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RESEARCH ARTICLE

Evaluation of the Cytotoxicity and Apoptotic Induction in Human Liver Cell Lines Exposed to Three Food Additives

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Abstract: ***Background*:** Rapid lifestyle, especially among people living in urban areas, has led to increasing reliance on the processed food market. Unfortunately, harmful effects caused by the excessive use of food additives in such type of industry are often neglected.

***Objective*:** This proposal investigates *in vitro* cytotoxic and apoptotic effects of three food preservatives commonly consumed in daily meals; sodium sulphite, boric acid, and benzoic acid.

***Methods*:** The effect of the three preservatives on cell viability was tested on two different cell lines; normal liver cell line THLE2 and human hepatocellular carcinoma cancer cell line HepG2 using MTT assay. Cell cycle arrest was measured using flow cytometry by propidium iodide. Measurement of expression levels of two central genes, p53 and bcl-2 that play key roles in cell cycle and apoptosis was carried out in HepG2 cells using real time-PCR.

***Results*:** Although the effect was more significantly realized in the HepG2 cell line, the viability of both cell lines was decreased by all of the three tested compounds. Flow cytometric analysis of HepG2 cells treated with sodium sulphite, boric acid, and benzoic acid has revealed an increase in G2/M phase cell cycle arrest. In Sodium sulphite and boric acid-treated cells, expression levels of p53 were up-regulated, while that of the Bcl2 was significantly down-regulated. On the other hand, Benzoic acid has shown an anti-apoptotic feature based on the increased expression levels of Bcl-2 in treated cells.

***Conclusion*:** In conclusion, all of the tested compounds have decreased the cell line viability and induced both cell cycle arrest and apoptotic events indicating their high potential of being cytotoxic and genotoxic materials.

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1. INTRODUCTION

Food additives are substances added to food either as preservatives to extend their lifetime by inhibiting the growth of microorganisms or to serve the purpose of enhancing the taste and appearance [1]. The allowed amounts of food additives are regulated by food and drug administration FDA in an attempt to reduce the possible overconsumption of these substances. However, the Longtime consumption of food additives even in small amounts, may harm the consumer [2]. Despite all the efforts, food additives are extensively used on a daily basis, ignoring that none are without some level of risk accompanied by abusive consumption of these materials, or their accumulation in the body [3]. In some developing countries, the situation is more complicated because in spite of regulation and restrictions by the prevention of the Food Adult reaction Act of 1954, the use of non-permitted food additives is still prevalent [4, 5]. It was well documented that certain types of foods and beverages in addition to the additives may pose toxic, genotoxic or carcinogenic hazards on human health [6, 7]. A few years ago, food additives have begun to attract attention as potential causes of various human diseases; they might be added to the factors responsible for the outbreak of cancer, mutagenesis, hepatic and nephritic failures and more [8-10].

All of the three tested compounds are well known for being used as preservatives in the food industry. Sodium

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sulphite is a preservative used to stop the browning and further ripening of fruits. Auerbach (1976) [11] reported that sodium sulphite motivates an array of cytological effects on *Vicia faba* root mitotic cells affecting all the stages of the mitotic cycle. Benzoic acid, a commonly used preservative as an antimicrobial substance in many food products, was found by Al-Tai (2014) [12], to be toxic to peripheral lymphocytes. Boric acid, an effective preservative against yeast and bacteria, was found to inhibit the proliferation of prostate cancer cell lines, DU-145 and LNCaP in a dose-dependent manner [13]. Borax, a salt of boric acid, had an inhibitory effect on HepG2 cell growth that induced apoptosis in a concentration-dependent manner [14].

Cytotoxicity tests are useful in detecting basal cytotoxic events common to various cells, but not in detecting organ-specific toxins [15]. The cytotoxicity test applied to evaluate food safety is expected to be useful for determining total cytotoxicity potential resulting from additive synergistic and antagonistic actions in the presence of different toxins [16]. Concerning cell culture applications, the first and most commonly used tetrazolium salt is MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to measure proliferation and cytotoxicity in high-throughput screening approaches in 96-well plates [17]. Tetrazolium salts are colorless or only weakly colored solutions which upon reduction change to a strongly colored formazan product [18]. The reduction of MTT salt to formazan colored crystals is based on the metabolic activity of living cells [19] that occurs only in viable cells by mitochondrial enzymes like oxidoreductases, dehydrogenases, oxidases, and peroxidases using NADH, NADPH, succinate, or pyruvate as an electron donor [20, 18]. MTT-based assay protocols usually include a cell lysis step and a formazan-dissolving step before spectrophotometric measurement can be performed. In spite of its advantages of being rapid and simple, the formation of an insoluble product and the necessity to dissolve it exclude this assay for any real-time assays [18]. Cytotoxic agents, radiation or drug-induced cell death could result in G2-M arrest. This phenomenon is usually combined with damaged or incomplete mitosis [21, 22]. Single time-point cell measurement is a flow cytometry technique that has been developed to analyze the cell cycle. It reveals cell percentage in G1 *vs*. S *vs*. G2/M without supporting information on cell cycle kinetics. The duration of each phase, however, can be deducted from the percentage of cells in this phase if the length of the cell cycle is known [23-25].

Apoptosis is a form of programmed cell death, a crucial pathway for regulating homeostasis, responding to DNA damage, and controlling cell proliferation. Apoptotic cells including cancerous ones, undergo a natural process of dying [26]. Two major pathways that regulate apoptosis are the cell death receptor-mediated extrinsic pathway and the mitochondrial-mediated intrinsic apoptotic pathway [27]. Three central and widely investigated as stress markers genes are p53, bcl-2 and bax because these genes play key roles in cell cycle and apoptosis [28, 29]. The P53 is a key tumor suppressor gene that has a crucial function in apoptosis [30] that also plays a critical role in the regulation of cell cycle arrest in the G2/M phase and is involved in DNA repair during activation of ribonucleotide reductase [31]. Apoptosis could be stimulated by the p53 gene through downregulation of Bcl-2 gene, activation of caspases [32] and contribution to the transcriptional activation of large numbers of target genes including Bax (pro-apoptotic protein) [33]. Bcl-2 family genes play a pivotal role in controlling the mitochondrial pathway of apoptosis [34, 35], which consists of anti-apoptosis genes, such as Bcl-2, Bcl-xl and pro-apoptosis genes, such as Bax, Noxa, Puma, Bim, [36].

The objective of this study was to evaluate the cytotoxic effects of three food additives; sodium sulphite, boric acid, and benzoic acid using MTT assay. The possible underlying anti-proliferation mechanisms were investigated, by studying the effect of the tested compounds on cell cycle arrest, cell apoptosis and apoptosis-related genes of the HepG2 cell line.

2. MATERIALS AND METHODS

2.1. Assessment of Cytotoxicity Using MTT Assay

The HepG2 and THLE2 cell lines [obtained from the American Type Culture Collection (ATCC), Manassas, Virginia, USA] were used in the present study. Cell proliferation and viability were estimated through the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) colorimetric assay (Mossman 1983 & Edmondson *et al.*, 1988).

A 100 µl cell suspension was added to each well of a 96-well microtitre plate (Corning Cell WellsTM, Corning, USA). The three tested compounds were added to the wells with different concentrations (6.25, 12.5, 25, 50, 100 and 200 µl) and incubated for 24 hours at 37°C in a 5% CO_2 humified incubator (Forma Scientific CO2 Water Jacketed Incubator). The same concentrations of cisplatin, a chemotherapeutic drug, were added as a reference and the microplates were incubated for a further 48 hours in DMEM medium (200 µL). Ice-cold PBS was used to wash the medium gently two times, followed by the addition of 200 µL MTT (Molecular probes, Eugene, Oregon, USA; Cat.no.V-13154)] to each well. The microplate was incubated at 37°C for another 4 hours in the CO_2 incubator. About 180 µL medium/MTT was removed from each well and replaced with 100 µL of acidified isopropanol to solubilize the produced formazan. Finally, the microplate was incubated at 37°C with shaking for 15 minutes. The absorbance of each well was measured at 630 nm using a microplate reader (ELX800, Biokit, spain). Assays were performed in triplicates in three independent experiments. Sigmoidal and dose-dependent curves were constructed to plot the results of the experiment. The concentration of the compounds inhibiting 50% of cells (IC50) was calculated using this sigmoidal curve.

2.2. Cell Cycle Analysis by Propidium Iodide (PI) Using Flow Cytometry

Treated HepG2 cells were digested using a mixture of 500 µl warm Trypsin-EDTA and 500 µl warm PBS-EDTA then incubated for 10 minutes at 37°C. Centrifugation was carried out at 450 rpm for 5 min followed by careful removal of the supernatant. The pellet was washed twice in warm PBS, then re-suspended in 500 µl of the warm buffered saline, centrifuged and supernatant was aspirated. A volume of

150 µl PBS and 350 µl ICE-cold absolute ethanol was added, mixed with a pipette then vortexed several times and incubated at 4°C for 1 hour to fix the cells. Ethanol was removed from the mixture by centrifugation at 350 rpm for 10 minutes and decant the supernatant. Again, the pellet was washed twice in warm PBS and the cell pellet was resuspend in 500 µl of the warm buffered saline, centrifuged and the supernatant was removed. The remaining pellet was resuspended in 100 µl PBS and stored at 4° for up to 4 days. Cells were stained with 100 µl of PI solution + 50 µl RNase A solution (100 µg/ml) and incubated in darkness for 30-60 min. The stained cells were analyzed using the Attune flow cytometer (Applied Bio-system, US).

2.3. Determination of the Expression Levels of Apoptosis-regulatory Genes

Total RNA was isolated from HepG2 cells using Gene JET RNA Purification Kit (Thermo Scientific, # K0731, USA) according to the manufacturer's protocol. Total RNA (5µg) was reverse transcribed using Revert Aid H Minus Reverse Transcriptase (Thermo Scientific, #EP0451, USA) to produce cDNA. The cDNA was used as a template to determine the relative expression of the apoptosis-related genes using StepOnePlus real-time PCR system (Applied Biosystem, USA).

Sequences of the primers for the amplification of the genes of interest were designed by Primer 5.0 software as follows; p53 forward 5'-CCCAGGTCCAGATGAAG-3', p53 reverse 5'-CAGACGGAAACCGTAGC-3', Bcl-2 forward5'-GGATGCCTTTGTGGAACTGT-3' and Bcl-2 reverse 5'-AGCCTGCAGCTTTGTTTCAT-3'. The housekeeping gene β-actin was used as a reference to calculate fold change in target gene expression. A 25-µL PCR mix was prepared by adding 12.5 µL of 2X Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, # K0221, USA), 2 µL of cDNA template, 1 µL forward primer, 1 µL reverse primer, and 8.5 µL of nuclease-free water. The thermal cycling conditions were programmed as follows: initial denaturation at 95°C for 10 min, 40-45 cycles of amplification of DNA denaturation at 95°C for 15 s, annealing at 60°C for 30 s, extension at 72°C for 30 s. At the end of the last cycle, the temperature was increased from 63 to 95°C for melting curve analysis. The cycle threshold (Ct) values were calculated for target genes and the housekeeping gene, then relative gene expression was determined using 2-ΔΔCt method.

2.4. Statistical Analysis

All data were expressed as means ± standard error (SE). The statistical significance was evaluated by one way ANOVA using SPSS 18.0 software. Values were considered statistically significant when $P \leq 0.05$. A comparison of means was carried out with Tukey's Honestly Significant Difference (Tukey's HSD) test.

3. RESULTS

3.1. *In Vitro* Assay for Cytotoxic Activity Human Cell Lines by MTT Assay

The effect of different concentrations (6.25, 12.5, 25, 50, 100 and 200 µg/ml) of each of the tested compounds; sodium sulphite, boric acid, and benzoic acid on the viability of HepG2 and THLE2 cell lines compared with cisplatin as a positive control was assessed using MTT assay. The obtained results showed that the viability of both cell lines was significantly reduced in a dose-dependent manner. Dose-response curves for cell viability are illustrated in Figs. (**1-3**). The effect of sodium sulphite on HpeG2 cell line viability was more drastic recording IC50 of 69.44 µg/ml compared to IC50 of 20.15 µg/ml recorded for cisplatin-treated cells. On the other hand, sodium sulphite treated THLE2 cells recorded IC50 value of 2094 µg/ml, while cisplatin-treated cells IC50 value was 645.7 µg/ml (Fig. **1**). The same effect was realized in boric acid and benzoic acid-treated cells. The IC50s were 64.27 µg/ml and 612.8 µg/ml for boric acid treated HepG2 and THLE2, respectively as (Fig. **2**). The value of IC50 for benzoic acid-treated HepG2 cells was 44.73 µg/ml, while that of THLE2 treated cells was 1884 µg/ml (Fig. **3**). The results suggest that all tested compounds are not toxic at 6.25 µg/ml concentration on HepG2 and THLE2, but significantly diminished the cell viability of

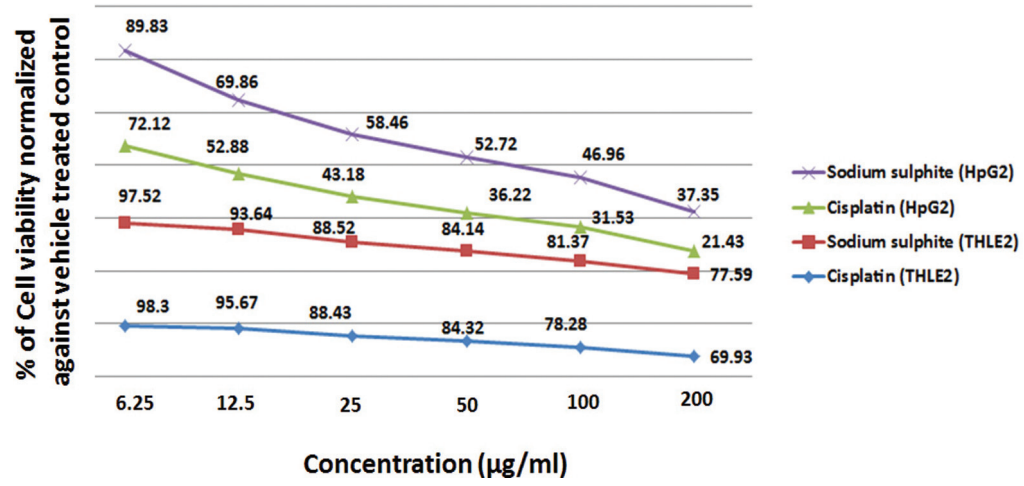

Fig. (1). Dose-dependent growth inhibition by cisplatin (positive control) and sodium sulphite on Human hepatocellular carcinoma (HepG2) and non-malignant human liver cells (THLE2). Cell viability as quantified by MTT assay. Results are mean ± (n = 3). *p<0.05 to compared to control. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

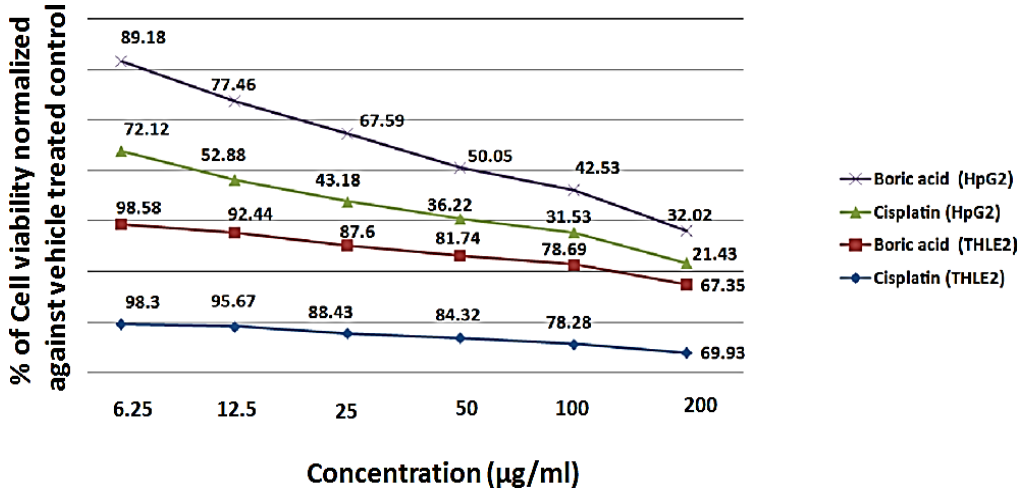

Fig. (2). Dose-dependent growth inhibition by cisplatin (positive control) and boric acid on Human hepatocellular carcinoma (HepG2) and non-malignant human liver cells (THLE2). Cell viability as quantified by MTT assay. Results are mean ± (n = 3). *p<0.05 to compared to control. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

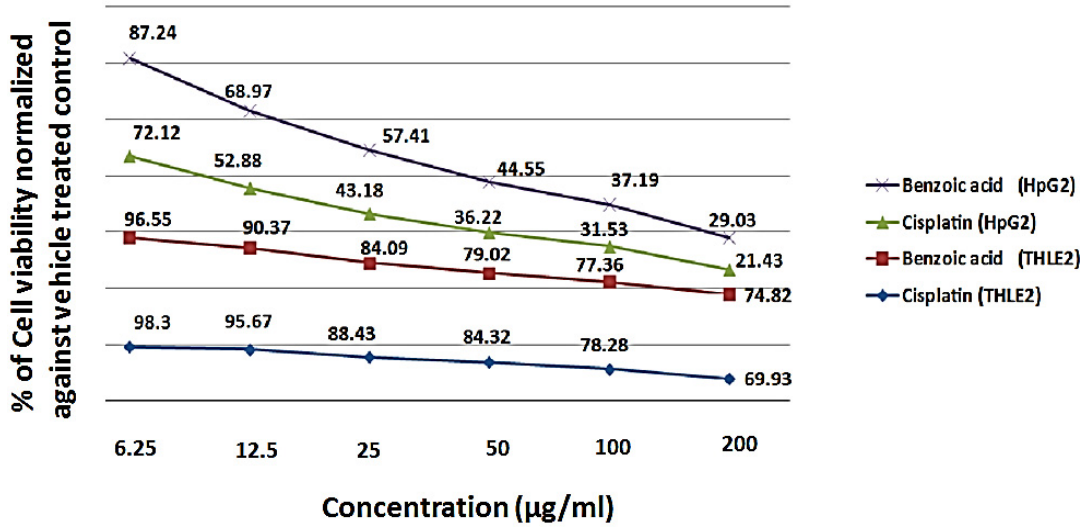

Fig. (3). Dose-dependent growth inhibition by cisplatin (positive control) and benzoic acid on Human hepatocellular carcinoma (HepG2) and non-malignant human liver cells (THLE2). Cell viability as quantified by MTT assay. Results are mean ± (n = 3). *p<0.05 to compared to control. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

HepG2 line cancer cells, beginning at 12.5 µ g/ml and increasing in a dose-dependent manner. These data indicated that sodium sulphite, boric acid, and benzoic acid had a potential cytotoxic effect on both malignant and non-malignant cells.

3.2. Cell Cycle Analysis by Propidium Iodide (PI) Using Flow Cytometry

Based on the cytotoxicity results obtained by MTT assay, the cytotoxic effect of the tested compounds was further investigated on the HepG2 cell line using cell cycle analysis by flow cytometry.

Compared to the control group, the IC50 concentration of sodium sulphite has affected the cell cycle distribution of HepG2 cells. Percentage of cells in G0/G1 had decreased from 60% to 52%, while the S-phase slightly decreased from 19% to 18% and finally the percentage of HepG2 cells at the G2/M phase was increased after sodium sulphite treatment from 21% to 30% as compared to control (Fig. **4**). Cells treated with IC50 concentration of boric acid has resulted in a drop in G0/G1 phase percentage which has decreased from 60% recorded in control to 43% in boric treated cells, while the S-phase was slightly increased from 19% to 20%. The percentage of HepG2 cells at the G2/M phase was increased after incubation with boric acid to 37% as opposed to 21% recorded in untreated cells (Fig. **5**). The same effect on cell cycle distribution was shown by benzoic acid-treated cells (Fig. **6**). The G0/G1 phase showed a decrease from 60% to 36%. The S-phase percentage was not affected by benzoic acid treatment. While the percentage of HepG2 cells at the G2/M phase was significantly increased after incubation with benzoic acid reaching 45% as opposed to the 21% recorded in untreated cells.

The obtained data exhibit clear accumulation of HepG2 cells at the G2/M phase, hence, confirming that sodium sulphite, boric acid and benzoic acid might have cytotoxic effect *via* induction of G2/M phase arrest of the cell cycle.

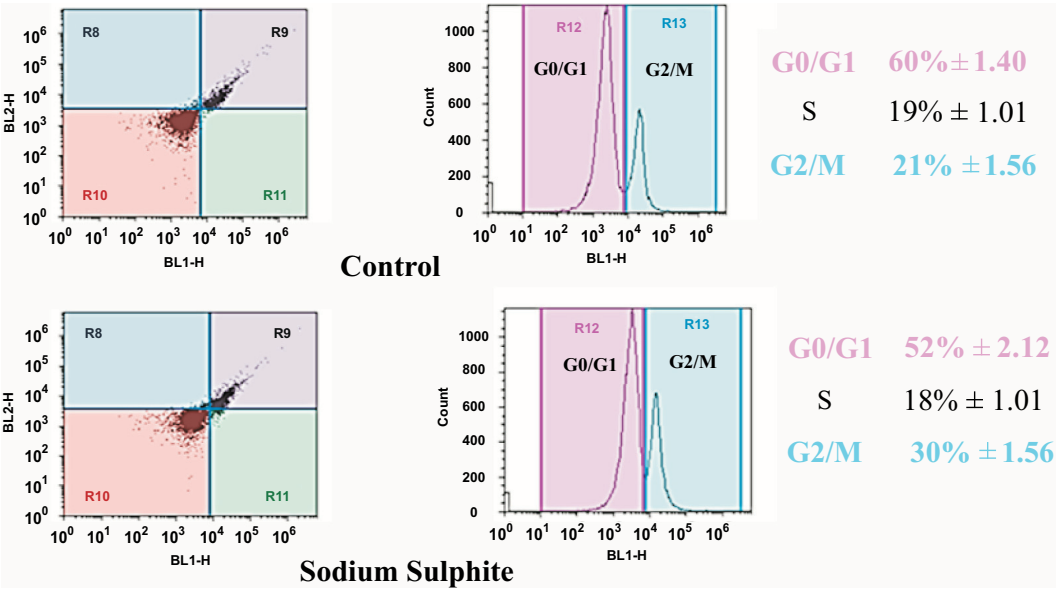

Fig. (4). Effect of Sodium sulphite on the G2/M cell cycle arrest. Liver cancer cells (HepG2) were treated with sodium sulphite at the concentration of 69.44 µg/mL in order to check the cell cycle distribution and then were analysed by flow cytometry. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

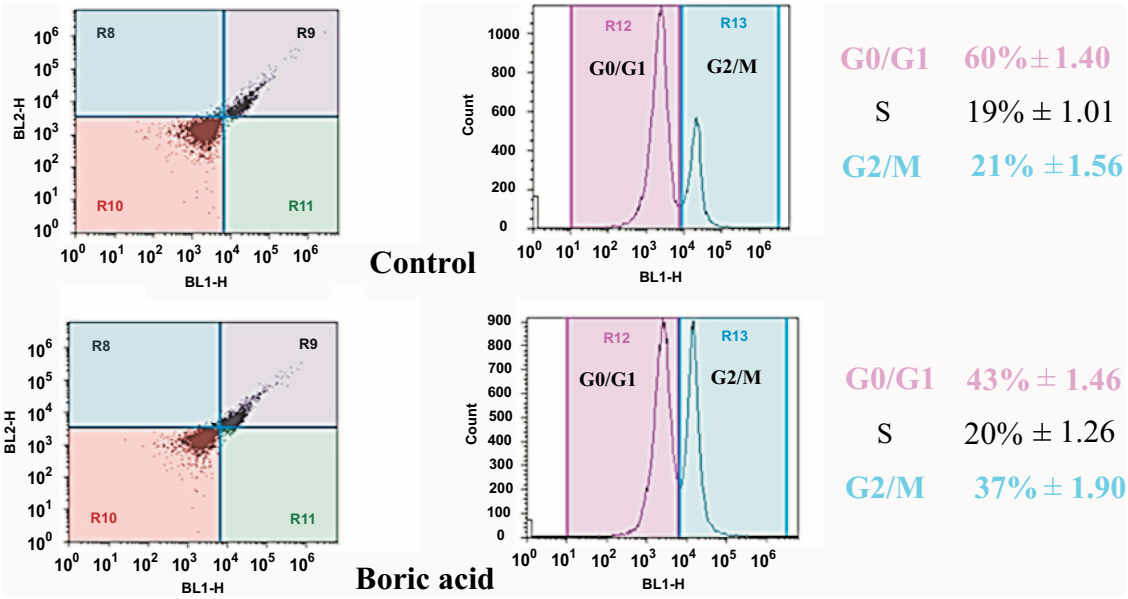

Fig. (5). Effect of Boric acid on the G2/M cell cycle arrest. Liver cancer cells (HepG2) were treated with boric acid at the concentration of 64.27 µg/mL in order to check the cell cycle distribution and then were analyzed by flow cytometry. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

3.3. Determination of the Expression Levels of Apoptosis-regulatory Genes

The expression levels of the apoptosis-related genes, p53 and Bcl-2 in HepG2 were determined by quantitative real-time PCR. Cell lines treated with both sodium sulphite and boric acid have shown an increase in the expression levels of p53 accompanied with a decrease in Bcl-2 expression levels compared to the control cells (Figs. **7** and **8**). The obtained results showed that boric acid had a higher apoptosis induction effect than sodium sulphite. In contrast, although the p53 gene expression levels were significantly increased after benzoic acid treatment, it was accompanied by a significant decrement in Bcl-2 gene expression levels (Fig. **9**).

The results indicated that both sodium sulphite and boric acid had initiated apoptosis mechanism mainly *via* the over-expression of p53 and downregulation of Bcl-2 genes. Benzoic acid might have exhibited anti-apoptotic features as indicated by Bcl-2 elevated expression levels.

4. DISCUSSION

The data obtained from MTT assay indicated that sodium sulphite, boric acid, and benzoic acid decreased mitochondrial activity and cell viability in both malignant and non-malignant hepatic cells indicating their cytotoxic effect. Boric acid and its derivatives are used as preservatives in food and medicines on a wide scale, however, these compounds

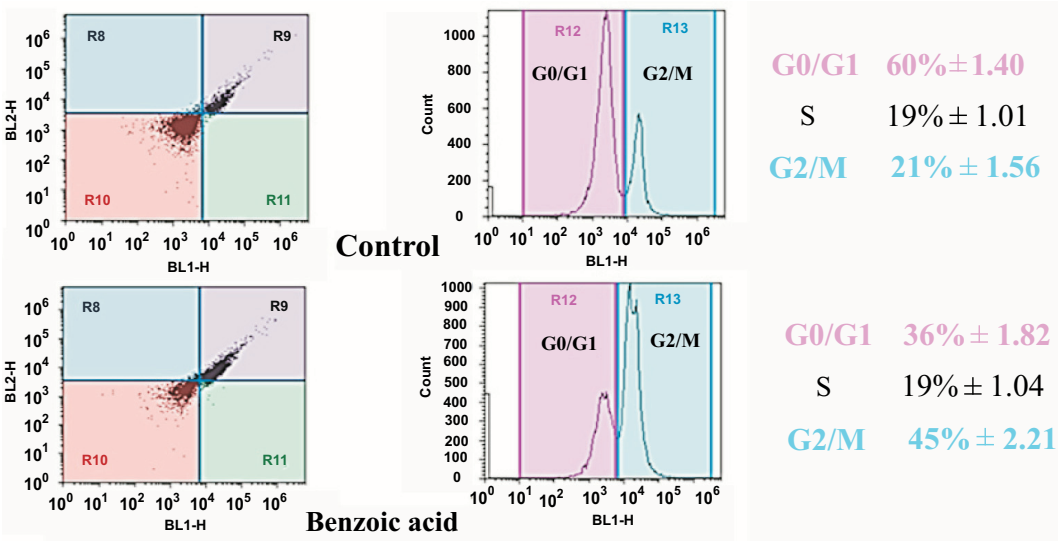

Fig. (6). Effect of Benzoic acid on the G2/M cell cycle arrest. Liver cancer cells (HepG2) were treated with benzoic acid at the concentration of 44.73 µg/mL in order to check the cell cycle distribution and then were analysed by flow cytometry. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

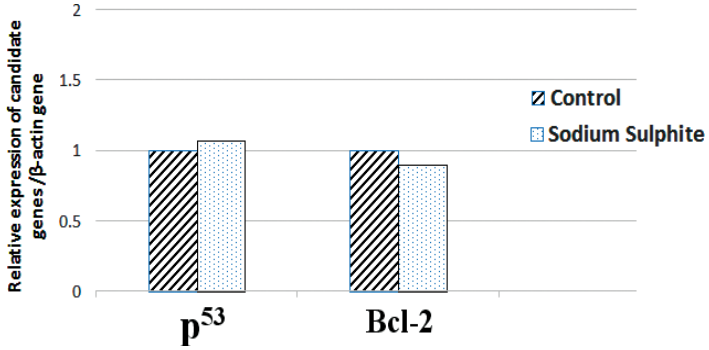

Fig. (7). Effects of sodium sulphite on apoptosis-related genes after exposure to 69.44 µg/mL, mRNA expression of p53 and Bax was assessed by quantitative RT-PCR *P<0.05, compared to the control group.

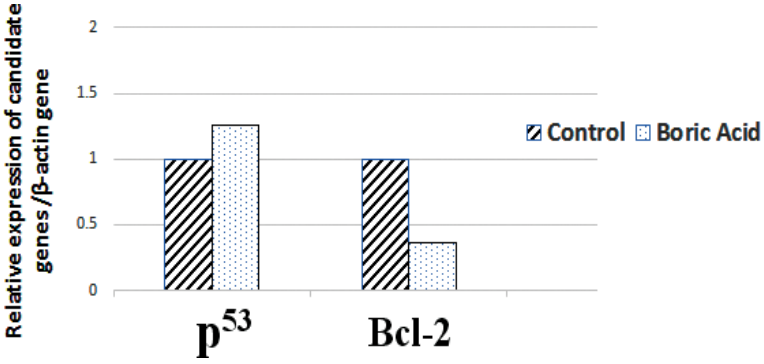

Fig. (8). Effects of boric acid on apoptosis-related genes after exposure to 64.27 µg/mL, mRNA expression of p53 and Bcl-2 was assessed by quantitative RT-PCR *P<0.05, compared to the control group.

were proven to be harmful to human health at high consumption [37]. The possible lethal doses in babies are in the range of 3-6 g, whereas 15-20 g were considered to be lethal in adults [38]. The obtained results revealed that boric affected both cancer and normal human cell lines. In this field, treatment of Hep-G2 cells with borax was proven to inhibit cell proliferation along with the promotion of apoptosis, using MTT and annexin V/P1 staining, respectively, [14]. Another study by Centurk *et al*. (2016) [39] recorded the cytotoxic effect of boric on both acute leukemia cell line (HL-60) and healthy human lymphocytes using MMT, Neutral Red, transmission microscope, and flow cytometry methods. On the other hand, Murmu *et al*. (2002) [40] reported the antitumor effect of the two new boron compounds, guanidine biboric acid and dihydroxy boron hydrochloride monohydrate adduct. Mice receiving 1.7 or 90 mg/kg daily dosage of boric showed a decrease in tumor size by 38% and 25%, respectively [41].

The current research results are in agreement with findings of Yilmaz and Karabay (2018) [42] that 6.25 mM of sodium benzoate is the starting concentration for reducing

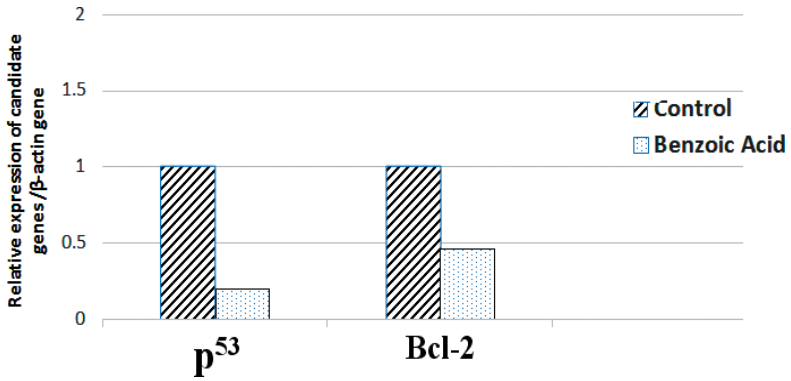

Fig. (9). Effects of benzoic acid on apoptosis-related genes after exposure to 44.73 µg/mL, mRNA expression of p53 and Bcl-2 was assessed by quantitative RT-PCR *P<0.05, compared to the control group.

cell viability of colon cancer cells. Sodium benzoate was also found to decrease the viability of rat cortical neuron cell [43]. Nevertheless, sodium benzoate at concentrations up to 1% had a very low cytotoxic activity on human fibroblast cells based on MTT assay results [45]. Yadav *et al*. (2016) [44] reported that sodium benzoate at concentrations up to 1 mg/ml was not cytotoxic on splenocytes.

Current data, obtained from flow cytometry has shown significant accumulation of HepG2 cells in the G2/M phase, and confirmed that sodium sulphite, boric acid and benzoic acid have cytotoxic effect *via* induction of G2/M phase arrest of the cell cycle. The cytotoxic agents or radiation induced cell death after G2-M arrest. This phenomenon was associated with damage or incomplete mitosis [21]. Wu *et al*. (2006) [22] also mentioned that many investigators have shown that different cytotoxic agents can induce G2/M phase accumulation. Sleiman and Stewart (2000) [46] suggested that cytotoxic molecules induce mitotic cell death (apoptosis) which occurs in parallel with G2/M arrest. Sinularin induced G2/M arrest by increasing the expressions of genes related to G2/M such as p53, and p21 was reported. Furthermore, sinularin stimulated apoptosis *via* decreased anti-apoptotic Bcl-2 expression and increased expressions of Bax [47]. Al-Senosy *et al*. (2018) [48] found that di-*n*-butyl phthalate (DBP) can induce cytotoxic activity and apoptosis *via* up-regulating p53 and Bax whereas, the transcription of Bcl2 was significantly down-regulated in human hepatocellular carcinoma (HepG2).

The results of real-time PCR indicated that both sodium sulphite and boric acid killed HepG2 cells through apoptosis mechanism, triggered *via* overexpression of p53 gene and downregulation of Bcl-2. On the other hand, benzoic acid has exhibited an anti-apoptotic feature based on Bcl-2 overexpression. Gene expression changes can be considered as one of the earliest responses to chemical exposure. Analysis of gene expression alterations that accompany chemical exposure is important for developing an understanding of toxicological processes initiated by particular chemical exposures [49]. Gene expression alteration of p53 dependent apoptosis is one of the genotoxicity signs, so any agent can cause induction of p53 might be considered as genotoxin [50, 51]. The p53 gene may modulate susceptibility of cells to apoptosis by downregulation of Bcl-2 and by causing up-regulation of Bax [52]. A number of studies have focused on the essential role of p53 in the balance between apoptosis and proliferation [53]. The p53 gene plays a key role in G2 checkpoint by suppressing the G2/M transition. It also regulates the balance between the pro-apoptotic Bax gene and the anti-apoptotic Bcl-2 gene through its transcriptional activities [54]. Bcl-2 genes play a crucial role in controlling the mitochondrial pathway of apoptosis [34], which consists of pro-apoptosis genes (Bax, Puma, Bim, Noxa, Bid), anti-apoptosis genes (Bcl-xl, Bcl-2), and one of the mitochondrial permeability transition pores [36]. Bcl-2 can stabilize the mitochondria permeability transition and avoid the release of cytochrome c to inactivate caspase [55].

Similar results were obtained by Wei *et al*. (2016) [14] who showed that Borax treatment induced an increase in the mRNA of the tumor suppressor p53 and pro-apoptotic Bax while it induced a decrease in the mRNA of the apoptosis regulator Bcl-2 in HepG2 cells. Raposa *et al*. (2016) [56] reported that sodium benzoate may cause apoptosis at low concentration, but anti-apoptotic activity was shown at a high concentration based on MAPK8 expression. Sodium benzoate induced caspase-3 activity in HCT116 colon cancer cells at concentrations of 12.5-50 mM compared to untreated cells. Moreover, it increased Bim expression but did not stimulate a significant difference in Bcl-xl levels, which may support the further induction of apoptosis signaling [42]. In a previous study, Gao *et al*. (2011) [57] reported tartrazine increased apoptotic characteristics in rats' brain tissue at low concentration, but at high concentration had anti-apoptotic effects.

CONCLUSION

It can be safely concluded from the present and the previous work that the tested compounds have a noticeable genotoxic and cytotoxic potential that cannot be ignored while using such chemicals in the food industry. Further thorough investigations are recommended before continuing using these substances in food and cosmetics as additives.

CURRENT & FUTURE DEVELOPMENTS

The present work has proven that the tested compound-shave a noticeable genotoxic and cytotoxic potential that cannot be ignored while using such chemicalsin food industry. Further thorough investigations are recommended before continuing using thesesubstances in food and cosmetics as additives.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this research are available within the article.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

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