JJBS

Volume 19, Number 2,June 2026
ISSN 1995-6673
Pages 261 – 271
https://doi.org/10.54319/jjbs/190209

Jordan Journal of Biological Sciences

Cyto-Genotoxic and Cell Cycle Arrest Assessment of a Synthetic Antioxidant Butylated Hydroxyanisole (BHA)

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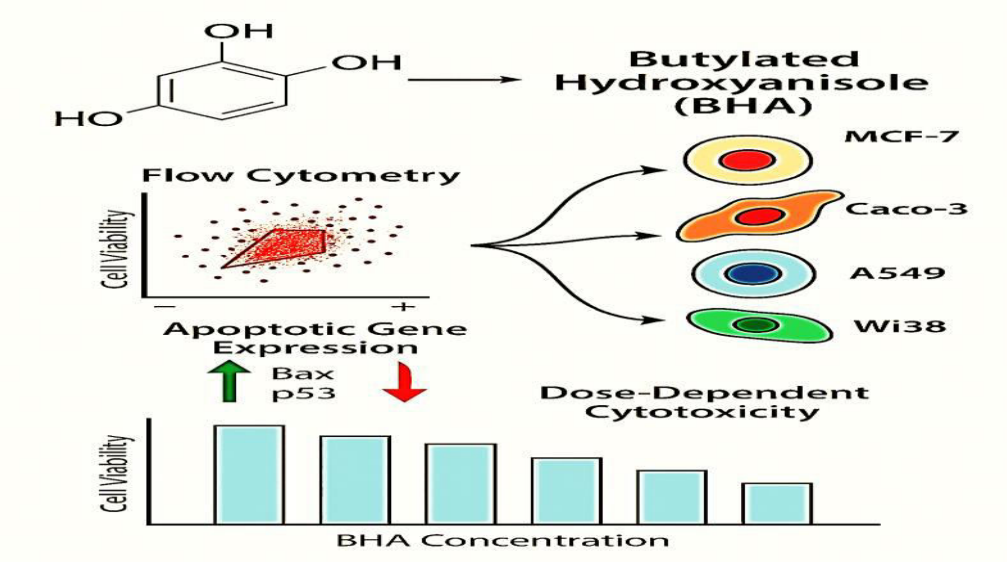
Received: May 22, 2025; Revised: October 31, 2025; Accepted: November 13, 2025

Abstract

In order to stop oxidative deterioration, butylated hydroxyanisole (BHA), a synthetic antioxidant, is widely used in the food, pharmaceutical, and cosmetic industries. Recent cellular and molecular research indicates that BHA may have cytotoxic and genotoxic effects, especially when used at higher concentrations or for longer periods of time, even though it is generally recognized as safe (GRAS) at low quantities. Using the MTT assay, this study examined the effects of different BHA doses (100, 50, 25, 12.5, and 6.25 µg/ml) on the viability of multiple human cell lines, including Wi38 (normal lung fibroblasts), A549 (lung carcinoma), Caco-2 (colon carcinoma), and MCF-7 (breast carcinoma). Propidium iodide (PI) staining labeling was used in flow cytometry to further assess cell cycle. The findings showed that BHA had a cytotoxic effect on every type of cell that was studied. For both malignant and non-malignant cells, the IC50 values were determined to be 58.55 µg/ml for MCF-7, 60.04 µg/ml for Caco-2, 93.61 µg/ml for A549, and 94.01 µg/ml for Wi38. These findings showed a concentration-dependent decrease in cell viability. Additional flow cytometric analysis showed that BHA treatment induced G2/M phase arrest and induced significant G2/M cell cycle arrest and altered the expression of apoptosis-related genes. Furthermore, it was discovered that increased BHA dosages had an impact on the expression of apoptosis-related genes. In particular, the anti-apoptotic gene *Bcl-2* was downregulated in both Caco-2 and ABC-1 cells, whereas the pro-apoptotic markers *Bax* and *p53* were upregulated. In conclusion, the results demonstrate that BHA can change apoptotic gene expression and promote programmed cell death in both healthy and malignant human cells, hence inducing cytotoxic and genotoxic reactions in a dose-dependent manner.

Keywords: Butylated hydroxyanisole (BHA); Cytotoxicity; Genotoxicity; Human cell lines; MTT assay; Apoptosis; Cell cycle arrest; Flow cytometry; Gene expression *Bax*; *p53*; *Bcl-2*.

Graphical Abstract



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1. Introduction

A common synthetic antioxidant used to stop oxidative rancidity in food items, cosmetics, and medications is butylated hydroxyanisole (BHA). 3-tert-butyl-4-hydroxyanisole and 2-tert-butyl-4-hydroxyanisole are its two isomeric constituents. BHA is frequently added to formulations that contain fat and oil in order to extend their shelf life because of its lipophilic qualities.

The toxicological profile of BHA has been extensively investigated through numerous in vitro studies employing both human and animal cell lines. HepG2 (human liver cancer), Caco-2 (human colon carcinoma), and A549 (human lung carcinoma) are cell lines that are often studied. Research indicates that exposure to BHA reduces cell viability, interferes with mitochondrial activity, and causes apoptosis (Lee et al 2012; Okamura et al 2023).

Characterizing BHA-induced apoptosis and changes in cell cycle progression has been made possible thanks in large part to flow cytometry. BHA treatment dramatically raised the sub-G1 population and Annexin V/PI staining in A549 cells, two markers of apoptosis. Additionally, treatment to BHA caused G0/G1 phase arrest, indicating that cell cycle progression was inhibited (Lee et al 2012; Tan et al 2022).

The application of quantitative real-time PCR (qRT-PCR) has been used to investigate the underlying molecular pathways. According to studies, BHA downregulates the anti-apoptotic gene *Bcl-2* while upregulating pro-apoptotic genes including *Bax* and Caspase-3. Furthermore, exposure to BHA has been shown to modulate the expression of several genes associated with oxidative stress and DNA repair pathways, including *p53*, GADD45, and XRCC1 (Demiray et al. 2021; Farhadi et al. 2024). Food additives' genotoxic and apoptotic effects on human cell lines were investigated by Rashad et al. (2018), with particular attention on how they affected genes linked to apoptosis and cell cycle regulation. Additional research by Rashad et al. (2019, 2021) showed that these substances hindered the growth of human cells, both malignant and non-cancerous, and were cytotoxic to Saccharomyces cerevisiae. The comet test was used to assess DNA damage and identify the ideal amounts of these food-related compounds in a variety of yeast knockout strains.

According to a different study by Rashad et al. (2022), gold nanorods (AuNRs) cytotoxically affected HepG2, Caco-2, A549, and CDD-19Lu cell lines, mostly by inducing cell cycle arrest in the G2/M phase. This implies that AuNRs have harmful effects on both healthy and cancerous cells. BHA has also been demonstrated to lower cell viability in a number of human cell lines. In a different study, Ali et al. (2024) discovered that doxorubicin caused PC3 prostate cancer cells to undergo apoptosis and G2/M arrest, along with increased *p53* and *Caspase-3* expression and decreased *Bcl-2* at higher dosages.

Tamarix nilotica ethyl acetate extracts were tested for their anticancer effects on A549 lung carcinoma cells by Abdel-Tawab et al. (2019). Normal Wi38 fibroblasts were largely unaffected by the extracts, whereas cancer cells showed notable cytotoxicity. The specific toxicity for cancer cells was validated by the Neutral Red assay.

El-Attar et al. (2019) used the MTT test to examine the cytotoxic activity of extracts from Salvadora palaestina and Ziziphus album. HepG2 cells treated with these plant extracts experienced G2/M phase arrest, according to flow cytometry. Additionally, qRT-PCR analysis revealed that, in comparison to untreated controls, the cell cycle regulators Cyclin B1 and CDK1 were downregulated.

According to the results of the MTT assay, SF6 significantly increased the cytotoxicity and induced apoptosis in HepG2, MCF-7, and HeLa cancer cells, while no toxicity was seen in normal lymphocytes. The results of qRT-PCR showed that SF6 upregulated the apoptosis regulating genes *Bax* and *p53* in cancer cells (Parveen and Varalakshmi 2023).

While single-nucleotide polymorphisms (SNPs) had negligible toxicity to normal fibroblast cells, they dramatically boosted calprotectin's toxicity against A549, MCF7, and PANC-1 cells at 0.75 µg/ml. It was also clear that co-incubation of SNPs and camptothecin successfully disrupted the pathways crucial for cancer cell survival because SNPs reduced the production of vascular endothelial growth factor (VEGF) and altered the profile of inflammatory markers and antioxidant enzymes (Alqaraleh et al. 2025). The chosen therapeutic leech species, the protein dosage or content of the leech saliva, and the applied cell line may all have different and variable impacts on the cell survival, migration, and death of different cell lines (Ünal et al., 2025). NTBF was identified as a novel inducer of cell death due to its cytotoxic activity, ability to arrest the cell cycle, and potent induction of apoptosis, primarily through activation of the caspase-dependent apoptotic pathway rather than the NRF2/GCLM antioxidant signaling pathway. Furthermore, NTBF strain ATCC-23745 might be a secret probiotic that is employed in future research on the therapy of colorectal cancer (Loniakan et al., 2023).

Using HepG2, A549, and Wi38 cell lines, Mousa et al. (2022a, 2022b) investigated the cytotoxic effects of different heavy metal concentrations. Significant DNA damage was found by the comet assay, especially in yeast strains, suggesting a high potential for genotoxicity.

The purpose of the study is to determine the underlying apoptotic mechanisms by assessing the cytotoxic and genotoxic effects of the synthetic antioxidant Butylated Hydroxyanisole (BHA) on human cell lines using cellular and molecular assays, such as RT-PCR and flow cytometry.

2. Materials and Methods

2.1. Materials

2.1.1. Butylated hydroxyanisole (BHA)

- Properties

The industrial antioxidants butylated hydroxyanisole (BHA) comes in two isomeric varieties: 2-tert-butyl-4-hydroxyanisole (2-BHA) and 3-tert-butyl-4-hydroxyanisole (3-BHA). BHA compositions must have at least 85% of the 3-BHA isomer, according to requirements set by the European Union and JECFA (EFSA, 2011).

- Concentrations

100, 50, 25, 12.5, and 6.25 µg/ml of BHA were utilized.

2.1.2. Cell lines

2.1.2.1. Mammalian Cell Lines

The American Type Culture Collection (ATCC, Rockville, MD, USA) provided the cell lines used in this study, including MCF-7 (human breast carcinoma), Caco-2 (human colon carcinoma), A549 (human lung carcinoma), ABC-1 (human lung adenocarcinoma), and Wi38 (normal human lung fibroblasts). Based on the cytotoxicity results, Caco-2 and ABC-1 cell lines were selected for further mechanistic investigations, including cell cycle analysis by flow cytometry and quantitative real-time PCR (qRT-PCR), to evaluate the molecular events associated with BHA-induced cytotoxicity and apoptosis.

2.1.2.2. Culture Media and Reagents

Lonza (Belgium) provided the necessary cell culture reagents, which included fetal bovine serum (FBS), RPMI-1640 medium, HEPES buffer, L-glutamine, gentamycin, and 0.25% Trypsin-EDTA.

2.1.2.3. Cell Line Maintenance

The cells were grown in RPMI-1640 media with 50 µg/ml of gentamycin and 10% heat-inactivated fetal bovine serum added. For the purpose of maintaining ideal growth circumstances, sub-culturing was carried out two or three times every week.

2.2. Methods

2.2.1. Cytotoxicity evaluation

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was used to assess cytotoxicity. Following a 24-hour incubation period, tumor cell lines were seeded into Corning 96-well plates at a density of 5×10^4 cells per well for antitumor testing. Following incubation, the 96-well plates were treated in triplicate with different doses of BHA. The control was a 0.5% solution of dimethyl sulfoxide (DMSO). According to Capone et al. (2014), the MTT test was used to assess cell viability after a 24-hour treatment period.

2.2.2. Cell cycle analysis by propidium iodide (PI) assay using flow cytometry

Warmed trypsin-EDTA combined with warm phosphate-buffered saline (PBS) with 0.25% ethylenediaminetetraacetic acid (EDTA) (500 µl trypsin + 500 µl PBS) were added to cell lines and maintained for 10 minutes at 37°C. The process adhered to Rashad et al.'s technique (2018, 2022). Attune flow cytometry was used to examine the labeled cell lines (Applied Biosystems, USA).

2.2.3. Cell Cycle Analysis by PI assay using flow cytometry

2.2.3.1. Cell Collection and Staining

The supernatant was removed following centrifugation at 1-5 × 10^5 cells. Following two rounds of washing in warm PBS solution, the recovered cells were once again suspended in 500 µl of 1X binding buffer. Following the protocol described by Vermes et al. (1995) and Ali et al. (2024), 5 µl of Propidium Iodide (PI) was added, and the cells were left to stand for 5 minutes at low temperatures in dark conditions.

2.2.3.2. Flow Cytometry Analysis

PI-stained cells was analyzed using an Attune Flow Cytometer (Applied Biosystems, USA).

2.2.4. Quantitative RT-PCR Analysis of Bax, Bcl-2, and p53 Gene Expression Following BHA Treatment

Total RNA was extracted from treated and untreated cell lines using the GeneJET RNA Purification Kit (Thermo Scientific, #K0731, USA) according to the manufacturer's instructions. RNA concentration and purity were determined spectrophotometrically. A total of 5 µg of RNA was reverse transcribed into complementary DNA (cDNA) using the RevertAid H Minus Reverse Transcriptase Kit (Thermo Scientific, #EP0451, USA). Quantitative real-time PCR (qRT-PCR) was performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA) to evaluate the expression levels of the apoptosis-related genes *Bax*, *Bcl-2*, and *p53* described by Rashad et al. (2026). *β-actin* was used as the housekeeping gene for normalization. Each PCR reaction was carried out in a total volume of 25 µL containing 12.5 µL of 2X Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, #K0221, USA), 2 µL of cDNA, 1 µL of each forward and reverse primer, and 8.5 µL of nuclease-free water. Thermal cycling conditions consisted of an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec. A melt curve analysis was performed from 63°C to 95°C to verify amplification specificity (Table 1 & Table 2). Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen (2001).

Table 1. The thermal cycler condition used during real time PCR.

Initial denaturation	Denaturation	Annealing	Extension	Cycles
95˚C/ 10 min	95˚C/ 15 sec	60˚C/ 30 sec	72˚C/ 30 sec	40

β-actin was used as the housekeeping gene for normalization of target gene expression levels. Relative gene expression was quantified using the comparative Ct ($2^{-\Delta\Delta Ct}$) method described by Livak and Schmittgen (2001). The threshold cycle (Ct) values of the target genes were normalized to the Ct value of the reference gene (*β-actin*) to obtain ΔCt values. The untreated control group served as the calibrator, whereas the treated groups were considered the test samples. Relative fold changes in gene expression were calculated using the following equations:

ΔCt (test) = Ct (target gene in treated group) − Ct (*β-actin* in treated group)

ΔCt (calibrator) = Ct (target gene in control group) − Ct (*β-actin* in control group)

$\Delta\Delta Ct = \Delta Ct$ (test) − ΔCt (calibrator)

Fold change = $2^{-\Delta\Delta Ct}$

Table 2. Forward and reverse primer sequences for *Bax*, *Bcl-2*, *p53*, and *β-actin* genes.

Gene	Forward primer (5′→ 3′)	Reverse primer (3′→ 5′)
Bax	CCTGTGCACCAAGGTGCCGGAACT	CCACCCTGGTCTTGGATCCAGCCC
Bcl-2	ATCGCCCTGTGGATGACTGAGT	GCCAGGAGAAATCAAACAGAGGC
p53	CCTCAGCATCTTATCCGAGTGG	TGGATGGTGGTACAGTCAGAGC
β-actin	CACCAACTGGGACGACAT	ACAGCCTGGATAGCAACG

2.2.5. Statistical analysis

Data are presented as mean ± standard error (SEM) of three independent experiments. With SPSS 18.0 software (2011), one-way analysis of variance (ANOVA) was used to establish statistical significance. The formula IC50 = (0.5 - b) / a, where b is the intercept and an is the slope factor, was used to determine the half-maximal inhibitory concentration (IC50), which shows how effective a substance is at blocking a particular biological or biochemical activity.

3. Results

3.1. Cytotoxicity evaluation

The MTT test was used to measure cytotoxicity. Cell viability gradually decreased as the BHA concentration rose, as Table 3 illustrates. Higher doses of BHA increased its cytotoxicity and toxicity, which reduced the viability of all cell lines. Table 3 and Figure 1 show the dose-response curves for cell viability and the concentration that causes 50% growth inhibition (IC50) for MCF-7, Caco-2, A549 and Wi38 cell lines.

The IC50 levels of tetrazolium, which served as a positive control in this investigation, were also examined in response to BHA dosages. The findings showed that all cancer cell lines had mild cytotoxicity when exposed to BHA concentrations. In particular, the IC50 values for human lung cancer (A549), human colon carcinoma (Caco-2), and human breast carcinoma (MCF-7) were 93.61 µg/ml, 60.04 µg/ml, and 58.55 µg/ml, respectively. With an IC50 of 94.01 µg/ml, BHA also demonstrated cytotoxic effects on human lung normal cells (Wi38) (Table 3 and Figure 1). These results demonstrate that BHA decreased the viability of both cancerous and healthy cells.

Table 3. Cytotoxic effects and IC_{50} values of BHA on MCF-7, Caco-2, A549, and Wi38 cell lines.

IC50	Conc. ug/m l	SD	Viability %	Toxicity %	ID
58.55	6.25	0	100	0	MCF-7
	12.5	0	100	0	[Breast cell line]
	25	0.02	65.39	34.61	
	50	0.022	81.62	18.38	
	100	0.012	85.47	14.53	
60.04	6.25	0	100	0	Caco-2
	12.5	0	100	0	[Human Colon cancer cell line]
	25	0.01	85	15	
	50	0.013	82	18	
	100	0.012	66	34	
93.61	6.25	0	100	0	A549
	12.5	0.01	100	0	[Human lung cancer cell line]
	25	0.009	86.3	13.7	
	50	0.205	81	19	
	100	0.004	76.11	23.89	
94.01	6.25	0	100	0	Wi38
	12.5	0.07	100	0	[Human lung normal cell line]
	25	0.065	86	14	
	50	0.004	81	19	
	100	0.061	76.1	23.9	

Sd= standard deviation **IC_{50}=**The half-maximal inhibitory concentration

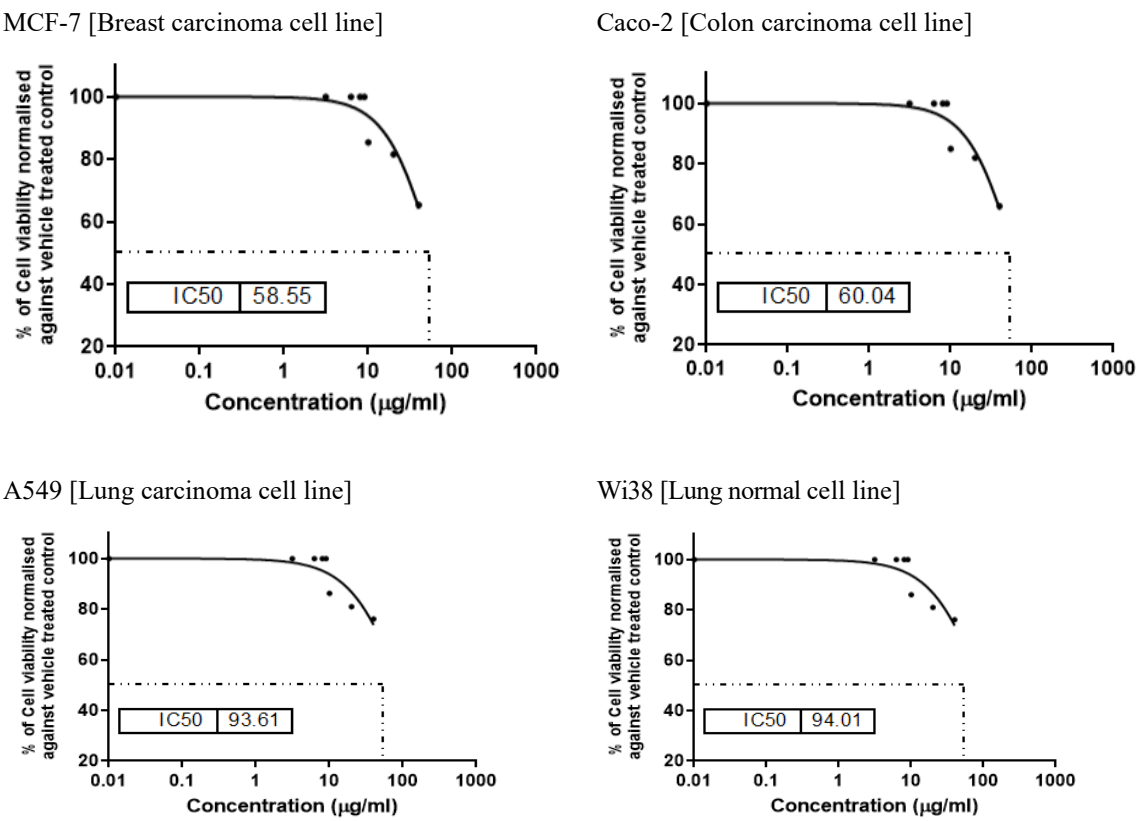

Figure 1. The non-malignant human's lung cell line Wi38 (used as a control) and the malignant human cell lines MCF-7, Caco-2, and A549 were utilized to assess the inhibitory effect of BHA on cell growth. The MTT assay was used to evaluate cell viability. In comparison to the untreated control group, statistical significance was established at *p < 0.05. The data are shown as mean values (n = 3). For every one of the four cell lines, the half-maximal inhibitory concentration (IC50) was determined.

3.2. Cell Cycle Analysis by PI Assay Using Flow Cytometry

The arrangement of DNA content in Caco-2 and ABC-1 cell lines was affected by the 50 µg/ml BHA exposure. After receiving BHA treatment, the average DNA content in the G0/G1 phase dropped from 60% ± 2.24 (Caco-2 control) and 62% ± 2.36 (ABC-1 control) to 15% ± 2.67 and 48% ± 2.27, respectively. Following treatment, the DNA content decreased from 24% ± 1.28 (Caco-2) and 21% ± 1.28 (ABC-1) to 20% ± 1.11 and 17% ± 1.50 in the S phase, showing a similar pattern.

On the other hand, after BHA treatment, the G2/M phase revealed a substantial increase in DNA content. Tables 4 and 5 show that G2/M DNA content averages for Caco-2 and ABC-1 cells treated with BHA were 65% ± 2.90 and 35% ± 2.32, respectively, while the untreated controls had G2/M DNA content averages of 16% ± 1.00 and 17% ± 1.47.

Overall, both cell lines' normal cell cycle progression was disturbed by treatment with BHA at 50 µg/ml, which resulted in a significant increase in the number of cells halted in the G2/M phase. According to these findings, BHA causes cell cycle arrest at the G2/M checkpoint, as shown in Figure 2, where both Caco-2 and ABC-1 cells exhibit a significant buildup of DNA. These alterations indicate that BHA induces cell cycle arrest at the G2/M phase, contributing to its cytotoxic effects.

Table 4. shows the percentage of DNA content in unprocessed Caco-2 cells (control) during various cell cycle phases in comparison to Caco-2 cells that received 50 µg/ml of BHA.

Groups	Average of DNA content % of cells in each cell cycle phase		
	G0/G1 phase (Mean± SEM)	S phase (Mean± SEM)	G2/M phase (Mean± SEM)
Caco-2-Control	60% ± 2.24^{a1}	24% ± 1.28^{a2}	16% ± 1.00^{c3}
Caco-2-Treated with BHA	15% ±2.67^{c3}	20% ±1.11^{a2}	65% ± 2.90^{a1}

Table 5 shows the variance of DNA content percentages in both handled (control) and stimulated (50 µg/ml BHA) ABC-1 cells across cell cycle cycles.

Groups	Average of DNA content % of cells in each cell cycle phase		
	G0/G1 phase (Mean± SEM)	S phase (Mean± SEM)	G2/M phase (Mean± SEM)
ABC-1 Control	62% ± 2.36^{a1}	21% ± 1.28^{a2}	17% ± 1.47^{c2}
ABC-1 Treated with BHA	48% ±2.27^{b1}	17% ±1.5^{a3}	35% ± 2.32^{a2}

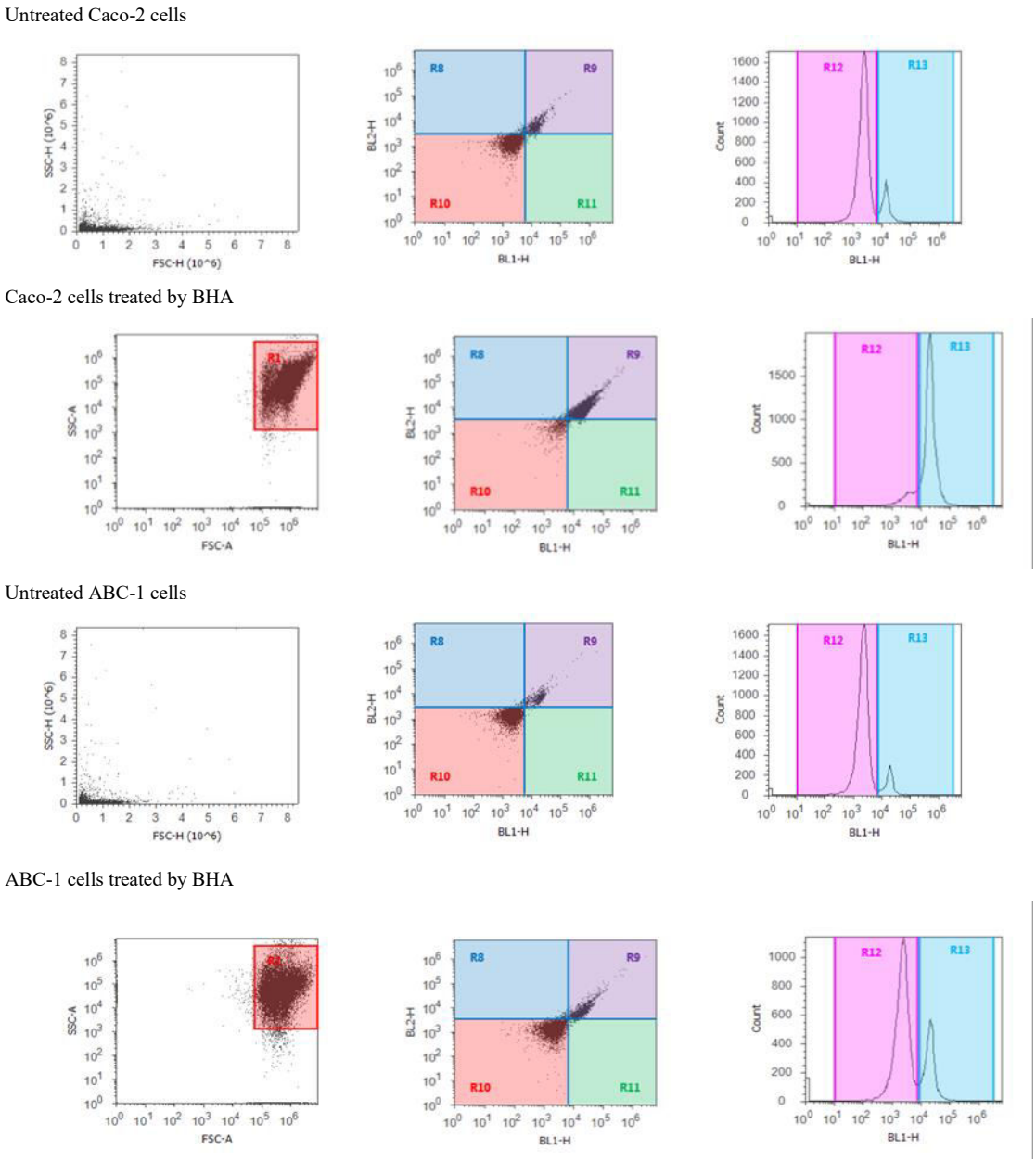

Figure 2. When comparing controls without treatment with cells treated with 50 µg/ml BHA, flow cytometric examination of the cell cycle distribution in the colon and lung cancer cell lines Caco-2 and ABC-1 reveals accumulation during the G2/M phase.

The findings of this investigation showed that BHA causes G2/M phase arrest in both Caco-2 and ABC-1 cell lines, hence causing cytotoxic effects. Flow cytometry confirmed an increase in the G2/M population after BHA treatment, and this arrest was seen across a dose range of 6.25 to 100 µg/ml. At doses of 6.25, 12.5, 25, 50, and 100 µg/ml, which correspond to physiologically significant exposure levels, a dose-dependent cytotoxic reaction was visible. The activation of *Bax* and the mitochondrial (intrinsic) route are two of the several routes that control the induction of apoptosis. Using cell culture models, the effect of BHA on cell death was further evaluated at a dose of 50 µg/ml. Figure 2 shows that BHA induced marked G2/M cell cycle arrest in both Caco-2 and ABC-1 cells.

3.3. Evaluation of BHA-Induced Genotoxicity by Quantitative RT-PCR Gene Expression Analysis of Bax, Bcl-2, and p53 in Caco-2 Cell Lines

Using quantitative real-time PCR (RT-PCR), the expression of important apoptosis-regulating genes, such as *Bax*, *p53*, and *Bcl-2*, was examined in order to assess the possible genotoxic effects of BHA on Caco-2 cell lines.

Pro-apoptotic genes were significantly upregulated after BHA therapy. In particular, compared to untreated controls, *p53* increased by 3.58 and *Bax* expression increased by 8.51-fold (Table 6). On the other hand, the expression of the anti-apoptotic gene *Bcl-2* dropped to 0.08 times that of the control group, indicating a considerable downregulation (Table 6). Figure 3 further demonstrates these results by confirming that the treated cells had increased expression of *p53* and *Bax*. The data presented reflect the relative gene expression levels

calculated according to the 2^−ΔΔCt method, where *β-actin* was used as the housekeeping gene for normalization. For each sample, the ΔCt value was obtained by subtracting the Ct value of *β-actin* (reference gene) from that of the target gene (ΔCt = Ct_target − Ct_reference). The ΔΔCt value was calculated by comparing the ΔCt of the treated group with the ΔCt of the control group (ΔΔCt = ΔCt_treated − ΔCt_control). The relative expression (fold change) was then determined using the equation 2^−ΔΔCt, where values greater than 1 represent gene upregulation, while values below 1 indicate downregulation.

According to the research, BHA mainly causes apoptosis in Caco-2 cell lines by downregulating survival signals mediated by *Bcl-2* and upregulating pro-apoptotic pathways (such as *p53* and *Bax*).

Table 6. RT-qPCR measurements of the relative mRNA expression levels of the *Bax*, *p53*, and *Bcl-2* genes in Caco-2 cell lines after BHA treatment.

Sample data	Groups TE& TE	Ct (*Bax*)values	*β-actin* ΔCT-HC	ΔΔCt (treated vs control)	Fold change (2^−ΔΔCt)	SEM
Sample code (*Bax*)	Caco-2 control	29.63	1.75	0.00	1.00	0.06
	Caco-2 treated	24.74	-1.34	-3.09	8.51	0.29
Sample data	Groups TE& TE	Ct *p53* values	*β-actin* ΔCT-HC	ΔΔCt (treated vs control)	Fold change (2^−ΔΔCt)	SEM
Sample code (*p53*)	Caco-2 control	28.13	0.25	0.00	1.00	0.07
	Caco-2 treated	25.49	-1.59	-1.84	3.58	0.21
Sample data	Groups TE& TE	Ct *Bcl-2* alues	*β-actin* ΔCT-HC	ΔΔCt (treated vs control)	Fold change (2^−ΔΔCt)	SEM
Sample code (*Bcl-2*)	Caco-2 control	28.96	1.08	0.00	1.00	0.06
	Caco-2 treated	32.64	4.66	3.58	0.08	0.01

Legend: Ct = cycle threshold for the target gene (*Bax*). ΔΔCt = (ΔCt_treated − ΔCt_control), where ΔCt = Ct_target − Ct_reference (reference = *β-actin*). Fold change = 2^−ΔΔCt. Values are shown as mean fold change ± SEM. Upregulation is indicated by fold change >1.

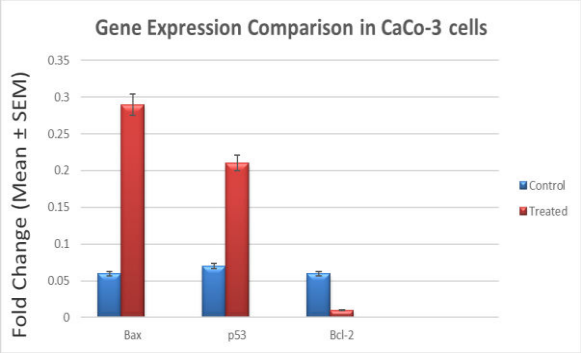

Figure 3: Comparative RT-PCR analysis of the expression levels of apoptosis-related genes (*p53*, *Bax*, and *Bcl-2*) in response to 50 µg/ml BHA treatment. *P less than 0.05 in relation to the untreated control.

3.4. Quantitative RT-PCR Analysis of BHA-Induced Genotoxicity on Genes Associated with Apoptosis in ABC-1 Cell Lines

The expression levels of important apoptosis-regulatory genes, including *Bax*, *p53*, and *Bcl-2*, were analyzed using quantitative real-time PCR (qRT-PCR) in order to ascertain the genotoxic effects of BHA on ABC-1 lung cancer cells. The pro-apoptotic genes' expression significantly increased after BHA treatment. In particular, *p53* and *Bax* expression were 4.20 and 3.56 times higher, respectively, than in the medicated control group (Table 7). On the other hand, the anti-apoptotic gene *Bcl-2*'s expression was drastically decreased, falling to 0.05 times that of control levels. Figure 4 depicts these alterations, emphasizing the increased expression of *p53* and *Bax* following therapy.

According to these findings, BHA mainly induces apoptosis in ABC-1 cells by upregulating the expression of genes linked to apoptosis and downregulating the expression of genes linked to survival, such *Bcl-2*.

Table 7. qRT-PCR analysis of the relative expression levels of the genes *Bax*, *p53*, and *Bcl-2* in ABC-1 cell lines following BHA therapy.

Sample data	Groups TE& TE	Ct (*Bax*) values	*β-actin* ΔCT-HC	ΔΔCt (treated vs control)	Fold change (2^−ΔΔCt)	SEM
Sample code (*Bax*)	ABC-1 control	27.53	-0.35	0.00	1.00	0.04
	ABC-1 treated	25.9	-2.18	-1.83	3.56	0.24
Sample data	Groups TE& TE	Ct *p53* values	*β-actin* ΔCT-HC	ΔΔCt (treated vs control)	Fold change (2^−ΔΔCt)	SEM
Sample code (*p53*)	ABC-1 control	31.27	2.39	0.00	1.00	0.04
	ABC-1 treated	25.4	0.32	-2.07	4.20	0.25
Sample data	Groups TE& TE	Ct *Bcl-2* values	*β-actin* ΔCT-HC	ΔΔCt (treated vs control)	Fold change (2^−ΔΔCt)	SEM
Sample code (*Bcl-2*)	ABC-1 control	26.78	0.90	0.00	1.00	0.06
	ABC-1 treated	32.33	5.25	4.35	0.05	0.01

Legend: Ct = cycle threshold for the target gene (*Bax*). ΔΔCt = (ΔCt_treated − ΔCt_control), where ΔCt = Ct_target − Ct_reference (reference = *β-actin*). Fold change = 2^−ΔΔCt. Values are shown as mean fold change ± SEM. Upregulation is indicated by fold change >1.

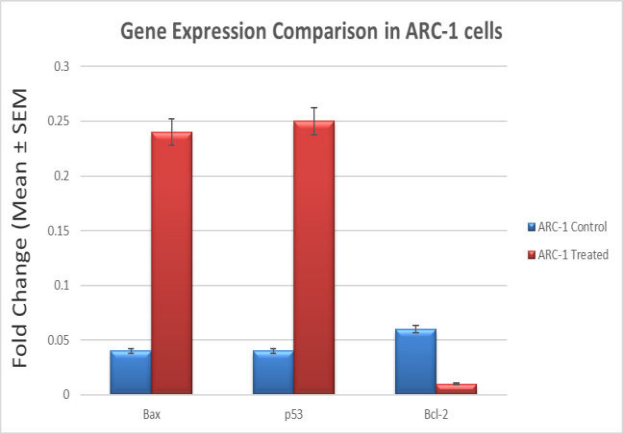

Figure 4: Quantitative RT-PCR was used to evaluate the effects of 50 µg/ml BHA on apoptotic-related genes, including mRNA expression of *Bax*, *Bcl-2*, and *p53*, with *P < 0.05 when compared to the control group.

In comparison to the untreated control group, the results showed a considerable elevation of *Bax* gene expression in both Caco-2 and ABC-1 cells after BHA treatment. Following exposure to the BHA chemical This increase in expression was clearly evident in both treated cell lines, as Tables 6 and 7 and Figures 3 and 4 demonstrate.

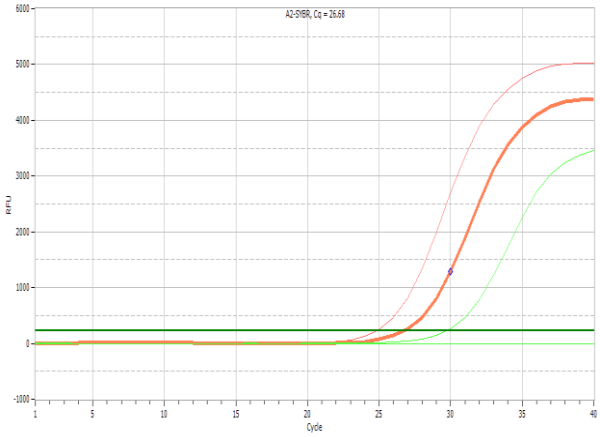

Figure 5 shows the cycle threshold (Ct) measurements for the expression of the *Bax* gene as linear (top) and logarithmic (bottom) amplified curves.

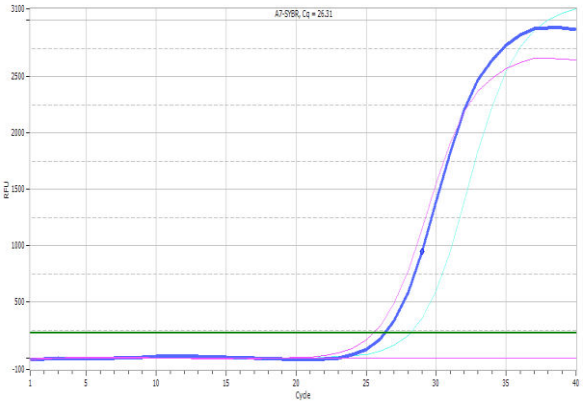

Figure 6. The quadratic (upper) and log (lower) amplification curves in illustrate the *p53* gene's frequency of transcription.

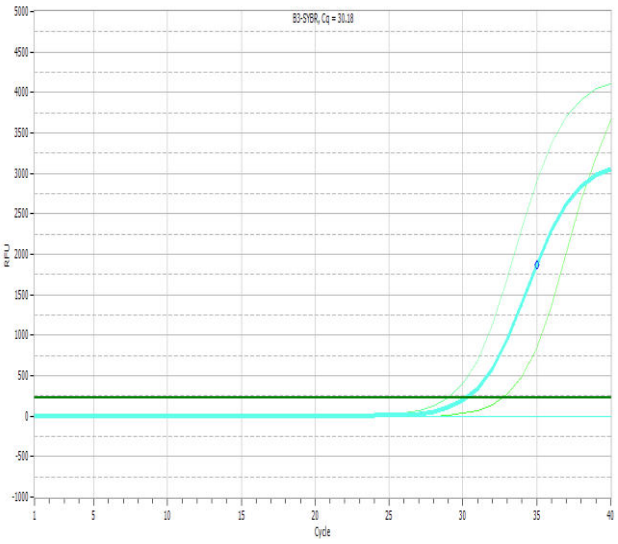

Figure 7 The log (lower) and logarithmic (upper) multiplying curves that match the Ct values for the *Bcl-2* gene are displayed.

4. Discussion

Butylated Hydroxyanisole's (BHA) cytotoxic and genotoxic impacts on human carcinoma cell lines, such as Caco-2 (colon cancer) and ABC-1 (lung cancer) cells, were the focus of this investigation. Using MTT tests, flow cytometry, and quantitative RT-PCR, we investigated the effects of BHA on cell viability, cell cycle progression, and the expression of genes linked to apoptosis (*Bax*, *Bcl-2*, and *p53*).

5. Cell viability and cytotoxicity

After BHA treatment, the MTT assay showed a dose-dependent decrease in cell viability. The MCF-7, Caco-2, A549, and Wi38 cell lines had IC50 values of 58.55 µg/ml, 60.04 µg/ml, 93.61 µg/ml, and 94.01 µg/ml, respectively, as indicated in Table 3. These findings imply that BHA has cytotoxic effects on cell lines that are malignant as well as those that are not. These results are in line with earlier research that documented BHA's cytotoxic effects on many cancers cell types (Capone et al 2014). To maximize its therapeutic potential for different cancer types, more research is necessary, as evidenced by the comparatively high amounts of BHA needed to produce noticeable cytotoxicity.

In a number of human cell lines, including MCF-7 (breast carcinoma), Caco-2 (colon carcinoma), A549 (lung carcinoma), and Wi38 (normal lung cells, used as a control), studies showed an important buildup and cytotoxic effect of different BHA concentrations. When compared to the other lines, BHA showed the strongest effect on Wi38 cell viability, suggesting greater sensitivity. MCF-7 cells, on the other hand, responded the least, showing just slight alterations in viability. These results are in line with research by Rashad et al. (2019, 2022), who found that different dietary additives had distinct effects on the morphology and survival of human cancer cell lines Caco-2, MCF-7, A549, and normal Wi38 cells. Their findings supported worries about these drugs' potential to cause cancer by highlighting notable differences between the treated and control groups. The employing of these models to evaluate cytotoxic potential was further validated by Mousa et al. (2022), who found that exposure to heavy metals significantly decreased the growth of HepG2, A549, and Wi38 cell lines as measured by the MTT assay.

6. Analysis of the Cell Cycle

Both Caco-2 and ABC-1 cell lines experienced G2/M phase arrest when exposed to BHA, according to flow cytometric monitoring of the cell cycle. The percentage of cells in the G2/M phase significantly increased, as seen in Figures 2, 3, and 5, suggesting that BHA impedes the progression of the cell cycle. These results are consistent with earlier research indicating that a number of cytotoxic drugs, such as BHA, induce G2/M arrest by interfering with the regular cell cycle and causing checkpoint activation or DNA damage (Rashad et al. 2022 & Rashad et al. 2025). A reduction in the G0/G1 and S phases and an increase in cells in the G2/M phase indicate that BHA impedes cell cycle progression, which in turn prevents cell division and proliferation.

$ZnSO_4$ substantially suppressed cell growth by causing G2/M cell cycle arrest, as demonstrated by Mousa et al. (2022a), who reported a considerable buildup of HepG2 cells in the G2/M phase. According to in vitro research, human cell lines exposed to this substance showed decreased viability, elevated cytotoxicity, and the start of apoptosis. Rashad et al. (2018) confirmed the inhibitory effects on cancer cell development by investigating gene expression in Caco-2 cells after apoptosis and cell cycle arrest. These results were corroborated by flow cytometric analysis, which revealed that HepG2 liver cancer cells had reduced viability. In a separate investigation, Rashad et al. (2022) confirmed the possible harmful mechanism through cell cycle arrest by observing G2/M phase accumulation in HepG2 cells exposed to dietary additives.

Furthermore, using flow cytometric analysis, Ali et al. (2024) showed that doxorubicin markedly caused apoptosis and G2/M phase cell cycle arrest in PC3 cells. These outcomes are consistent with what we have discovered thus far.

Cadmium chloride was demonstrated to cause apoptosis in HepG2 cells, with a progressive rise in annexin-positive cells in comparison to untreated controls, in line with these previous investigations (EL-Attar et al. 2019; Mousa et al 2022b). The current study's use of flow cytometry further demonstrated that BHA caused both cell cycle arrest and apoptosis. BHA treatment specifically increased the sub-G1 population and annexin V/PI staining in A549 cells, demonstrating the pro-apoptotic impact. Furthermore, BHA treatment hindered normal cell cycle progression by causing arrest at the G0/G1 phase (Abdel-Tawab et al 2019; Tan et al 2022).

These results are mostly in line with the research of Mizobuchi et al. (2022), who found that BHA-sensitive and BHA-resistant cell types responded differently to genotoxic stress. Their research demonstrated BHA's capacity to regulate cellular stress responses and apoptotic pathways by connecting these differences to differences in reactive oxygen species (ROS) formation, DNA damage, and apoptosis levels after UV exposure.

7. Gene Expression and Apoptosis

The molecular processes behind BHA-induced cell death were clarified by the RT-PCR investigation. The pro-apoptotic gene *Bax* and the tumor suppressor gene *p53* were significantly upregulated in both Caco-2 and ABC-1 cell lines following BHA treatment, as indicated in Table 7 and Figures 3 and 4. In Caco-2 and ABC-1 cells, for example, *Bax* expression increased 8.51 and 3.56 times, respectively, but *p53* expression increased 3.58 and 4.20 times. These results imply that BHA triggers apoptosis by triggering signaling pathways that promote apoptosis, such as the mitochondrial pathway. Moreover, the anti-apoptotic gene *Bcl-2* was significantly downregulated upon BHA treatment, with expression levels falling to 0.08-fold in Caco-2 cells and 0.05-fold in ABC-1 cells. The idea that BHA encourages apoptosis by blocking survival signals that shield cells from programmed cell death is further supported by this downregulation of *Bcl-2* (Esazadeh et al 2024).

The downregulation of *Bcl-2* and the overexpression of *p53* and *Bax* suggest that BHA mainly triggers apoptosis in cancer cells via activating pro-apoptotic pathways.

These findings are consistent with earlier research showing that BHA can induce mitochondrial-dependent apoptosis through altering the expression of important proteins that regulate apoptosis (Luan and Honma, 2022). Moreover, the substantial increase of *p53* implies that BHA might trigger DNA damage reactions, which would then trigger apoptotic signaling. The current study's findings are consistent with other investigations showing that cadmium chloride causes HepG2 cells to undergo apoptosis. Cadmium chloride-treated samples showed a progressive rise in annexin-positive cells relative to untreated controls, indicating apoptotic progression. Furthermore, Mousa et al. (2022a) found that differing degrees of DNA damage and apoptosis indicated that malignant and normal cell lines were differently sensitive to doxorubicin-induced genotoxic stress. At 96 hpf, a greater number of apoptotic cells was seen in larvae exposed to BHA. Oxidative stress was indicated by a decrease in the activity of glutathione peroxidase (GPx), glutathione-S-transferase (GST), catalase (CAT), and superoxide dismutase (SOD) (Veshaal et al. 2025).

According to recent research compiled in this description, high gastrointestinal tract consumption of synthetic antioxidants such TBHQ, BHA, and PG can have cytotoxic, genotoxic, and perhaps carcinogenic consequences. According to thermodynamic analyses, these chemicals' binding to albumin is mostly mediated by hydrophobic interactions. Moreover, molecular modeling demonstrates that the predominant binding site for TBHQ, BHA, and PG is subdomain IIA of albumin. Together, these findings lend credence to the idea that, in order to lower any health concerns, dietary consumption of TBHQ, BHA, and PG from food additives should be kept to a minimum (Esazadeh et al 2024). Elevated levels of 8-OHdG have been linked to exposure to BHA and TBHQ, suggesting oxidative DNA damage. The evidence points to a possible function for BHA and/or chemicals with numerous hydroxyl groups in inducing apoptosis through enhanced formation of reactive oxygen species (ROS) (Baran et al 2020).

8. Conclusions

The results of this investigation show that butylated hydroxyanisole (BHA) has dose-dependent cytotoxicity and can stop the growth of human cell lines. The evidence makes it abundantly evident that BHA causes human cells to undergo both apoptosis and toxicity. The distribution of DNA content in the cell cycle phases of Caco-2 and ABC-1 cell lines was considerably impacted by exposure to BHA at a concentration of 50 µg/ml, according to flow cytometry analysis. This was especially evident in the G0/G1 phase population, which was reduced when compared to untreated controls.

The expression of important apoptosis-related genes was assessed using quantitative real-time PCR (qPCR) in order to better investigate BHA-induced apoptosis. After BHA treatment, the anti-apoptotic gene *Bcl-2* was significantly downregulated, but *Bax* and *p53* were upregulated in both Caco-2 and ABC-1 cell lines. The notion that BHA predominantly causes cell death by activating pro-apoptotic pathways and suppressing anti-apoptotic signals is supported by these molecular studies.

All of these findings point to the possibility that BHA, a widely used food ingredient, may damage human cell lines by causing apoptosis and interfering with normal cell cycle development. Because BHA has been shown to have cytotoxic and genotoxic properties, its use in consumer goods should be strictly controlled. To reduce any health concerns and maintain public safety, stringent allowable limits must be established.

In conclusion, our work offers strong evidence that BHA and other food additives can have detrimental effects on human health by lowering cell viability and changing apoptosis-related gene expression. These results underline how crucial it is to assess the biosafety of food additives and back the necessity of strict regulations to reduce human exposure from dietary and manufacturing sources.

Acknowledgments

I sincerely thank the anonymous reviewers for their helpful comments and their constructive feedback.

Conflicts of Interest

Author declares no conflict of interests.

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