



# Combination of Cytochalasin H and zinc oxide nanoparticles in human breast cancer: an insight into apoptosis study

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## Abstract

Nanoparticle-mediated combination therapies have significant impact on improving the therapeutic efficacy of cancer. The aim of the current work was to investigate the apoptotic efficacy of Zinc Oxid nanoparticles (ZnONPs) in combination with Cytochalasin H (CytoH) in human MCF-7 breast cancer cells. The anti cancer activity of combined treatment of CytoH and ZnONPs was performed using MTT assay, Annexin V-propidium iodide staining, Caspase assay, and Hoechst 33342 staining. Quantitative real-time polymerase chain reaction was conducted to detect of *p53* as a tumor suppressor gene expression in cancerous cells. Also, measurement of ROS generation was performed using flow cytometry. According to the MTT assay results, the combination of CytoH and ZnONPs significantly decreased the viability of cancer MCF-7 cells than either CytoH or ZnONPs alone as compared with normal HEK293 cells. The experiments, such as caspase 8 and 9 assay and Hoechst 33342 staining, showed that the combined treatment of CytoH and ZnONPs significantly increased the level of cell apoptosis pathway as compared with breast cancer cells treated with CytoH or ZnONPs alone. As demonstrated by annexin V/PI detection assay, the percentage of apoptotic MCF-7 cells was significantly increased, following combined treatment with CytoH and ZnONPs as compared with CytoH or ZnONPs alone. Moreover, by comparing the combination of CytoH and ZnONPs to CytoH and ZnONPs alone the ability of combined treatment to up-regulate *p53* gene expression was observed. The results of the present study showed that combination of CytoH and ZnONPs could have a powerful synergistic cytotoxic effect on breast cancer cell line. Moreover, this is the first report indicating a new insight into how CytoH and ZnONPs can enhance apoptosis and cell growth in breast cancer cells.

**Keywords** Cytochalasin H · Breast cancer · ZnONPs · Apoptosis

## Abbreviations

ANOVA	One-way analysis of variance
CytoH	Cytochalasin H
DCF-DA	2',7'-dichlorofluorescein diacetate
DCF	2', 7' –dichlorofluorescein
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

HEK293	Human embryonic kidney 293
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	Phosphate-buffered saline
ROS	Reactive oxygen species
ZnONPs	Zinc Oxid nanoparticles

## Introduction

Breast cancer is one of the most frequent malignant tumors, with increasing incidence over the past 20 years among women (Yuan et al. 2016; Wang et al. 2017). The classical approach for the treatment of breast cancer is through surgery, chemotherapy, followed by radiotherapy (Powell et al. 2017; Kutanzi et al. 2011). One of the major drawbacks of drug administration strategies is the side effects due to killing of non specific target or healthy cells instead of eliminating cancerous cells (Lee et al. 2017). In view of their undesirable

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devastation of non-cancer cells, a novel alternative means of cancer treatment is needed. Therefore, identifying alternative promising therapeutic modalities in decreasing the mortality rate of tumor cells is essential.

Cytochalasins, a mycogenic toxin, are effective agents that exhibit promising anticancer activity (Natarajan et al. 2000). Moreover, Cytochalasin H, a member of the cytochalasin family, is biologically derived from active secondary metabolites of fungal (Wells et al. 1976). Also, according to previous study, Cytochalasin H isolated from the ethanol extract of *Gleditsia sinensis* thorns demonstrated in vitro anti-angiogenic properties, such as suppressed mobility and cell growth in human umbilical vein endothelial cells (HUVEC) (Lee et al. 2014).

To increase therapeutic effectiveness, combination therapy may be an attracting strategy to eliminate undesired side effects (Hu et al. 2010). In cancer treatments, nanoparticles-mediated combination therapy may be a synergistic approach to enhance effective therapeutic effects as compared with single-drug treatment (Mignani et al. 2015).

Recently, the fabrication of noble metals nanoparticles, which exhibit unique biological, chemical, and physical activities, is gaining remarkable attention in the field of cancer therapy (Jain et al. 2005; Sonvico et al. 2005). Among different metallic nanoparticles, Zinc Oxide nanoparticles (ZnONPs) have been widely applied in different field such as varnishes, paints, solar cells, plastics, and pharmaceuticals (Hayat et al. 2011; Ann et al. 2014; Zak et al. 2011; Vafaei and Ghamsari 2007). They have been extensively examined as biomedical agents due to their high

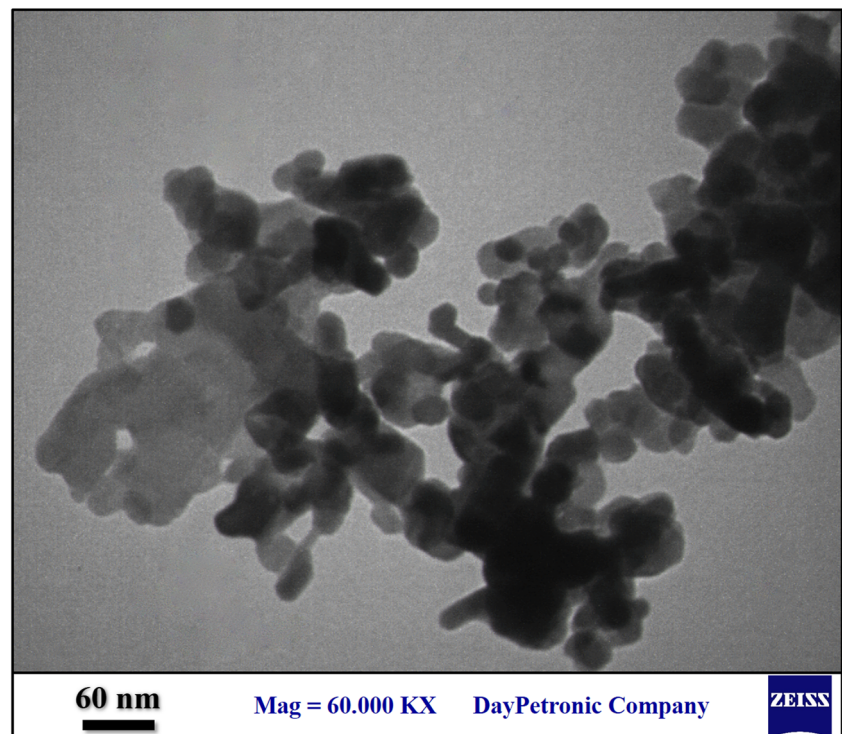
surface energy and high surface-to-volume ratio (Sharon et al. 2010), but their use in medicine remains a huge challenge (Rasmussen et al. 2010). Moreover, ZnONPs are known to initiate cytotoxicity via apoptosis in various cancer cell lines. Therefore, the development of potential ZnONPs for combining chemotherapy with other high efficient of therapy is essential. There are several approaches regarding the combination of different chemotherapeutic agents in various types of cancer cell lines. Particularly, no study has been conducted regarding the combination of ZnONPs and Cytochalasin H in human breast cancer cells. To determine the effect of anticancer property, we investigated the cytotoxic properties of Zinc Oxide nanoparticles and Cytochalasin H in MCF-7 cells. The final aim of this study was to investigate the mechanism of Cytochalasin H and ZnONPs-induced apoptosis in human breast MCF-7 cancer cells.

## Materials and methods

### Materials

ZnONPs (99% pure, ranging from 10 to 30 nm in diameter; the structure is depicted in Fig. 1) were obtained from the US Research Nanomaterials, Inc. (Twig Leaf Lane, Houston, USA). The human breast cancer cell lines (MCF-7) and human embryonic kidney (HEK293) cells were collected from the National Cell Bank Pasteur Institute (Tehran, Iran). RPMI1640 medium, trypsin-EDTA solution, Penicillin–

**Fig. 1** TEM image of ZnONPs



streptomycin solution, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and fetal bovine serum (FBS), were purchased from Thermo Fisher Scientific. Cytochalasin H and the Hoechst 33342 staining were obtained from Sigma-Aldrich Co. (St Louis, MO, USA). Caspase 8/9 assay kit was purchased from Invitrogen Co kit. The annexin V-FITC and PI kit were purchased from Hoffman-La Roch Ltd. (Basel, Switzerland). Moreover, the cellular reactive oxygen species (ROS) were evaluated according to cellular ROS detection assay Kit (Abcam Inc., USA).

### Characterization of ZnONPs

The ZnONPs were suspended in sterilised distilled water and sonicated for 10 min. The stability and dispersion of nanoparticles was evaluated using Malvern Zeta sizer (Nano ZS90, UK) instrument.

### Cell culture and in vitro cell viability assay

To clarify the cytotoxicity effect of the desired samples, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) reduction assay was assessed. Briefly,  $1 \times 10^4$  cells per well were seeded into 96-well plates containing RPMI<sub>1640</sub> medium supplemented with 10% (v/v) FBS at 37 °C in humidified atmosphere of 5% CO<sub>2</sub>. After 24 h incubation, the attached cells were treated with 100 µL of media containing cytochalasin H ( $0-10^{-8}$  M) or ZnONPs ( $0-4500$  µg/mL) and continuously cultured for 24 h. The untreated cells that were not exposed to cytochalasin H or ZnONPs served as the control. After incubation for 24 h, the 20 µL of MTT solution (5 mg/mL in PBS) was added to each sample-treated well. After 4 h of incubation at 37 °C, the medium was then removed and 100 µL of dimethyl sulfoxide (DMSO) was transferred to each well of cultured cells, followed by incubation at 37 °C for 30 min. Absorbance was calculated at 570 nm, using Epoch microplate reader (BioTek, USA).

### Analysis of tumor suppressor p53 gene expression

The cells at a density of  $1 \times 10^5$  cells/mL were seeded into six-well plates and allowed to grow and attach to the bottom of the plate. After 24 h of incubation, the total RNA was isolated from cells treated with Cyto H, ZnONPs, or Cyto H and ZnONPs for 24 h using the RNA-isolation kit (TransGen Biotech AE301–02, China) following the manufacturer's instructions. The complementary DNA synthesis was conducted using the PrimeScript™ first-strand cDNA synthesis kit (Takara, Japan) based on the manufacturer's protocol. Primers sequences corresponding to the genes were designed using Primer Express software version 3.0 and the characteristics of the primers were qualified using the BLAST program. The sequence of forward and reverse specific primers for p53 transcripts amplification during real time PCR

was (F: 5'- CTCAGCCTGTATCAAAGTCACCA -3') and (R: 5'-CCCACGCCGATGAAGACATA -3'). The mRNA expression levels for target genes were normalized to the expression levels of housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). *GAPDH* amplification was carried out using forward and reverse primers (F: 5'- CCCACTCC TCCACCTTTGAC -3') and (R: 5'- CATACCAGGAAATG AGCTTGACAA -3'). The real-time quantitative polymerase chain reaction was conducted using SYBR GREEN® (non-specific DNA-binding factors) and an ABI7300 real-time PCR system (Applied Biosystems Company, USA). The expression level of p53 gene as compared with *GAPDH* was measured using the formula: (Ratio formula =  $2^{-\Delta\Delta C_t}$ ).

### Measurement of caspase 8 and 9 activity

The evaluations of caspase 8 and 9 activity were carried out based on the method reported earlier (Khalili et al. 2017). The MCF-7 cells were treated with ZnONPs, CytoH, or both CytoH and ZnONPs with the addition of caspase-8 and 9 reagent.

### In vitro apoptosis/necrosis assay

Treated and untreated cells were stained with annexin V-FITC and PI kit (Hoffman-La Roch Ltd., Basel, Switzerland) according to the manufacturer's instruction. Thereafter, flow cytometric analysis was performed for the determination of apoptosis and necrosis as reported previously (Salehi et al. 2016) and the contents were measured using a flow cytometer (FACS Calibur, BD).

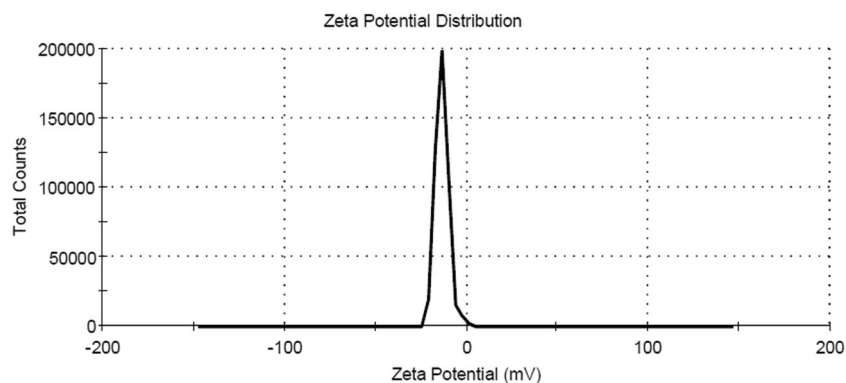
### Nuclear morphological analysis

To analyze the changes in apoptotic characteristics of MCF-7 cells after ZnONPs, CytoH, or both CytoH and ZnONPs treatment, nuclear Hoechst 33342 (Sigma, St. Louis, MO) staining was performed. The MCF-7 cells were cultured in 6-well plates, and IC<sub>50</sub> concentrations of samples were added for 24 h. After incubation period, the cells were exposed to Hoechst 33342 nuclear staining dye (1 µg/mL) for 15 min at room temperature, followed by washing three times with phosphate-buffered saline (PBS). The cancerous stained cells were observed under a fluorescence microscope (Nikon, Tokyo, Japan).

### Measurement of cellular ROS

Cellular reactive oxygen species (ROS) were evaluated according to the ab113851 2',7'-dichlorofluorescein diacetate (DCFDA) cellular ROS detection assay Kit (Abcam Inc., USA). DCFDA can diffuse into cells and then reacts with ROS and turn to 2', 7' -dichlorofluorescein (DCF) as fluorescent compound. For the measurement of the amount of produced ROS, maximum

Fig. 2 Zeta potential of ZnONPs



excitation and emission spectra of DCF fluorescence intensity were detected at 495 and 529 nm, respectively. MCF-7 cells were incubated with samples at  $IC_{50}$  concentrations for 24 h. At the end of the exposure, cells were incubated with 20  $\mu$ M DCF-DA at 37 °C for 30 min. Finally, ROS generation was measured using flow cytometry.

### Statistical analysis

All assays were presented as mean  $\pm$  SD of at least 3 independent experiments. The treated and control data

were statistically evaluated using one-way analysis of variance (ANOVA).

## Results and discussion

### ZnONPs zeta potential analysis and cell viability results

The zeta potential value of ZnONPs was determined for the measurement of stability and particles surface charges. This

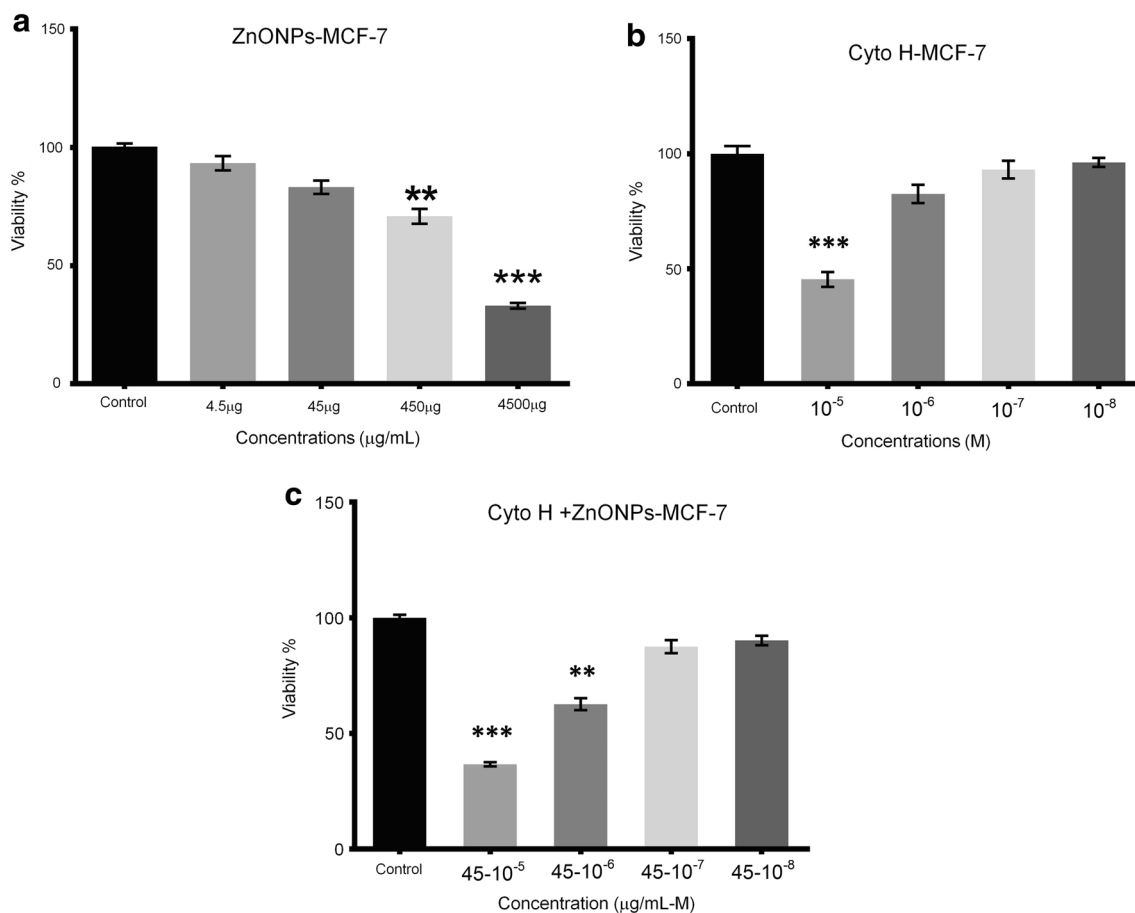


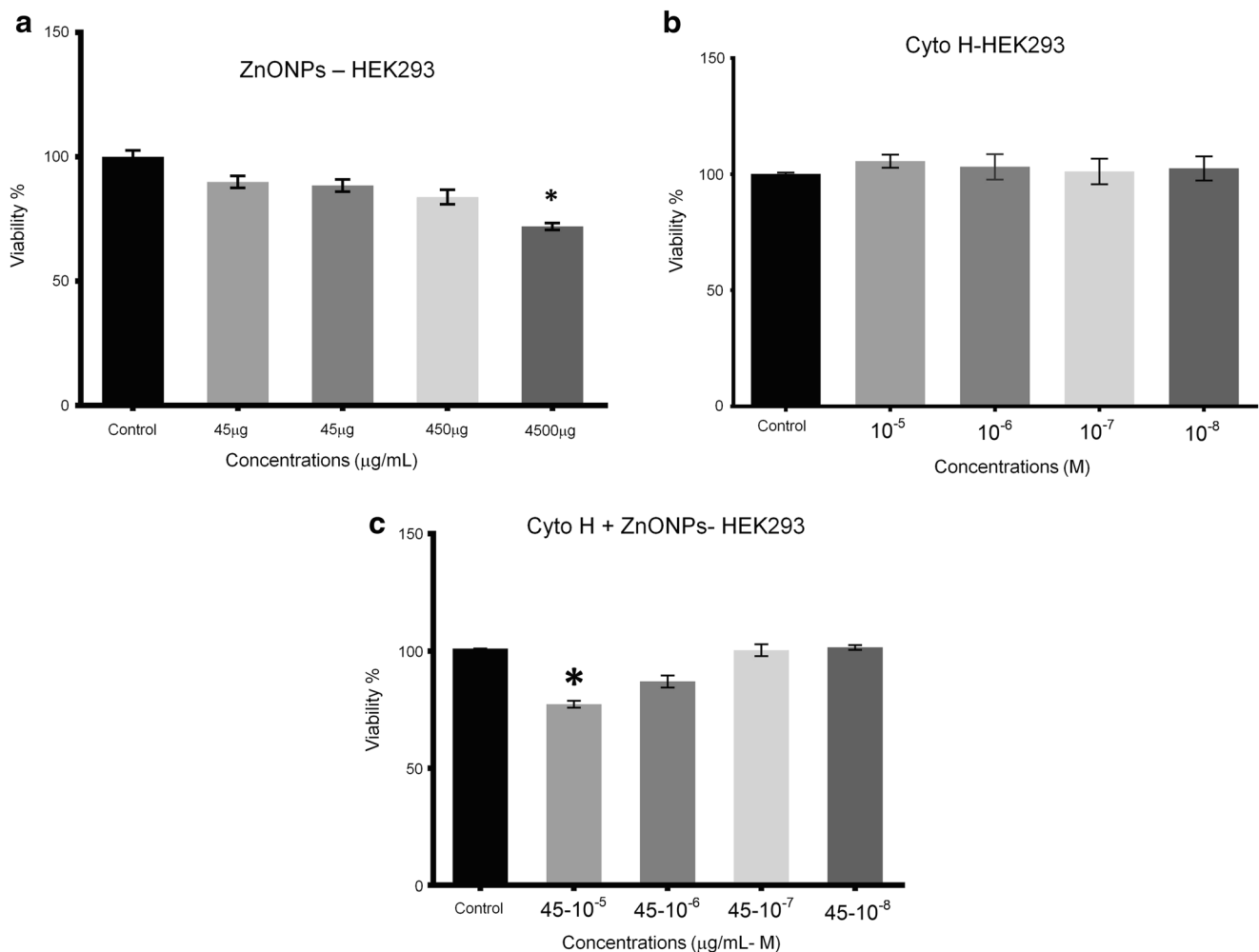
Fig. 3 Effect of ZnO NPs (a) Cyto H (b), and combination treatment of CytoH and ZnONPs (c) on the viability of human breast cancer MCF-7 cells

resulted in negative zeta potential of  $-13.4$  mV which indicates good stability, as shown in Fig. 2. The effect of CytoH and ZnONPs on cell viability was measured by MTT assay (Figs. 3 and 4). Initially, the human breast cancer MCF-7 and HEK293 cell lines were treated with different concentrations of CytoH ( $0$ – $10^{-8}$  M) or ZnONPs ( $0$ – $4500$   $\mu\text{g/mL}$ ) for 24 h. The CytoH and ZnONPs induced significant cell death in breast MCF-7 cancer cells than normal HEK293 cells at given concentrations. The cell growth inhibition indicated a concentration-dependent pattern in MCF-7 cells. Both CytoH and ZnONPs induced toxic effects on breast cancer cells (MCF-7); thus, it could be a promising therapeutic approach for combating breast cancer. The cytotoxicity effect of MCF-7 cells treated with ZnONPs decreased significantly than that of HEK293 cells as concentration increases. Also, the results showed that higher concentration of CytoH reduced the MCF-7 cell viability more than HEK293 cells. This finding indicated that ZnONPs as compared with CytoH were more toxic against MCF-7 cells.

To evaluate the combined cytotoxicity effect of CytoH and ZnONPs, we calculated the  $\text{IC}_{50}$  values of CytoH ( $10^{-6}$  M) and ZnONPs ( $750$   $\mu\text{g/mL}$ ) for cell viability assay. Also, a

fixed concentration of ZnONPs ( $45$   $\mu\text{g/mL}$ ) was calculated with simultaneous addition of CytoH ( $10^{-5}$  M,  $10^{-6}$  M,  $10^{-7}$  M, and  $10^{-8}$  M) in the MCF-7 and HEK293 cell lines. The CytoH and ZnONPs combination exposure was more potent as the cell viability ( $35\%$ ) decreased by enhancing the cell toxicity as compared with either ZnONPs or CytoH alone (Fig. 3). The data indicate that higher concentrations of CytoH induce significantly cell death more than lower concentrations. In this study, it was shown that a  $45$   $\mu\text{g/mL}$  of ZnONPs was suitable to powerfully synergize with CytoH to reduce cell viability in breast cancer MCF-7 cells as compared to HEK293 cells.

Hackenberg et al. reported the anticancer activity of photo-stimulated zinc oxide nanoparticles combined with cisplatin or paclitaxel in three human squamous cell carcinoma (HNSCC) cell lines, including HLaC-78, Cal-27, and PJ-41. They concluded that the anticancer activities of combination approach including chemotherapeutic agents (paclitaxel and cisplatin) and UVA-1-irradiation with ZnONPs were more potent in eliminating HNSCC with low toxicity and safe therapy regimes (Hackenberg et al. 2012).



**Fig. 4** Effect of ZnO NPs (a) Cyto H (b), and combination treatment of CytoH and ZnONPs (c) on the viability of normal HEK293 cells



In similar synergistic cytotoxic effects, Guo et al. analyzed the effect of combination therapy with daunorubicin and different sized ZnO nanoparticles under UV irradiation on leukemia cancer cells. They showed that Zn Oxid particles could greatly exert cell-killing effect and UV irradiation can increase this effect (Guo et al. 2008). While the results of the present study showed that the combination of CytoH and ZnONPs synergistically enhanced cytotoxicity in MCF-7 cells.

### Combination of CytoH and ZnONPs enhances apoptosis

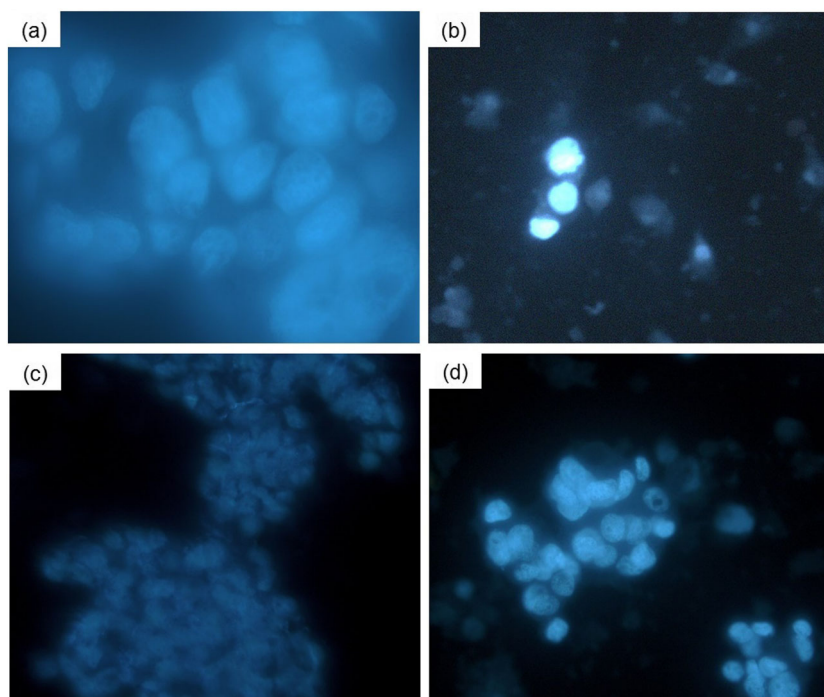
Cell death through apoptosis is characterized by different typical feature such as cell shrinkage, DNA fragmentation, nuclear condensation, and other distinctive biochemical changes (Kitazumi and Tsukahara 2011). Herein, the cells were treated with cytoH, ZnONPs, or their combination using Hoechst 33342 staining. Thus apoptosis was determined after a 24-h exposure. CytoH alone significantly enhanced apoptosis as compared with ZnONPs, whereas, the combination of CytoH and ZnONPs also induced apoptosis effects as compared with single treatment (Fig. 5). It was observed that ZnONPs can enhance the apoptotic effects of cytochalasin H against breast MCF-7 cancer cells via DNA fragmentation. Also, the annexin V and PI staining was applied to analyse the apoptotic and necrotic cells after treatment with CytoH, ZnONPs, or the combination of both. For untreated MCF-7 cells, the rate of viable was 81.4%. ZnONPs caused induction level of both early and late apoptosis to be 49.94%, which was 36.6% higher than untreated cells. The highest percentages in

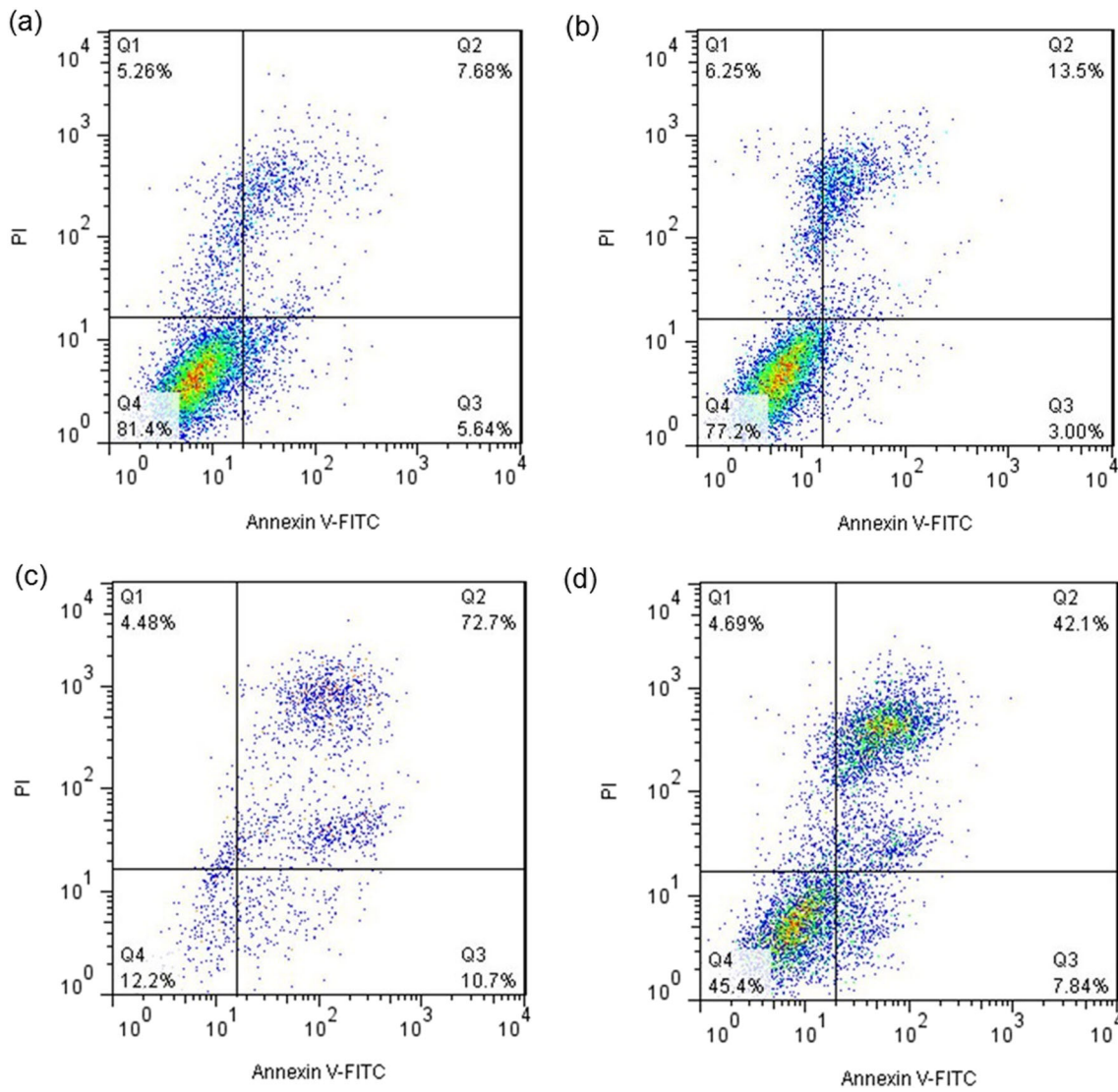
total apoptosis were obtained by combination of CytoH and ZnONPs, which were 10.7 and 72.7%, respectively; therefore, the total percentage of apoptosis distribution was 70.1%. As a result, a significant enhancement in apoptotic cells was observed for the combination of CytoH and ZnONPs exposure. In contrast, no increase in apoptosis rate was observed for CytoH alone (Fig. 6).

In addition, it was found that ZnONPs can induce apoptosis via nuclear fragmentation, membrane blebbing, and formation of apoptotic body in murine cancer cell lines, (Namvar et al. 2015) none-small cell lung cancer (A549) hepatocellular carcinoma (HEPG2), and human prostate cancer (PC3) cell lines (Hassan et al. 2017). In a similar study, it was reported that Cytochalasin H, an active constituent of *G.sinensis* thorns extract, showed in vivo anti-tumor activity against lung cancer cells (Yi et al. 2015). In a study by Yuan L, it was observed that a combination of Isoorientin with ZnONPs can trigger the apoptotic effect on HepG2 cancer cells through mitochondrial dysfunction. Also, it was reported that the ZnONPs were taken up by cells through endocytic pathway and increased the cellular uptake of Isoorientin (Yuan et al. 2014).

Caspase proteins play important roles in the regulation of signaling pathway in apoptosis. While caspase-8 protease plays a significant role in triggering apoptotic signaling through receptor pathway (extrinsic pathway). The intrinsic pathway (mitochondrial pathway) is induced by different signals in cells, such as DNA damage and cellular stresses, and translocates apoptotic mitochondrial molecules for the activation of caspase-9 proteins (Kitazumi and Tsukahara 2011; Kominami et al. 2012). In this study, we evaluated whether

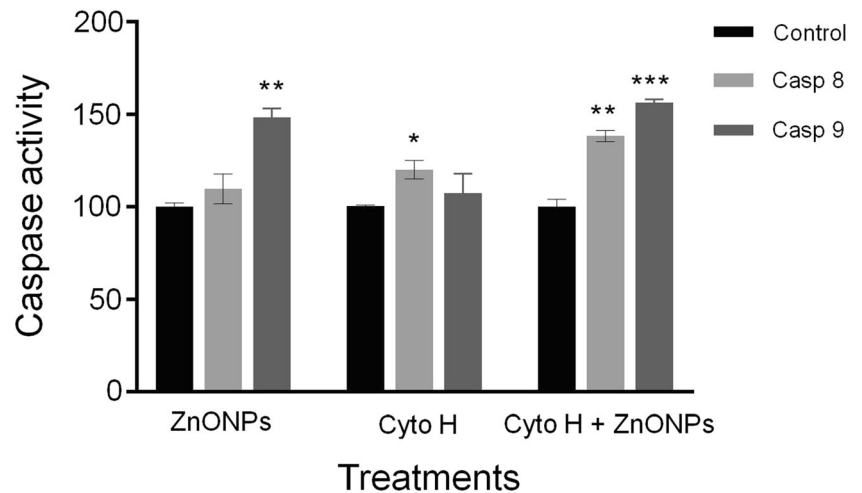
**Fig. 5** Affects of control (a) CytoH (b) or ZnONPs (c) alone or in combination (d) on apoptosis in breast MCF-7 cells. Apoptosis was performed by Hoechst 33342 staining

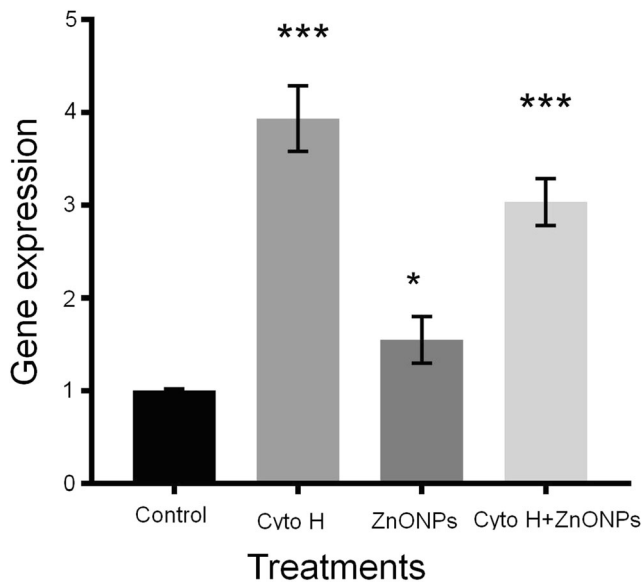




**Fig. 6** Dot plots of annexinV/PI flow cytometry of MCF-7 cells. Cells without using any samples (a) CytoH (b), combination treatment of CytoH and ZnONPs (c) and ZnONPs alone

**Fig. 7** Effect of CytoH or ZnONPs alone or the combination effect of CytoH and ZnONPs on apoptosis in human breast cancer MCF-7 cells using caspase assay



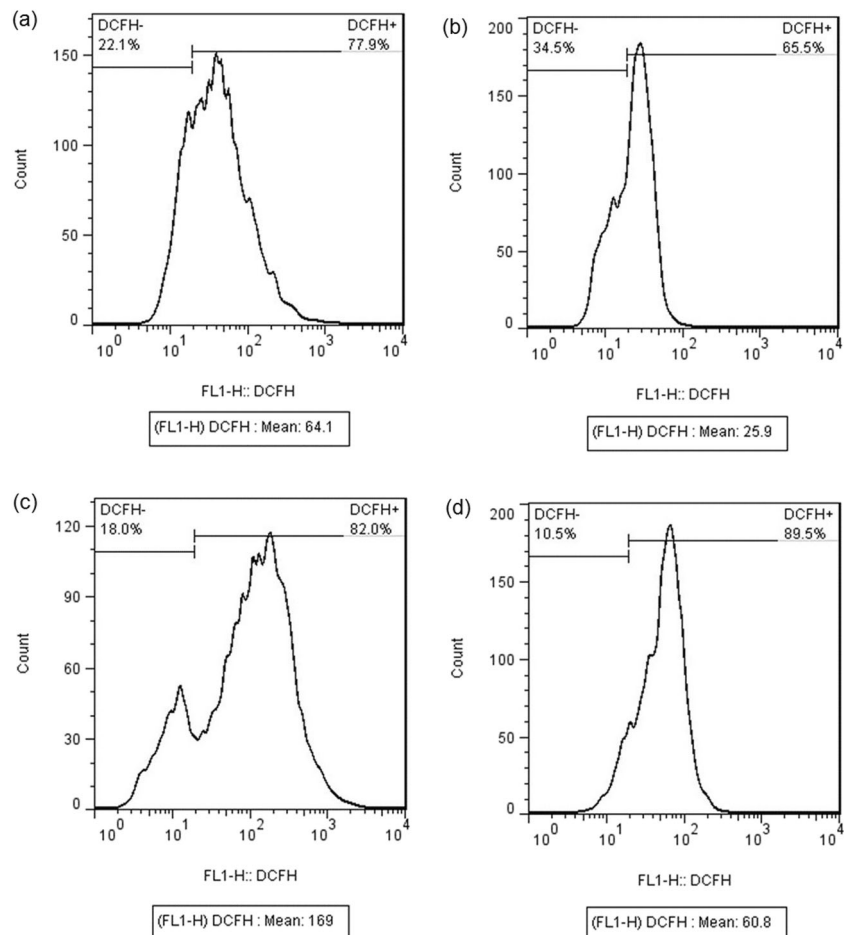


**Fig. 8** Quantitative real-time PCR analysis of mRNA levels of *p53* in human breast MCF-7 cancer cells. Cells were exposed to Cyto H, ZnO NPs, and combination treatment of CytoH and ZnONPs

CytoH and ZnONPs could trigger cell death via caspase-8 and caspase 9-dependent or independent pathways (Fig. 7). To confirm the apoptotic potential of CytoH and ZnONPs, the impact of CytoH and ZnONPs alone, or combination of CytoH and ZnONPs on caspase-8 and caspase-9 activation was measured. CytoH alone induced significant caspase-8 activity in MCF-7 cells; while the activation was higher than that of ZnONPs alone. The cells treated with ZnONPs alone showed significant increase in caspase 9 activation as compared with the control. Also, treatment with both CytoH and ZnONPs exhibited significant caspase-8 and caspase-9 activation.

The study of the apoptotic effects of our samples was intensified by evaluating the *p53* gene expression. The tumor suppressor gene *p53* is a transcription factor that suppresses tumor growth and regulates different cellular response such as autophagy; apoptosis or cell cycle arrest (Freeman and Espinosa 2012, Sullivan et al. 2018). To investigate if the tumor suppressor *p53* is a potent factor for triggering apoptosis pathway, we examined the expression of *p53* by treatment of CytoH, ZnONPs, or the combination of both using quantitative RT-PCR. The results showed that *p53* was up-regulated

**Fig. 9** Intracellular ROS generation in MCF-7 cells. Fluorescence intensity detected using flow cytometry for MCF-7 cells treated with ZnONPs (b) or CytoH (c) alone or in combination (d) for 24 h





by 1.5 and 4-fold in ZnONPs and CytoH-treated cells, respectively, whereas the combined treatment of ZnONPs and CytoH resulted in a 3-fold increase (Fig. 8). This study showed that treatment of ZnONPs and CytoH induced p53-mediated apoptosis in MCF-7 human breast cancer cells.

### Induction of ROS production

ROS production has been regarded as significant modulators of apoptosis pathway (Su et al. 2013; Akhtar et al. 2012). The overproduction of ROS can cause damage to cellular constituents such as lipids DNA, and proteins, thereby causing injury to various cellular organelles (Ma et al. 2014).

After MCF-7 cells were treated with CytoH and ZnONPs or combination of CytoH and ZnONPs for 24 h, ROS production was evaluated using DCFDA as a fluorescent probe.

In the present study, CytoH triggered ROS-mediated apoptosis with maximum yield of 169% in MCF-7 cells as compared with other samples. The fluorescence intensity decreased to 25.9% by ZnONPs and to 60.8% by combination of CytoH and ZnONPs after 24 h (Fig. 9).

### Conclusion

In summary, the present study showed that the Cytochalasin H and ZnONPs combination exposure effectively decreased cell viability by enhancing the cell toxicity as compared with either single treatment. Treatment with combination of cytochalasin H and ZnONPs induced apoptosis in human breast MCF-7 cancer cells, resulting in up regulation of *P53* gene expression, nuclear fragmentation, and formation of apoptotic body, increase in apoptosis percentage rather than necrosis, and enhancement of caspase 8 and caspase 9 activation. Thus, our results suggest that the combination of ZnONPs with cytochalasin H might provide an effective alternative strategy for the treatment of breast cancer.

### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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