics	Code	Antibiotic group
Ciprofloxacin	CIP (5 µg)	Fluoro-quinolone
Erythromycin	ERYC (15 µg)	Macrolide
Ceftriaxone	CRO (30 µg)	Third generation cephalosporin
Trimethoprim-sulfamethoxazole	SXT (25 µg)	Folate-pathway inhibitor

2.3 Isolation of Bacteria

The identified Gram negative bacteria used in this study are *Escherichia coli*, *Enterobacter cloacae*, *Serratia marcescens*, *Pseudomonas aeruginosa, Klebsiella pneumoniae, Cronobacter sakazakii* and *Aeromonas hydrophila.* These bacteria were isolated from drinking water sources of Islamabad, Pakistan and API 20 E kit was used to identify the bacteria.

2.4 Antibacterial Assay of ZnO NPs

Antibacterial assay of ZnO NPs was performed using agar well diffusion method (Perez *et al.,* 1990). Culture of bacteria was grown on nutrient agar plates and bacterial suspension was made after 24 hours. 0.5% Mcfarland standard solution was used to measure the turbidity of bacterial suspension. Bacterial lawn was made on sterile nutrient agar plates using cotton swabs. 9 mm wells were made using sterile borer on nutrient agar plate. Working volume 60 µl of different concentrations i.e. 0, 100, 200, 300 & 500 mg/ml of ZnO NPs sample was poured in separate wells. Also different working volumes i.e. 10, 20, 30, 40, 50 & 60 µL of ZnO NPs of same concentration (500 mg/ml) was poured in separate wells respectively. After 24 hours of incubation at 37ºC diameter of zone of inhibition was measured. Experiment was performed three times under same conditions for better accuracy of results.

2.5 Antibiotic Susceptibility Assay

Antibiotic susceptibility assay was performed in triplicate by Kirby Bauer's disc diffusion method (Bauer *et al.,* 1966). Culture of bacteria was grown on nutrient agar plates and bacterial suspension was made after 24 hours. 0.5% Mcfarland standard solution was used to measure the turbidity of bacterial suspension. Bacterial lawn was made on sterile nutrient agar plates using cotton swabs. Pre sterilized disc of 5 mm of antibiotics ciprofloxacin (CIP-5 µg), ceftriaxone (CRO-30 µg), trimethoprim-sulfamethoxazole (SXT- 25 µg) and erythromycin (15 µg) were placed on the surface of the bacterial lawn. The plates were incubated at 37 ºC for 24 hours. Diameter of zone of inhibition (DZI) was measured after 24 hours.

2.6 Evaluation of synergistic antibacterial activity

The disc diffusion assay was performed by Kirby Bauer's disc diffusion method (Bauer *et al.,* 1966) on nutrient agar plates for standard antibiotics discs listed in Table- I. In order to evaluate the synergistic effect, ZnO nanoparticles at a concentration of 500 mg/ml were loaded on each standard antibiotic disc. Culture of bacteria was grown on nutrient agar plates and bacterial suspension was made after 24 hours. 0.5% Mcfarland standard solution was used to measure the turbidity of bacterial suspension. Bacterial lawn was made on sterile nutrient agar plates using cotton swabs and standard antibiotics discs along with test discs containing ZnO nanoparticles were placed on the surface of bacterial lawn. After incubation at 37°C for 24 h, the inhibition zones were measured. All experiments were repeated three times.

3. RESULTS

3.1 Growth and Morphological studies of isolated Bacteria

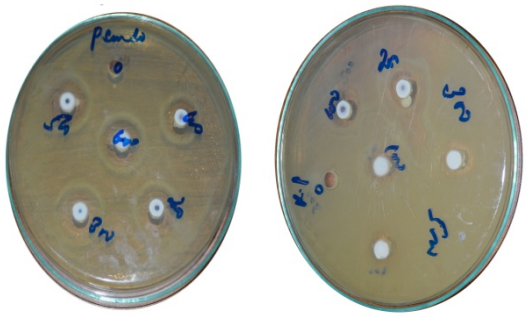
Bacteria isolated from drinking water supplies of different sectors of Islamabad, Pakistan were cultured on Nutrient Agar and Eosin Methylene Blue (EMB) agar. The plates were incubated for 24 hours at 37°C. Growth pattern was observed after 24 hours. *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* showed round colourless colonies on nutrient agar plates and round pink colour colonies were observed on EMB agar plates as shown in Fig. 1.

(a) *P. aeruginosa* **(b) *K. pneumoniae***

Fig. 1 Representation of bacterial growth (a) *Pseudomonas aeruginosa* (b) *Klebsiella pneumoniae* On Eosine Methylene Blue agar (EMB)

3.2 Antibacterial Assay of ZnO Nanoparticles

Antibacterial activity of ZnO nanoparticles was tested against identified bacteria i.e. *Pseudomonas aeruginosa and Klebsiella pneumoniae* isolated from drinking water supplies of Islamabad, Pakistan. ZnO nanoparticles showed marked antibacterial activity against both bacteria as shown in Fig. 2.



(a) *P. aeruginosa* **(b) *K. pneumoniae***

Fig. 2 Antibacterial Activity of ZnO NPs against (a) *Pseudomonas aeruginosa* (b) *Klebsiella pneumoniae*

Antibacterial activity of ZnO NPs was tested using different working volumes i.e. 10, 20, 30, 40, 50, 60 µl of ZnO nanoparticles of concentration 500 mg/ml. When these volumes were poured in wells, highest zone of inhibition observed against *P. aeruginosa* was 23 mm and 22 mm against *K. pneumoniae* at 60 µl as shown in Fig 3. Zones of inhibition showed increasing trend with the increase in volume as can be observed by graph (Fig. 3) against *P. aeruginosa* and *K. pneumoniae.* Minimum zone of inhibition was observed at 10 µl volume. *P aeruginosa* showed 19 mm zone of inhibition at this working volume while *K. pneumoniae* showed 18 mm diameter of zone of inhibition.

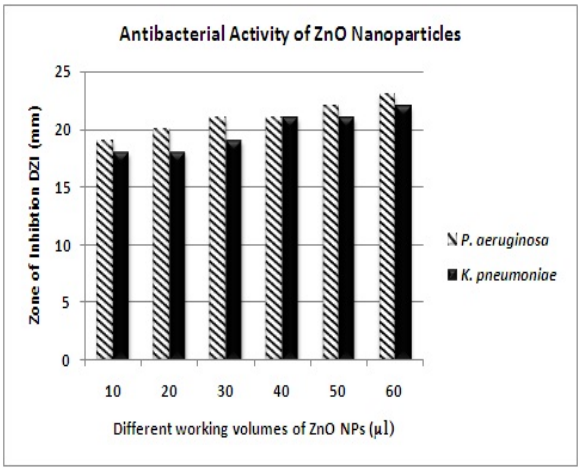


Fig. 3 Antibacterial effect working volumes (10-60 µL) of ZnO NPs on water borne bacteria

Antibacterial activity of ZnO NPs of average size 35 nm was also tested using different concentrations (100, 200, 300 & 500 mg/ml) of ZnO nanoparticles. When these concentrations were poured in wells highest zone of inhibition was observed at 500 mg/ml showing 25 mm diameter of zone of inhibition against *P. aeruginosa* and 20 mm diameter of zone of inhibition against *K. Pneumonia* as shown in Fig. 4.

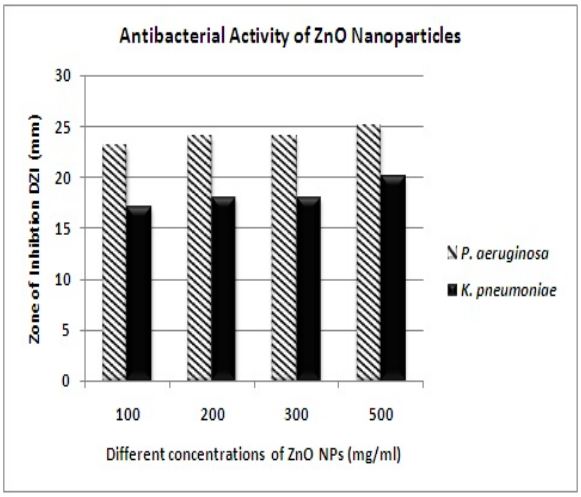


Fig. 4 Antibacterial effect of various concentrations of Zinc Oxide nanoparticles on water borne bacteria

3.3 Antibiotic Susceptibility Assay

The bacterial isolates were also tested for susceptibility against antibiotics which include; ceftriaxone, ciprofloxacin, trimethoprim-sulfamethoxazole and erythromycin. The growth of *E. coli* was inhibited by all antibiotics showing highest zone of 32 mm against ciprofloxacin (CIP) while the minimal activity of 11 mm was observed against erythromycin (ERYC). While ceftriaxone (CRO) and trimethoprim-sulfamethaxazole (SXT) showed intermediate antibacterial activity against *E. coli* as shown in Fig. 5

As given in graph (Fig. 5) erythromycin (ERYC) showed minimum zone of inhibition against *E. cloacae* showing only 7 mm diameter of zone of inhibition. While ciprofloxacin (CIP), Ceftriaxone (CRO) and trimethoprim-sulfamethaxazole (SXT) showed 36, 21 and 20 mm of zone of inhibition against the same bacteria. Erythromycin (ERYC) showed highest zone of inhibition against *S. marcescens* having a diameter of 17 mm. Growth of *S. marcescens* was strongly inhibited by all other antibiotics as well showing DZI 34, 25 and 19 mm against CIP, CRO and SXT respectively.

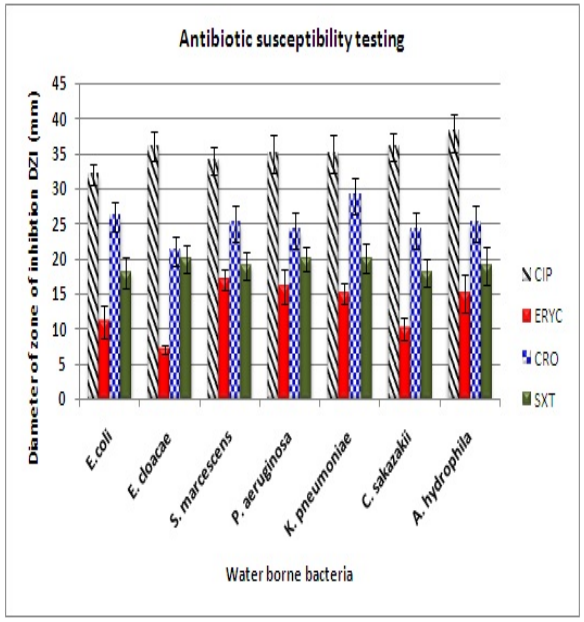


Fig. 5 Antibacterial activity of different Antibiotics on water borne Bacteria

Of all the tested antibiotics, Ciprofloxacin (CIP) showed strong antibacterial activity against all the tested bacteria. CIP showed its highest Zone of inhibition against *A. hydrophila* where 38 mm of zone of inhibition was observed. Trimethoprim-sulfamethaxazole (SXT) showed intermediate antibacterial activity as compared to the ciprofloxacin (CIP) and cefriaxone (CRO) showing its highest zone of inhibition of 20 mm against *E. cloacae, P. aeruginosa* and *K. pneumoniae*.

K. pneumoniae showed 29 mm zone of inhibition against ceftriaxone (CRO) while *E. cloacae* showed

21 mm zone of inhibition against the same antibiotics. *E. coli, S. marcescens, P. aeruginosa, C. Skazakii* and *A. hydrophila* were also susceptible to the ceftriaxone as indicated by the graph shown in Fig. 5.

3.4 Synergistic Antibacterial Activity

The combined effect of ZnO nanoparticles with standard antibiotic discs i.e. ciprofloxacin (CIP), erythromycin (ERYC), ceftriaxone (CRO) and trimethoprim- sulfamethaxazole (SXT) was also studied against isolated bacteria. Antibiotic efficacy of Erythromycin (ERYC) was enhanced by the addition of ZnO NPs (5 µl) of 500 mg/ml. Erythromycin showed only DZI of 7 mm against *E. cloacae* but the same antibiotic along with ZnO NPs showed zone of inhibition of 12 mm against *E. cloacae* as shown in Fig. 6.

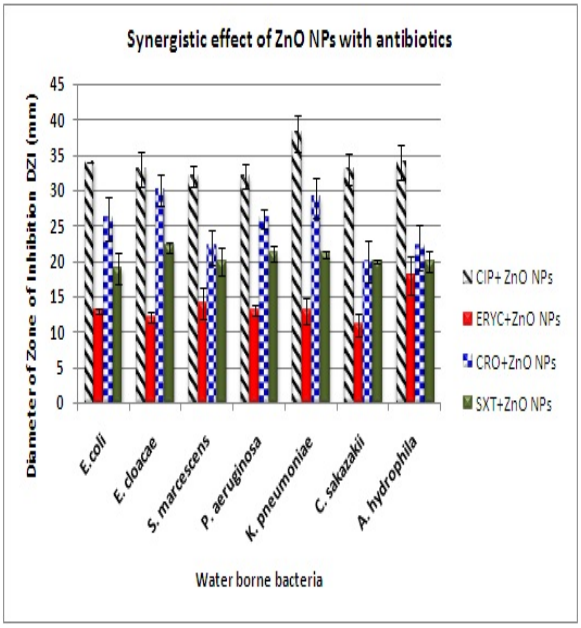


Fig. 6 Synergistic effect of ZnO NPs and Antibiotics

The antibacterial activity of erythromycin ERYC was also enhanced against *E. coli, C. sakazakii* and *A. hydrophila* where zone of inhibition showed increasing trend from 11, 10, 15 mm to 13, 11 and 18 mm respectively by the addition of ZnO NPs. The antibacterial activity of erythromycin is also reported to increase when used in combination with silver nanoparticles against *E. coli* (Shahverdi *et al.,* 2007).

Trimethoprim-sulfamethaxazole showed a positive response against all bacteria when used in combination with Zinc oxide nanoparticles. The antibacterial activity was increased against all tested bacteria. When ciprofloxacin (CIP) was used in combination with zinc oxide nanoparticles a slight increase in antibacterial activity was observed against *E. coli* and *K. pneumoniae*. CIP showed 32 mm zone of inhibition against *E. coli* while Zinc oxide nanoparticles in combination with CIP showed a 34 mm zone of inhibition. Similarly the zone of inhibition of CIP against *K. pneumoniae* was 35 mm which also showed slight increase to 38 mm when CIP was used in combination with ZnO nanoparticles. It is reported that antibacterial activity of ciprofloxacin was enhanced in the presence of ZnO nanoparticles against various clinical strains of *E. coli* and *S. aureus* (Maryam *et al.,* 2010).

The antibacterial effect of ceftriaxone (CRO) increased significantly when used in combination with ZnO nanoparticles against *E. cloacae*. This bacterium showed 21 mm diameter of zone of inhibition against CRO which was increased up to 30 mm diameter of zone of inhibition when CRO was used in combination with zinc oxide nanoparticles which showed 9 mm increase in diameter of zone of inhibition. The antibacterial activity was also increased against *P. aeruginosa* when CRO was used in combination with ZnO nanoparticles showing slight increase in diameter of zone of inhibition from 24 to 26 mm. There was no effect observed on the zone of inhibition of *E. coli* and *k. pneumoniae* (Fig. 6).

CONCLUSION

Zinc oxide nanoparticles are effective against all the tested strains of bacteria isolated from drinking water supply of Islamabad. Bacterial isolates which showed resistance against antibiotics was susceptible to zinc oxide nanoparticles as in case of *E. cloacae.* This bacterium was found to be resistant against antibiotic Erythromycin (ERYC) showing 7 mm DZI; whereas it was sensitive and showed a 24 mm zone of inhibition against ZnO nanoparticles (Rubab *et al.,* 2015) Zinc oxide nanoparticles when used in combination with antibiotics enhanced the antibacterial efficiency of antibiotics as in case of erythromycin. This antibiotic showed only DZI of 7 mm against *E. cloacae* but the same antibiotic when used in combination with ZnO NPs showed zone of inhibition of 12 mm against *E. cloacae.* The standard deviation in diameter of zone of inhibition (DZI) measurements of three repetitions in any experiment was maximum $\pm$ 2.5 mm. These results will be

helpful in understanding the usefulness of nanoparticles against problematic and antibiotic resistant bacteria in future. In future we intend to explore the antibacterial effect of zinc oxide nanoparticles of different sizes against water borne bacteria and also their combined effect with various antibiotics. The synergistic effect of ZnO and other nanoparticles with antibiotics opens a new direction of research for clean drinking water and for the treatment of water borne infectious diseases.

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REFERENCES

1. AR. Shahverdi, A. Fakhimi, HR. Shahverdi and S. Minaian (2007). Synthesis and effect of silver nanoparticles on the antibacterial activity of different antibiotics against *Staphylococcus aureus* and *Escherichia coli*. Nanomedicine. 3: 168–171.
2. AW. Bauer, WM. Kirby, JC. Sherris and M. Truck (1966). Antibiotic susceptibility testing by standardized singles disc method. *Am. J. Clin. Pathol.* 45: 493-496.
3. B. Fei, Z. Deng, JH. Xin, Y. Zhang and G. Pang (2006). Room temperature synthesis of nano rods and their applications on cloth. *Nanotechnology*. 17:1927–1931.
4. B. Maryam, S. Sepideh, NE. Zeinab, JF. Parisa, RS. Hamid, M. Ali, MM. Kamyar and RS. Ahmad (2010). ZnO nanoparticles enhanced antibacterial activity of ciprofloxacin against *Staphylococcus aureus* and *Escherichia coli*. Wiley InterScience. DOI: 10.1002/jbm.b.31615. 557-561
5. C. Lok, C. Ho, Q. He, W. Yu, H. Sun, PK. Tam, J. Chiu and C. Che (2007). Silver nanoparticles: partial oxidation and antibacterial activities. J Biol Inorg Chem. 12: 527–534.
6. C. Perez, M. Pauli and P. Bazerque (1990). An antibacterial assay by agar well diffusion method. Acta *Bio Et Med Exp*. 15: 113-115.
7. F. Rubab, MF. Chaudhary and NM. Butt (2015). Antibacterial effect of zinc oxide nanoparticles against waterborne bacteria. TechConnect Brief, Vol. 1: 344-348.
8. G. Ren (2009). Characterization of copper oxide nanoparticles for antimicrobial applications. J Antimicrob Agents. 33: 587–590.
9. J. Fabera (2009). Silver nanoparticle impact on bacterial growth: Effect of pH, concentration, and organic matter. J Environ Sci Technol. 43: 7285–7290.
10. JH. Xin, WA. Daoud and YY. Kong (2004). A new approach to UV-blocking treatment for cotton fabrics. *Textile Research Journal*. 4:97–100
11. M. Rai, A. Yadav and A. Gade (2009). Silver nanoparticles as a new generartion of antimicrobials. Biotech Adv. 27: 76–83.
12. MB. Rashmi, CN. Khobragade, RS. Mane and S. Bhande (2013). Enhanced synergism of antibiotics with zinc oxide nanoparticles against extended spectrum b-lactamase producers implicated in urinary tract infections. J Nanopart Res. 15: 1413 DOI 10.1007/s11051-012-1413-4
13. N. Jones, B. Ray, KT. Ranjit and AC. Manna (2008). Antibacterial activity of ZnO nanoparticle suspensions on a broad spectrum of microorganisms. FEMS Microbiol. Lett. 279(1): 71-76.
14. N. Vigneshwaran, S. Kuma, AA. Kathe, PV. Varadarajan and V. Prasad (2006). Functional finishing of cotton fabrics using zincoxide-soluble starch nanocomposites. *Nanotechnology*. 17: 5087–5095.
15. PM. Bennett (2008). Plasmid encoded antibiotic resistance: Acquisition and transfer of antibiotic resistance genes in bacteria. Br J Pharmacol.153: S347–S357.
16. S. Baruah and J. Dutta (2009). Nanotechnology applications in pollutionsensing and degradation in agriculture: a review. *Environ. Chem.Lett*. 7(3): 1-14.
17. VK. Sharma, RA, Yngard and Y. Lin (2009). Silver nanoparticles on *Escherchia coli*. Adv Colloid Interface Sci.145: 83–96.
18. Y. Liu, L. He, A. Mustapha, H. Li, ZQ. Hu and M. Lin (2009). Antibacterial activities of zinc oxide nanoparticles against Escherichia coli O157:H7. J.Appl. Microbiol. 107: 1193-1201