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Research Article
Metallocenes-induced Apoptosis in Human Hepatic Cancer HepG2 Cells: The Prodigy of Zamzam Water

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Abstract

Background and Objective: Hepatocellular carcinoma (HCC) is a public health problem and one of the common causes of mortality around the world. Zamzam water (ZW) contains metallocenes that were reported as promising anticancer agents by blocking DNA replication. The purpose of the current study was to explore the antiproliferative effect of ZW against HCC *in vitro*. **Materials and Methods:** To achieve this goal, elemental analysis by inductively coupled plasma mass spectrometry (ICP-MS) was used for metal testing in ZW and tap water (TW), as well as metal-free water sources, deionized water (DW) and double deionized water (DDW). Water was applied *in vitro* on hepatic cancer HepG2 cells to test their cytotoxic effects and the underlining apoptotic mechanism. One-way ANOVA was used as a statistical method to compare between treated and untreated groups. **Results:** It was found that ZW is explicitly rich in Na, Ca and Mg ions which are essential for biological homeostasis in human beings. Moreover, it was reported that ZW has an oncolytic property, owing to the presence of V, Fe and Se, where it produces the highest killing effect with about 35% of cell death associated with mitochondrial impairment with a dose dependent decrease in cell viability of HepG2 cells upon treatment. To test the underlining mechanism of the anti-cancer effect of ZW, apoptosis was performed to investigate the apoptotic signals. It was found that ZW killed the HepG2 cells through apoptosis. Mechanistically, 0.84% of the pro-apoptotic cells increased significantly to be 38% post-treatment with 50% ZW v/v. **Conclusion:** The balanced nutritional composition of ZW could be of great interest, synergizing with other anti-cancer components to thwart cancer progression.

Key words: Zamzam water, metal signals, ICP-MS, liver cancer, HepG2 cells, cytotoxicity

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Zamzam water (ZW), as a great gift of God, is described in holy books of the three known religions, the old testament of the Tawrah, the Bible and the Quran[1]. The well of Zamzam is approximately 30.5 m deep, with an internal diameter up to 2.66 m. It was reported that Zamzam water is different from others, because it is a sterile water, where there is no bacterial or fungal contamination, no changes in color, taste and smell[2]. Four essential metal ions (sodium "Na", potassium "K", magnesium "Mg" and calcium "Ca") and seven transition metal ions (vanadium "V", manganese "Mn", iron "Fe", cobalt "Co", copper "Cu", zinc "Zn" and molybdenum "Mo") are well-known to be required for normal biological functions in humans[3-6].

Advanced approaches for elemental investigations of ZW were performed by Naeem's group, who analyzed 34 metal ions in ZW including calcium "Ca", magnesium "Mg" and sodium"Na" in higher concentrations than natural water[7]. Scientists suggested that high level of Ca ions makes food has certain healthier characteristics, accordingly the high Ca ions level of ZW provides an advantage to the human health[1]. Normal range of Ca ions in drinking water is 50 mg L^{-1} as reported by World Health Organization[8].

Very recently, it was reported that Ca ion is a cell cycle commander and controller of cancer cell death-mediated apoptosis to drives cancer cell differentiation and/or apoptosis in a phenomenon called "diffpoptosis"[9]. Calcium is already known as a promising chemopreventive agent[10-12].

Authors suggested that decreased calcium-containing diet is associated with increased risk of pre neoplastic intestinal lesions *in vivo*. Accordingly, calcium-enriched diet significantly suppressed cancer features in diseased mice[13]. Together, these observations showed the significance of underscoring the mechanistic pathways through which Ca ions drives cancer progression. Thus, this could be of great interest for designing of chemoprevention protocols, focusing on possible strategies of Ca ions at genomic and non-genomic levels to control the tumorigenesis[9].

In addition, less than 0.01 ppm of metal ions including antimony "Sb", cobalt "Co" and molybdenum "Mo" were found in Zamzam water. On the other hand, few traces of manganese "Mn" and titanium "Ti"ions were appeared in the samples of ZW[1]. Although arsenic "As", cadmium "Cd", lead "Pb" and selenium "Se"ions are known to be cytotoxic in high and moderate doses, their synergistic effect might assist cancer eradication if they were much below the danger level for human consumption[1].

The three most effective carcinogenic metal ions are Nickel "Ni", chromium "Cr" and cadmium "Cd". Nickel subsulfide, Ni_3S_2, found in many nickel-containing ores, has been extensively studied and shown to be carcinogenic in humans and animals, causing disturbances in DNA replication and repair machineries. Chromium is most carcinogenic as chromate ion (CrO^{2-}_4), which enters cells and damages its nucleic acid. The exact molecular mechanisms of metal-DNA interactions are still obscure. New approaches become recently available for studying these mechanisms of oncogenesis. Since cancer is a genetic disease, metal-DNA chemistry is likely to be prominent in such mechanisms[14,15].

Intriguingly, metallocenes and their halides (vanadium "V" and iron "Fe" ions) reported to be anticancer drugs[16], noticing that they should be in drinking water normal ranges (Up to 0.1 mg $L^{-1}$of V ions[17] and up to 0.3 mg L^{-1} of Fe ions[18,19]). Studies of Ehrlich ascites tumor cells treated with vanadium complex *in vitro* revealed selective inhibition of tumor progression, by blocking DNA replication. Also, metallocenes undergo rapid hydrolysis in aqueous media, forming aqua complexes that may have a higher affinity for phosphate oxygen atoms than the heterocyclic nitrogen atoms of the DNA bases. The exact mechanism of how the ferrocenium ion binds to DNA is still obscure, although partial metallo-intercalation and groove binding are more understandable[20].

Selenium (Se) is well-known as acrucial trace element with antioxidative, antimutagenic, antiviral and anticarcinogenic properties[21], noticing that it should be in the normal range of drinking water (0.01 mg L^{-1})[17,18]. Se is an important component of antioxidant enzyme glutathione peroxidase (GPx), which is critical for scavenging reactive oxygen species (ROS) and maintaining the oxidation-reduction (redox) balance. Recent studies have shown that oxidative stress is related to unrepaired DNA damage, mutating tumor suppressor genes, carcinogenesis and tumor's angiogenesis[22].

This study aimed at investigating the mitochondrial-based cytotoxic effects of different doses of Zamzam water (ZW) and ordinary tap water (TW) as water metal sources, as well as deionized water (DW) and double deionized water (DDW) as ions-free water sources on hepatic cancer HepG2 cells compared to untreated control cells. In addition, the current work is assessing the metallocenes-mediated apoptotic cell death underlying machinery upon ZW treatment.

MATERIALS AND METHODS

HepG2 cell culture: Human hepatoma (HepG2) cell line was purchased from VACSERA Co. (Egypt) which purchased it from American Type Culture Collection (ATCC, USA). HepG2 cells were cultured using a Roswell Park Memorial Institute

(RPMI-1640) medium, with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S) and 1% L-glutamine (Life Technologies, Gibco, Grand Island, NY). Cells were cultured in 5% CO_2 at 37°C and then treated with 0.25% (w/v) trypsin/EDTA to subculture cells for next passaging.

Growth inhibition assay: The cytotoxic effects of the Zamzam water (ZW)obtained from Saudi Arabia against tap water(TW) as metal-containing water sources as well as deionized water (DW) and double deionized water (DDW) as metal-free water sources (Milli Q Water System, Germany) at the concentrations (0, 25 and 50% v/v) were investigated on HepG2 (human liver cancer cell line) using the sulphorhodamine B (SRB) assay according to the Skehan's method[23]. In details, 0, 25 and 50% of water (ZW, TW, DW, or DDW) were completed to 100% with RPMI-1640 medium before treatment of HepG2 cancer cells.

Briefly, the cells were seeded in 96 well microtiter plates at a concentration of 1×10^5 cells/well and left for cell attachment on the plate for 24 h in 5% CO_2 at 37°C. After 24 h, cells were incubated for 4 h with the mentioned concentrations of the ZW, DW, DDW and TW. After incubation, the medium were discarded, the cells were fixed with 10% trichloroacetic acid (TCA) 150 µL/well for 1 h at 4°C (TCA reduce SRB protein binding). Then, the cells were washed with distilled water 3 times. Wells were stained for 30 min at room temperature with 50 µL of 0.4% SRB dissolved in 1% acetic acid at room temperature (25±2°C) and kept in dark place. After incubation, the SRB solution was poured off and the plates were washed with 1% acetic acid to remove unbound dye and to leave only the cell adhered dye. Then, the plates were air dried and the dye was solubilized with 150 µL/well of 10 mM tris base solution (pH 7.4) and the mixture was shaken for 5 min at room temperature (25±2°C). The optical density (OD) of each well was measured at 545 and 600 nm using a microplate reader (BIORad Instrument Inc., USA). The experiment was performed in triplicate and the percentages of cell viability were calculated.

Mitochondrial activity: The 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) from (Sigma, St Louis, MO, USA) is based on the conversion of MTT into formazan crystals by living cells, which determines mitochondrial activity[24].The effects of ZW on the mitochondrial activity were estimated by MTT assay using HepG2 cancer cells. Briefly, the cells were cultured in 96-well plates at a density of 1×10^4 cells/well. Wide range (5, 10, 20, 30, 40, 50% v/v) of ZW was added/well in RPMI-1640 over HepG2 cells. Also, media without treatment was used as control. After 4 h incubation, MTT dissolved in PBS was added to each well at a final concentration of 5 mg mL^{-1} and the samples were incubated at 37°C for 4 h. Water-insoluble crystals of formazan that formed during MTT cleavage in actively metabolizing cells were then dissolved in dimethyl sulfoxide (DMSO) using 100 µL/well. Absorbance was measured at 455 nm using a microplate reader (BIORad Instrument Inc., USA). The mitochondrial activity (%) was calculated and compared with the control.

Apoptosis assay: Annexin-V assay, based on the binding of the fluorescently-conjugated annexin-V to phosphatidylserine which has translocate to the cell membrane exterior during apoptosis. Briefly, a 24-well tissue culture plate was seeded with 5×10^4 cells/well in 500 µL culture medium and the cells were incubated at 37°C and 5% CO_2, until confluence is reached overnight. Cells were treated with 25 and 50% v/v of Zamzam water (ZW). We used 2 controls: Control (I) represents medium over HepG2 cells and Control (II) represents a mixture of 75% RPMI-1640 medium plus 25% double deionized water (DDW) over HepG2 cells. The measurements of annexin V were measured in triplicate using the fluorescence module of BMG LABTECH, Germany.

Disposal procedure of cell culture biohazards: Strictly disposal procedure of cell culture biohazards was applied. Where, all cell culture biohazards generated during this research were deactivated by autoclaving (if solid) or chemically treated (if liquid) before final disposal by sterility assurance using NEWSter NW5 machine.

Metal testing: Elemental analysis by inductively coupled plasma mass spectrometry (ICP-MS) was used for metal testing in Zamzam water (ZW) and ordinary tap water (TW) as water source of ions, as well as deionized water (DW) and double deionized water (DDW) as ions-free water sources (Can-Alt Health Laboratories, Canada).

Analytical technique used for elemental determination: ICP-MS (ELAN-DRC II) is an analytical technique used for elemental determination in ZW and TW, using DW and DDW as metal-free water sources. ICP-MS has the ability to handle simple matrices in each water candidate with a minimum of matrix interferences due to its high-temperature.

CanAlt's quality assurance understands that the health practitioner depends on reliable data to provide the best possible assessments for their clients. CanAlt's quality assurance System is based on: (1) Method validation program to assure reliability and reproducibility of data,

(2) Use of standard reference materials, (3) Instrument controls, (4) Inter batch controls and (5) Process blanks.

CanAlt Health Labs uses state-of-the-art ICP-MS technology (ELAN-DRC II) for accurate and reliable results. Combines Dynamic Reaction Cell™ technology and performance enhance Axial Field Technology™, providing interference correction for uncompromised sensitivity and performance. It used ion-molecule chemistry for improved detection limits, such as Fe, Ca, Mg, As, Se, Cr, V and others. It has exceptional sensitivity and stability using collisional focusing for relative standard deviations for isotope ratios of less than 0.03%. The quadrupole used in DRC technology provides high and low-mass cutoffs defining a precise mass band pass window preventing the formation of new species and interferences, thereby, eliminating false positives.

Statistical analysis: All biological experiments were performed in triplicate for 3 independent times (n = 3). Comparisons between treated and untreated groups were made using one way ANOVA (OriginPro 8 software) and values of $p<0.05$ were considered statistically significant.

RESULTS

Metal analysis: Inductively coupled plasma mass spectrometry (ICP-MS) was used for analyzing the elemental composition of Zamzam water (ZW), ordinary tap water (TW), deionized water (DW) and double deionized water (DDW). ZW analysis shows that it contains the highest composition of essential elements (sodium "Na" and calcium "Ca" ions) compared to TW. In ZW, Na and Ca ions show high concentrations of 85 and 47 mg L^{-1}, respectively, compared to TW which shows 25 mg L^{-1} for both ions as illustrated in (Fig. 1 and 2a).

The results observed that ZW has 0.5 and 0.3 mg L^{-1} of strontium "Sr" and boron "B" ions, respectively, compared to TW with concentrations of 0.25 and 0.02 mg L^{-1}, respectively, as illustrated in (Fig. 2b). EPA recommends that drinking water levels of stable strontium should not be more than 4 mg L^{-1}. WHO recommends that drinking water levels of boron should be less than 0.5 mg L^{-1}.

Few traces of selenium "Se", copper "Cu" and iron "Fe" ions also were found in ZW with concentrations 0.0031 mg L^{-1} of Se ions, 0.0035 mg L^{-1} of Cu ions, 0.0048 mg L^{-1}

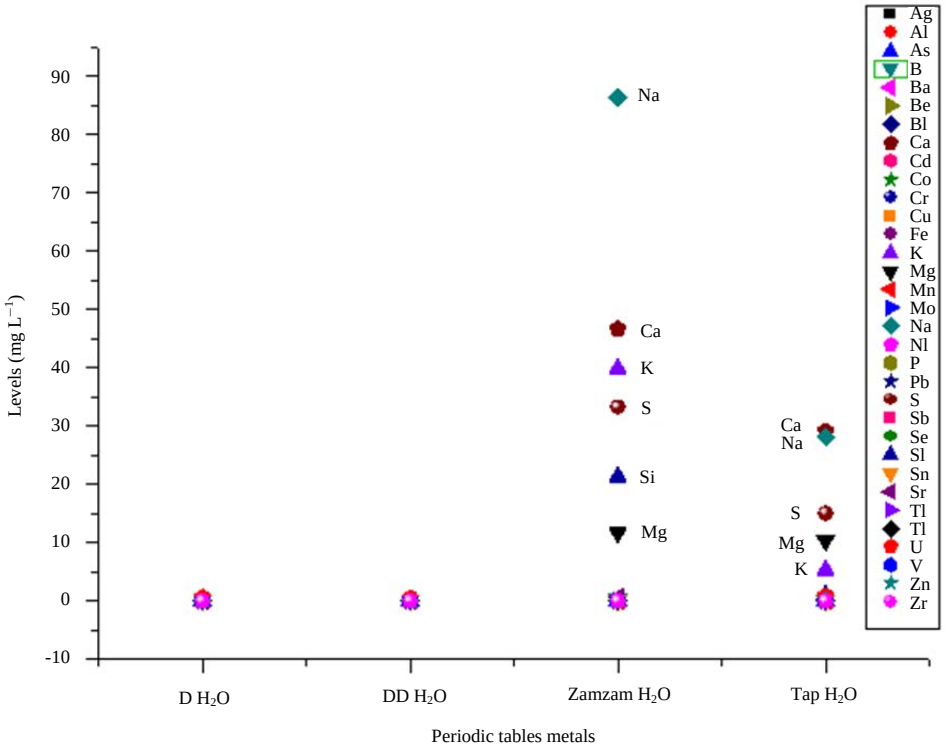

Fig. 1: An overview of the elemental analysis of Zamzam water (ZW) and tap water (TW)
Deionized water (DW) and double deionized water (DDW) were used as metal-free water sources. This analysis was performed using ELAN-DRC II ICP-MS

Table 1: Concentration ranges of essential elements and metallocenes in deionized water (DW), double deionized water (DDW), zamzam water (ZW) and tap water (TW)

	mg L^{-1}					
Elements	*BECs	DW	DDW	ZW	TW	WHO[17,18]
Sodium	0.22×10^{-6}	**ND	0.00007	86.301	28.152	Up to 200
Magnesium	0.18×10^{-6}	0.0097	0.0069	11.68	10.3	Up to 30
Calcium	0.1×10^{-6}	ND	ND	46.649	29.087	Up to 50
Vanadium	0.04×10^{-6}	0.00012	ND	0.001666	0.00267	Up to 0.1
Iron	0.4×10^{-6}	ND	ND	0.00484	0.00569	Up to 0.3
Selenium	1×10^{-6}	ND	ND	0.0031	ND	Up to 0.01

* BECs : Background equivalent concentrations and detection capabilities. The ELAN DRC II can dramatically reduce or eliminate these interferences, providing enhanced analytical capabilities. **ND: Lower than background equivalent concentrations and detection capabilities (BECs), DW: Deionized water, DDW: Double deionized water, ZW: Zamzam water, TW: Tap water

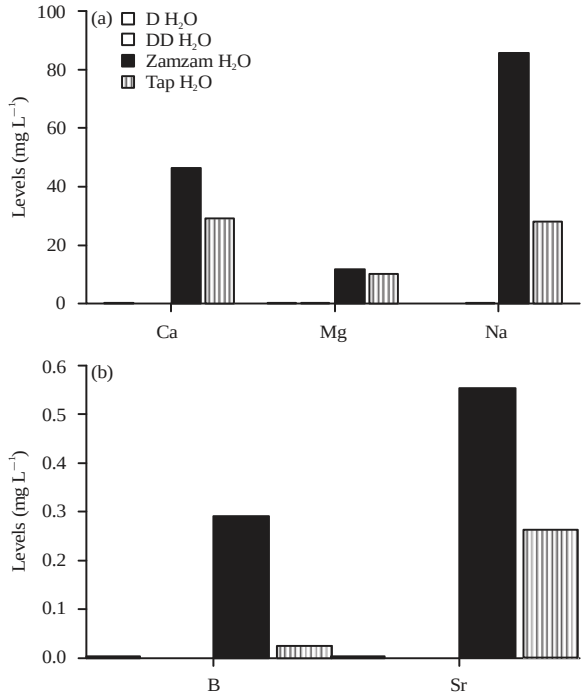

Fig. 2(a-b): The elevated essential elements in Zamzam water compared to tap water (TW)

Deionized water (DW) and double deionized water (DDW) were used as metal-free water sources. This analysis was performed using ELAN-DRC II ICP-MS

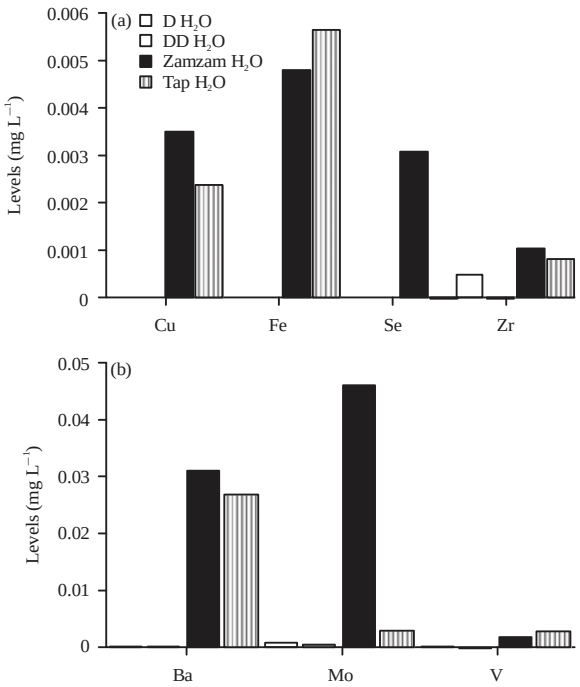

Fig. 3(a-b): The traces and some anticancer elements (Se, V and Fe) in Zamzam water compared to tap water (TW)

Deionized water (DW) and double deionized water (DDW) were used as metal-free water sources. This analysis was performed using ELAN-DRC II ICP-MS

of Fe ions compared to TW in concentrations where, Se ions is not detected, 0.0024 mg L^{-1} of Cu ions and 0.0056 mg L^{-1} of Fe ions (Table 1). Se and Fe ions showed values less than 0.4×10^{-6} in DW and DDW, due to the ELAN DRC II can dramatically reduce or eliminate matrix-based interferences, providing enhanced analytical capabilities. Interference removal provides superior detection capabilities. Many elements suffer from common matrix-based interferences that can degrade background equivalent concentrations (BECs) and detection capabilities as illustrated in (Fig. 3a and Table 1).

In addition, ZW has traces of molybdenum "Mo", barium "Ba" and vanadium "V" ions levels in concentrations 0.046 mg L^{-1} of Mo ions, 0.031 mg L^{-1} of Ba ions and 0.0166 mg L^{-1} of V ions. These results compared to TW which scored 0.0028 mg L^{-1} of Mo ions, 0.026 mg L^{-1} of Ba ions and 0.0026 mg L^{-1} of V ions. Also, it was noted that the drinking water acceptable values are 0.07 mg L^{-1} of Mo ions, 0.7 mg L^{-1} of Ba ions and 0.1 mg L^{-1} of V ions, as illustrated in (Fig. 3b and Table 1).

Table 2: Concentration ranges of cytotoxic elements in deionized water (DW), double deionized water (DDW), zamzam water (ZW) and tap water (TW) compared with WHO and USEPA ranges

		mg L^{-1}						
Elements	Symbol	*BECs	DW	DDW	ZW	TW	WHO[17,18]	USEPA[19]
Beryllium	Be	0.87×10^{-6}	0.0001	0.00001	0.00003	0.00001	0.00	0.004
Cadmium	Cd	0.11×10^{-6}	0.00007	ND	ND	0.00006	0.003	0.005
Aluminum	Al	0.09×10^{-6}	ND	ND	0.005	0.035	0.20	0.05-0.2
Lead	Pb	0.09×10^{-6}	ND	ND	ND	ND	0.01	-
Arsenic	As	1.6×10^{-6}	ND	ND	0.005	0.001	0.01	0.005
Antimony	Sb	0.08×10^{-6}	ND	ND	0.002	0.002	0.02	0.006
Nickel	Ni	0.2×10^{-6}	ND	ND	ND	0.019	0.02	-

*The ELAN DRC II can dramatically reduce or eliminate matrix-based interferences, providing enhanced analytical capabilities. DW: Deionized water, DDW: Double deionized water, ZW: Zamzam water, TW: Tap water

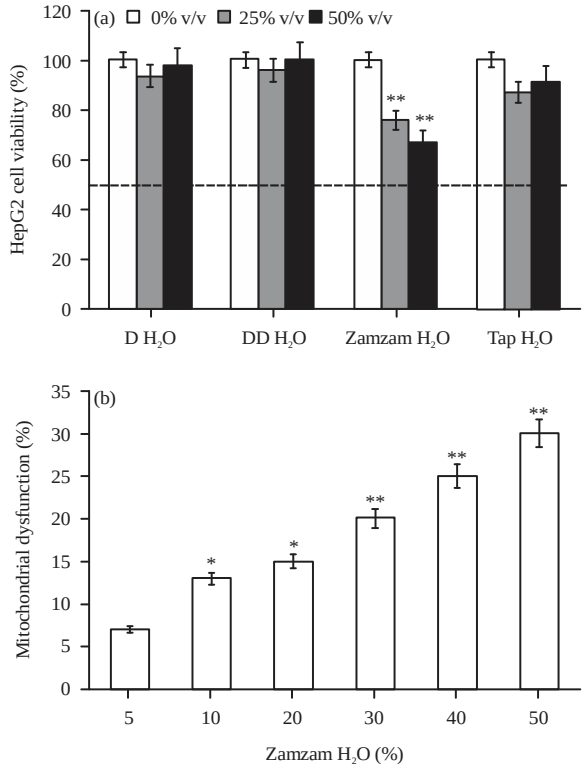

Fig. 4(a-b): Zamzam metallocenes induce (a) HepG2 cytotoxicity via triggering and (b) Mitochondrial dysfunction

(a) SRB-based cytotoxicity assay of HepG2 cells treated with 0, 25 and 50% v (ZW)/v (RPMI-1640 medium) and (b) MTT-based assay was performed to test the effect of different ZW doses (5, 10, 20, 30, 40 and 50% v/v) on HepG2 mitochondrial function. *Statistical significant differences ($p>0.05$) when comparing treatments with control. **High statistical significant differences ($p>0.01$) when comparing treatments with control

Although there are some elements considered to be cytotoxic if over-presented in the drinking water, the World Health Organization "WHO" and the United States Environmental Protection Agency "USEPA" reported the elemental limits of the healthy drinking water including these toxic elements. Accordingly, Table 2 showed the results of these proposed toxic elements (beryllium "Be", cadmium "Cd", aluminum "Al", lead "Pb", arsenic "As", antimony "Sb" and nickel "Ni" ions) and found that all of them in ZW and TW are within the allowable range of WHO and USEPA (near to the absolute zero). Similarly, we tabulated (Table 1) the normal ranges in drinking water of essential elements (sodium "Na", magnesium "Mg" and calcium "Ca"), metallocenes (vanadium "V" and iron "Fe") and selenium "Se" ions. In this study found that all of these elements are in the normal ranges required for healthy individuals.

Zamzam water induces mitochondrial dysfunction-associated cell death: In Fig. 4a, compared the effectiveness of different doses of ZW, TW, DW and DDW on the proliferative ability of the human HepG2 liver cancer cells compared to the untreated cells as a control. ZW, compared with other water types, produced remarkably superior effects with 25 and 50% v/v doses after 4 h of incubation. Interestingly, 50% v/v of ZW has higher cytotoxic effect than 25% v/v dose, with approximately 35% SRB-based cell death. These results showed a dose dependent decrease in cell viability of HepG2 cells after Zamzam water treatment versus other water types. The SRB assay results were confirmed by the MTT-based assay, which reflect the gradual occurrence of mitochondrial dysfunction of HepG2 cancer cells as illustrated in (Fig. 4b).

Zamzam water drivesapoptotic-mediated cell death: Next, determined the underlying mechanism of the anti-cancer effect of Zamzam water. We observed a gradual apoptotic induction of HepG2 cells treated with 25 and 50% ZW for 4 h of incubation (Fig. 5). Figure 5 shows that ZW metallocenes and anti-cancer elements may trigger apoptosis after

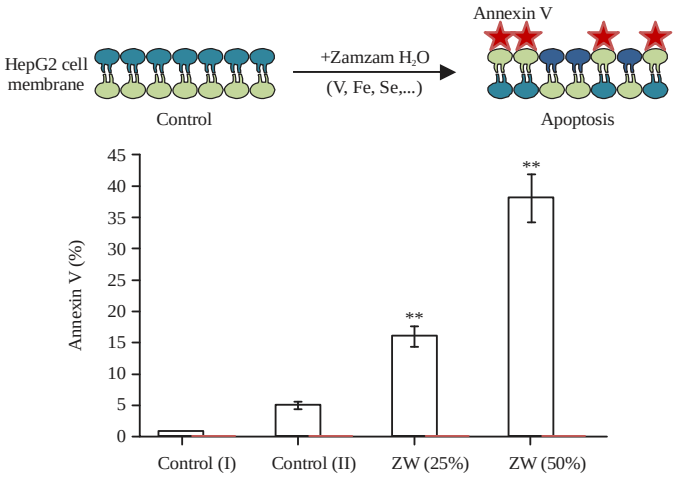

Fig. 5: Zamzam metallocenes triggers apoptosis after treatment with Zamzam water (ZW)

For apoptosis detection, Annexin V binds to the flipped phospholipids in HepG2 cellular membrane. There was gradual induction of apoptosis by increasing ZW doses (25 and 100% v/v). Control (I) represents RPMI-1640 medium over HepG2 cells, while Control (II) represents a mixture of 75% RPMI-1640 medium plus 25% double deionized water (DDW) over HepG2 cells. The measurements of annexin V were measured in triplicate using (BMG multiplate reader). **High statistical significant differences (p>0.01) when comparing treatments with controls

treatment with 25 and 50% of ZW. For apoptosis detection, annexin V binds to the flipped phospholipids in HepG2 cellular membrane (Scheme of Fig. 5). There were gradual inductions of apoptosis (16 and 38%) by increasing ZW doses (25 and 50%). Control (I) represents medium over HepG2 cells, while: Control (II) represents a mixture of 75% RPMI-1640 medium plus 25% double deionized water (DDW) over HepG2 cells.We hypothesized control (II) to be as a negative control in order to see the effect of ions-free water versus ZW on HepG2 cells, where we reported that there were no significant difference between control (I) and control (II). Eventually, it was found that ZW is apoptosis-relevant anti-cancer water, where HepG2 cell population was shifted to be apoptotic cells upon treatment with ZW, recording the highest effect at 50% administration of ZW.

DISCUSSION

In the current study, found that Zamzam water is explicitly rich, but within normal ranges, in essential elements (Na, Ca, K and Mg ions), metallocenes (V and Fe ions), which have anticancer properties [16,17] and Se, which is well-known as an anticancer agent[19,20]. This observation is in agreement with previous studies indicated that Na, K, Mg and Ca are essential metals and required for biological homeostasis in human beings[3,5].

Very recently, it was reported that Ca ions, in its normal range (50 mg L^{-1}), are a cancer modulator[9] and it was suggested that Ca ions are a promising chemopreventive agent[10-12]. Cells in the early post-initiation stages of cancer are probably proliferatively restrained by elements flowing into them from their normal neighbors through their functional gap junctions. However, gap junctions are designed to be slammed shut by Ca ions. This is meant to prevent the draining of components from the cellular network through a damaged cell[25]. Calcium ion was chosen as the damage detector and drain plugger simply because its accumulation is a universal consequence of cell injury. The closing of gap junctions by supra-optimal Ca ions loading would release the initiated colon cells from the restraining influences of their normal neighbors. However, the importance of natural and essential elements in cancer chemoprevention[26], it was reported that, in few studies, Ca ions from dietary supplements increases the risk of advanced prostate cancer[27], if taken by very high doses.

After several mutations in the carcinoma cells have completed the divorce from control by external Ca ions. The extent of this divorce is indicated by the fact that while the cells in the early hyperplastic stage up to the well-differentiated adenocarcinoma stage still express Ca receptors[28,29].

The presence of metallocenes (V and Fe) in Zamzam water (ZW), within their normal ranges, gave it the oncolytic power against HepG2 cells, where we found that ZW at 50% v/v dosage post 4 h of incubation produces the highest tackling effect against cancer cells with about 35% of cell death associated with mitochondrial impairment with a dose dependent decrease in cell viability of HepG2 cells upon treatment.

Some medical experts consider vanadium "V" supplements as anticancer drug, because of its potential toxic effects[16,17]. However, V deficiency is rare in human, if there is a deficiency, it may cause the following: (1) Elevation of molybdenum, calcium and magnesium levels, (2) Reduced growth and reproductive ability, (3) Elevation of LDL "bad cholesterol". These studies are only in the preliminary stage and more research is required to confirm their results. However, the ranges of daily vanadium needed, it was suggested that vanadium works best with calcium, magnesium and selenium.

Directive 2002/46/EC of the European Parliament and of the Council on the approximation of the laws of the Member States relating to food supplements recorded the intake of vanadium from normal food which is estimated to be of the order of 10-20 μg/day. This daily intake is at least three orders of magnitude below the lowest doses reported to cause adverse effects. In the case of supplements used by athletes and body builders, however, the intake can be similar to the doses causing adverse effects in rats and humans. Therefore, a risk can be expected to result from the prolonged ingestion of such supplements.

Mechanistically, the minute traces of Seions, within its normal range, provided a plus advantage to ZW, where in that concentration range, Se much below the danger level for human consumption and acts as a signaling stimulator for anti-cancer cascade through apoptosis[19,20]. This finding supports the current study indicated that ZW is an apoptotic anti-cancer candidate, where 0.84% of the apoptotic cells in the control increased significantly to be 38% post-treatment.

Recent studies found that Seions may be a potential chemopreventive agent for breast and colon cancer[30-32]. However, the underlying mechanisms of tumor chemoprevention by Seions remain to be elucidated. Additionally, acute therapy of breast and colon cancer cells has been investigated to result in apoptosis[21,33], which in agree with our expectation indicating that Se ions in ZW may be one of reasons causes HepG2 cell apoptosis-mediated cell death.

To date, most experimental studies have investigated only the acute effects of Se ions on cell growth inhibition and cytotoxicity. The possible mechanisms by which Se ions is postulated to decrease the incidence of cancer include inhibition of oxidative damage to DNA, recharging of cellular proliferation, modulation of apoptosis and alteration of cellular gene expression[31].

The adaptable mechanisms of cell cycle arrest and apoptosis are extremely complex and for Seions they mainly involve a mitochondrial pathway and protein kinases[34]. This confirms our SRB protein-based cytotoxicity and MTT-based mitochondrial cell death results. Also, Se ions may be related in part to the caspase-3 activation and Bax cleavage mediated by caspase-dependent calpain activation, resulting inhibit tumor promotion and inducing apoptosis[35].

The presence of V and Fe ions metallocenes in our ZW provide a favor over other water types, because previously it was reported that it act as anticancer intervention[16,17]. This observation is supported by a previous study on Ehrlich ascites tumor cells treated with V complex revealed strong anti-tumor effect by DNA interfering[18]. In addition, authors confirmed that Fe binds to the genetic material making metallo-intercalation at DNA grooves[36].

Although there are some elements considered to be cytotoxic if over-presented in the drinking water, the WHO and the USEPA reported the elemental limits of the healthy drinking water including these toxic elements. Our results of these cytotoxic elements (beryllium "Be", Cadmium "Cd", mercury "Hg", aluminum "Al", lead "Pb", arsenic "As", antimony "Sb" and nickel "Ni" ions) indicated that they are in ZW and TW within the allowable range of WHO and USEPA (near to the absolute zero). Synergizing of the traces in ZW is useful for triggering multiple biological signaling cascades in human beings[7,8].

It was reported that ZW has strong anti-cancer and anti-inflammatory activities, where it down-regulates cancer cell growth-mediated genes, integrin, insulin like growth factor, nuclear factor of kappa light polypeptide gene enhancer in β-cells 1 (p105) and interleukin 12A[37]. Otherwise, Zamzam water has oncolytic action through an indirect influence on endocrine immunology and the growth system of the body[38]. To study that action, samples were taken from tumor tissues from colon cancer-induced models before and after feeding the mice with 500 cc of Zamzam water daily for a month[39]. The authors found a significant $p<0.05$ reduction in the tumor size, owing to the elemental nature of Zamzam water and that it directly affects the lymphocytes residing in the colon, which in turn affect tumorgenesis by specific immune mechanisms, resulting in decreased tumor size[40] all of these observations support our current findings of that Zamzam water may act as a natural anti-cancer candidate like other recently reported natural compounds[41-44].

Very recently, two publications[45,46] supported the current results, where the authors indicated that ZW treatment reduced cell viability of colon cancer HCT 116[45] and lung cancer A549[46] cell lines. In parallel with the current results, it was suggested that MTT assay showed a significant $p<0.05$ reduction in cell viability after treatment with ZW and cell death has occurred through apoptotic pathway.

Ali *et al.*[47], treated 50 healthy fertile females with 750 cc of Zamzam water daily for a month and other 50 healthy fertile females with ordinary tap water. They observed significant $p<0.05$ increases in three oncopreventive markers, Bikunin, Lunasin and Bowman Brik.

Evaluation of antitumor properties of organometallic metallocene complexes[48] of Se, V, Fe and Ca ions that were detected in the current study on ZW as new approaches for cancer treatment is warranted to be performed in our next experimental study using ZW. Finally, Zamzam water metallocenes could be of great interest to be studied against viral-originated cancers, because some minerals work as signals for certain human hormones, like growth hormone, that associated with viral response[49].

CONCLUSION

Zamzam water (ZW) is a prodigy. ZW inhibits liver cancer cells proliferation *in vitro* using HepG2 cell line. Numerous gains of ZW have been elucidated, but a few scientific papers were published for interpreting this important issue. However, *in vitro* and *in vivo* studies have showed great benefits of ZW in fighting various human ailments, especially cancer. These publications supported by the current study reported the balanced mineral and nutritional composition of ZW, which synergizing with other anti-cancer components to thwart cancer progression. Comprehensive research regarding ionic complexes in ZW and their impact *in vivo* cancer models are warranted to be explored in ZW in the future studies to test its unique mysteries.

SIGNIFICANCE STATEMENTS

This study discovers the possible synergistic effect of metallocenes in Zamzam water that can be beneficial for treatment of hepatocellular carcinoma. This study help the researchers to uncover the critical area of hepatic cancer apoptosis-mediated cell death through Zamzam water metallocenes that many researchers were not able to explore. Thus, a new theory on these unique elemental components found in Zamzam water and possibly other combinations, may be arrived at.

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