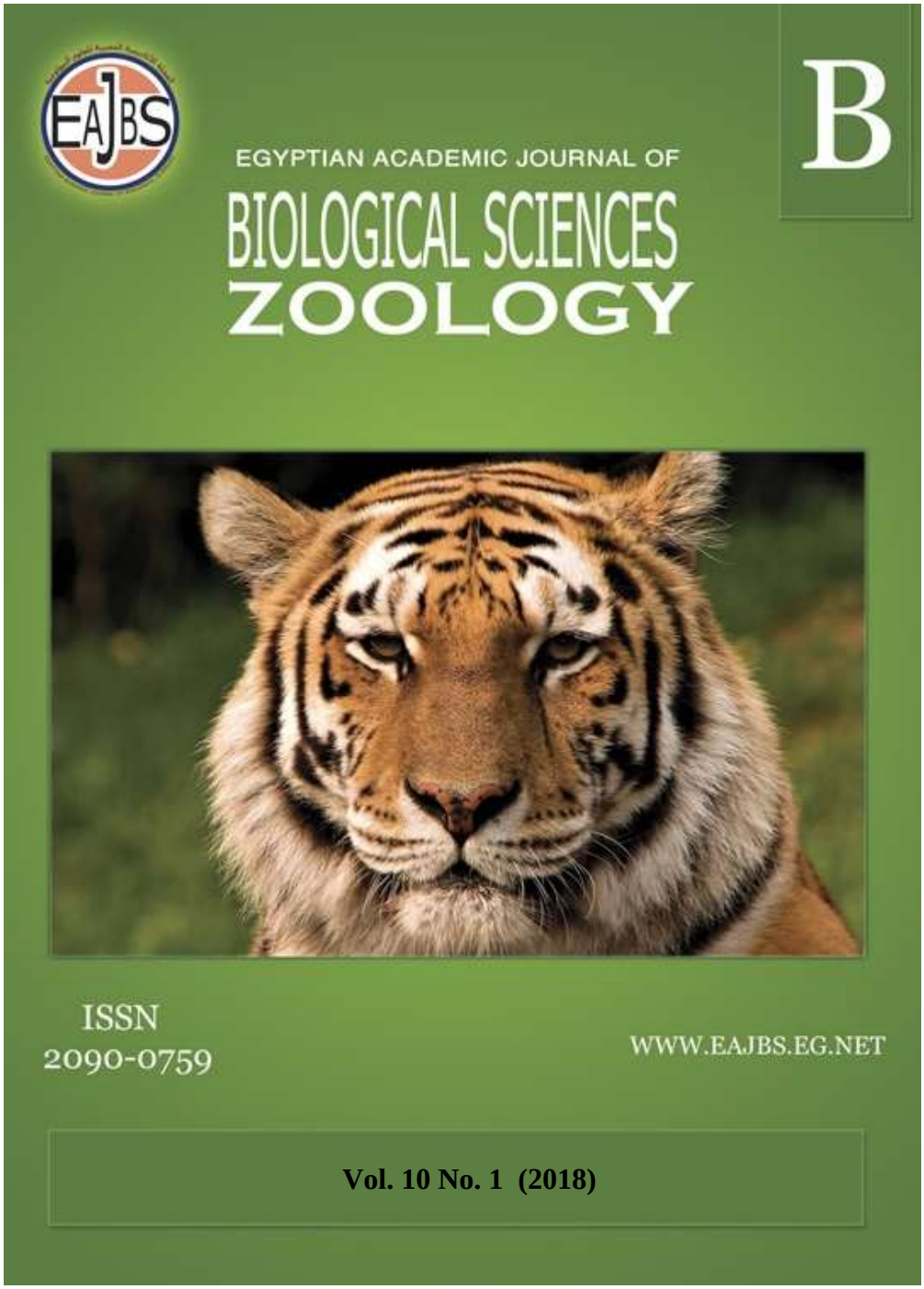

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Evaluation of The Cytotoxicity, Cell Cycle Perturbations and Apoptotic Induction in Human Normal and Cancer Liver Cell Lines Exposed to Potassium Nitrate and Sodium Benzoate

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ABSTRACT

This proposal investigates *in vitro* cytotoxic and apoptotic effects of two food preservatives commonly consumed in daily meals; potassium nitrate and sodium benzoate. The effect of these two 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. Although the effect was more significantly realized in HepG2 cell line, the viability of both cell lines was decreased by both of the tested compounds. Flow cytometric analysis of the HepG2 treated cells has revealed an increase in G2/M phase cell cycle arrest. Measurement of expression levels of three central genes; p53, bcl-2 and bax that play key roles in cell cycle and apoptosis were carried out in HepG2 using real time-PCR and western blot. In conclusion, both of the tested compounds have decreased the cell line viability and induced both cell cycle arrest and apopototic events indicating their high potential of being cytotoxic and genotoxic materials.

INTRODUCTION

Food additives have been defined by the Joint WHO/FAO Committee in 1955 as substances 'which are added intentionally to food, generally in small quantities, to improve its appearance, flavour, texture, or storage properties' (Pressman *et al*., 2017).

Multiple Recent research results have proven that the consumption of food products processed by additives might have raised the risk of cancer in human despite the respected legal limits of these additives by the manufactures. (Gülsoy *et al*., 2015). During the last few years, food additives have attracted the attention as potential causes of various human diseases; they might be added to the factors responsible for the outbreak of cancer, hepatic and nephritic failures, and mutagenic potential (Tanaka 2007; Turkoglu 2007; Demir *et al*. 2008).

Food additives were also found to be genotoxic and may cause genomic instability and damaging effects which are associated with an increased rate of cancer (Saatei *et al*., 2016; Patel and Ramani, 2017). Some food preservatives can contribute to the activation of inflammatory pathways favoring the development of cancers (Raposa *et al*., 2016).

Potassium nitrate (E252) is commonly used as a preservative in various kinds of the food industry including meat, cheese, vegetables and fruits (Binkerd and Kolari, 1975). The carcinogenic potential of nitrates and nitrites used as preservatives and color-enhancing agents in meats was confirmed in a study by Nujić and Habuda-Stanić (2017). Human peripheral blood cells treated with potassium nitrate *in vitro* has shown a significant decrease in mitotic index (MI) compared to control (Mpountoukas *et al.* 2008).

Exposure of HepG2 to nitrate has resulted in down-regulation of genes that initiate cell cycle machinery and the up-regulation of genes involved in protein degradation which may indicate that potassium nitrate may induce apoptotic events in the exposed cells. It also induced a significant down-regulation of DNA cross-link repair 1C gene. The down-regulation of DNA repair genes may indicate that potassium nitrate act as mutagens with the potential to induce cancer (Bharadwaj *et al*., 2005). It was also found to initiate up-regulation of proliferating cell nuclear antigen (PCNA) levels which are associated with the progression of cancer and high levels are detected in numerous malignant cancers such as lymphomas (Takahashi *et al*., 1997) and breast cancer (Baldi *et al*., 1998).

Sodium benzoate is one of the synthetic additives that are widely used as a preservative against fungi, yeast and bacteria in food and soft drinks industry and is generally recognized as safe (GRAS) (Hong *et al*., 2009; Onyemaobi *et al*., 2012; Zhang and Ma 2013; Lennerz *et al*., 2015; Hanes 2017).

Sodium benzoate treatment has resulted in chromosomal aberrations (CAs), sister chromatid exchange, DNA damage and micronuclei formation in human peripheral lymphocytes. This indicates that sodium benzoate is clastogenic, mutagenic and cytotoxic to human lymphocytes *in vitro* and it may cause cancer (Mamur *et al*., 2010; Zengin *et al*., 2011; Soares *et al*., 2015). Sodium benzoate produced cytotoxic and mutagenic activities in splenocytes and lymphocytes inducing micronucleus formation and chromosome breaks (Pongsavee, 2015; Yadav *et al*., 2016). Yilmaz and Karabay (2018) investigation results revealed that sodium benzoate treatment reduced cell viability and induced apoptosis in HCT116 colon cancer cell lines.

The use of *in vitro* models reduces the use of experimental animals and they have an additional advantage that multiple tests can be performed with a relatively low amount of sample (Andrioli *et al*., 2017). The majority of human and mammals genotoxic carcinogens require metabolic activation to become genotoxic (Kirkland *et al*., 2014). I*n vitro* genotoxic studies should ideally use human cell lines originating from tissue most involved in biotransformation, including liver and intestine (Khoury *et al*., 2016).

Over the years several tetrazolium salts have been developed for various applications in histochemistry, cell biology, biochemistry, and biotechnology (Berridge *et al*., 2005). 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 (Mosmann, 1983). MTT is reduced only by mitochondrial enzymes of viable cells (Lewis *et al*, 1999; Berridge *et al*., 2005).

Studies of borax effect on the viability of HepG2 using MTT assay have proved that different concentrations of borax could inhibit cell growth (Wei *et al*., 2016). MTT assay revealed a significant reduction of viability in leukemia (K562) cells treated with 2-(6-(2-thieanisyl)-3(Z)-hexen-1,5-diynyl)alanine (THDA) (Wu *et al*., 2006). Chen *et al*. (2017) found that morphine can reduce the cell viability, growth and colony formation rate of MCF-7 cells using MTT assay.

Single time-point cell measurement is a flow cytometry technique that has been developed to analyze the cell cycle. It reveals cells 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 (Crissman and Hirons, 1994; Darzynkiewicz *et al*., 1996; Larsen *et al*., 2001).

Different cytotoxic agents, radiation or drug-induced cell death can induce G2/M phase accumulation (Bonelli *et al*., 1996). Cell cycle analysis showed G2/M phase arrest and apoptosis in K562 cells after 24 h exposure to THDA (Wu *et al*., 2006). Morphine was found to induce cell cycle arrest at the G0/G1 and G2/M phase accompanied by breast cancer cell line (MCF-7) apoptosis (Chen *et al*., 2017). Apoptosis is a form of programmed cell death, a crucial pathway for regulating homeostasis, responding to DNA damage and controlling cell proliferation (Esmaeili *et al*., 2014).

The P53 is a key tumor suppressor gene that has a crucial function in apoptosis (Roshan *et al*., 2014). It 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 (Levine, 1997). Apoptosis could be stimulated by p53 gene through down-regulation of Bcl-2 gene, and activation of caspases (Haupt and Haupt 2004). It has been known that p53 contributed to the transcriptional activation of large numbers of target genes (Mirzayans *et al*., 2012). Bcl-2 family genes play a pivotal role in controlling the mitochondrial pathway of apoptosis (Dewson and Kluck, 2009; Chen and Lesnefsky, 2011; Wu *et al*., 2013).

The aim of this investigation was to assess the potential cytotoxic and apoptotic effects of potassium nitrate and sodium benzoate on human cell growth. Cell cycle arrest, apoptosis-related genes of the HepG2 cell line was also evaluated.

MATERIALS AND METHODS

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).

A 100 µl cell suspension was added to each well of a 96- well microtitre plate (Corning Cell WellsTM, Corning, USA).The two tested compounds were added to the wells with different concentrations (6.25, 12.5, 25, 50, 100 and 200 µl) for 24 hours at 37 °C in a 5% CO_2 and a humified incubator (Forma Scientific CO2 Water Jacketed Incubator). In addition, 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 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 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 triplicate 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.

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 supernatant. The pellet was washed twice in warm PBS, then re-suspended in 500 µl of the warm buffered saline and centrifuged. The supernatant was removed. 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 decanting the supernatant. Again, the pellet was washed twice in warm PBS and the cell pellet was re-suspend in 500 µl of the warm buffered saline, centrifuged and the supernatant was removed. The remaining pellet was re-suspended in 100 µl PBS and was 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 analysed using Attune flow cytometer (Applied Bio-system, US).

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 Step One Plus real-time PCR system (Applied Biosystem, USA).

Sequences of the primers designed for the amplification of the genes of interest by Primer 5.0 software were as follows; p53 forward

5'-CCCAGGTCCAGATGAAG-3', p53 reverse

5'-CAGACGGAAACCGTAGC-3', Bcl-2 forward

5'-GGATGCCTTTGTGGAACTGT-3' and Bcl-2 reverse

5'-AGCCTGCAGCTTTGTTTCAT-3' And Bax forward was

5'-TTTGCTTCAGGGTTTCATCC-3', and the reverse was

5'-CAGTTGAAGTTGCCGTCAG A-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, and the relative gene expression was determined using $2^{-\Delta\Delta Ct}$ method .

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 $P \leq 0.05$. Comparison of means was carried out with Tukey's Honestly Significant Difference (Tukey's HSD) test.

Western Blotting:

The HepG2 cells were lysed in RIPA buffer and the protein concentration was determined by the Bradford method. Equal volumes of protein were loaded and separated on a 10% SDS-PAGE gels. Proteins were transported to a 0.45 µm polyvinylidene fluoride membrane (Millipore). After incubation with the primary antibodies overnight at 4°C, membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibodies (1: 5,000; Santa Cruz Biotechnology, Inc.) for 1 hour at room temperature. The specific protein bands were developed using tetramethylbenzidine (TMB, Sigma Chemical Company, St. Louis, MO, USA). The density of each band was normalized by β-actin. Sources and dilution factors of primary antibodies were TGFb (1:150; Bioworld), phosphatidylinositol-3-kinase (PI3K, 1:100, Abcam), and ERK (1:100, Santa Cruz Biotechnology).

RESULTS AND DISCUSSION

Cytotoxic Activity on HepG2 and THLE2 Cells Lines by MTT Assay:

The cytotoxic effect of potassium nitrate and sodium benzoate was measured as a function of their influence at different concentrations (6.25, 12.5, 25, 50, 100 and 200 µg/ml) on the viability of HepG2 cell line and THLE2 cell line using MTT assay. Cisplatin, a metal-based anticancer drug, was used as a positive control.

The results showed that the viability of HepG2 and THLE2 was reduced gradually as the concentration of potassium nitrate, sodium benzoate and cisplatin increased. Analysis of the data revealed that both potassium nitrate and sodium benzoate are not toxic at 6.25 µg/ml concentration on HepG2 and THLE2. Potassium nitrate and sodium benzoate significantly affected the cell viability of HepG2 cell line cancer cells starting 12.5 µg/ml concentration. It required higher concentrations to affect the viability of THLE2 cell line. Data analysis also indicated that potassium nitrate and sodium benzoate decreased cell viability in malignant and non-malignant cells revealing a cytotoxic effect.

The IC50 of potassium nitrate was 38.91 µg/ml, 5257 µg/ml In case of HepG2 and THLE2 respectively while that of cisplatin was 20.15 µg/ml. and 645.7µg/ml as represented in figures (1& 2).

In sodium benzoate treatment the IC50 was 40.91 µg/ml in HepG2 cells and 1475 µg/ml in THLE2 cells figures (3& 4)

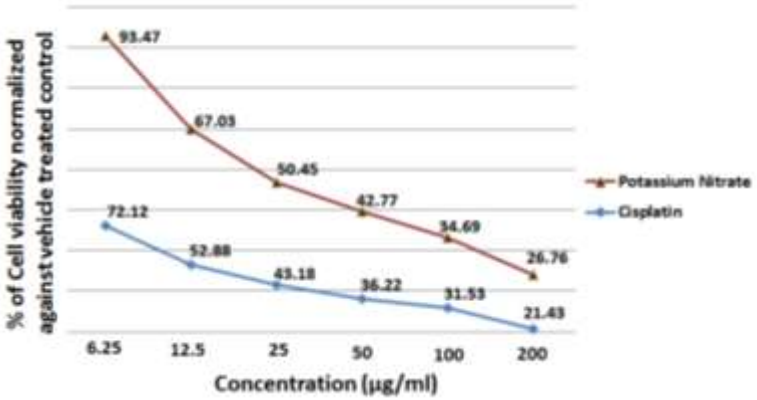

Fig. (1). Dose-dependent growth inhibition by Cisplatin (Positive control) and potassium Nitrate on *HepG2* cell line. Cell viability was quantified by MTT assay. Results are mean ± (n = 3). *$p < 0.05$ to compared to control.

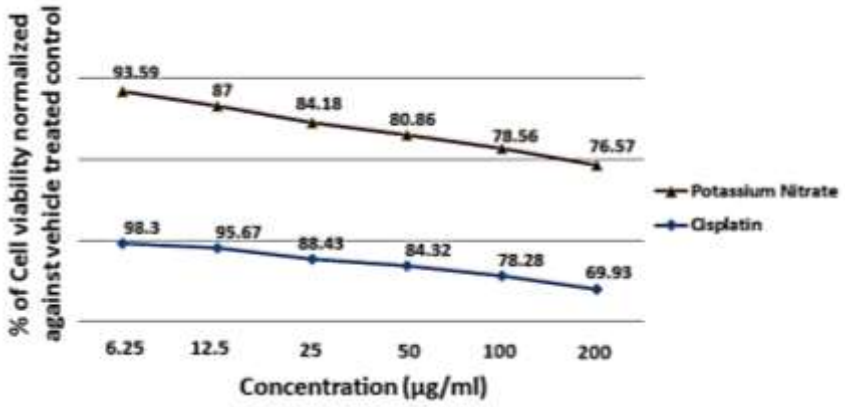

Fig. (2). Dose-dependent growth inhibition by Cisplatin (positive control) and Potassium Nitrate on *THLE2* cell line. Cell viability was quantified by MTT assay. Results are mean ± (n = 3). *p < 0.05 to compared to control.

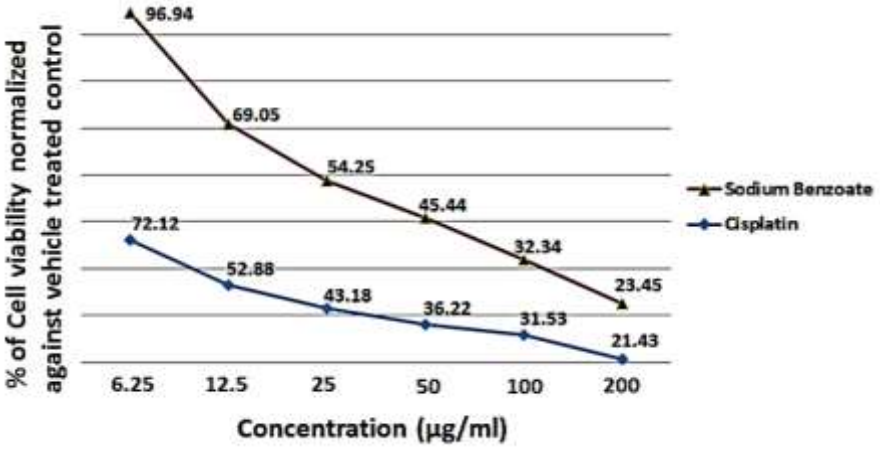

Fig. (3). Dose-dependent growth inhibition by Cisplatin (positive control) and Sodium Benzoate on *HepG2* cell line. Cell viability was quantified by MTT assay. Results are mean ± (n = 3). *p < 0.05 to compared to control.

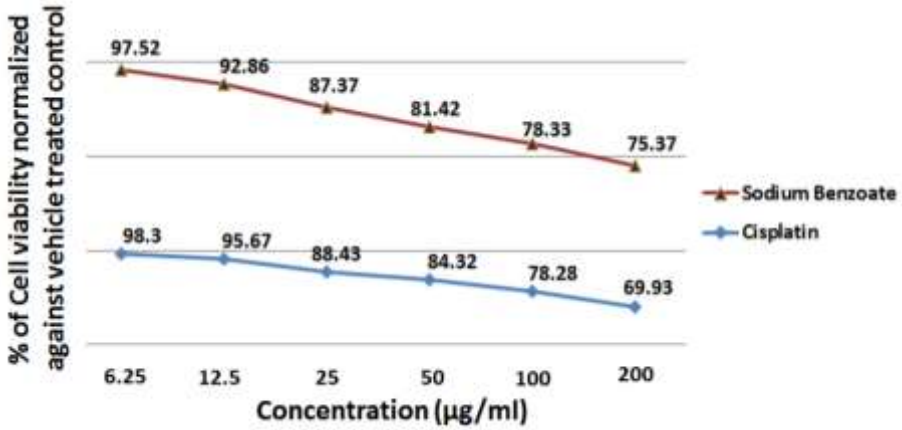

Fig. (4). Dose-dependent growth inhibition by Cisplatin (positive control) and Sodium Benzoate on *THLE2* cell line. Cell viability was quantified by MTT assay. Results are mean ± (n = 3). *p < 0.05 to compared to control.

The current results are consistent with the findings of Yilmaz and Karabay (2018) except for that they found that the 6.25 mM concentration of sodium benzoate is the starting concentration for reducing cell viability in colon cancer cells. Park *et al*. (2011) showed that sodium benzoate decreased the viability in rat cortical neuron cell. Moreover, El Hefny *et al*. (2017) found the toxic effect for benzoic acid at 300 µg/ml on normal lung cell (Wi38).

Bharadwaj et *al*., (2005) evaluated the cytotoxicity of potassium nitrate on human hepatocellular carcinoma (HepG2) using the neutral red-viability assay. They found that potassium nitrate treatment caused a non-significant decrease in HepG2 viability after 24 h treatment with concentrations ranging from 3 to 1000 mg/L. Yadav *et al*. (2016), reported that sodium benzoate at concentrations up to 1 mg/ml is not cytotoxic on splenocytes. Furthermore, Spindola *et al*. (2018), using human fibroblast cells by MTT assay found that sodium benzoate at concentrations up to 1% has very low cytotoxic activity.

Cell Cycle Analysis Using Flow Cytometry Assay:

After realizing the high inhibition rate of HepG2 cell viability by potassium nitrate and sodium benzoate, it was necessary to assess cytotoxicity effect of both of the tested compounds on cell cycle arrest using flow cytometry based on cell cycle distribution. figure (5) showed that compared with control group, potassium nitrate treatment at 38.91 µg/ml affected the cell cycle distribution on HepG2 cells showing reduction in the G0/G1 phase, slight increase in S-phase and a significant increase in the percentage of HepG2 cells at G2/M phase to be about 2.14 folds as compared to control. Regarding sodium benzoate, the concentration of 40.91 µg/ml affected the cell cycle distribution on HepG2 cells. Like in potassium nitrate, the G0/G1 phase decreased from 60% in control to 34% in sodium benzoate treatment but the S-phase was slightly increased from 19% to 20% as represented by figure (6). While, the G2/M phase was more than twice in sodium benzoate treatment as compared with control. These results showed significant accumulation of HepG2 cells in the G2/M phase, and confirmed that potassium nitrate and sodium benzoate have cytotoxic effect via induction of G2/M phase arrest of the cell cycle.

Multiple cytotoxic molecules induce mitotic cell death (apoptosis) which occurs in parallel with G2/M arrest (Bonelli *et al*., 1996; Sleiman and Stewart, 2000; Wu *et al*., 2006).

Sinularin, a marine anti-cancer agent, was found to induce G2/M arrest by increasing the expressions of genes related to G2/M such as p53, and p21 (Chung *et al*., 2017).

Al-Senosy *et al*., (2018) found that di-*n*-butyl phthalate (DBP) can induce G2/M arrest and apoptosis via up-regulating p53 and Bax whereas, the transcription of Bcl2 was significantly down-regulated in HepG2 cell line.

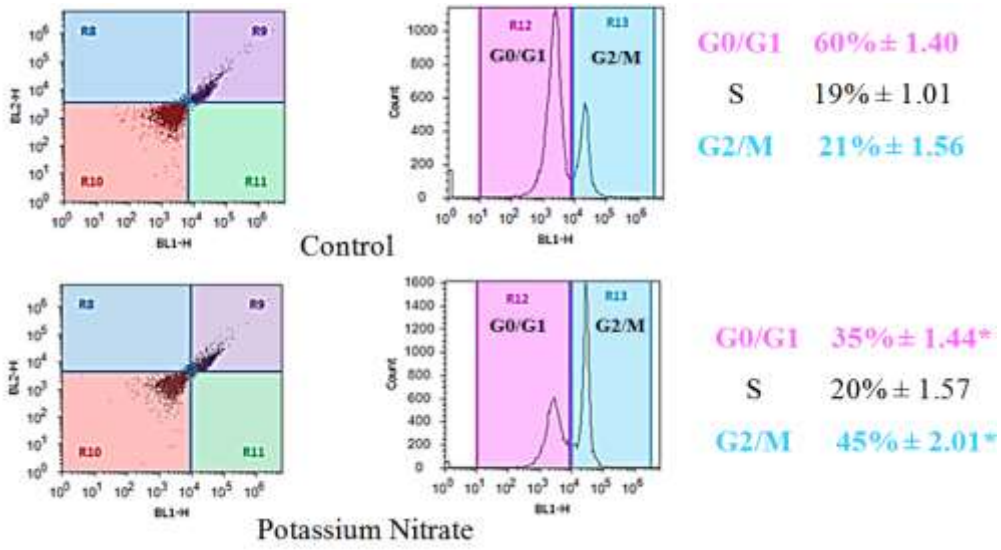

Fig. (5). Flow cytometry analysis for the effect of 38.91 μg/mL potassium nitrate treatment on the G2/M cell cycle arrest in HepG2 cell line.

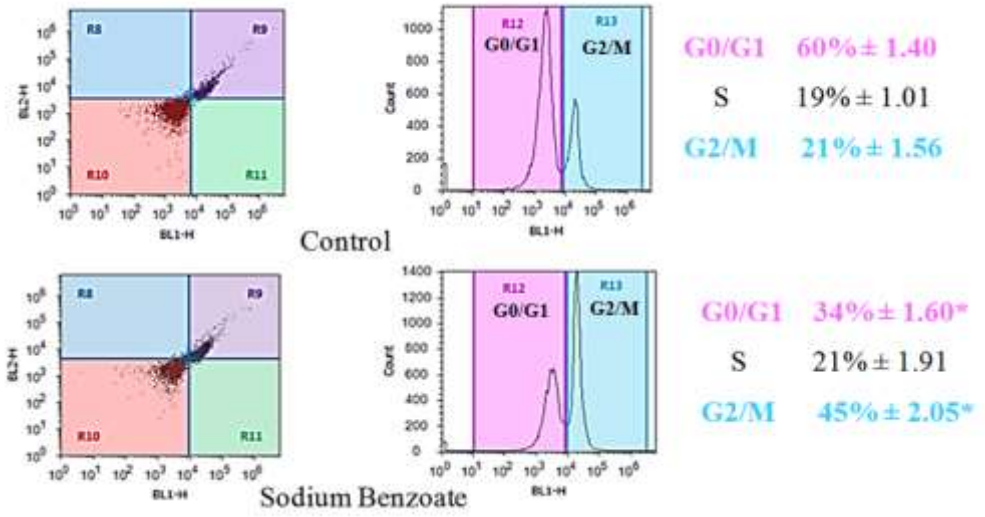

Fig. (6). Flow cytometry analysis for the effect of 40.91 μg/mL sodium benzoate treatment on the G2/M cell cycle arrest in HepG2 cell line.

Determination of the Expression Levels of Apoptosis-Regulatory Genes:

The expression levels of some apoptosis-related genes; p53, Bcl-2 and Bax in HepG2 were determined by quantitative real-time PCR. figures (7 & 8) reveals that, compared to the control, the expression levels of p53 and Bax were increased, whereas that of Bcl-2 were decreased. The results indicated that either potassium nitrate or sodium benzoate killed HepG2 cells through apoptosis mechanism mainly via the over-expression of p53 and Bax genes, while Bcl-2 was down-regulated.

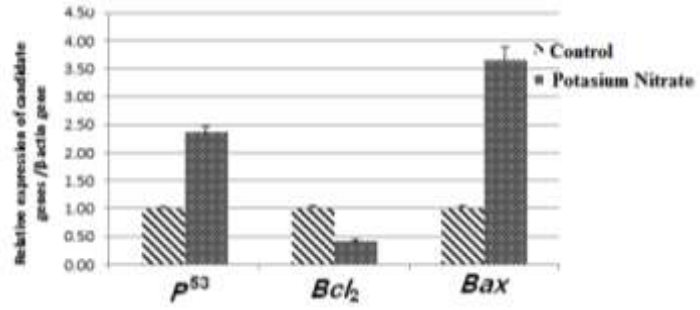

Fig. (7). Effect of potassium nitrate on apoptosis-related genes after exposure to 38.91 μg/mL, mRNA expression of p53, Bcl-2, and Bax was assessed by quantitative RT-PCR *P < 0.05, compared to the control group.

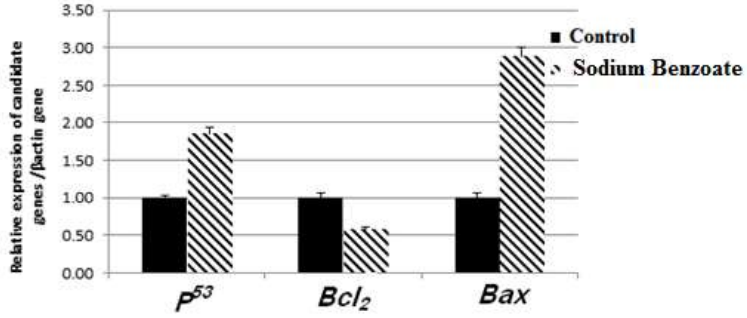

Fig. (8). Effect of sodium benzoate on apoptosis-related genes after exposure to 40.91 μg/mL, mRNA expression of p53, Bcl-2, and Bax was assessed by quantitative RT-PCR *P < 0.05, compared to the control group.

Determination of the Expression Levels of Apoptosis-Related Proteins in HepG2 Cells Using the Western Blotting Technique:

To confirm the mechanism of apoptosis induction by potassium nitrate and sodium benzoate in HepG2 cells, the protein expression levels of Bax, Bcl-2 and p53 were analysed by western blotting technique (Fig.9). The p53 and Bax, protein were clearly up-regulated as compared to the control, while the Bcl2 protein expression level was down-regulated by the two preservatives.

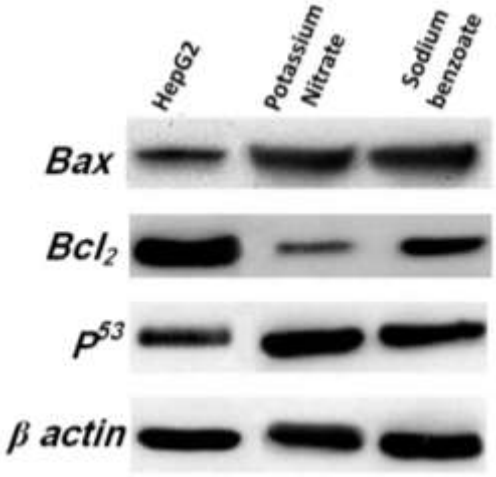

Fig. (9). Effect of potassium nitrate and sodium benzoate on protein expression of p53, Bcl-2, and Bax as evaluated by the western blotting technique in HepG2 cells compared to the control group.

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 (Aardema and MacGregor, 2005).

Gene expression alteration of p53 dependent apoptosis is one of the genotoxicity signs. In other words, an agent able to cause induction of p53 expression might be considered as a genotoxin (Strasser *et al*., 1994; Kirsch-Volders *et al*., 2003). The p53 gene may modulate susceptibility of cells to apoptosis by down-regulation of Bcl-2 and be causing up-regulation of Bax (Choi *et al*., 2000).

A number of studies focused on the essential role of p53 in the balance between apoptosis and proliferation (Polager and Ginsberg, 2009). The p53 gene plays a key role in G2 checkpoint suppressing G2/M transition. Besides, it regulates the balance between the pro-apoptotic Bax gene and the anti-apoptotic Bcl-2 gene through its transcriptional activities (Leu *et al*., 2004). Bcl-2 genes play a crucial role in controlling the mitochondrial pathway of apoptosis (Dewson ans Kluck, 2009), 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 pore (Chen and Lesnefsky, 2011). Bcl-2 can stabilize the mitochondria permeability transition and avoid the release of cytochrome c to inactivate caspase (Li *et al*., 1997).

The obtained results indicated that the potassium nitrate and sodium benzoate induced apoptosis in HepG2 cells mainly via the over-expression of p53 and Bax genes, while Bcl-2 was down-regulated.

These results are in agreement with Bharadwaj *et al*., (2005) who reported that potassium nitrate may cause apoptosis in HepG2 cells because it induced the down-regulation of genes that initiate cell cycle machinery and the up-regulation of genes involved in protein degradation. Moreover, it induced significant down-regulation of DNA repair genes and may act as mutagens with the potential to induce cancer. Similar results were obtained by borax treatment which 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 (Wei *et al*., 2016).

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 (Yilmaz and Karabay 2018).

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