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N-acetyl cysteine substantially enhances the antimicrobial activity of zinc oxide nanoparticles against multidrug resistant pathogens

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Abstract

Aim: to evaluate the antimicrobial activity of Zinc Oxide nanoparticles (ZnO NPs) and N-acetylcysteine (NAC) alone and in combination against MDR pathogens. **Material & Methods:** ZnO NPs were tested against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* standard strains besides 50 clinical isolates from cancer patients. The antimicrobial and cytotoxic effects of ZnO NPs alone and combined with NAC were studied. **Results:** The MIC values of ZnO NPs against tested bacteria ranged from (60-100) µg/ml while it was 500µg/ml for *C. albicans*. Interestingly, the antimicrobial effect of ZnO NPs was substantially enhanced by addition of NAC. ZnO NPs completely abrogated respiratory dehydrogenases of tested pathogens and released muramic acid content from *S. aureus* in culture. No cytotoxicity was observed at MIC values of ZnO NPs. **Conclusions:** ZnO NPs exhibited robust antimicrobial activity against MDR pathogens without provoking cytotoxicity to HepG2. We report for the first time that NAC has a robust synergistic effect with ZnO NPs against MDR pathogens which could pave the way towards a paradigm shift in treating infectious diseases and combating the problem of increasing drug resistance.

Keywords: Nanoparticles, *Staphylococcus aureus*, *E. coli*, *Candida albicans*, MDR, Synergism.

Introduction

Recently, nanotechnology research has grown enormously with tremendous applications including drug delivery systems, imaging, gene therapy, diagnosis of infectious diseases and infection control (Ae Jung Hu 2011). Nanomaterials have fascinating properties demonstrating antimicrobial activity by themselves (Li et al. 2008) or enhancing the effectiveness and safety of antibiotics administration, hence are termed "nanoantibiotics"(Yadav A 2006; Abeylath and Turos 2008).

Metal oxides nanoparticles represent a novel class of extremely impressive materials that are extensively and continuously being developed for research and health-related applications Metal oxides such as zinc oxide (ZnO) have been receiving mounting attention not only because they are stable, but also due to their safety to human beings and animals (Stoimenov 2002; Roselli et al. 2003; Fu et al. 2005).

Zinc Oxide nanoparticles (ZnO NPs), which are non-toxic and biocompatible, have been utilized as drug carriers, cosmetics ingredients, and medical filling materials (J. Zhou 2006; Wang et al. 2007). Recently, Zn ONPs have been shown to have antibacterial activity against food borne pathogens, such as enterohemorrhagic *E. coli* (O157:H7) and enterotoxigenic *E. coli* (Brayner et al. 2006; Liu et al. 2009; Kairyte et al. 2013).

ZnO NPs are believed to demolish lipids and proteins of bacterial cell membrane leading to leakage of intracellular constituents and eventually to bacterial cell death (J. Zhou 2006; Huang et al. 2008). Additionally, generation of H_2O_2 and Zn^{+2} ions was proposed to be the key antibacterial mechanisms of ZnO NPs (Gold and Moellering 1996) while increased membrane permeability, cellular internalization and intracellular structural (Gold and Moellering 1996).
Multi-drug resistance (MDR) has been dramatically increasing particularly among Gram-negative bacteria which are capable to develop various mechanisms for antimicrobial resistance, often rendering these pathogens unresponsive to such drugs and accordingly termed MDR pathogens (Nataro and Kaper 1998). Most strains of *E. coli* are normal inhabitants of the small intestine and colon and are non-pathogenic, nonetheless, some variants (e.g. *E. coli* O157:H7) produce verotoxins (Nataro and Kaper 1998; Zhu et al. 2002). *E. coli* O157:H7 can survive in acidic foods with a low infective dose of 10–100 cells, and infection with such pathogen can lead to inflammation of the colon (Al-Holy 2006; Li et al. 2008).

Recently, a large proportion of *S. aureus* strains isolated from hospitals were resistant to methicillin (MRSA) (Stefani et al. 2012; Asencio et al. 2014; Kim et al. 2015) and some were found to be vancomycin resistant (VRSA) (Cesur et al. 2012; Di Gregorio et al. 2015). Currently, treating VRSA is a global and daunting medical challenge because vancomycin is the latest generation of antibiotics and is assumed at the moment to be the most effective for *S. aureus* infection (Perichon and Courvalin 2009; Chakraborty et al. 2010). There have been considerable efforts in searching for new natural product-derived antibiotics to control infections caused by MRSA, VRSA and other MDR pathogens (Hemaiswarya et al. 2008).

Antibiotic resistance is also a crucial in antifungal therapy especially due to the structural and metabolic resemblance with eukaryotic host cells. Candida species represent one of the most common fungal pathogens associated with hospital-acquired sepsis with a mortality rate of up to 40% (Kaufman 2007).

The aim of this work is to examine the antimicrobial and cytotoxic activities of ZnO NPs as well as their mode of action against MDR pathogens. Furthermore, this work aimed at exploring the impact of addition of NAC to ZnO NPs on its antimicrobial activity.

Materials and Methods

Microorganisms

Microorganisms used in this study were: *Escherichia coli* O157:H7 (ATCC 51569) and *Candida albicans* (ATCC 90028) were purchased from Egyptian Microbial Culture Collection (EMCC) while *Staphylococcus aureus* (ATCC 9144) was a gift from Department of Microbiology and Immunology, Faculty of Pharmacy, Tanta University. Additionally, 50 clinical isolates were collected from National Cancer Institute (NCI, Cairo, Egypt). All chemicals and media used were purchased from Sigma-Aldrich (St Louis, MO, USA).

Preparation and characterization of Zinc Oxide Nanoparticles

The zinc oxide nanoparticles were prepared by wet chemical method as previously described using zinc nitrate and sodium hydroxide as precursors and soluble starch as stabilizing agent (Yadav A 2006). The prepared nanoparticles were characterized using UV-Vis spectrophotometry ($T80^+$ PG instrument) and their size was determined using FEI Tecnai G2 F20 X-TWIN Transmission Electron Microscope (TEM).

Bacterial identification and *in vitro* susceptibility testing

The 50 clinical isolates were identified using Microscan technique at NCI, Cairo, Egypt. These clinical isolates as well as standard strains of tested pathogens were recruited in the *in vitro* antimicrobial susceptibility testing to ZnO NPs.

Assessment of the antimicrobial activity of ZnO NPs by disc diffusion test

The antimicrobial susceptibility of bacterial and fungal standard strains as well as clinical isolates to ZnO NPs was determined by standard disc diffusion method as described by the clinical laboratory standards institute (CLSI) (Institute 2008; 2012). Briefly, colonies from an overnight culture of all tested bacteria grown on nutrient agar were suspended in 3 ml saline and turbidity was adjusted to 0.5 McFarland ($1\text{-}2x10^8$ CFU/ml). The samples were inoculated (10^8 CFU/ml) in petri dishes with Nutrient agar or Sabaroud agar. Afterwards, paper discs of 6 mm diameter were laid on the inoculated test organism, which was instilled with different concentrations (500, 250, 125 and 62.5 µg/ml) of ZnO NPs. Petri dishes were incubated

at 37°C for 24h and antimicrobial activity was investigated by measuring the zones of inhibition around each disc. All experiments were done in triplicate.

Determination of minimum inhibitory concentration (MIC) of ZnO NPs

The MIC of ZnO NPs against all tested bacteria was estimated by standard broth microdilution method as described by the clinical laboratory standards institute (CLSI) (Institute 2012). Bacterial suspensions were prepared as previously described under disc diffusion test then diluted 1:1000 to give (1-2 x 10^5 CFU/ml). Tested bacteria were grown on nutrient broth in shaking incubator (150 rpm) and MIC values of ZnO NPs were determined using the two fold serial dilution technique at concentration ranging from 31.25 to 500 µg/ml. The cultures were incubated at 37°C for 18-20h. Results were estimated by measuring optical density (OD) at 600nm. MIC value of ZnO NPs against Candida was determined by the same technique using Sabaroud dextrose broth.

Evaluation of the antimicrobial activity of N-acetyl cysteine alone and in combination with ZnO NPs

MIC values of NAC alone were determined as previously described using two fold serial dilution technique against tested standard strains and clinical isolates. at concentrations ranging from (50 mg/ml to 0.78mg/ml. The impact of NAC addition to ZnO NPs was determined using two fold serial dilution technique with the ranges tested being 25-0.78 mg/ml for NAC and 125-3.9 µg/ml for ZnO NPs. The cultures were incubated at 37°C for 18-20h and the results were assessed by measuring OD at 600nm. MIC of ZnO NPs against *C. albicans* was determined by the same technique using Sabaroud dextrose broth.

Assay of respiratory chain dehydrogenase activity

Dehydrogenase activity was determined according to the iodonitrotetrazolium (INT) chloride method (Iturriaga et al. 2001). Different concentrations (500, 250,125, 100 and 60 µg/ml) of ZnO NPs were mixed with 1x10^5 CFU/ml of tested standard strains in nutrient broth and incubated for fixed contact time (100 min). Negative control was bacterial and fungal cells that have been boiled for 20 min to inactivate the enzymes completely. Experiments were conducted without boiling and in absence of ZnO NPs as a positive control. One milliliter culture was sampled separately from the cultures and centrifuged at 12,880g, then the supernatants were discarded and the cell pellet was washed twice in phosphate buffered saline (PBS) and resuspended in 0.9 ml PBS. INT solution (0.1 ml of 0.5%) was added, the culture was incubated at 37°C in dark for 2h, and 50µl of formaldehyde was added to terminate the reaction. The culture was centrifuged to collect the bacteria, and 250µl of 50% v/v acetone and ethanol were used to distill the iodonitrotetrazolium chloride-formazan (INF) twice. The supernatants were subsequently combined and the dehydrogenase activity was then calculated according to the maximum absorbance of INF at 490nm. The same test was performed using fixed concentration (100µg/ml) of ZnO NPs at various time intervals.

Effect of ZnO NPs on bacterial cell wall stability

Muramic acid is the main cell wall component of the Gram positive *S. aureus* and is commonly used as an indicator for cell wall stability. Muramic acid release in the culture medium subsequent to treatment with ZnO NPs was determined using liquid chromatography–mass spectrometry (LC-MS). Bacterial suspension (prepared as previously described under MIC experiment) was probed with 60 µg/ml of ZnO NPs for 18h then the biomass of the resulting suspension, cells without any treatment (negative control) and standard muramic acid in fresh culture medium (positive control) were centrifuged for 20 min at 3220 g, filtered through 0.2 mm sterilized syringe filter, dried under reduced pressure and kept at 4^{o}C until analysis. The following LC protocol was used: Mobile phase, A: 0.1% formic acid, B: Acetonitrile, run time: 3 min, flow rate: 250 µl /ml. Mass spectrometric analysis was carried out using a TSQ Quantum Access MAXtriple quadrupole system. Data acquisition and processing were performed using Thermo Scientific Xcalibur 2.1 software.

Evaluation of ZnO NPs cytotoxicity using human hepatocellular carcinoma cells in absence and presence of NAC.

Human hepatocellular carcinoma cells (HepG2), purchased from ATCC (USA), were cultured at 0.5x10^5 cells/well in Dulbecco's minimal essential medium (DMEM) and plated in a flat bottom 96-well microplate. Cells were treated with 20µl of increasing concentrations (0.012-100 mg/ml) of ZnO NPs for 48h at 37º C, in a humidified 5% CO2 atmosphere. After incubation, media were removed and 40µl MTT solution were added to each well and incubated for an additional 4h. MTT crystals were solubilized by adding 180 µl of acidified isopropanol to each well and plate was shacked at room temperature,

followed by photometric determination of the absorbance at 570 nm using microplate ELISA reader. The same experiment was repeated using higher concentrations (0.5, 1 and 3 mg/ml) of ZnO NPs alone or in combination with increasing concentrations (5, 10, 20, 30, 40 and 50 mg/ml) of NAC. Data are expressed as the percentage of relative viability compared with the untreated cells.

Results

Preparation and characterization of ZnO NPs

ZnO NPs, prepared according to established protocol (Yadav A 2006), were characterized by measuring their absorption spectra, size and morphology. Absorption spectra of ZnO NPs as depicted in Figure 1 shows a distinct absorption peak at 370nm and TEM image revealed nearly spherical shaped nanoparticles with an average size of 50nm size as shown in Figure 2.

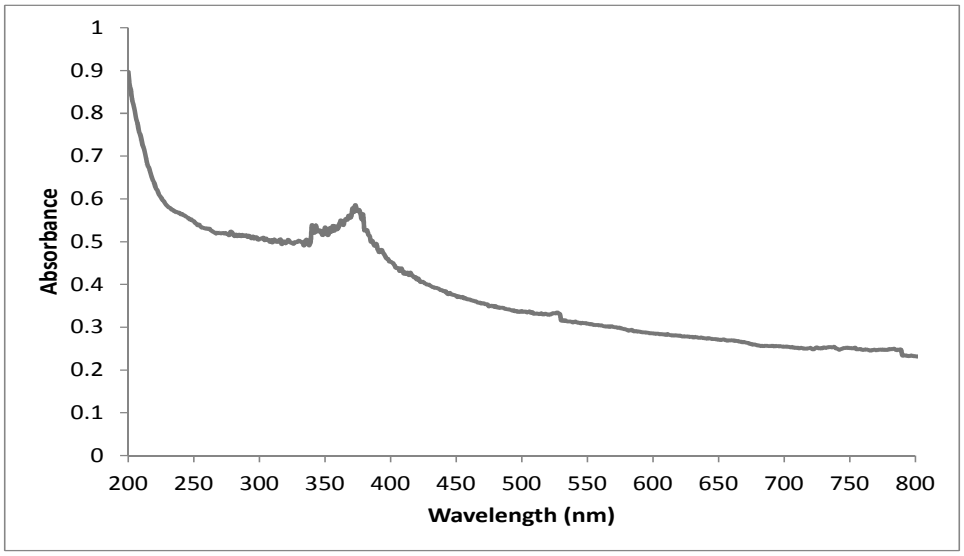


Figure 1. The absorption spectra of ZnO NPs.
The prepared ZnO NPs nanoparticles were characterized using UV-Vis spectrophotometry demonstrating a characteristic peak at 370nm

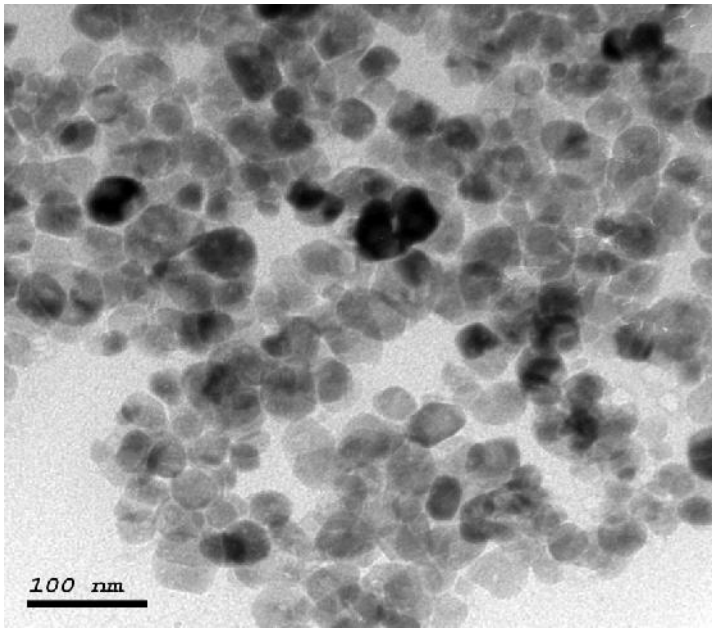


Figure 2. Transmission electron microscope (TEM) image of ZnO NPs.
This shows nearly spherical particles shape with an average particle size of about 50 nm.

Identification of clinical isolates

The 50 clinical isolates were identified using Microscan technique. Identification revealed the presence of 13 *E. coli*, 25 *S. aureus* including 7 MRSA and 12 *C. albicans.* All identified isolates were found to be MDR pathogens (table 1).

Table 1. Antibiotic resistance patterns of *E. coli* O157:H7 ATCC 51569 and *Staphylococcus aureus* ATCC 9144 standard strains as well as 38 clinical isolates belong to *E. coli* and *S. aureus*.

Antibiogram showing the resistance profile of the 38 clinical isolates and two standard strains bacteria that have been recruited in the current study

Microorganism		Type	Antibiotic resistance pattern
E. coli	O157:H7	Standard strain	TE,E,DA
	1	Clinical isolate	CPE,CP,CFZ,CRM,GM,CFT,AM,GM,LVX,TE,TO, PI,ESBL+
	2	Clinical isolate	AM,AUG,CF,CFT,CFZ,CF,CP,CPE,GM,LVX,TE, ESBL+
	3	Clinical isolate	AM,CFX,CPE,CF,CFT,CFZ,CF,CP,CPE,GM,TE, ESBL+
	4	Clinical isolate	AM,CFX,CPE,CF,CFT,CFZ,CF,CP,CPE,GM,LVX,TE, ESBL+
	5	Clinical isolate	AM,CFX,CPE,CF,CFT,CFZ,CF,CP,CPE,GM,IMP,LVX ,TE,AZT,AUG,CAX,CAZ, ESBL-
	6	Clinical isolate	AM,CFX,CPE,CF,CFT,CFZ,CF,CP,CPE,GM,LVX,TE, ESBL+
	7	Clinical isolate	AM,CFX,CPE,CF,CFT,CFZ,CF,CP,CPE,GM,LVX,TE, ESBL+
	8	Clinical isolate	AM,CFX,CPE,CF,CFT,CFZ,CF,CP,CPE,GM,LVX,TE, ESBL+
	9	Clinical isolate	AM,CFX,CPE,CF,CFT,CFZ,CF,CP,CPE,GM,LVX,TE, ESBL+
	10	Clinical isolate	AM,CFX,CPE,CF,CFT,CFZ,CF,CP,CPE,GM,LVX,TE, ESBL+
	11	Clinical isolate	AM,CFX.CPE,CF,CFT,CFZ,CF,CP,CPE,GM,LVX,TE, ESBL+
	12	Clinical isolate	AM,CFX,CPE,CF,CFT,CFZ,CF,CP,CPE,GM,IMP,LVX ,TE,AZT,AUG,CAX,CAZ, ESBL-
	13	Clinical isolate	AM,CFX,CPE,CF,CFT,CFZ,CF,CP,CPE,GM,LVX,TE, ESBL-
Staphylococcus aureus	ATCC 9144	Standard strain	SENSTIVIVE
	1	Clinical isolate	AUG,AZI,C,CAX,CD,CF,CFT,CFZ,CP,CPE,E,GAT,IM P,MXF,OFL,OX,RIF,T/S,BL+,TFG+
	2	Clinical isolate	AUG,AZI,C,CAX,CD,CF,CFT,CFZ,CP,CPE,E,GAT,IM P,MXF,OFL,OX,RIF,T/S,TE,VA,BL+,TFG+
	3	Clinical isolate	AUG,AZI,C,CAX,CD,CF,CFT,CFZ,CP,CPE,E,GAT,IM P,MXF,OFL,OX,RIF,T/S,BL+,TFG+
	4	Clinical isolate	AUG,AZI,C,CAX,CD,CF,CFT,CFZ,CP,CPE,E,GAT,IM P,MXF,OFL,OX,RIF,T/S,TE,VA,BL+,TFG+
	5	Clinical isolate	AUG,AZI,C,CAX,CD,CF,CFT,CFZ,CP,CPE,E,GAT,IM P,MXF,OFL,OX,RIF,T/S,TE,VA,BL+,TFG+
	6	Clinical isolate	AUG,AZI,C,CAX,CD,CF,CFT,CFZ,CP,CPE,E,GAT,IM P,MXF,OFL,OX,RIF,T/S,BL+,TFG+
	7	Clinical isolate	AUG,AZI,C,CAX,CD,CF,CFT,CFZ,CP,CPE,E,GAT,IM P,MXF,OFL,OX,RIF,TE,T/S,BL+,TFG+

	8	Clinical isolate	AUG,AZI,C,CAX,CD,CF,CFT,CFZ,CP,CPE,E,GAT,IMP,MXF,OFL,OX,RIF,TE,T/S,BL+,TFG+
	9	Clinical isolate	AUG,AZI,C,CAX,CD,CF,CFT,CFZ,CP,CPE,E,GAT,IMP,MXF,OFL,OX,RIF,TE,T/S,BL+,TFG+
	10	Clinical isolate	AUG,AZI,C,CAX,CD,CF,CFT,CFZ,CP,CPE,E,GAT,IMP,MXF,OFL,OX,RIF,TE,T/S,BL+,TFG+
	11	Clinical isolate	AUG,AZI,C,CAX,CD,CF,CFT,CFZ,CP,CPE,E,GAT,IMP,MXF,OFL,OX,RIF,TE,T/S,BL+,TFG+
	12	Clinical isolate	AUG,AZI,C,CAX,CD,CF,CFT,CFZ,CP,CPE,E,GAT,IMP,MXF,OFL,OX,RIF,TE,T/S,BL+,TFG+
	13	Clinical isolate	AUG,AZI,C,CAX,CD,CF,CFT,CFZ,CP,CPE,E,GAT,IMP,MXF,OFL,OX,RIF,T/S,BL+,TFG+
	14	Clinical isolate	AUG,AZI,C,CAX,CD,CF,CFT,CFZ,CP,CPE,E,GAT,IMP,MXF,OFL,OX,RIF,TE,T/S,BL+,TFG+
	15	Clinical isolate	AUG,AZI,C,CAX,CD,CF,CFT,CFZ,CP,CPE,E,GAT,IMP,MXF,OFL,OX,RIF,T/S,BL+,TFG+
	16	Clinical isolate	AUG,AZI,C,CAX,CD,CF,CFT,CFZ,CP,CPE,E,GAT,IMP,MXF,OFL,OX,RIF,T/S,BL+,TFG+
	17	Clinical isolate	AUG,AZI,C,CAX,CD,CF,CFT,CFZ,CP,CPE,E,GAT,IMP,MXF,OFL,OX,RIF,TE,T/S,BL+,TFG+
	18	Clinical isolate	AUG,AZI,C,CAX,CD,CF,CFT,CFZ,CP,CPE,E,GAT,IMP,MXF,OFL,OX,RIF,TE,T/S,BL+,TFG+
	19	Clinical isolate	AUG,AZI,C,CAX,CD,CF,CFT,CFZ,CP,CPE,E,GAT,IMP,MXF,OFL,OX,RIF,TE,T/S,BL+,TFG+
	20	Clinical isolate	AUG,AZI,C,CAX,CD,CF,CFT,CFZ,CP,CPE,E,GAT,IMP,MXF,OFL,OX,RIF,TE,T/S,BL+,TFG+
	21	Clinical isolate	AUG,AZI,C,CAX,CD,CF,CFT,CFZ,CP,CPE,E,GAT,IMP,MXF,OFL,OX,RIF,TE,T/S,BL+,TFG+
	22	Clinical isolate	AUG,AZI,C,CAX,CD,CF,CFT,CFZ,CP,CPE,E,GAT,IMP,MXF,OFL,OX,RIF,TE,T/S,BL+,TFG+
	23	Clinical isolate	AUG,AZI,C,CAX,CD,CF,CFT,CFZ,CP,CPE,E,GAT,IMP,MXF,OFL,OX,RIF,TE,T/S,BL+,TFG+
	24	Clinical isolate	AUG,AZI,C,CAX,CD,CF,CFT,CFZ,CP,CPE,E,GAT,IMP,MXF,OFL,OX,RIF,TE,T/S,BL+,TFG+
	25	Clinical isolate	AUG,AZI,C,CAX,CD,CF,CFT,CFZ,CP,CPE,E,GAT,IMP,MXF,OFL,OX,RIF,TE,T/S,BL+,TFG+

AM=Amikin, AUG=Amoxacillin/clavulinic acid, AZI=Azithromycin, C= Chloramphenicol, CP=Ciprofloxacin, CPE= Cefepeme, CFZ=Cefzolin, CRM=Cefuroxime, CFT=Ceftriaxone, CD=Clindamycin, CAX=Ceftriaxone, CAZ=Ceftazidime, E=Erythromycin, GM=Gentamycin, GAT=Gatifloxacin, OX=Oxacillin, OFL=Ofloxacillin, LUX=Levofloxacin, IMP=Imipenim, MFX=Moxifloxacin, PI=Pipercillin, RIF-Rifampicin, TE=Tetracycline, TO=Toberamycin, T/P=Tazobactam/Piperacillin, T/S=Trimethoprime/Sulphamethoxazole, V=Vancomycin

Antimicrobial effect of ZnO NPs

ZnO NPs were tested for their antimicrobial activity against *S. aureus*, *E. coli* and *C. albicans* standard strains using Kirby Bauer disc diffusion method according to CLSI guidelines. Results as shown in table 2 revealed that ZnO NPs (500 µg/ml) showed zones of inhibition of 14, 19 and 11 mm for *E. coli* (O157:H7) ATCC 51569, *S. aureus* ATCC 9144 and *C. albicans* ATCC 90028, respectively.

Table 2. Zones of inhibition obtained by disc diffusion test for various concentrations of ZnO NPs against standard strains of *E. coli*, *S. aureus* and *C. albicans*.

Discs instilled with different concentrations (500, 250, 125 and 62.5 µg/ml) of ZnO NPs were placed in plates containing tested pathogens in corresponding media. Plates were incubated at 37°C for 24h and antimicrobial activity was investigated by measuring the zones of inhibition around each disc.

Concentration of ZnO NPs (µg/ml)	Zone of Inhibition (mm)		
	E. coli O157:H7 ATCC 51569	*S. aureus ATCC 9144*	*C. albicans ATCC 90028*
500	14	19	11
250	10	15	No zone
125	8	11	No zone
62.5	No zones	No zones	No zones

Data are presented as means ± standard deviations

Determination of MIC values of ZnO NPs against tested pathogens

MIC values of ZnO NPs against various tested pathogens were determined using standard microdilution technique according to CLSI guidelines. Results obtained demonstrated that 100% inhibition of bacterial growth occurred at 60,100 and 500µg/ml of ZnO NPs for *S. aureus*, *E. coli* and *C. albicans*, respectively for both standard strains and clinical isolates. Figure 3 demonstrates the antibacterial effect of ZnO NPs against OD values of microbial suspensions with increasing ZnO NPs concentration.

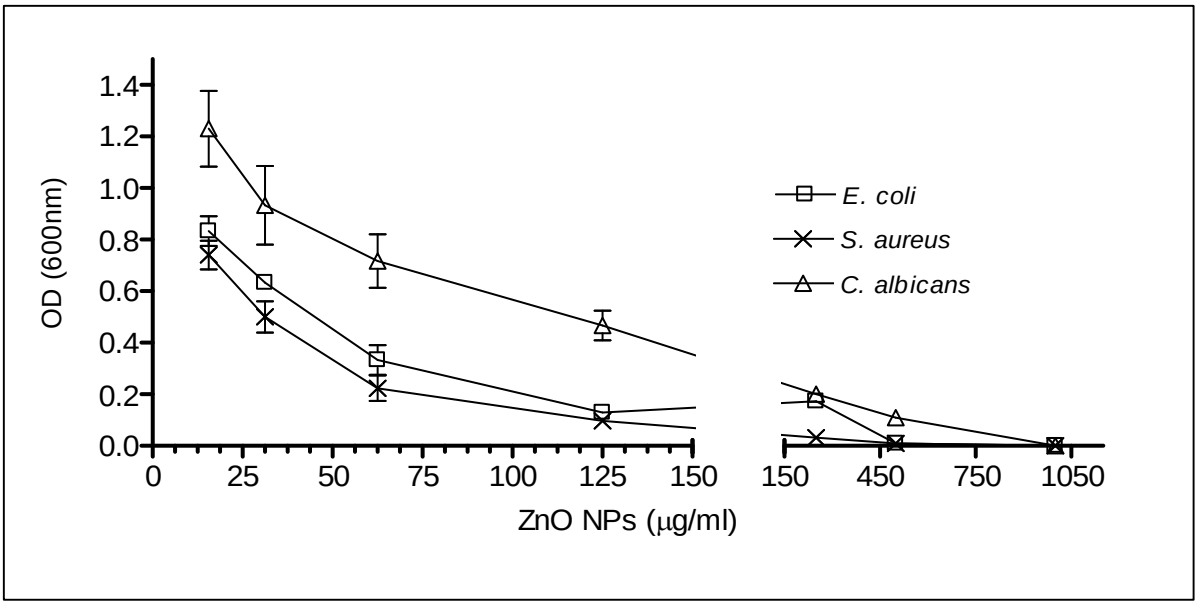


Figure 3. The optical densities of *E. coli*, *S. aureus* and C. *albicans* standard strains subsequent to treatment with increasing concentrations of ZnO NPs.

Tested bacteria were grown on corresponding media in shaking incubator (150 rpm) and MIC values of ZnO NPs were determined using the two fold serial dilution technique at concentration ranging from 500 to 31.25 µg/ml. The cultures were incubated at 37°C for 18-20h and MIC values were estimated by measuring (OD600nm). Concentration-dependent reduction in OD values was obtained using increasing concentrations of ZnO NPs. n=3 experiments; means ± standard deviations are shown.

Synergism between NAC and ZnO NPs

The impact of addition of NAC to ZnO NPs has been evaluated. Results as shown in table 3 demonstrate substantial reductions (15, 16 and 25 fold) in MIC values of ZnO NPs when it was used in combination with 0.78 mg/ml of NAC (this concentration was previously selected based on titration experiments) against tested *S. aureus*, *C. albicans* and *E. coli* standard strains, respectively. Similar results were obtained for clinical isolates.

Table 3. MIC values for N-acetyl cysteine (NAC) alone (mg/ml), ZnO NPs alone (µg/ml) and for ZnO NPs in combination with NAC against standard strains of *E. coli*, *S. aureus* and *C. albicans*.

Microorganisms	MIC of NAC (mg/ml)	MIC of ZnO NPs (µg/ml)	MIC of ZnO NPs (µg/ml) + NAC (0.78 mg/ml)
E. coli	4.7	100	3.9
S. aureus	1.2	60	3.9
C. albicans	12.5	500	31.25

MIC values of NAC alone were determined at concentrations ranging from 50 to 0.78 mg/ml. The impact of NAC addition to ZnO NPs on MIC values was determined at concentration range of 0.78-25 mg/ml for NAC and 3.9-125 µg/ml for ZnO NPs. The cultures were incubated at 37°C for 18-20h and the results were assessed by measuring OD600nm. Combination of ZnO NPs and NAC substantially reduce MIC values by 15, 16, 25 fold for *S. aureus*, *C. albicans* and *E. coli*, respectively.

Studying the proposed mechanism of action of ZnO NPs as antimicrobial

Effect of ZnO NPs on respiratory chain dehydrogenase

The effect of ZnO NPs on respiratory chain dehydrogenases of tested pathogens has been examined. Results obtained demonstrate that ZnO NPs exhibited a concentration-dependent inhibitory effect on the activity of respiratory chain dehydrogenase with a more considerable inhibition effect in case of *S. aureus* compared to that obtained with *E. coli* or *C. albicans*. The dehydrogenase activity in positive control cells increased as the incubation time increased, while enzymatic activity of cells treated with ZnO NPs was almost reduced in a dose-dependent manner as shown in Figures 4 and 5.

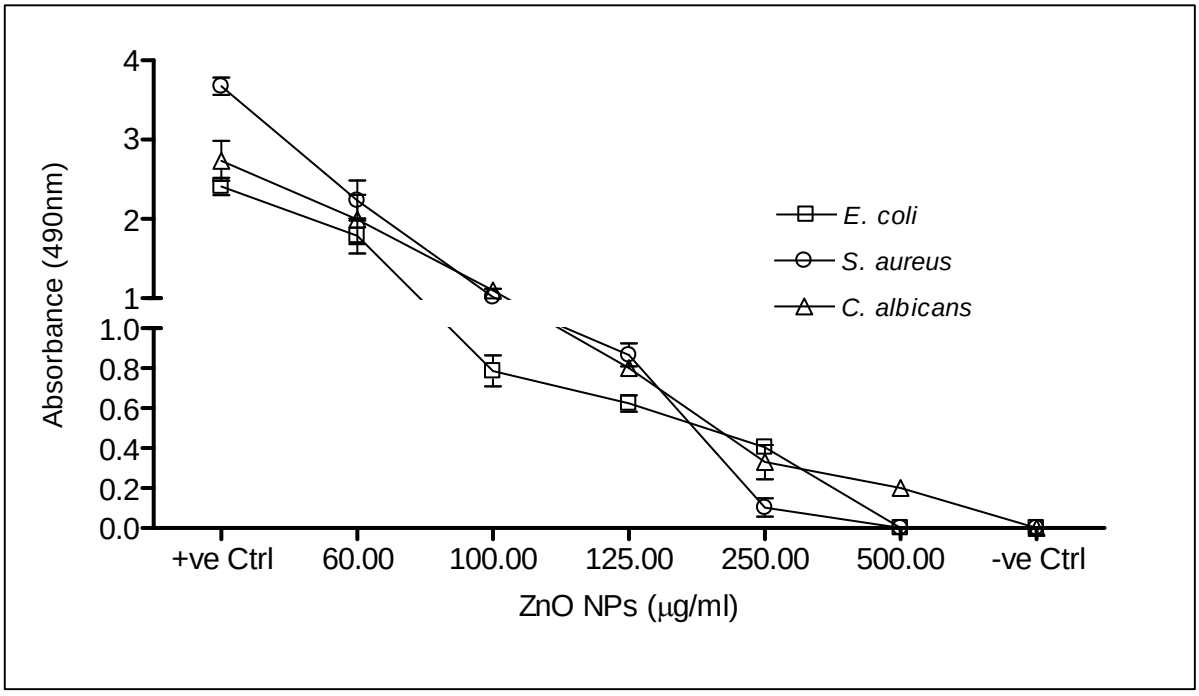


Figure 4. Respiratory chain dehydrogenase activity of increasing concentrations of ZnO NPs at fixed contact time (100 min).

$1x10^5$ CFU/ml of tested pathogens were incubated with different concentrations of ZnO NPs for 100 min. Cell pellets were mixed with iodonitrotetrazolium (INT) solution, incubated at 37°C in dark for 2h, and 50µl of formaldehyde was added to terminate the reaction. 250µl of 50% v/v acetone and ethanol were used to distill the INF. Absorbance of was read at 490nm. The -ve Ctrl and +ve Ctrl represent the boiled and not boiled bacterial cells, respectively. n=3 experiments; means ± standard deviations are shown.

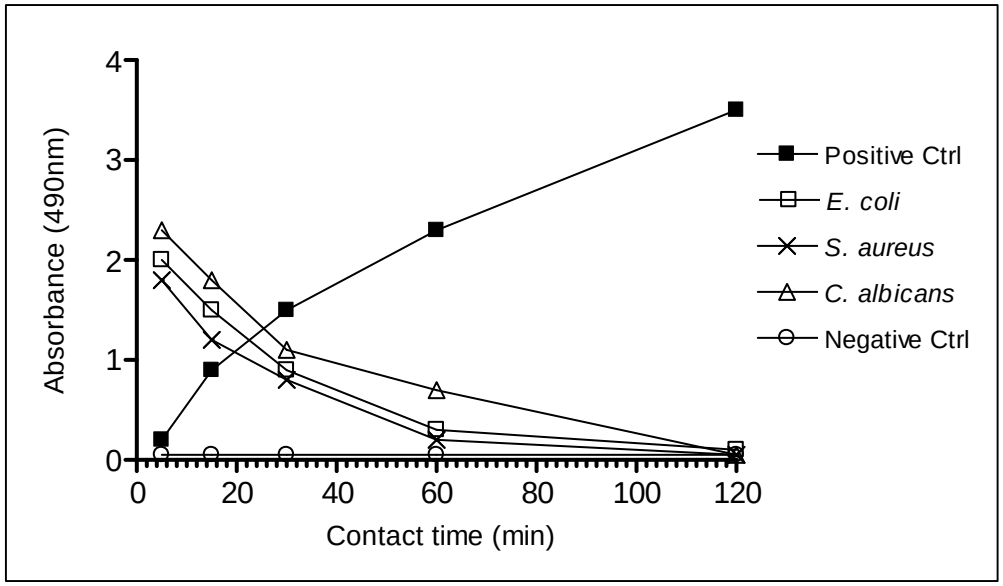


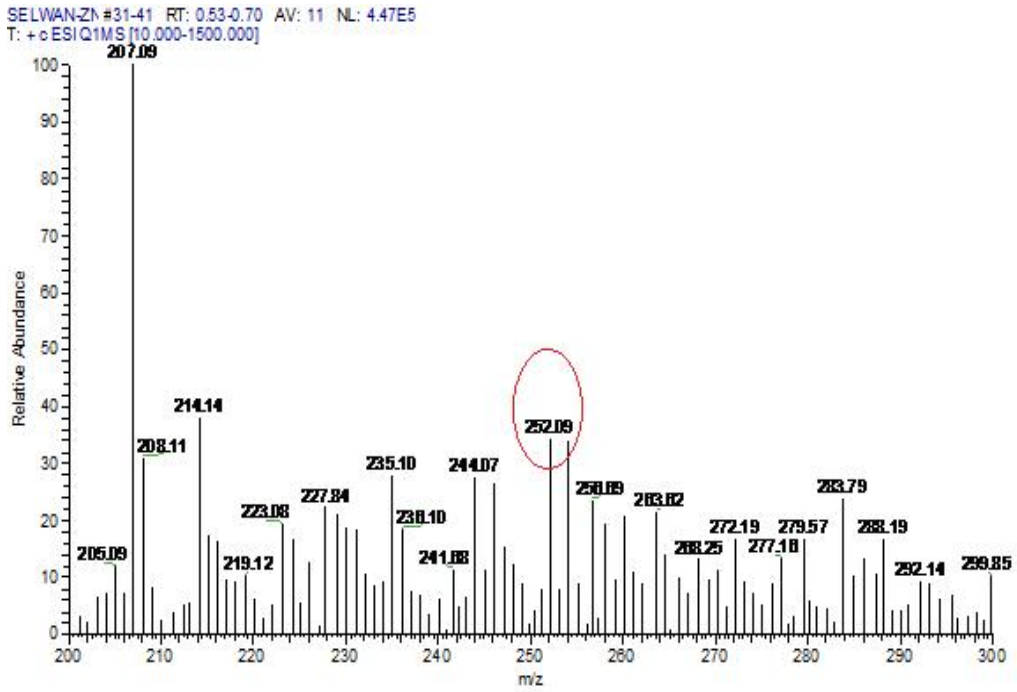
Figure 5. Effect of ZnO NPs (100µg/ml) on respiratory chain dehydrogenase activity of tested pathogens at different incubation times.

Tested pathogens ($1x10^5$ CFU/ml) were incubated with fixed concentration (100µg/ml) of ZnO NPs at various time intervals. Cell pellets were mixed with iodonitrotetrazolium (INT) solution, incubated at 37°C in dark for 2h, and 50µl of formaldehyde was added to terminate the reaction. 250µl of 50% v/v acetone and ethanol were used to distill the INF and absorbance of was read at 490nm. n=3 experiments; means ± standard deviations are shown; means ± standard deviations are shown.

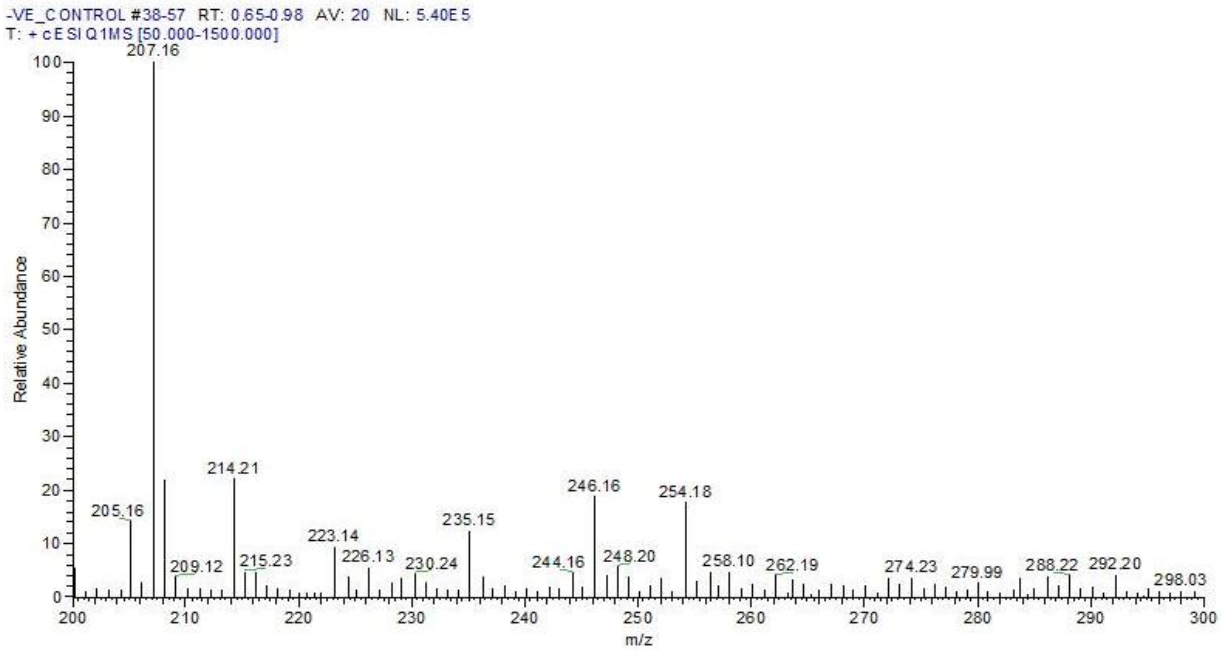
Effect of ZnO NPs on cell wall stability of *S. aureus*

ZnO NPs (60 µg/ml) were tested for their effect on cell wall stability against standard *S. aureus* and this has shown the release of muramic acid content in the culture media. Muramic acid was determined using LC/MS and the chromatogram peak is shown in Figure 6.

6 (A)



6 (B)



6 (C)

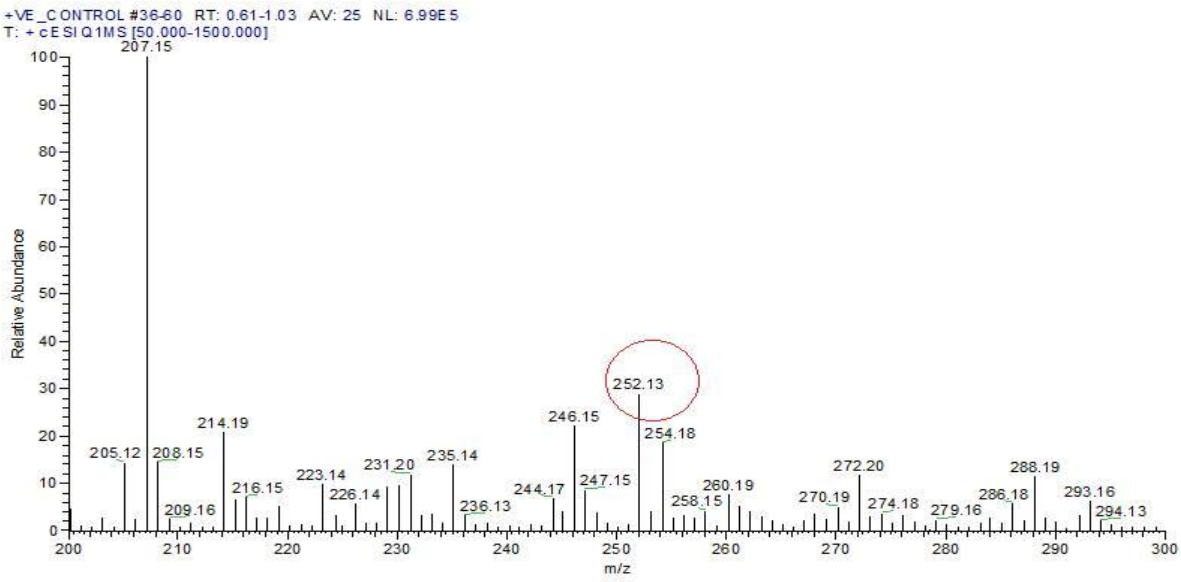


Figure 6: LC/MS chromatogram demonstrating the release of muramic acid from *S. aureus* in culture medium subsequent to treatment with 60µg/ml of ZnO NPs.

Bacterial suspension was probed with ZnO NPs (60 µg/ml) for 18h. Suspension was centrifuged, filtered, dried and analyzed by LC-MS. A; cells treated with ZnO NPs, B; cells without any treatment (negative control) and C; standard muramic acid in fresh culture medium (positive control).

Investigating the cytotoxicity of ZnO NPs

In order to address the cytotoxic effect of ZnO NPs, HepG2 cells have been recruited. Results obtained as shown in Figure 7 indicate a concentration dependent reduction in cell viability was obtained by increasing concentrations of ZnO NPs as cell proliferation was decreased with increasing ZnO NPs concentration and no cytotoxic activity has been detected for ZnO NPs at their MIC values. NAC was added to examine its adjuvant effect on cytotoxicity of ZnO NPs at high concentrations. Results obtained as shown in Figure 8 demonstrate that NAC reduced the cytotoxic effect of ZnO NPs at high concentrations.

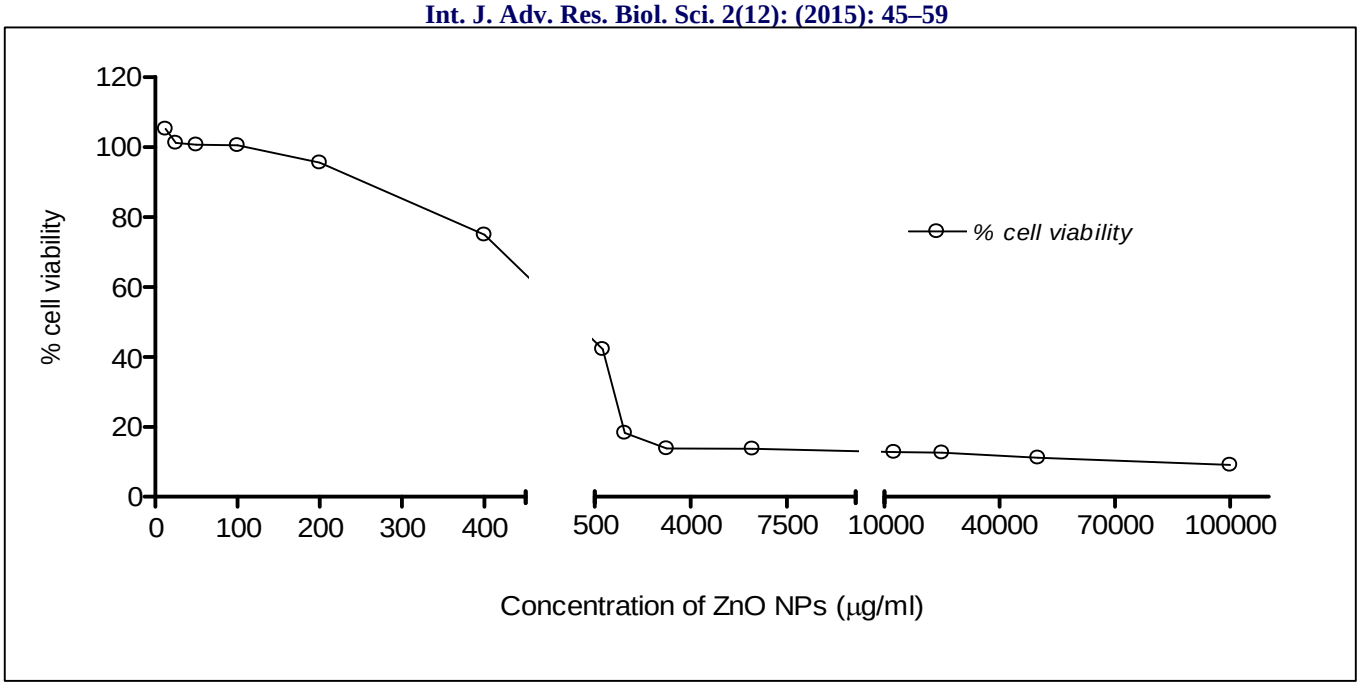


Figure 7: Effect of various concentrations of ZnO NPs on cell viability and proliferation of human hepatocellular carcinoma cells (HepG2).

HepG2 cells were treated with 20µl of increasing concentrations (0.012-100 mg/ml) of ZnO NPs for 48h at 37ºC. 40µl of MTT solution were and incubated for an additional 4h and 180 µl of acidified isopropanol were added. Absorbance was measured at 570nm and data are expressed as the percentage of relative viability compared with the untreated cells. No cytotoxicity was observed at MIC values of ZnO NPs. n=3 experiments; means ± standard deviations are shown; means ± standard deviations are shown.

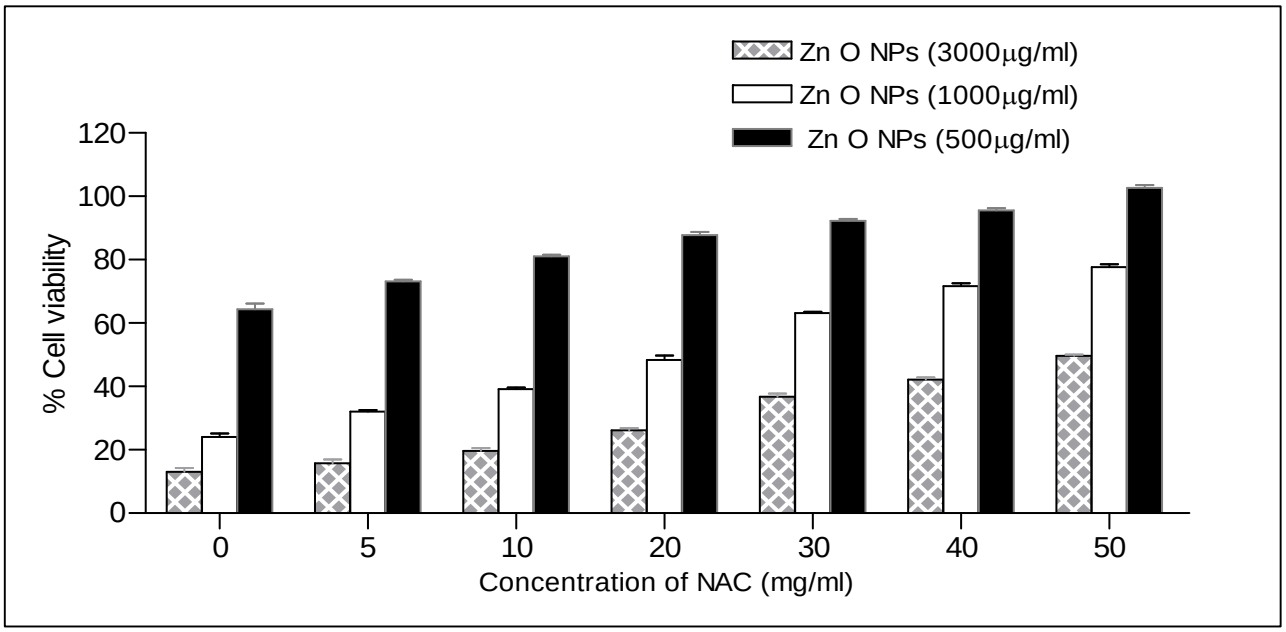


Figure 8: Effect of increasing the concentrations of ZnO NPs and N-acetyl cysteine (NAC) on cell viability of human hepatocellular carcinoma cells (HepG2).

HepG2 cells were treated with 20µl of increasing concentrations (0.5, 1 and 3 mg/ml) of ZnO NPs alone or in combination with increasing concentrations (5, 10, 20, 30, 40 and 50 mg/ml) of NAC and were incubated for 48h at 37ºC. 40µl of MTT solution were and incubated for an additional 4h and 180 µl of acidified isopropanol were added. Absorbance was measured at 570nm and data are expressed as the percentage of relative viability compared with the untreated cells. NAC reduced the cytotoxic effect of ZnO NPs at high concentrations. n=3 experiments; means ± standard deviations are shown; means ± standard deviations are shown.

Discussion

With chaotic use of antibiotics and the emergence of MDR pathogens, a serious threat has been posed to human health worldwide; therefore antibiotics are no longer the magic drugs as they were (Roux et al. 2012). Thus, there is a burning desire and an urgent demand for development of innovative strategies to combat infections caused by MDR pathogens. Nanobiotechnology represents a revolutionary super-cutting-edge field that could pave the way to develop innovative antibacterial interventions (Baba 2006; de Morais et al. 2014).

ZnO has been shown to protect against some bacterial infections cause by a specific phenotype of *E. coli* (ETEC) (Roselli et al. 2003). Moreover, ZnO has long been used as an active ingredient in skin preparations (e.g. lotions, creams and ointments) owing to its antibacterial properties (Sawai 2003). We therefore, thought o evaluate the antimicrobial activity of ZnO NPs against Gram positive and Gram negative bacteria as well as fungi therefore, standard strains of *S. aureus*; *E. coli* and *C. albicans* in addition to 50 clinical MDR isolates have been recruited in this study. All tested pathogens were susceptible to ZnO NPs which inhibited the growth in a concentration-dependent manner. MIC values of ZnO NPs against tested pathogens showed that ZnO NPs have a better effect on the Gram positive pathogen *S. aureus* than on the Gram negative pathogen *E. coli*, while *C. albicans* required higher concentration of ZnO NPs to be fully inhibited.

NAC, conventionally used as antioxidant, was examined for its antimicrobial activity against tested pathogens. This has shown that NAC had a weak antimicrobial activity (table 3) which is in line with previously reported data (Ahmed Mohsen 2015). Likewise, NAC has previously been reported to weakly augment effect of some β- lactam antibiotics such as ampicillin (Goswami and Jawali 2010). This gave us the impetus to test whether it has a similar effect when used concomitantly with ZnO NPs. For the first time, our data provide compelling evidence that NAC has a robust synergistic effect with ZnO NPs which substantiate their action as antimicrobials. It is noteworthy that alcohol-based and chlorine-based disinfectants are the most commonly used surface disinfectants in hospitals, nonetheless, they have many side effects including allergic reactions and irritations, also their disinfection capacity on a mass of resistant microorganisms is disputatious (Gojova et al. 2007; Fatemah Ahmadi 2012).

It is well known that there is a substantial difference in cell permeability between Gram-positive and Gram-negative pathogens owing, at least in part, to the presence of the lipopolysaccharide (LPS) in Gram-negative bacteria (Kim et al. 2007; Silhavy et al. 2010). LPS plays an imperative role in bacterial defense, providing negative charge and stabilization to the cell membrane (Schleifer and Kandler 1972; Nikaido 2003; Nanda and Saravanan 2009). The difference in MIC values of ZnO NPs against bacteria and fungi is most likely attributed to the divergence between the bacterial and yeast cell type. Owing to their more simple structure, prokaryotic cells are unable to cope with the toxic action of ZnO NPs as effectively as the eukaryotic cells that can tolerate higher doses of ZnO NPs thanks to their superb cell organization and composition as well as their excellent detoxification system (Panacek et al. 2009).

Several mechanisms for ZnO NPs as antimicrobials have been proposed including induction of reactive oxygen species (ROS) (e.g. H_2O_2), which is a strong oxidizing agent detrimental to bacterial cells (Sawai 2003; Ghule K. 2006; Reddy et al. 2007), and damage of the cell membrane and interaction of intracellular contents with ZnO NPs (Jones et al. 2008). Irrespective of the aforementioned mechanisms, the exact detailed mechanisms of action of ZnO NPs as antimicrobial agents have not been fully understood. Thus, we have investigated other mechanisms that might, at least in part, be involved in the antimicrobial activity of ZnO NPs. First, we set out to investigate the effect of ZnO NPs on respiratory chain dehydrogenases of tested pathogens through INT chloride assay. This showed that ZnO NPs exhibited a concentration-dependent inhibitory effect on respiratory chain dehydrogenases of tested pathogens with a more considerable inhibition effect on *S. aureus* compared to *E. coli* or *C. albicans*.(Figures 4 and 5).

Afterwards, we examined the effect of ZnO NPs on cell wall stability of the standard *S. aureus* and this has shown the release of muramic acid content in the culture media as detected by LC-MS. By analyzing the LC-MS data, we could conclude that the cell wall damage was in the vicinity of the glycan strands as well as the peptide branches. We proposed that ZnO NPs split glycan strands which in turn released small amounts of muramic acid and amino sugars into culture media.

The cytotoxicity of ZnO NPs dispersions has already been visited and some published reports indicate that ZnO NPs can exhibit some toxic effects on mammalian cells (Sawai 2003). Reddy and coworkers proposed that the mechanism of toxicity of ZnO NPs involves the generation of ROS (Reddy et al. 2007). Zhou and Wang suggested cell-specific behavior where cancer cells producing higher inducible levels of ROS than their normal counterparts following exposure to ZnO NPs (J. Zhou 2006). In order to address the cytotoxic effect of ZnO NPs, HepG2 cells have been chosen. Results obtained (Figure 7) indicate that cell proliferation was decreased with increasing ZnO NPs concentration. Nevertheless, no cytotoxic activity of ZnO NPs has been detected at their MIC values. NAC was added to examine its adjuvant effect on cytotoxicity of ZnO NPs at higher concentrations and this showed that NAC was able to reduce the cytotoxic effect of higher concentrations ZnO NPs (Figure 8). As such, ZnO NPs could play a dual role being an important candidate for cancer curing in addition to its antibacterial activity and NAC could be co-administered as a protecting agent for normal cells.

To conclude, ZnO NPs have been shown to exhibit a robust antimicrobial activity against *S. aureus*, *E. coli* and *C. albicans*. Interestingly, we reported for the first time a robust synergistic effect between NAC and ZnO NPs which substantially enhanced the antimicrobial activity of ZnO NPs and would in turn help to reduce the dosing of ZnO NPs. Moreover, No cytotoxicity has been observed for ZnO NPs at their MIC values. We have also shown that ZnO NPs inhibit the respiratory chain dehydrogenases and cause cell wall damage through release of muramic acid content of *S. aureus*. Altogether, this study emphasizes the potential of ZnO NPs as a new promising non-cytotoxic nanobiocide against MDR pathogens which could pave the way towards a paradigm shift in treating infectious diseases and combating the problem of increasing drug resistance. Further investigations are required to address the *in vivo* efficacy of ZnO NPs as antimicrobial agents. Moreover, further work is required to identify and fully understand the mechanism whereby NAC synergizes with ZnO NPs.

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