Supporting Information

Defects Mediated ROS Generation in Mg Substituted ZnO Nanoparticles: Efficient Nanomaterials for Bacteria Inhibition and Cancer Therapy

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1.1 Characterization

X-ray diffraction (XRD) patterns were recorded on a PANalytical's X'Pert PRO diffractometer with Cu Kα radiation. The crystallite size and the lattice strain were estimated from the X-ray line broadening using Sherrer formula of X'Pert High Score Plus software. The transmission electron micrographs were taken by FEG Jeol-JEM-2100F TEM. The samples were dispersed in ethanol and UV-visible spectra were recorded with a UV-visible spectrophotometer (Cecil, Model No. CE3021). The photoluminescence (PL) spectra of ZnO and MgZnO NPs were recorded at RT following excitation with a continuous wave He-Cd laser (λ= 325 nm).

1.2. Antibacterial Study of ZnO and MgZnO NPs:

The antibacterial activity of ZnO and MgZnO NPs toward *E. coli* was performed using culture turbidity as a qualitative measure of cell growth and also by plating counting assays. The bacterial growth behavior of *E.coli* was performed in nutrient broth culture medium (NB) with various concentrations of ZnO and MgZnO NPs. The bacterial growth curves were achieved by measuring the optical density (OD_{600}) of the cultures with respect to time. OD measurements were taken using a multiplate plate reader Varioskan Flash instrument at OD_{600} nm and the background was eliminated by taking blank readings (nutrient broth culture medium (NB) without inoculums and ZnO and MgZnO NPs in the nutrient broth culture medium). The bacteria for each experiment were freshly prepared by inoculating a single colony into 10 ml of sterile nutrient broth in a glass tube. The bacteria were allowed to grow overnight in an incubator maintained at 37 °C and shaken at 200 rpm. Stock solution of ZnO and MgZnO NPs were freshly prepared by mixing the calculated amount of ZnO powder in sterile 0.9 % NaCl solution. Appropriate concentrations of ZnO and MgZnO NPs suspensions (400 to 12.5 µg/ml) were added to the test tube and placed in a shaker at 37 °C for 8 h at 200 rpm. 100 µl was taken and the OD_{600} nm of this suspension (containing the bacteria with ZnO or MgZnO NPs) was periodically monitored at fixed interval of times (2 h). For the plate counting assay, 100 µl of overnight culture solution containing 4.1×10^9 colonies was added in NB with an appropriate concentration of ZnO and MgZnO NPs suspension in Nutrient Broth and maintains a total volume of 5 ml and incubate them at 37 °C for 2 h at 200 rpm. A tube of bacteria without ZnO and MgZnO NPs served as a control. The solutions were incubated by a rotary shaker at 200 rpm for 24 h in the dark and the numbers of colonies were counted.

1.3. Live dead and membrane potential analysis of E. coli

For the live and dead analysis, the bacterial suspensions (~ 10^8 to 10^9 CFU/ml) were treated with appropriate concentration of MgZnO NPs in nutrient media for 2 h.[1] After the treatment with nanomaterials, 1 ml of the bacterial cells suspension (*E. coli*) were stained with SYTO 9 and propidium iodide (PI) following the instruction given in the Live/Dead *Bac* Light bacterial viability kit (Molecular Probes). Treated bacterial suspension has been analysized by using FACS. The green fluorescence of the SYTO 9 dyes was recorded fluorescence spectra using excitation wavelength 485 nm and emission at 530 nm. The red fluorescence emitted from PI was recorded fluorescence spectra using excitation wavelength 488 nm and emission at 630 nm.

1.4. Determination of reactive oxygen species (ROS) in E. coli:

The generation of ROS due to interaction ZnO NPs and MgZnO NPs in the culture media were measured by using TBARS assay and DCFH-DA dye. In this typical experiment, 1 ml of treated culture was collected and added 200 µl of 10% (w/v) trichloro acetic acid. This mixture kept 30 min. Then 2 ml of freshly prepared thiobarbituric acid (TBA) buffer reagent was added to the above mix and incubated at 95 °C for 60 min. After cooling at room temperature and centrifuged at 3500 rpm for 15 min. The absorption of the supernatant was measured at 532 nm to estimate the formation of TBARS adduct. Generation of intracellular ROS in the bacterial cells was calculated using dichlorofluorescein diacetate (DCFH-DA). For this experiment, 1 ml of treated and untreated culture was collected and centrifuged at 5000 rpm for 15 min. the supernatant was removed and added 20 µg/ml of the DCFH-DA and incubate for 1h at 37 °C. Fluorescence intensity was measured at excitation 485 and emission 530 nm using micro plate reader.

1.5. Cytotoxicity Study

Sulforhodamine B (SRB) assay was performed to evaluate cytotoxicity of MgZnO NPs with normal fibroblasts cells (L929), human cervical cancer cells (HeLa) and KB cells.[2] The cells were seeded into 96 well plates at density of 1×10^4 cells per well for different time periods. Different concentrations of ZnO and MgZnO NPs were added to the cells and incubated for different time periods at 37 °C and 5 % CO_2. Thereafter, the cells were washed thrice with phosphate buffer saline (PBS) and processed for SRB assay to determine the cell viability. Further, cells were fixed with a solution of 10% trichloroacetic acid and stained with 0.057% SRB and washed with 1% acetic acid. Cell-bound dye was extracted with 10 mM tris buffer solution (pH 10.5) and then absorbance was measured at 560 nm using a plate reader. The cell viability was calculated using the following formula:

$$\% \text{ Viability} = (\text{Absorbance of sample}/\text{Absorbance of control}) \times 100$$

1.6. Measurement of Reactive Oxygen Species (ROS)

Intracellular ROS is also measured using 2, 7, dichlorodihydrofluorescein diacetate (H_2-DCFDA) and the images were captured by confocal laser scanning microscopy (CSLM). Briefly, 5 µM H_2-DCFDA solutions in DMEM were used. After incubation with 50 µg/ml of MgZnO NPs for 24 h, the cells were washed with phosphate buffered saline (PBS) and fixed with 4 % paraformaldehyde. For another experiment for detection of ROS, KB cells and L929 cells were seeded in 96-well plates at a concentration of 1×10^4 cells/ml for 24 h for the attachment of the cells followed by treatment with different concentration of MgZnO NPs. ROS is determined by adding H_2DCFDA to pretreated cell cultures and recording the 2',7'-dichlorofluorescein (DCF) fluorescence at 530 nm. Detection of ROS measurement was repeated 3 times independently. After the background subtraction, fluorescence intensity of DCF is recorded by the plate reader

and expressed as ratio of fluorescence of DCF of treated cell to the fluorescence of DCF of untreated cell.

1.7. Evaluation of death mechanisms in L929 and KB cells by Annexin V-FITC and PI assay

An Annexin V–FITC and PI assay is used for the detection of apoptotic cells.[2] The assay procedure is according to the instruction of Annexin V-FITC apoptosis detection kit (Invitrogen). 5×10^5 cells per experiment have been taken. After 24 h, cells were treated with 25, 50 and 100 µg/ml of MgZnO NPs for next 24 h. Then, cells are washed with cold PBS, centrifuged and the supernatants were removed. Cell plates were again resuspended in 100 µl of binding buffer and added 5 µl of Annexin V-FITC and 1 µl of propidium iodide (PI) at room temperature in the dark. After incubation for 30 min at room temperature in the dark, stained cells were diluted by the same binding buffer and directly analyzed by the fluorescence-activated cell sorting method (FACS, BD FACS Aria, USA).

1.8. Cell cycle analysis

The effect of MgZnO NPs on the cell cycle of L929 and KB cells was analyzed by staining the DNA with propidium iodide and using flow cytometry. In brief, 5×10^5 cells were seeded in the 6-well plate. After 24 h, cells were treated with the 25, and 50 µg/ml of MgZnO NPs for 24 h. After treatment, Cells were tripsinized, washed with ice-cold PBS and fixed in ice-cold 70% ethanol for 1 h at 4 °C. Before flow cytometry analysis, cells were washed in PBS and stained with RNase (20 µg/ml) for 1 h at 37 °C and PI was added (20 µg/ml) for 30 min and analyzed by flow cytometric measurement. Collected Data was analyzed using Modfit software.

1.9. Mitochondrial membrane potential assessment and Lactate dehydrogenase (LDH) release

Mitochondrial membrane potential (MMP) was assessed by using JC-10 dye mitochondrial membrane potential assay kit having (Sigma-Aldrich). In this typical experiment,

1×10^4 cells were seeded in 96 well plates and treated with different concentration of MgZnO NPs for 24 h. After treatment, the cells were washed with PBS and the JC-10 dye was added according to the instruction given in JC-10 dye mitochondrial membrane potential assay kit. The ratio of red/green fluorescence intensity was used to determine the MMP. Similar experiment has also been performed with L929 and KB cells seeded into the 6 well plates with 1×10^6 cells per well and treated with 50 µg/ml of MgZnO NPs for 24 h. After the treatment cells have been trypsinized and stained with JC-10 dyes for 30 min. After 30 min cells have been washed with 1X PBS and resuspended in the same buffer. These stained cells have been analyzed by FACS. For confocal microscopic analysis, KB cells have been seeded on cover slips and treated with 50 µg/ml of MgZnO NPs for 24 h. The cells have been stained with JC-1 and fixed using 4 % paraformaldehyde.

For the LDH activity in extracellular medium due to membrane damage was assessed by using LDH kit (Sigma-Aldrich). For this experiment, 5×10^5 cells were seeded in 6 well plates and treated with different concentration of MgZnO NPs for 24 h. LDH present in extracellular medium was done according to the instruction given in the LDH kit. LDH release and absorbance was measured in a micro plate spectrophotometer at 490 nm.

1.10. Detection of Hydroxyl radical

Hydroxyl radicals were estimated by fluorescence spectroscopy using Terephthalic acid (TA).[1] Terephthalic acid reacts with hydroxyl radicals and forms a complex of 2-hydroxyl terephthalic acid which gives fluorescence. The fluorescence intensity is a direct associated with the generation of hydroxyl radical concentration. In a typical experiment, to aqueous suspensions of ZnO and MgZnO NPs (10 mg/100 ml), 2 mM of TA was added and stirred under dark and

ambient light. At regular intervals, 2 mL aliquots were taken and centrifuged and fluorescence spectra of supernatant was recorded at excitation wavelength of 312 nm and emission at 425 nm.

1.11. Detection of superoxides

Superoxide radicals ($\bullet O_2^-$) were detected by UV−visible spectroscopy in aqueous suspensions of ZnO and MgZnO NPs using nitro blue tetrazolium (NBT). NBT after reaction with superoxide radicals convert into the monoformazan and diformazan. The experiment was carried out both in ambient light and dark according to the reported earlier. [1]

1.12. Detection of singlet oxygen

For the determination of singlet oxygen, 2,2,6,6-tetramethylpiperidin (TEMP) is used as spin trapper by electron spin resonance (ESR). We measured singlet oxygen from MgZnO NPs (10 mg/ml) with TEMP (4.7 mM). [1]

1.13. Dissolution study of ZnO and MgZnO NPs

Dissolution study was carried out using ICP-AES analysis. In the experiment, 1mg of ZnO and MgZnO NPs in 1 mL centrifuge tube (triplicates) were dispersed in two different pH (7.4 and 5.4) and kept it at 37 °C for 24h. The solution of centrifuge at 10000 rpm for 20 min and supernent was taken in a separate tube and volume was made up 10 ml by adding 9 ml of water.

1.14. Statistics

The data are expressed as the mean ± SD (standard deviation) from three independent experiments (n=3). One tailed unpaired Student's t-test (Microsoft excel) was used for significance analysis, and $p < 0.05$(*), 0.001(**) and 0.001(***) are considered significant as compared to control.

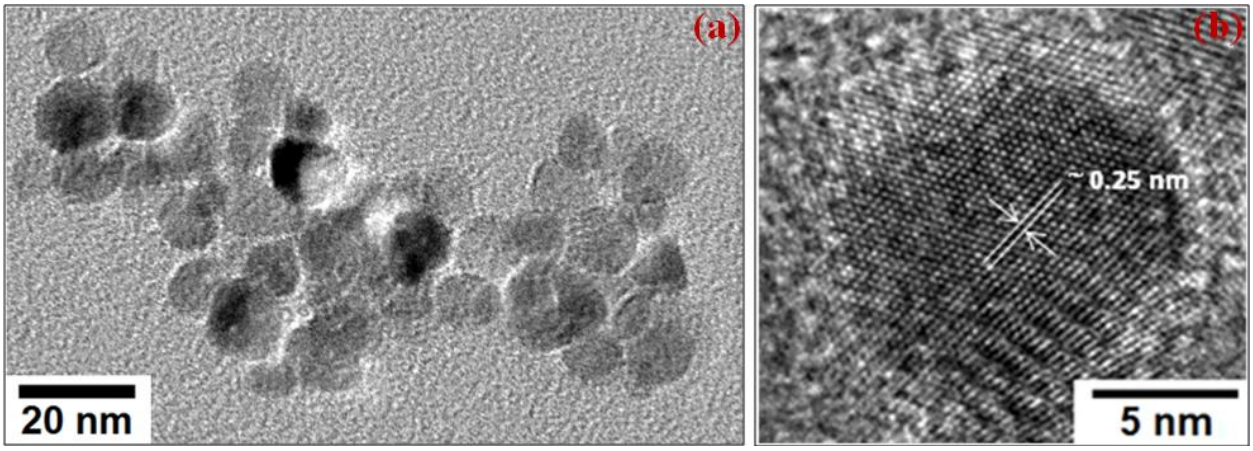

Figure S1 TEM micrographs of ZnO nanoparticles (a) Low resolution image and (b) High resolution image.

ICP-AES analysis and EDX analysis of MgZnO NPs, given in Table S1.

Sample name	ICP-AES analysis (wt. %)		EDX analysis (wt. %)	
	Zn	Mg	Zn	Mg
MgZnO NPs	34.5	3.16	22.02	1.95

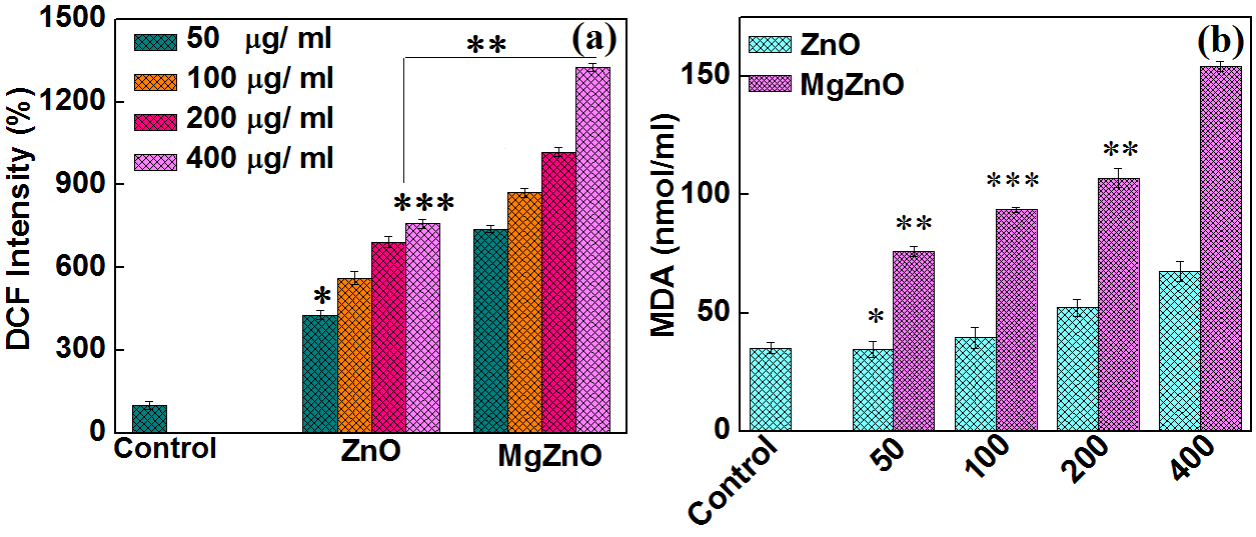

Figure S2 (a) Generation of ROS and (b) Malondialdehyde (MDA) equivalent in *E. coli* after 4 h treatment with different concentration of ZnO and MgZnO NPs.

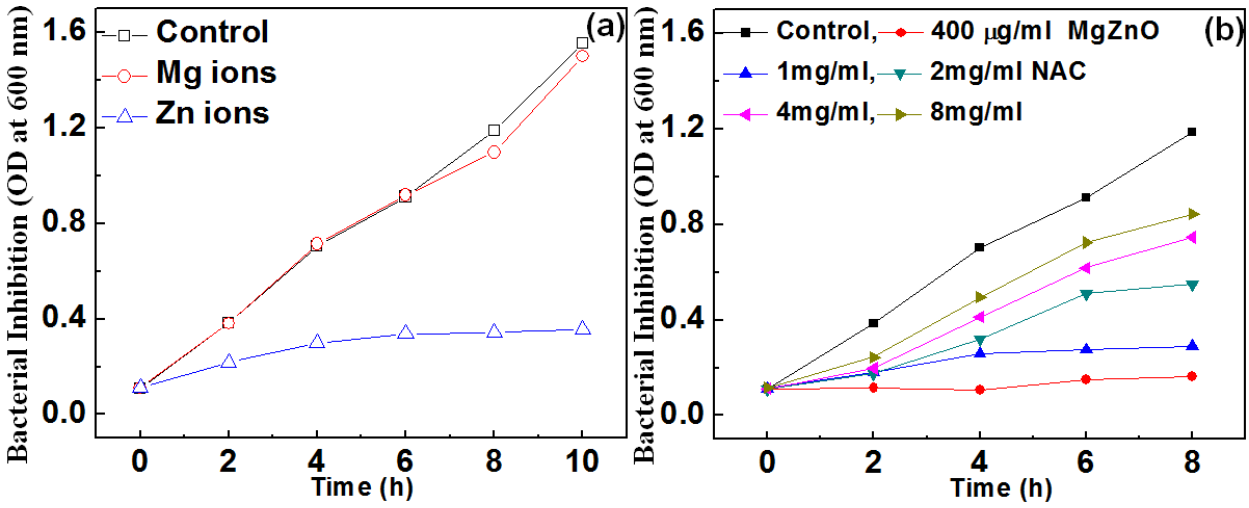

Figure S3 (a) Growth inhibition Curve of *E. coli* in presence of Mg and Zn ions, (b) Growth inhibition Curve of *E. coli with* 400 µg/ml of MgZnO NPs in presence of different concentrations of NAC.

Effect of MgZnO NPs on membrane potential measurement of E. coli

To confirm the cell membrane disruption, membrane potential of *E. coli* has been analyzed by the FACS analysis using $DiOC_2$ dye. Figure S4 shows the membrane potential of *E. coli* on treatment with (400-50 µg/ml) MgZnO NPs for 2 h stained with $DiOC_2$ which exhibits green emission. But the fluorescence shifts toward red emission as the dye molecules self associate at the higher cytosolic concentrations caused by larger membrane potentials. Figure S4 (a) shows the change in the membrane potential of the control *E. coli* without any treatment and Figure S4 (b to e) shows the change in the membrane potential on treatment with different concentration of MgZnO NPs. Depolarization of bacterial cell membranes has been observed as a shift of $DiOC_2$ fluorescence from red to green. This shift is higher in MgZnO NPs with respect

to control. An increased concentration caused a higher rate of reduction, which confirmed that MgZnO NPs could indeed damage the membrane integrity of *E. coli*.

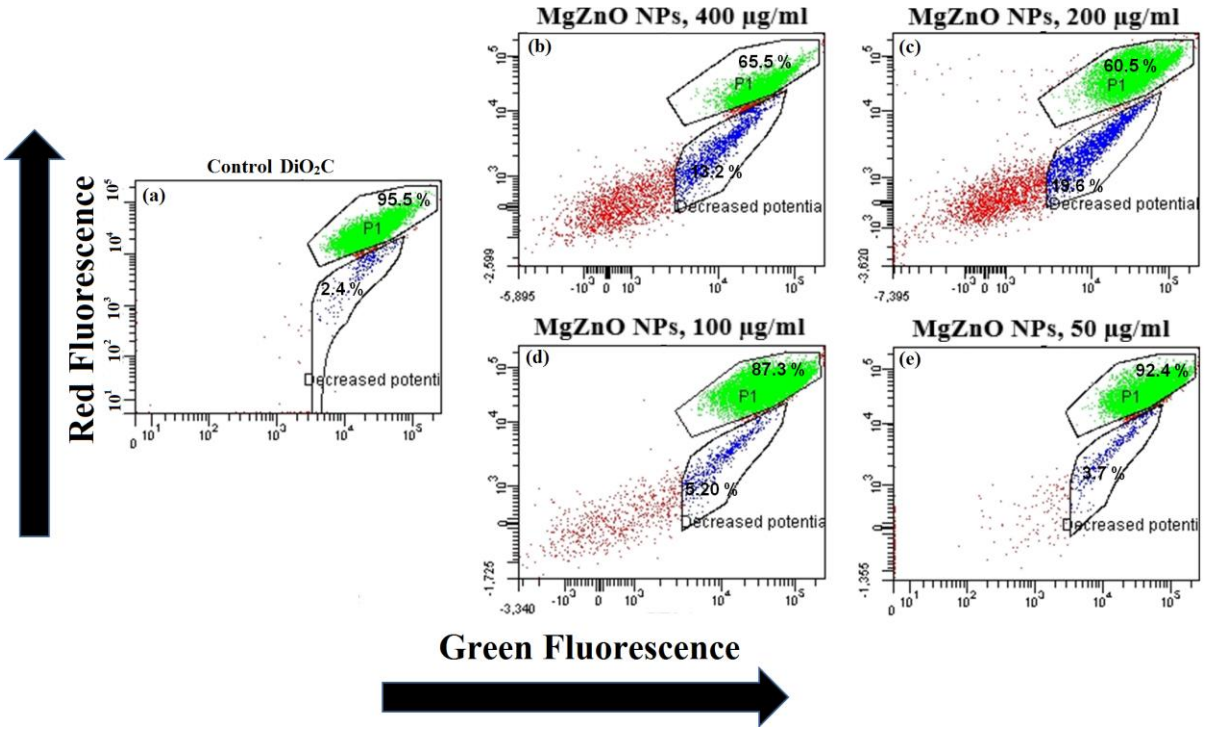

Figure S4 Flow cytogram depicting the changes in (a) membrane potential of *E.coli* untreated and (b-e) treated with 400 to 50 µg/ml of MgZnO NPs for 2 h. In the flow cytogram, P1 and P2scatter region (blue and red dots) indicate cells with polarized membrane potential while P2 scatter region (red dots) denotes cells with depolarized membrane.

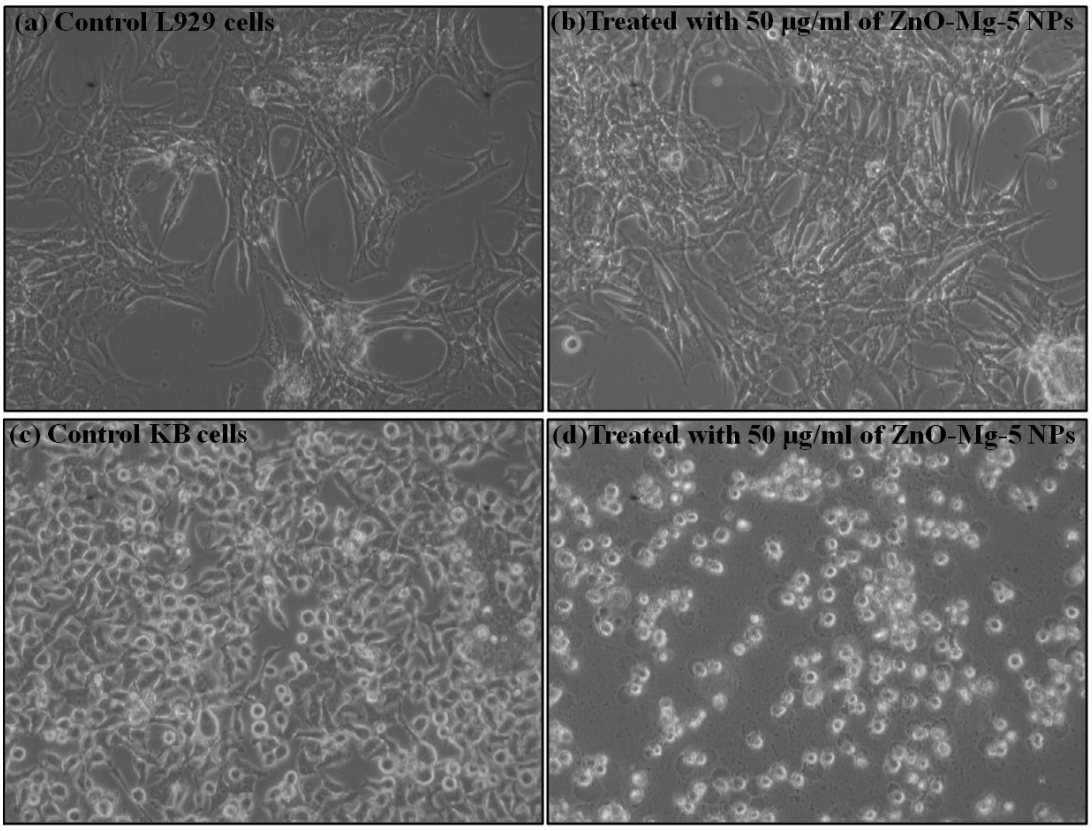

Figure S5 Optical microscopic images of L929 (a) Control, (b) Treated cells with 50 µg/ml of MgZnO NPs, (c) Control KB cells, and (d) KB cells treated with 50 µg/ml of MgZnO NPs. Image was taken at 10X.

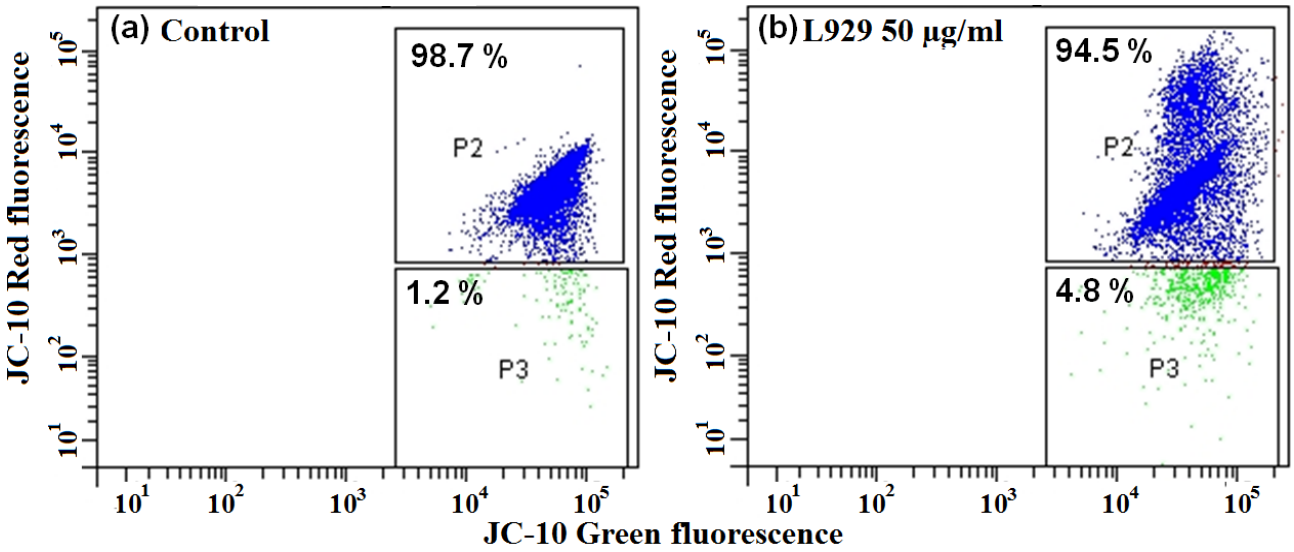

Figure S 6 Flow cytogram depicting the changes in (a) Mitochondrial Membrane Potential (ζ) of L929 cells untreated (b) treated with 50 µg/ml of MgZnO NPs for 24 h. In the flow cytogram, P2 scatter region (blue dots) indicate cells with polarized mitochondrial membrane while P3 scatter region (green dots) denotes cells with depolarized mitochondrial membrane.

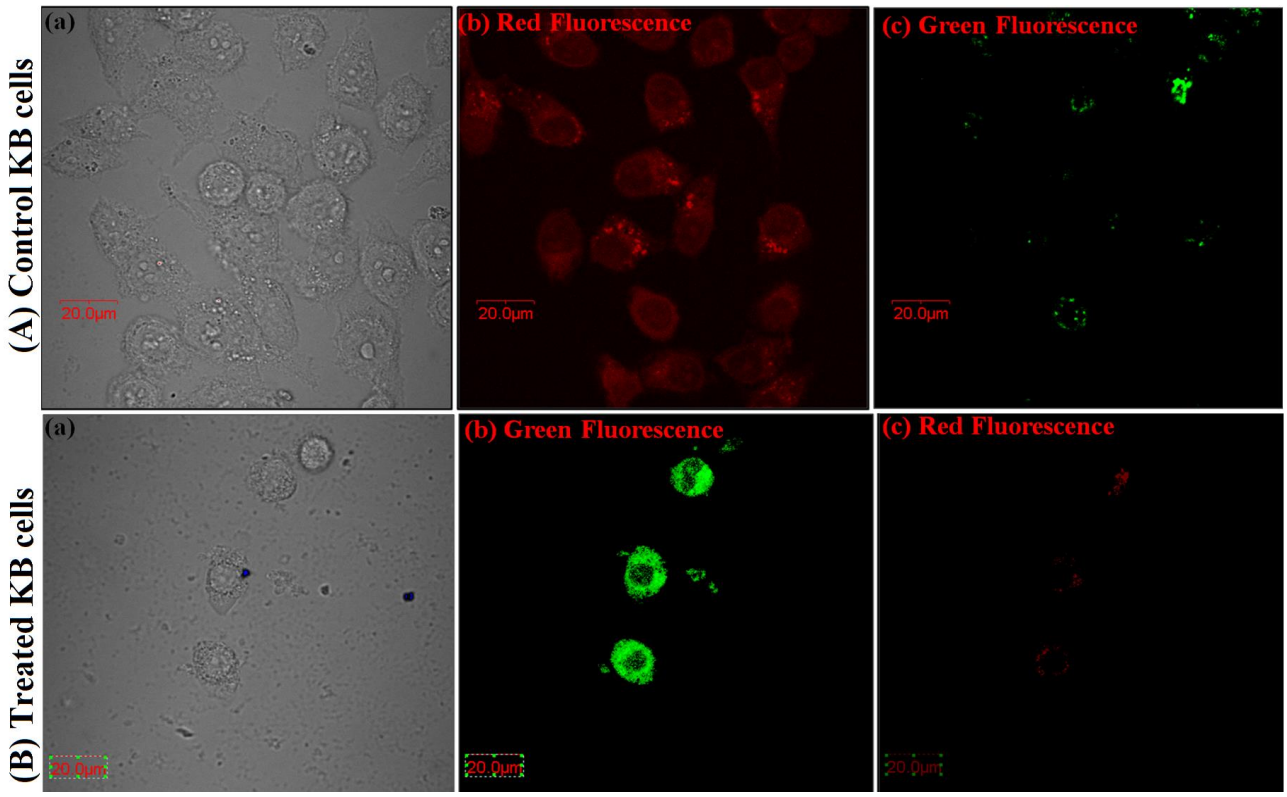

Figure S7 (A) Confocal images of KB cell depicting the changes in mitochondrial membrane potential (ζ) of KB cells image treated without NPs for 24 h (a) dark field and (b) red fluorescence and (c) green fluorescence. Figure S7 (b) Confocal images of KB cells depicting the changes in mitochondrial membrane potential (ζ) treated with 50 µg/ml of MgZnO NPs for 24 h (a) dark field image, (b) green fluorescence and (c) red fluorescence.

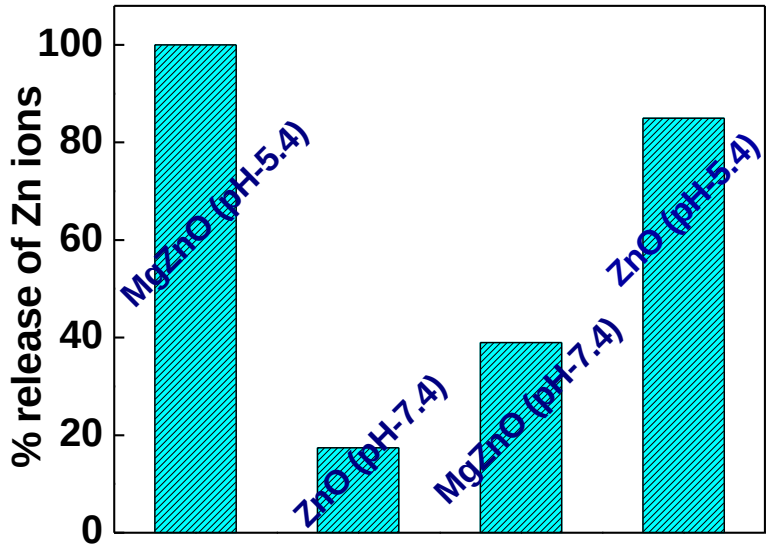

Figure S 8 Release of Zn ions from ZnO and MgZnO NPs at two different pH

Reference:

1 Gupta, J.; Bahadur, D., Visible Light Sensitive Mesoporous Cu-Substituted ZnO Nanoassembly for Enhanced Photocatalysis, Bacterial Inhibition, and Noninvasive Tumor Regression. *ACS Sustainable Chem. Eng.* **2017**, *5* (10), 8702-8709.

2 Gupta, J.; Bhargava, P.; Bahadur, D., Fluorescent ZnO for imaging and induction of DNA fragmentation and ROS-mediated apoptosis in cancer cells. *J. Mater. Chem. B* **2015,** *3* (9), 1968-1978.