DETERMINATION OF GENOTOXIC EFFECTS OF SOME FOOD ADDITIVES ON SOME HUMAN CANCER CELLS BY FLOW CYTOMETRY ANALYSIS

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Any substance added to food is apt to change any of its attributes from the beginning of the production phase and ending to the consumer, it is added in order to improve preservation or sensory qualities or reduction of exposing consumers to poisoning and other health hazards**.**

Cann *et al*. (2007) some investigations aimed at determining the effect of mono sodium glutamate (MSG) on body organs showed that it inflicted DNA damage at all concentrations in isolated human lymphocytes after 1 h *in vitro* exposure. It was demonstrated that MSG is genotoxic to the human peripheral blood lymphocytes (Ataseven *et al.*, 2016). Osfor *et al.* (1997) indicated that kidney, liver, brain, and heart weight significantly increased in rats treated with MSG. MSG in high doses produced neuroendocrine abnormalities and neuronal degeneration (Moreno, 2005), and oxidative damage in different organs (Farmobi and Onyema, 2006; Pavlovic *et al*., 2007). Furthermore, glutamate in high concentrations, particularly in postnatal period, could act as a neurotoxin (excitotoxin) (Eweka *et al*., 2011).

The genotoxic potential of sodium benzoate (SB) was associated with chromosomal aberration, sister chromatid exchanges and cell cycle proliferation index analysis in the cultured human peripheral lymphocytes. *In vitro* genotoxicity tests detected that it can induce genetic damage, directly or indirectly, by different mechanism (Patel and Ramani, 2017).

Although, many studies have investigated the effect of saffron and its active constituents in the prevention and treatment of cancer, so far, the exact mechanism of action has not been cleared yet on anticancer and toxic effects of saffron and its constituents as a chemopreventive herb and its mechanisms of action was reported (Milajerdi *et al*., 2016). Saffron could induce apoptosis in MCF-7 cells in which it was dependent on caspase activation. It has been suggested that saffron could cause MCF-7 cell death, in which apoptosis or programmed cell death plays an important role.

Flow cytometry analysis indicated inhibition of the rate of liver cancer (HepG2) cell viability, it was necessary to assess cytotoxic effect of DBP (Di-Butyl

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Phthalate) on cell cycle arrest based cell cycle distribution. The results showed significant accumulation of HepG2 cells in G2/M phase, and confirmed that DBP has cytotoxic effect via induction of G2/M phase arrest of the cell cycle (Al-Senosy *et al*., 2018).

The quantification real-time polymerase chain reaction (PCR) has been used to measure the mRNA levels of *p53, Bax, and Bcl-2* genes. The data showed that DBP changed transcriptional levels of these appoptosis-related genes. The mRNA expression of *p53* and *Bax* were up-regulated, while of that *Bcl-2* was significantly down-regulated compared to the control. The toxic and apoptotic effects of DBP were detected in human cell lines (Al-Senosy *et al*., 2018).

The objective of this study was to assess the potential cytotoxic and apoptotic effects of three common food additives on human cell growth.

MATERIALS AND METHODS

1. Materials

1.1. Human cell lines and cultures

Cancerous colon carcinoma (Caco-3), breast carcinoma (MCF7), lung carcinoma (A549) and normal lung cell line (Wi38) was used as the control [obtained from the American Type Culture Collection (ATCC), Manassas, Virginia, USA]. The plates with human cell lines were incubated at 37°C in 5% CO_2 for 24 hours to obtain monolayer confluence.

1.2. Food additives

Three different types of food additives (MSG, SB and saffron) were used in this study [obtained from Sigma Chemical Company, St. Louis, USA].

2. Methods

2.1. Cell cycle analysis using flow cytometry

The cells were digested with warm Trypsin-EDTA + warm Phosphate Buffered Saline (PBS)- Ethylen diamin tetra acetate (EDTA) (0.25%) (500 µl + 500 µl) with incubation for 10 minutes at 37°C. The mixture was centrifuged 450 rpm for 5 min, then supernatant was carefully removed. The mixture was washed twice in warm PBS and the cell pellet was re-suspended in 500 µl warm PBS, centrifuged and supernatant was removed. A volume of 150 µl PBS + 350 µl ice-cold 70% ethanol was added and incubated at 4°C for 1 hour to fix the cells. To remove ethanol, the mixture was centrifuged at 350 rpm for 10 minutes and then the supernatant was carefully removed. The mixture was washed twice in warm PBS and the cells were re-suspended in 500 µl warm PBS, then centrifuged and the supernatant was removed. The cells were re-suspended in 100 µl PBS and stored at 4°C for up to 4 days in darkness. The cells were stained with 100 µl of PI (Propidium Iodide) solution + 50 µl RNase A solution (100 µg/ml), and incubated in darkness for 30-60 min. The stained cells were read in Attune flow cytometry (Applied Biosystem, USA).

2.2. Quantitative RT-PCR analysis

Total RNA was isolated from rat liver using Gene JET RNA Purification Kit (Thermo Scientific, # K0731, USA) according to the manufacturer's protocol. Total RNA (5 µg) was reverse transcribed using Revert Aid H Minus Reverse Transcriptase (Thermo Scientific, #EP0451, USA) to produce cDNA as previously described (El-Magd *et al*., 2013). 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 Bio system, USA). The primers were designed by Primer 5.0 software (Table 1). 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 MM (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 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 relative gene expression (Table 1) was determined using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

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

RESULTS AND DISCUSSION

1. Flow cytometry

Saffron at 3.98 µg/ml affected the cell cycle distribution on Coca-3 cells. The G0/G1 phase showed a shier decrease from control 61% to 16% in saffron treatment. Similarly, the S phase percentage also exhibited a slight decrease from 21% to 20% in the control and saffron treatment, respectively (Fig. 1). While, the percentage of Coca-3 cells at the G2/M phase highly increased after incubation with the control (18%) as compared to saffron (64%). These results showed significant accumulation of Coca-3 cells in the G2/M phase, and confirmed that saffron has marked cytotoxic effect via induction of G2/M phase arrest of the cell cycle (Table 2).

SB at 15.01 µg/ml affected the cell cycle distribution on Coca-3 cells. The G0/G1 phase showed a decrease from 61% to 42%. Similarly, the S phase percentage also exhibited a decrease from 21% to 15% in the control and SB, respectively (Fig. 1). While, the percentage of Coca-3 cells at the G2/M phase highly

increased after incubation with the control (18%) as compared to SB (43%) treatment. These results showed significant accumulation of Coca-3 cells in the G2/M phase, and confirmed that SB has marked cytotoxic effect via induction of G2/M phase arrest of the cell cycle (Table 2).

Subsequent to the occurrence of substantial inhibition rate of colon cancer cell line (Coca-3) viability, an attempt was made to assess cytotoxic effect of the food additives on cell cycle arrest using flow cytometry based on cell cycle distribution. Figure (1) showed that, compared with the control group, MSG at 33.92 µg/ml affected the cell cycle distribution of Coca-3 cells. The G0/G1 phase showed a decrease from 61% to 43%. Similarly, the S phase percentage also exhibited a decrease from 21% to 17% in the control and MSG, respectively. While, the percentage of Coca-3 cells at the G2/M phase highly increased due to MSG (40%) compared with the control (18%). These results showed significant accumulation of Coca-3 cells in the G2/M phase, and confirmed that MSG has marked cytotoxic effect via induction of G2/M phase arrest of the cell cycle (Table 2).

Sleiman and Stewart (2000) suggested that many cytotoxic molecules induce mitotic cell death (apoptosis) which occurs in parallel with G2/M arrest. In addition, the sinularin induced G2/M arrest by increasing the expressions of genes related to G2/M such as *p53*, and *p21*. Furthermore, sinularin apoptosis was observed via decreased anti-apoptotic *Bcl-2* expression whereas; it increased the expressions of *Bax* (Chung *et al*., 2017).

Flow cytometry analysis caused inhibition of the rate of liver cancer (HepG2) cell viability, it was necessary to assess cytotoxic effect of Di-Butyl Phthalate (DBP) on cell cycle arrest based cell cycle distribution. The results showed significant accumulation of HepG2 cells in G2M phase, and confirmed that DBP has cytotoxic effect via induction of G2M phase arrest of the cell cycle (Al-Senosy *et al*., 2018).

1. qRT PCR

1.1. MSG induced genotoxicity of some related genes p53, Bcl-2 and Bax in Caco-3 cells

The role of apoptosis in MSG induced cytotoxicity on colon cancer cell lines (Caco-3) was studied. The expression levels of apoptosis-related genes such as *p53*, *Bcl-2* and *Bax* in Caco-3 cells were estimated by real time PCR (qRT-PCR). Figure (2) **s**howed that, compared to the untreated group (control), the expression levels of *p53* and *Bax* increased, whereas that of *Bcl-2* gene was decreased. These results indicated that the MSG killed Co-Ca-3 cells through apoptosis mechanism mainly *via* over expression of *p53* and *Bax* genes, while *Bcl-2* down regulated.

1.2. SB induced cytotoxicity related genes such as p53, Bcl-2 and Bax in Caco-3 cells

SB induced cytotoxicity on colon cancer cell lines (Caco-3). The expression

levels of apoptosis-related genes such as *p53*, *Bcl-2* and *Bax* in Caco-3 cells were estimated by real time PCR (qRT-PCR). Figure (3) showed that compared to the untreated group, the expression levels of SB with *p53* and *Bax* were increased, whereas that of SB with *Bcl-2* gene was decreased. These results indicated that the SB killed CoCa-3 cells through apoptosis mechanism mainly via over expression of *p53* and *Bax* genes, while *Bcl-2* was down regulated.

1.3. The role of apoptosis in Saffron induced cytotoxicity related genes such as p53, Bcl-2 and Bax in Caco-3 cells

Saffron induced cytotoxicity on colon cancer cell lines (Caco-3) was studied. The expression levels of apoptosis-related genes such as *p53*, *Bcl-2* and *Bax* in Caco-3 cells were estimated by real time PCR (qRT-PCR). Figure (4) showed that, compared to the untreated group, the expression levels of Saffron with *p53* and *Bax* were increased, whereas that of Saffron with *Bcl-2* gene was marked by decreased. These results indicated that the Saffron killed CoCa-3 cells through apoptosis mechanism mainly via over expression of *p53* and *Bax* genes, while *Bcl-2* was down-regulated.

1.1. Effect of Saffron, MSG and SB administration on the relative expression of Bax gene in Caco-3 cells

Several investigators pointed to the essential role of *p53* in the balance between proliferation and apoptosis (Polager and Ginsberg, 2009); as the *p53* gene plays a key role in G2 checkpoint, by halting G2/M transition. In addition, *p53* regulates the balance between the proapoptotic gene *Bax* and the anti apoptotic gene *Bcl-2* through its transcriptional activities (Leu *et al*., 2004).

The obtained results revealed significant upregulated of *Bax gene* expression level in treated Caco3 cells compared with untreated control. This expression was significantly upregulated following administration of Saffron, MSG and SB compounds, with relatively low expression in MSG and highest in saffron followed by SB (Tables 3, 4 and Figs 5 and 6).

1.2. Effect of Saffron, MSG and SB administration on the relative expression of P^{53} in Caco-3 cells

The obtained results revealed significant upregulation of *p53* gene expression level in treated Caco-3 cells (Tables 5 and 6 and Figs 7 and 8). This expression was significantly up-regulated following administration of Saffron, MSG and SB compounds, with highest expression in Saffron, SB and lowest in MSG compared with the untreated control.

1.3. Effect of MSG, SB and Saffron administration on the relative expression of Bcl2gene in Caco-3 cells

The obtained results revealed significant up-regulation of *Bcl2*gene expression level in untreated Caco3 cells (Tables 7 and 8 and Figs. 9 and 10), while its

expressions were significantly down-regulated following administration of MSG, SB and Saffron compounds, with lowest expression in Saffron and highest in MSG.

SUMMARY

Toxicogenomics is the study of the relationships between the structure and activity of the genome and the adverse biological effects of exogenous agents. Different kinds of additives are widely applied in food industry. Yeast cells were grown with the food additives, and culture growth. This study aimed to select and genetically determine the possible genotoxic effects of three food additives (MSG, SB and Saffron) on human cell lines. Flow cytometric analysis demonstrated that treatment of human hepatocellular carcinoma cells (Caco-3) cells with food additives increased G2/M phase cell cycle arrest. The quantitative real time-PCR was used to measure the mRNA levels of *p53*, *Bax*, and *Bcl-2* genes. The data showed that food additives changed transcriptional levels of these related genes. The mRNA expression of *p53* and *Bax* were up-regulated, but, the transcription of Bcl2 was significantly down-regulated compared to the control. Protein-protein interaction maps provided a valuable framework for a better understanding of the functional organization of the proteome. These data indicated that food additives decreased cell viability in malignant and non-malignant cells as well as confirmed the occurrence of their cytotoxic effects.

It is evident from the aforementioned discussion that the studied food additives could inflect some serious health hazards if they are added haphazardly in foods and preveradges. Therefore, strict control of their use in accordance with the special regulations set by the Food and Drug Administration (FDA) and World Health Organization (WHO) to insure their biosafety for human consumption.

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Table (1): Forward and reverse primer sequences for and *β-actin* genes.

Gene	Forward primer (/5 ------ /3)	Reverse primer (/5 ------ /3)
p53	TAACAGTTCCTGCATGGGCGGC	AGGACAGGCACAAACACGCACC
Bax	CCTGTGCACCAAGGTGCCGGAACT	CCACCCTGGTCTTGGATCCAGCCC
Bcl2	AGGAAGTGAACATTTCGGTGAC	GCTCAGTTCCAGGACCAGGC
β-actin	CACCAACTGGGACGACAT	ACAGCCTGGATAGCAACG

Table (2): Average% of cells in each cell cycle phase using Caco3 cells treatments.

Groups	Percentages of cells in each cell cycle phase		
	G0/G1 phase (Mean± SEM)	S phase (Mean± SEM)	G2/M phase (Mean± SEM)
Caco-3	61% ± 2.36^{a1}	21% ± 2.42 ^{a2}	18% ± 1.05^{c3}
Saffron	16% ±2.11^{c3}	20% ±2.23^{a2}	64% ± 3.60 ^{a1}
MSG	43% ±2.37^{b1}	17% ± 2.94^{a2}	40% ± 3.85^{b1}
SB	42% ±2.18^{b1}	15% ± 2.90 ^{a2}	43% ± 2.91 ^{b1}

Different superscript letters in the same column indicate significant results at $p < 0.05$
Different superscript numbers in the same row indicate significant results at $p < 0.05$

Table (3): Changes in relative expression of *Bax* gene in Caco-3 cells following treatment by Saffron, MSG and SB compounds.

Group^{c}	Fold change mean	SEM
Caco3	1.00^{c}	0.06
Saffron	3.05 ^{a}	0.23
MSG	1.65 ^{b}	0.12
SB	2.55 ^{a}	0.16

Means within the same column carrying different superscript letters are significantly different ($P \leq 0.05$).

Table (4): Effect of Saffron, MSG and SB compounds administration on the relative expression of *Bax* gene in Caco3 cells.

	Bax Ct values	Delta Ct	Delta delta Ct	Relative quantification	SEM
Caco3	26.12	-1.76	0.00	1.00	0.06
Saffron	23.21	-3.37	-1.61	3.05	0.23
MSG	25.88	-2.48	-0.72	1.65	0.12
SB	24.45	-3.11	-1.35	2.55	0.16

Table (5): Changes in relative expression of *p53* gene in Caco-3 cells following treatment by Saffron, MSG and SB compounds.

Group	Fold change mean	SEM
Caco3	1.00 ^{c}	0.05
Saffron	2.58 ^{a}	0.14
MSG	1.45 ^{b}	0.10
SB	2.13 ^{a}	0.09

Means within the same column carrying different superscript letters are significantly different ($P \leq 0.05$).

Table (6): Effect of MSG, SB and Saffron compounds administration on the relative expression of P^{53} gene in Caco3 cells.

	p53 Ct values	Delta Ct	Delta delta Ct	Relative quantification	SEM
Caco3	32.94	5.06	0.00	1.00	0.05
Saffron	29.07	3.69	-1.37	2.58	0.14
MSG	32.18	4.52	-0.54	1.45	0.10
SB	31.73	3.97	-1.09	2.13	0.09

Table (7): Changes in relative expression of *Bcl2* gene in Caco-3 cells following treatment by MSG, SB and Saffron compounds.

Group	Fold change mean	SEM
Caco3	1.00 [a]	0.04
Saffron	0.24 [c]	0.03
MSG	0.62 [b]	0.06
SB	0.30 [c]	0.02

Means within the same column carrying different superscript letters are significantly different ($P \leq 0.05$).

Table (8): Effect of Saffron, MSG and SB compounds administration on the relative expression of Bcl2 gene in Caco3 cells.

	Bcl2 Ct values	Delta Ct	Delta delta Ct	Relative quantification	SEM
Caco3	26.9	-0.98	0.00	1.00	0.04
Saffron	29.04	1.06	2.04	0.24	0.03
MSG	27.77	-0.29	0.69	0.62	0.06
SB	28.54	0.78	1.76	0.30	0.02

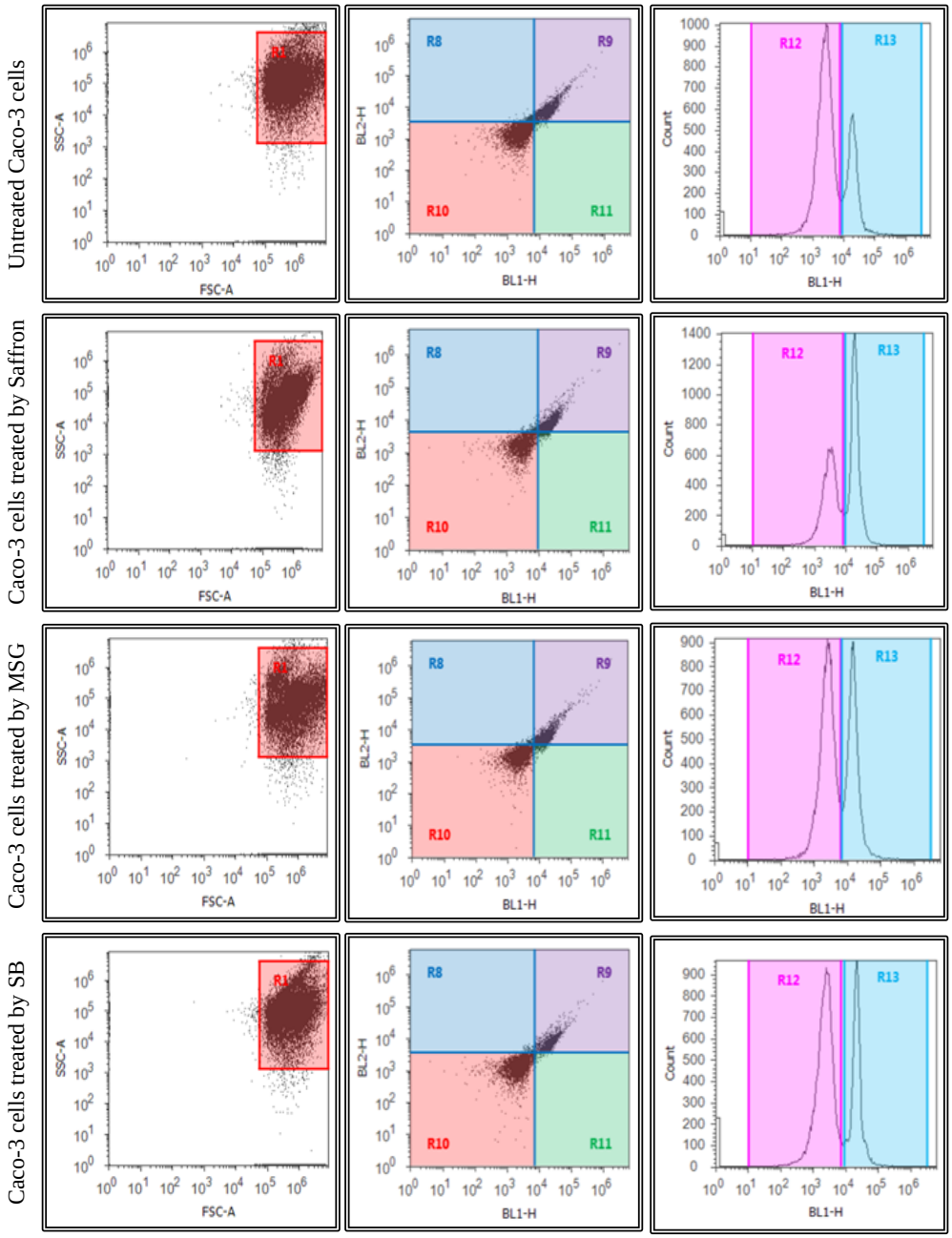

Fig. (1): Effects of MSG, SB and Saffron on G2/M cell cycle arrest. Colon cancer cells line (Caco-3) was treated with food additives at the concentration of (33.92, 15.01 and 3.98 g/mL) in order to check the cell cycle distribution and then were analyzed by flow cytometry.

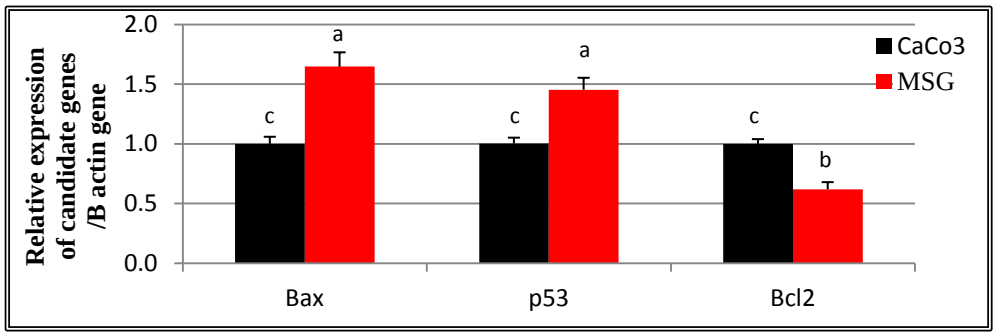

Fig. (2): Effects of MSG on apoptosis-related genes after exposure to 27.77 g/mL, mRNA expression of *p53*, *Bcl-2*, and *Bax* was assessed by quantitative RT-PCR *P < 0.05, compared to the control group.

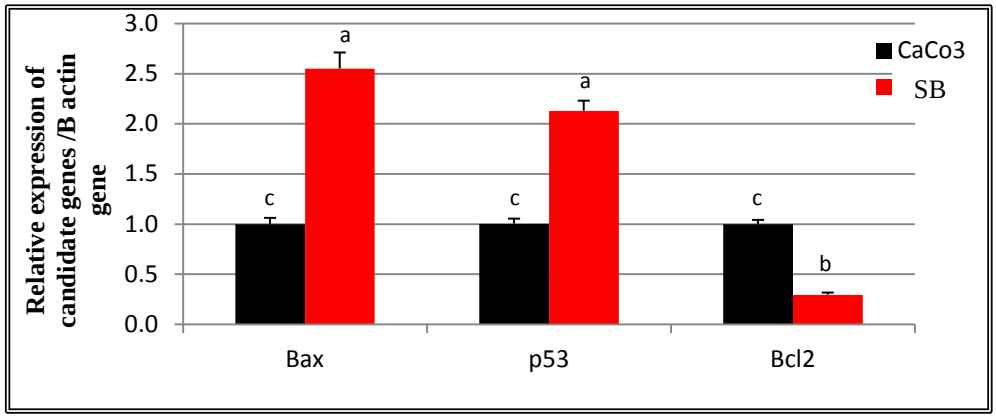

Fig.(3): Effects of SB on apoptosis-related genes after exposure to 28.54 g/mL, mRNA expression of *p53*, *Bcl-2*, and *Bax* was assessed by quantitative RT-PCR *P < 0.05, compared to the control group.

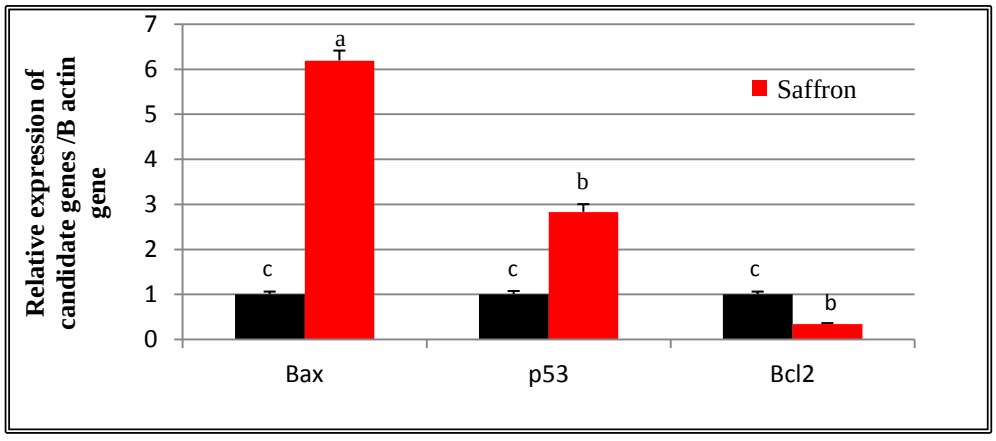

Fig. (4): Effects of Saffron on apoptosis-related genes after exposure to 29.04 g/mL, mRNA expression of *p53*, *Bcl-2*, and *Bax* was assessed by quantitative RT-PCR *P < 0.05, compared to the control group.

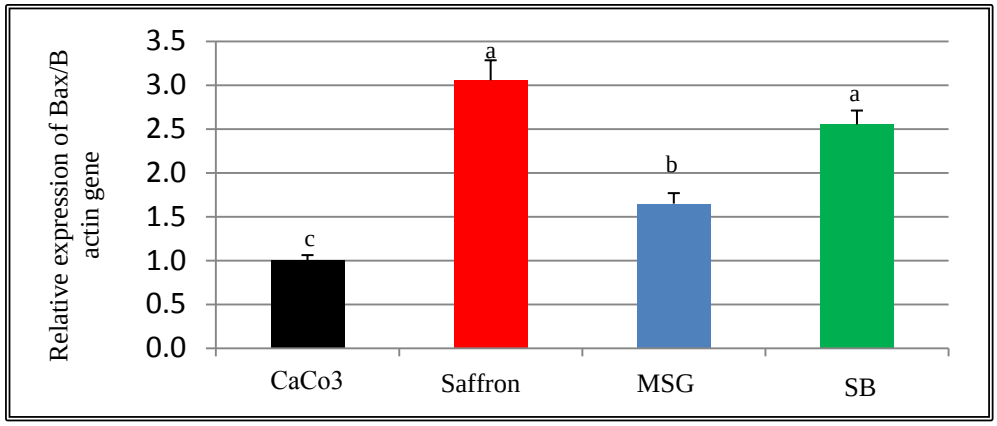

Fig. (5): Graphical presentation of real-time quantitative PCR analysis of the expression of *Bax* gene in Caco3 cells.

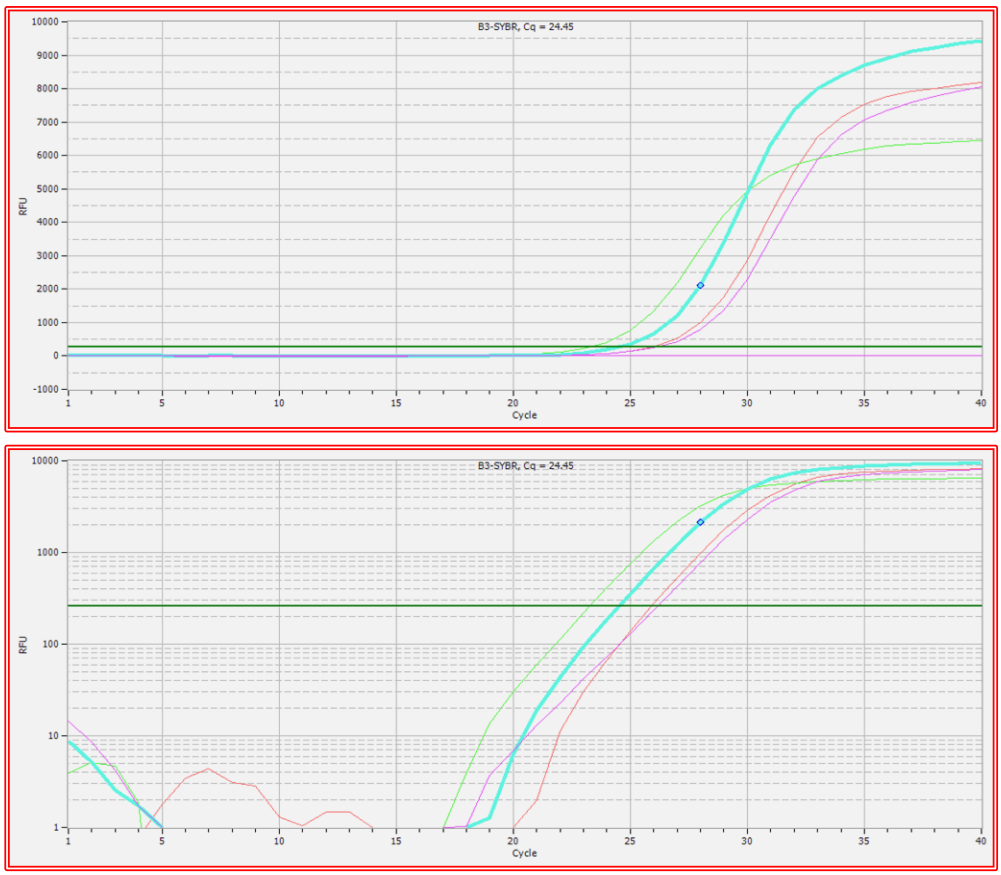

Fig. (6): The linear (upper) and log (lower) amplification curves representing the Ct values of *Bax* gene.

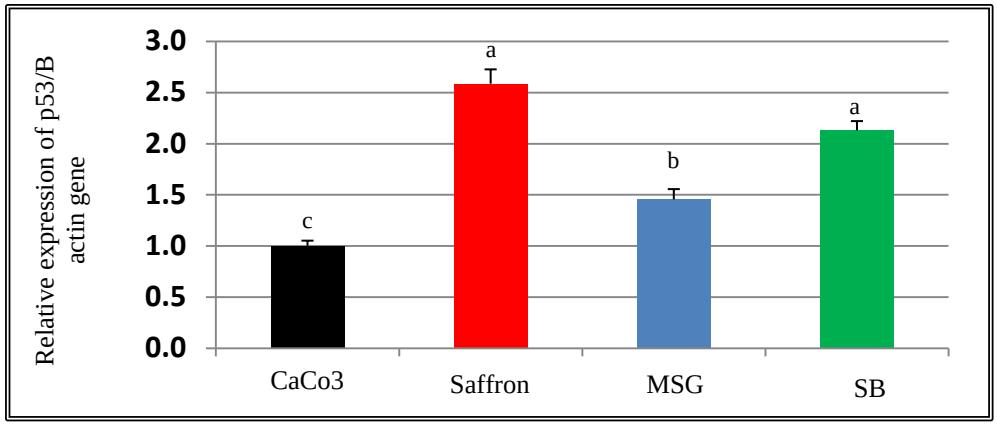

Fig. (7): Graphical presentation of real-time quantitative PCR analysis of the expression of *p53* gene in Caco3 cells.

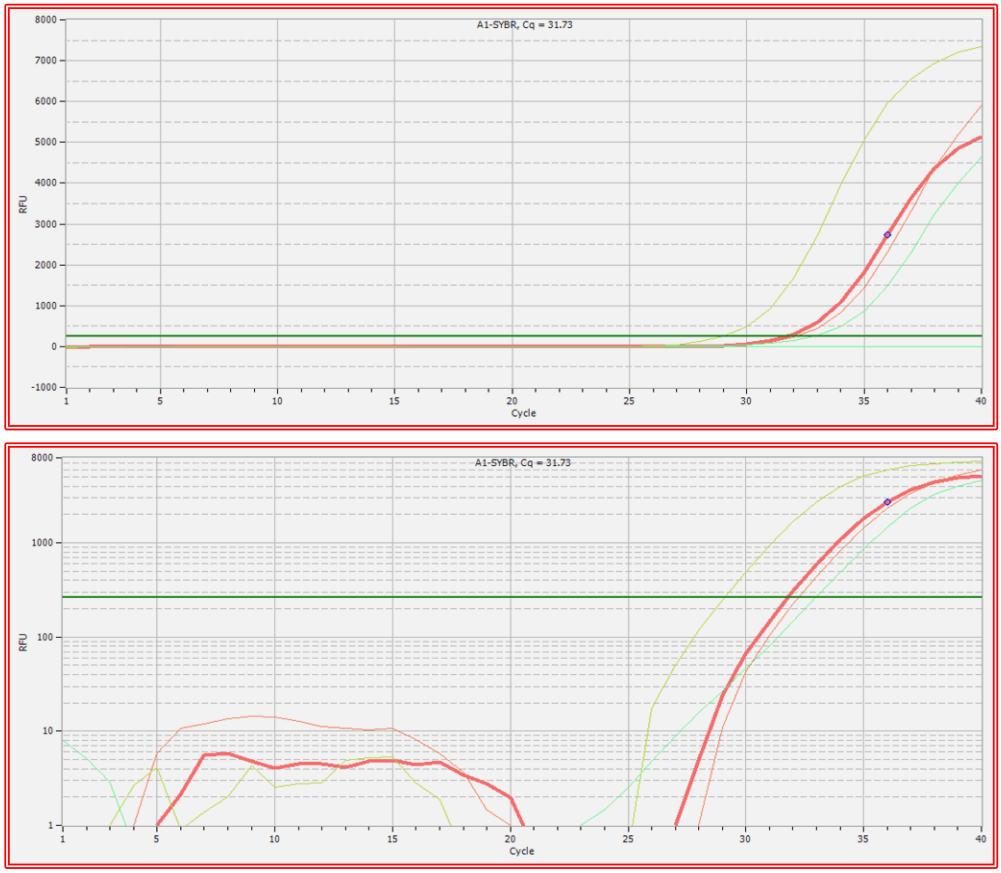

Fig. (8): The linear (upper) and log (lower) amplification curves representing the Ct values of *p53* gene.

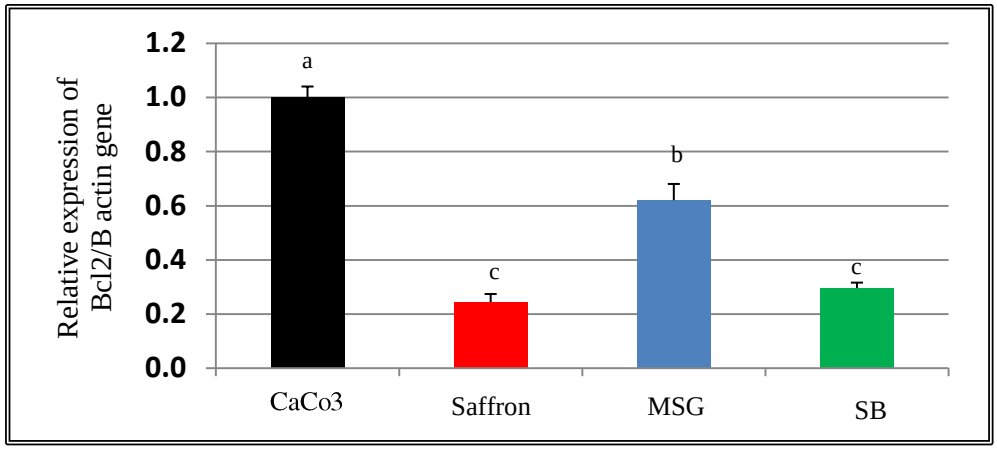

Fig. (9): Graphical presentation of real-time quantitative PCR analysis of the expression of *Bcl2* gene in Caco3 cells.

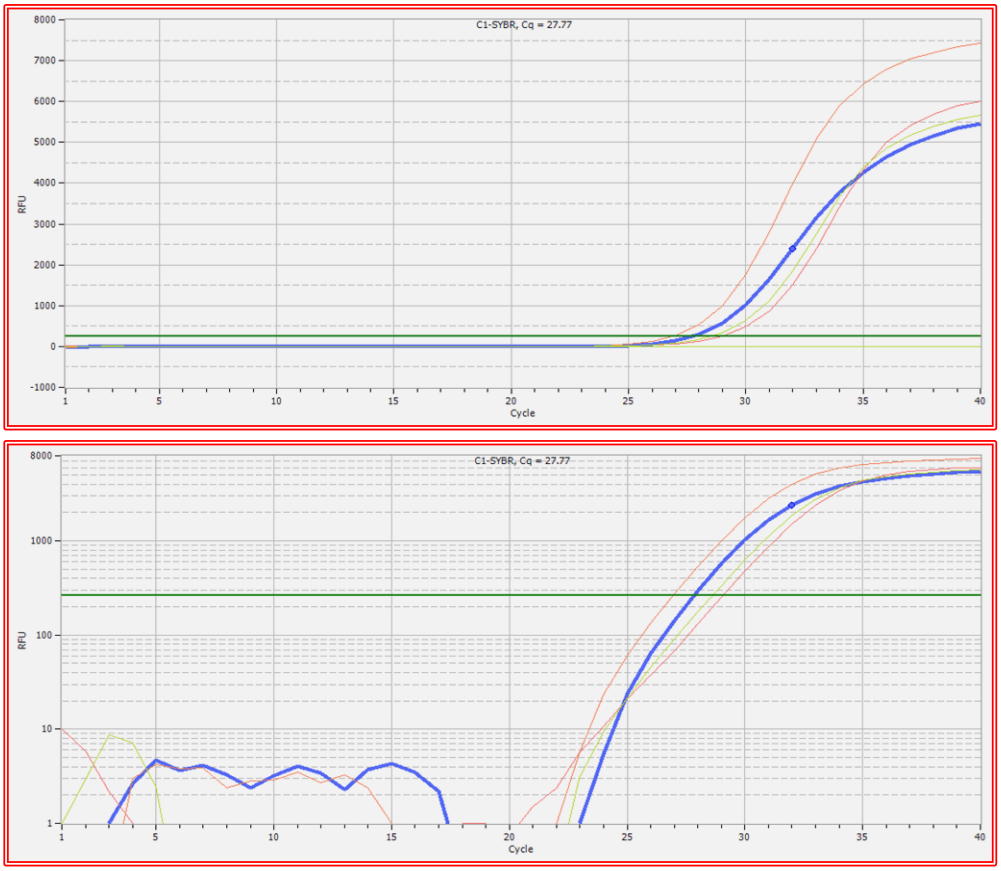

Fig. (10): The linear (upper) and log (lower) amplification curves representing the Ct values of *Bcl2* gene.