

## Original Article

# In Vitro Cytotoxic and Anticancer Effects of Zamzam Water in Human Lung Cancer (A594) Cell Line

Ulfat M. OMAR, Huda A. AL DOGHAITHER, Sawsan A. RAHIMULDDIN, Shiekhah M. AL ZAHRANI, Ayat B. AL-GHAFARI

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Department of Biochemistry, Faculty of Sciences, King Abdulaziz University, Jeddah, Saudi Arabia

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## Abstract

**Background:** Zamzam water is naturally alkaline and rich in a variety of minerals which may represent a powerful tool for cancer therapy. In this research, the cytotoxic effects of Zamzam water were investigated in human lung cancer (A549) cell line and compared with human skin fibroblasts (HSF).

**Methods:** Two different preparations of Zamzam water were used: Z1, with pH adjusted to 7.2 and Z2, with no pH adjustment. The effects of both treatments on the morphology of the A549 and HSF cell lines were investigated. The cell viability of HSF and A549 cells was identified by the MTT assay and trypan blue exclusion. Detection of apoptotic cells and cell cycle analyses were determined using flow cytometry. Moreover, reactive oxygen species (ROS) were measured for both cell lines.

**Results:** Both Zamzam water treatments, Z1 and Z2 showed reductions in the cell viability of A549 cells. Cell death occurred via necrosis among cells treated with Z2. Cell cycle arrest occurred in the G0/G1 phases for cells treated with Z2. Cellular and mitochondrial ROS productions were not affected by either treatment.

**Conclusion:** Our findings indicate that Zamzam water might have potential therapeutic efficacy for lung cancer.

**Keywords:** alkaline water, Annexin V, cell cycle, cytotoxic, lung cancer

## Introduction

Lung cancer is a life-threatening disease and has currently become the number one killer among cancers worldwide (1). It is the leading cause of cancer-related mortality in the United States, China and the Arab countries (2). Lung cancer has a dismal five-year survival rate of 15% (3). Timely detection, as well as diet and food supplements have been associated with an increase or decrease in lung cancer risk.

Water has been used historically as a healer due to its many properties, such as a high

mineral content and high pH value (4). Roman bath water and the Dead Sea water were used to heal many skin problems, and deep-sea water has been mentioned as hydrotherapy for various body systems (5) and for inhibiting metastasis in human breast cancer cell lines (6). Moreover, alkaline water was reported to reduce oxidative stress and hemorrhagic gastric lesions in rats (7).

Zamzam water is naturally alkaline and it comes from an ancient well located in Makkah, Saudi Arabia. Millions of people have used it since the time of Prophet Ibrahim as a holy natural water for its exceptional healing

properties. It is rich in a variety of minerals and has a high pH value (average pH of 8) (8). Moreover, no bacteria can form at the source of or in Zamzam water (9). It was reported that Zamzam water has the ability to prevent the formation of renal stones in kidney-stone induced rats (10) and to reduce the oxidative stress in type 2 diabetic patients (11).

Oxidative stress is associated with many diseases and their complications. One reason of such an oxidant/antioxidants imbalance is dietary deficiencies in essential elements, which are important components of antioxidant enzymes (12). The aim of this study is to illustrate the cytotoxic and anticancer properties of Zamzam water in human lung cancer cell line (A549).

## Materials and Methods

### Chemicals

High glucose Dulbecco's Modified Eagle's medium powder (DMEM), fetal bovine serum (FBS) and trypan blue were purchased from Hyclone, USA. Propidium iodide (PI), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and L-glutamine were purchased from Sigma-Aldrich Co, Poole, UK. Trypsin/EDTA, antibiotics (penicillin/streptomycin), and non-essential amino acids (NEAAs) were purchased from Gibco, Canada. Phosphate-buffered saline (PBS) tablets were purchased from Oxoid, Hampshire, UK. RNase and Annexin V-FITC Apoptosis Detection Kit were purchased from Abcam, UK. MitoSOX red and 2',7'-Dihydrodichlorofluorescein diacetate (CM-H2DCFDA) were obtained from Invitrogen, Paisley, UK.

### Cell lines

Human lung cancer cell line (A549) was purchased from the immunology laboratory, King Fahad Medical Research Center (KFMRC), and normal human skin fibroblast (HSF) cell line was purchased from the virology laboratory, King Abdulaziz University Hospital, Jeddah, Saudi Arabia. The study was approved by the Biochemistry Department and the Permanent committee for academic council's affairs at King Abdulaziz University (91831/37/D).

### Zamzam water source

Zamzam water was obtained directly from Zamzam well, which is located within the Holy Mosque in Makkah, Saudi Arabia, 20 m (66 ft) east of the Kaaba. The well is about 30 m (98 ft) deep and 1.08 to 2.66 m (3 ft 7 in to 8 ft 9 in) in diameter. The water was stored in sterilised glass bottles and kept at room temperature until it was used.

### Preparation of culture media

DMEM growth media stock was supplemented with 10% FBS, 1% L-glutamine, 1% antibiotics, and 1% NEAAs.

### Preparation of Zamzam water treatments and the negative control

In this study, two different Zamzam water samples, designated Z1 and Z2, and one negative control were prepared. To prepare Z1 and Z2 treatments, a PBS tablet was dissolved in 800 ml of Zamzam water and the pH was either adjusted to 7.2 (Z1) or not adjusted (pH = 8, Z2). The negative control was PBS only.

### Morphological changes

A549 and HSF cells were seeded in 25 cm<sup>2</sup> flasks at a concentration of 8×10<sup>5</sup> cells/ml. After 24 h of incubation, changes in cell morphology were photographed using a Nikon Eclipse microscope at 10x magnification for Z1 and Z2 and were compared to the control.

### Cell viability assay

#### 1. Trypan blue exclusion

A549 and HSF cells were treated under the same conditions described in the (morphological changes) section. After 24 h, when the cultured cells were 60%–70% confluent, they were treated with trypan blue dye (0.2%) and counted with a hemocytometer.

#### 2. MTT assay

Three independent experiments were performed to assess the cytotoxic effects of Zamzam water on A549 and HSF cells. A549 and HSF cells were seeded in a 96-well tissue culture plate at a concentration of 1×10<sup>4</sup> cells/well and incubated for 24 h at 37 °C/5% CO<sub>2</sub>. After, the culture medium was replaced with 200 µl of Z1 or Z2 or with 200 µl of PBS only as a negative control. MTT assay was performed by adding 20 µl of MTT dye and incubated for 2 h at 37 °C/5% CO<sub>2</sub>. Then, treatments were discarded

and the precipitates were dissolved in 200 µl of isopropyl. The developed colour intensity was measured at 490 nm using a BioTek microplate reader.

#### *Determination of cell death type by annexin-V/PI assay*

To detect early and late apoptosis in A549 and HSF cell lines,  $8 \times 10^5$  cells/ml were seeded in 25 cm<sup>2</sup> flasks until they became 70% confluent. After, cells were incubated with Z1 or Z2 and with the negative control medium for 24 h. Then, the annexin-V/PI assay was carried out according to the manufacture instructions using a Beckman Coulter flow cytometer to acquire a minimum of 10,000 events in the gated regions. For cells labelled with annexin V-FITC, the emission wavelength was 520 nm; meanwhile, for cells labelled with PI, an emission wavelength of 620 nm was used.

#### *Cell cycle analysis*

A549 and HSF cells were seeded in 25 cm<sup>2</sup> flasks at a concentration of  $8 \times 10^5$  cells/ml. When cells were 60%–70% confluent, they were treated with Z1 and Z2 for 24 h followed by trypsinisation. Pellets were washed twice with PBS then cells were fixed with 1ml of ice-cold fixing buffer (70% ethanol in PBS) and incubated overnight at 4 °C. After 24 h, the fixation buffer was discarded and cells were re-suspended in 500 µl PBS followed by 5 µl of 5mM RNase. Cells were incubated for 30 min at 37 °C, dyed with 5 µl PI (1 mg/ml), and analysed with the BD FACSCalibur flow cytometer. At least, 10,000 events were acquired in the list mode using an emission wavelength of 620 nm.

#### *Measurement of cellular reactive oxygen species (ROS)*

ROS levels in A549 were measured using the H<sub>2</sub>DCFDA dye. A549 cells were seeded in 25 cm<sup>2</sup> flasks at a density of  $8 \times 10^5$  cells/ml and treated with Z1 and Z2 culture media for 24 h. Cells were incubated in the dark with 5 µl of DCFDA for 30 min at 37 °C/5% CO<sub>2</sub>. DCF fluorescence was read at an excitation wavelength of 485 nm and at an emission wavelength of 528 nm using the BioTek fluorescence microplate reader.

#### *Measurement of mitochondrial reactive oxygen species (mtROS)*

The superoxide levels in A549 cell line incubated with Z1 and Z2 were measured with

MitoSOX dye using the BioTek fluorescence microplate reader according to the manufacture's instructions. The resulting red fluorescence was detected at excitation and emission wavelengths of 530 nm and 590 nm, respectively.

#### *Statistical analysis*

The data were analysed using GraphPad Prism software version 6. All experiments were performed at least three times to ensure reproducibility. Significant differences were evaluated using *P*-values by the Kruskal–Wallis test followed by Dunn's multiple comparison tests. The cut-off level for significance was < 0.05.

## Results

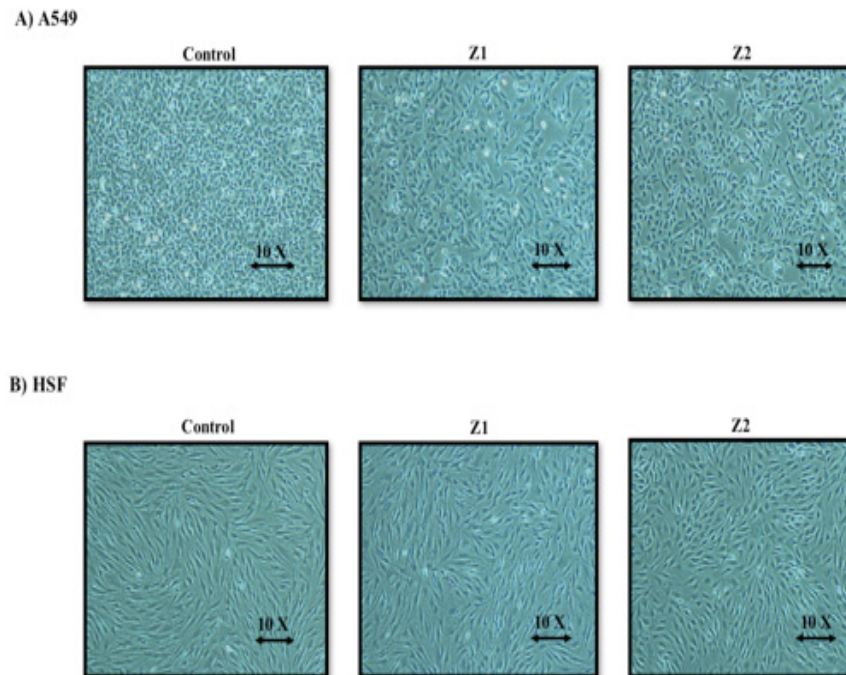
#### *Morphological changes*

The antiproliferative effects of the two Zamzam treatments (Z1 and Z2) on A549 and HSF cell lines are shown in Figures 1A and 1B. The changes in the cell membranes of A549 cells were more obvious in the high-alkaline medium (Z2) than in the adjusted pH treatment (Z1). In contrast, the same treatments had no effect on HSF cells, which appeared intact when compared to the control.

#### *Cell viability assessment by trypan blue exclusion and MTT assay*

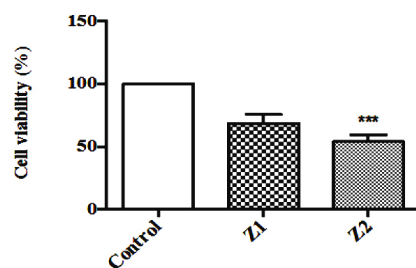
The effects of Zamzam water treatments on cell viability were assessed using a trypan blue cell count and MTT assay. In both assays, only Zamzam water treatment (Z2) was found to decrease cancer cell viability significantly in A549 cell line compared to the untreated control. In trypan blue cell count, A549 cell viability was reduced to 68.66% (Z1 treatment) and 54% (Z2 treatment) (Figure 2A), whereas HSF cells showed no significant differences in the cell viability when incubated with Z1 and Z2 (103.66% and 105.8%, respectively).

MTT assay showed that the cancer cell viability was decreased to 79% and 73% after treatment with Z1 and Z2, respectively when compared to the untreated A549 cells (Figure 2B). The same treatment showed insignificant effects on the viability of the HSF cells (95.02% and 97.9% with Z1 and Z2, respectively).

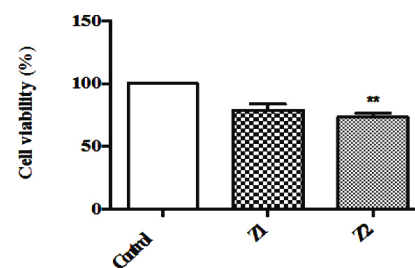


**Figure 1.** The effects of Zamzam water on A549 and HSF cells incubated for 24 h compared to untreated cells. A) A549 cells incubated with Z1 and Z2 compared to untreated cells. B) HSF cells treated with Z1 and Z2 treatments compared to untreated cells. Images were captured using light microscopy at 10× magnification

### A) Trypan blue



### B) MTT



**Figure 2.** The cytotoxic effects of Zamzam water on A549 cell line by trypan blue and MTT assay. A) Trypan blue cell count of A549 cells treated with Z1 and Z2 compared to untreated cells. B) MTT results of A549 cells treated with Z1 and Z2 compared to untreated cells. The data represents the mean of three independent experiments ( $n = 3 \pm \text{SEM}$ ). Comparisons of means were made using the Kruskal–Wallis test followed by Dunn’s test (\*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ )



*Determination of cell death type*

The necrosis phenomenon was significantly induced only in A549 cells treated with Zamzam water treatment (Z2) compared to the untreated control and Z1 treatment. The percentage of early apoptosis was 0.75%, 0.7%, and 1% in the control, Z1, and Z2 treatments, respectively. In the late apoptotic stage, there was no significant increases between the untreated control cells and Z1-treated cells (1.5% and 1.4%, respectively), whereas in Z2-treated cells, the percentage of apoptosis increased to 3.9% compared to the untreated control. Regarding necrosis, only Z2 treatment showed a significant effect compared to the untreated control and Z1 treatment. The percentages of necrosis in A549 cells were 1.9%, 4.7%, and 6.65% ( $P < 0.05$ ) in the control, Z1, and Z2, treatments, respectively (Figures 3A and 3B). For normal HSF cells, Zamzam water treatments did not show any significant effect in all apoptotic and necrotic stages. The percentage of early apoptosis in HSF remained as low as 3%, 3.5%, and 2.8% for the untreated control, Z1, and Z2 treatments, respectively, whereas in the late apoptotic stage, the percentages were 0.78%, 1.32%, and 0.85%, respectively. In the necrotic stage, the lowest percentages were, 0.2%, 0.12%, and 0.08% for the control and treated cells, respectively (Figures 3C and 3D).

*Effect of Zamzam treatments on cell cycle*

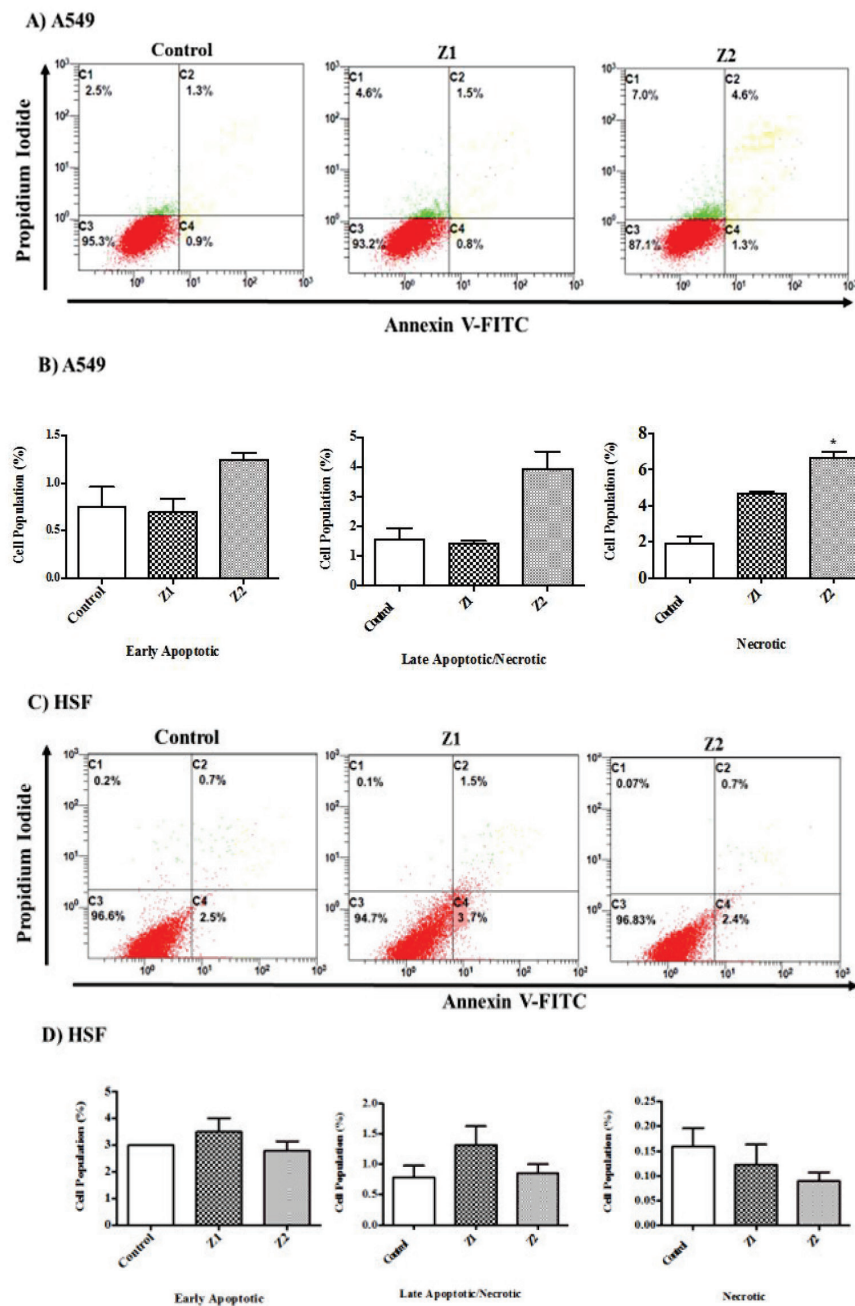
For the cell cycle analysis, Z2 treatment showed a significant effect on A549 cells. There was a significant increase in the cell population

treated with Z2 (arrest in the G0/G1 phase) while the S phase was not affected by the treatment. The percentages of A549 cells in the G0/G1 phase were 38.6%, 37.9%, and 52% for the control, Z1, and Z2 treatments, respectively. Moreover, the percentages of cells in the S phase were 18.3%, 17%, and 17.2%, respectively, while in the G2 phase, the percentages were 10.8%, 20%, and 10.4% for the control, Z1 and Z2 treatments, respectively (Figure 4B).

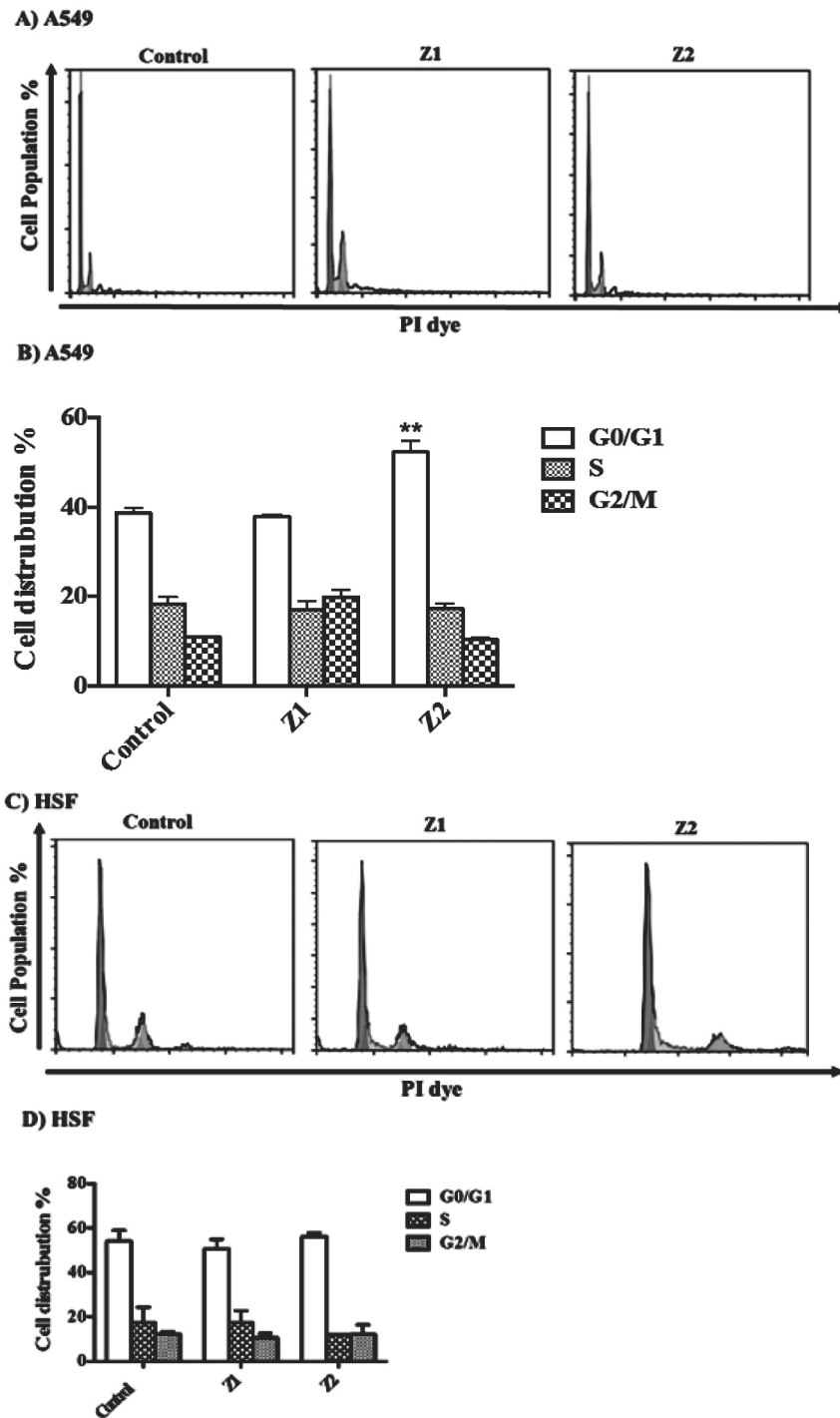
In contrast to A549 cells, the same Zamzam water treatment used on normal HSF cells showed no significant effect ( $P > 0.05$ ) in the G1, S, and G2 phases compared to the control, as presented in Figure 4D. The percentages of HSF cells in the G1 phase were 54.1%, 52.75%, and 56.26% for the control, Z1, and Z2 treatments, respectively. The percentages of cells in the S phase were 17.33%, 17.33%, and 12%, while in the G2 phase, the percentages were 12.38%, 10.7%, and 12.22% for the controls, Z1, and Z2 treatments, respectively.

*Cellular and mitochondrial ROS measurement*

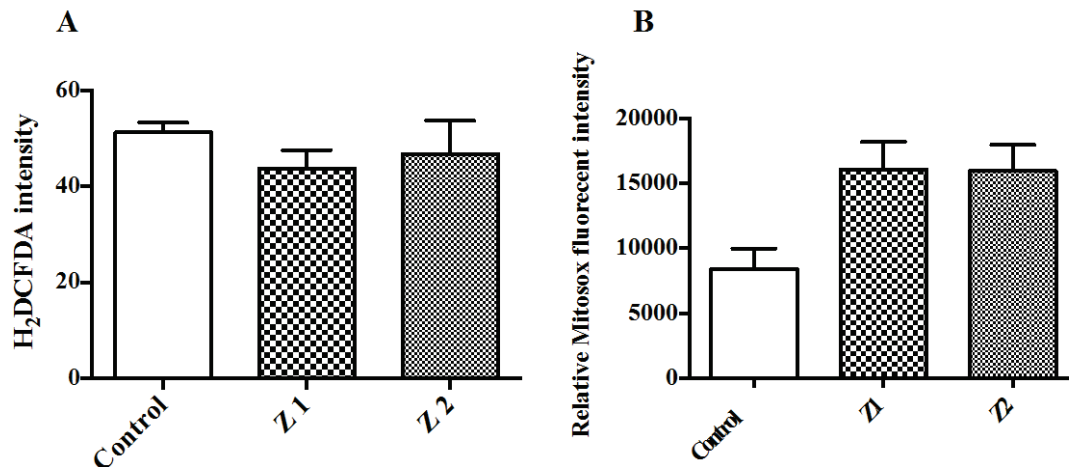
Cellular and mitochondrial ROS (superoxide anion) productions were not affected by Z1 and Z2 treatments when compared to the untreated control cells ( $P > 0.05$ ) (Figure 5A). The intensity of cellular ROS formation by Z1 was 43.75 RFU, while the cellular ROS production of Z2 treatment was 46.75 RFU compared to the control, which was 51.25 RFU. Moreover, the florescent intensity of the mtROS formation of Z1 treatment was 16,067 RFU while the mtROS



**Figure 3.** Detection of cell deaths of A549 and HSF cells after treatment with Z1 and Z2 for 24 h by flow cytometry. Represented values are the means of three independent experiments ( $n = 3 \pm \text{SEM}$ ). Comparisons of means were made using the Kruskal–Wallis test followed by Dunn’s test ( $*P < 0.05$ ). A) Flow cytometric analysis of annexin V/PI in A549 cells treated with Z1 and Z2 treatments for 24 h. C1– C4 quadrants indicate: C1 = Cells stained with PI; C2 = Cells conjugated with annexin V and stained with PI; C3 = Healthy cells; and C4 = Cells conjugated with annexin V. B) Percentage of A549 cells in early apoptosis, late apoptotic/necrotic cell death, and necrotic cell death pathways after treatment with Z1 and Z2 for 24 h. C) Flow cytometric analysis of annexin V/PI in HSF cells treated with different Z1 and Z2 for 24 h. C1– C4 quadrants indicate: C1 = Cells stained with PI; C2 = Cells conjugated with annexin V and stained with PI; C3 = Healthy cells; and C4 = Cells conjugated with annexin V. D) Percentage of HSF cells in early apoptosis, late apoptotic/necrotic cell death, and necrotic cell death pathways after treatment with Z1 and Z2 for 24 h



**Figure 4.** Cell cycle arrest in A549 and HSF cells treated with Z1 and Z2 for 24 h by flow cytometry. Represented values are the means of three independent experiments ( $n = 3 \pm \text{SEM}$ ). Comparisons of means were made using the Kruskal–Wallis test followed by Dunn’s test (\*\* $P \leq 0.01$ ). A) A549 cells treated with Z1 and Z2 for 24 h. B) Effects of Z1 and Z2 treatments on A549 cell cycle distribution after 24 h. Growth phase (G1 phase), DNA synthesis phase (S phase), and growth 2 phase (G2 phase). In each phase, cell percentages were measured by flow cytometry. C) HSF cells treated with Z1 and Z2 for 24 h. D) Effects of Z1 and Z2 on HSF cell cycle distribution after 24 h. G1 phase (growth phase), S phase (DNA synthesis phase), and G2 phase (growth 2 phase)



**Figure 5.** Measurement of cellular and mitochondrial ROS in A549 cells treated with Z1 and Z2 for 24 h. The data represent the mean of three independent experiments ( $n = 3 \pm \text{SEM}$ ). Comparisons of means were made using the Kruskal–Wallis test followed by Dunn’s test. A) A549 cells treated with Z1 and Z2 for 24 h. Cells were loaded with CM-H<sub>2</sub>DCFDA. B) A549 cells treated with Z1 and Z2 for 24 h. Cells were loaded with MitoSOX stains. Fluorescence measured using the BioTek fluorescence microplate reader to monitor cellular and mitochondrial ROS formation. Data from both assays revealed that both Zamzam treatments (Z1 and Z2) have no significant effect on ROS formation

production of Z2 treatment was 15,965 RFU, compared to the control, which was 8,398.2 RFU (Figure 5B).

## Discussion

Lung cancer incidence is considered the highest among malignant tumors. It has become the leading cause of cancer-related deaths worldwide, including in Saudi Arabia (13). Resistances to treatment with anticancer drugs and toxic side effects have resulted in the need for new anticancer treatments with little or no side effects (14). To date there are limited studies on the effects of alkaline water on cancer cells. In this study, the cytotoxic and apoptotic effects of Zamzam water on A549 cells were investigated for the first time and compared to the effects of Zamzam water on normal HSF cells.

Our results showed that the incubation of A549 cells with Zamzam water inhibited growth and proliferation and changed the morphology of A549 cells. Although it is unclear which component of Zamzam water affects the proliferation of cancer cells, it is presumed that the combined ionic actions of several minerals such as calcium, magnesium, lead,

arsenate, lithium, cadmium-zinc combinations and selenium may play an important role in mediating the inhibition of proliferation of cancer cells. Our results were in contrast to previous studies that have shown that minerals such as lead, arsenate (15), lithium (16), cadmium-zinc combinations (17), and selenium (18), are capable of inhibiting the proliferation of normal and cancerous cells. Interestingly, the proliferation of HSF cells was not affected by treatment of Zamzam water, suggesting that this water might have selective cytotoxic effects, but the exact mechanism is unclear.

Pettersson et al. (19) have found that As<sub>2</sub>O<sub>3</sub> induced small cell lung carcinoma (SCLC) cytotoxicity and has been shown to involve several different cell death pathways (apoptosis and necrosis). Moreover, cell death is mainly due to caspase-independent necrotic cell death, whereas the involvement of apoptosis is more cell line-dependent (19). Our results indicate that Z1 treatment did not induce cell death in A549 lung cancer cells. On the other hand, Z2 treatment caused necrotic cell death. These effects could be due to the unique combination of minerals in Zamzam water, arsenic and cadmium might be responsible for inducing cell death in A549 cells. Furthermore, the high pH value of Zamzam water is another important feature, as



the pH of Zamzam ranges from 7.9–8 compared to the ordinary water, which ranges from 6.5–8 (11, 12). Studies on cancer have shown that the oral administration of sodium bicarbonate increases the extracellular pH of tumors and reduces the formation of spontaneous metastasis (20). The extracellular pH of malignant tumors is acidic (pH 6.5–6.9) compared to the pH of normal tissues, which is more alkaline (pH 7.2–7.5) (21).

In normal cells, ROS plays an important role in regulating various biological pathways. The cell balances the generation of ROS thereby controlling it. In our study, ROS level in A549 cell line was investigated to assess the role of ROS in the induction of apoptosis after exposure to Zamzam treatments. Our data demonstrated that cellular ROS formation and mitochondrial ROS production were not significantly induced in A549 cells.

To further elucidate the possibility of cell growth arrest, cell cycle phases were evaluated using a flow cytometry assay. Our data showed that cells were arrested in the G<sub>0</sub>/G<sub>1</sub> phase for Z<sub>2</sub>-treated cells, while Z<sub>1</sub>-treated cells were unaffected. The percentages of A549 cells in the G<sub>0</sub>/G<sub>1</sub> phase were 38.6%, 37.9%, and 52% for the control, Z<sub>1</sub>, and Z<sub>2</sub> treatments ( $P \leq 0.01$ ), respectively. The percentage of cells in the G<sub>2</sub>/M phase increased in cells treated with Z<sub>1</sub>, with percentages of 10.8%, 20%, and 10.4% for the control, Z<sub>1</sub>, and Z<sub>2</sub> treatments, respectively. It was reported previously that cancer cells treated with arsenic trioxide were arrested at either the G<sub>1</sub> or the G<sub>2</sub>/M phase (22). In addition, it has been found that arrest in G<sub>2</sub>/M phase that occurs in human cancer cell lines by arsenic trioxide is due to an increase in cyclin B level, a regulatory protein involved in mitosis (23, 24). De Groot and his colleagues (25) observed a significant accumulation of renal cells in the G<sub>2</sub> phase after lithium treatment when compared to their controls. It is well known that cyclin D1 and cyclin E are regulatory proteins that control the transition from G<sub>1</sub> to S phase, whereas, cyclin B1 regulates the progression of the G<sub>2</sub>/M phase. Recently, a study reported that a low-concentration of inorganic arsenic inhibits the proliferation of C<sub>2</sub>C<sub>12</sub> myoblasts cells by inducing G<sub>1</sub> and G<sub>2</sub>/M phase cell-cycle arrest due to the decrease in the protein expressions of cyclin D1, cyclin E, and cyclin B1 (26). In this study, it was assumed that the arresting effect of Z<sub>1</sub> and Z<sub>2</sub> in A549 cells might be due to the decreased levels of cyclin D1, cyclin E, and cyclin B1 caused by both the effect of both the alkaline

pH and the elements found naturally in Zamzam water. Our results revealed that HSF cells were unaffected by both Zamzam water treatments. It was demonstrated that the concentrations of calcium and magnesium ions in Zamzam water were almost double the concentrations found in bottled water (8). Calcium has been implicated in the induction of apoptosis via the activation of caspase 12. Previous reports have suggested that the intake of calcium reduces the risk of colon and breast cancers (27, 28). Moreover, magnesium deficiency is also associated with the metastasis of cancer cells. Thus, Zamzam water may be a beneficial source, providing the proper ratio of calcium and magnesium and other elements. However, previous research studies have reported that alkaline-reduced water has potent antioxidant activity and anticancer effects and it has been shown to protect DNA from oxidative damage (29).

## Conclusion

In conclusion, according to the results obtained from all previous experiments, Zamzam water might be a promising anticancer agent. Further researches are needed on the different types of cancer cell lines to investigate the cytotoxic and anticancer effects of Zamzam water on these cancers. In addition, in vivo studies are necessary to investigate the effects of Zamzam water on experimental animals with induced cancers.

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## Conflicts of Interests

None

## Authors' Contributions

Conception and design: UO  
Analysis and interpretation of the data: UO  
Drafting of the article: SR  
Critical revision of the article for important intellectual content: HAD  
Final approval of the article: AA  
Provision of study materials: HAD  
Statistical expertise: AA

Administrative, technical, or logistic support:  
SMA

## Correspondence

Dr Ulfat M. Omar  
Assistant Professor  
MSc (King Abdulaziz University), PhD (University of Surrey)  
King Abdulaziz University,  
Faculty of Sciences, Biochemistry Department,  
Jeddah, P.O. Box 40288,  
Zip code 21499, Saudi Arabia.  
Tel: +966503675685  
E-mail: uomer@kau.edu.sa

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