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ORIGINAL RESEARCH

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The protective effect of gallic acid against endocrine disrupting damages of Bisphenol A and possible mechanism

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ABSTRACT

Cancer is uncontrolled and/or abnormal cell growth and proliferation as a result of DNA damage. When the normal cells are exposed to DNA damage, they try to prevent this damage through various mechanisms. But in cancer, repair mechanisms of the cell are disrupted. Cells grow excessively and abnormally, and this threatens the life of organism. There are many factors that lead to carcinogenesis. One of these factors is endocrine disruptors.

Endocrine disruptors can damage the body mechanisms to mimic certain hormones in the body. Bisphenol-A (BPA), which is one of the endocrine disrupting substances, is found in many products, such as plastic bottles, including primarily packaged foods. Exposure to BPA causes estrogen-mimetic feature which may lead to carcinogenesis through estrogen metabolism in cells. Phenolic compounds are widely present in plants which had beneficial effects on the body, and generally considered as health nutrients for human. We think, the phenolic compounds could have some positive effects on estrogen metabolism against BPA. To demonstrate that gallic acid (GA)—which is a prototype of phenolic compounds—, BPA and pure estrogen receptor blocker [fulvestrant (FV)] were applied to DU-145 (malign prostatic cancer cell) and HGF-1 (human gingival fibroblast cell) cell lines. FV was used to detect estrogen receptors related efficacy. We used 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) test to measure the cytotoxicity of chemical substances that we used. Effects of BPA and GA on estrogen metabolism were investigated by real-time polymerase chain reaction (PCR) method.

In our study, MTT test showed that the GA decreased the cytotoxicity of BPA in cells. Using FV in combination with GA or BPA, also changed the percent of cell viability in MTT test, but these changes were negligible amount. Therefore, it could be considered that BPA and GA related effects are not only via estrogen receptor alpha and estrogen receptor beta.

As a result of studies conducted by RT-PCR, we determined that GA and BPA had effects on enzymes of intracellular estrogen metabolism. FV caused changes on the effects of GA and BPA. This shows us that the these substances may show their effects via estrogen receptors. One of the most promising results of this study was: GA increased the expression of mRNA expression of glutathione S-transferase enzyme which increases detoxification and quinone reductase enzyme that decreases the DNA adducts. This showed us GA can also do protective effects to carcinogenic properties as well as endocrine disrupting effects of chemicals like BPA. GA also increased the expression of mRNA expression of poly (ADP-ribose) polymerase enzyme in benign cells, but not in malign cells specifically, which is an important step in DNA repair mechanism. We planned a new RT-PCR and Western Blot study in order to determine the possible role of estrogen receptors and estrogen metabolism related enzymes in the mechanism of the preventive effect of phenolic compounds against endocrine disrupters more precisely, in over cancer, mammary cancer and prostate cancer cell lines, and we believed that this would be very informative for the understanding the underlying mechanism of endocrine disrupters and cancer.

ARTICLE HISTORY

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KEYWORDS

Bisphenol A; polyphenolic compound; gallic acid; endocrine disruptors

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Introduction

The endocrine disrupters are the substances that mimic the hormones synthesized by the endocrine system and which prevent their functioning. The substances called endocrine disruptors can be found naturally in our environment or they can be included in synthetic products[1,2]. Endocrine disrupters are found in the content of a large number of products, such as synthetic hormones, disinfectants, food additives, plastic products, beverages, cleaning agents, and chemical agents used in the chemical industry.

Endocrine disrupting agents can mimic estrogen, androgen, and thyroid hormones in the body [1,2]. As a result; (a) by binding to receptors in cells, inhibiting the recognition of the corresponding endogenous hormone, (b) they cause disruptions in the normal hormonal communication mechanism in the body by reducing, increasing or modifying the effect of the respective hormones [1,2].

Bisphenol A (BPA, 4,4-hydroxy-22,2-diphenylpropane), one of the endocrine disrupting agents, is found in the structure of many products in our enviroment. It was first produced as a synthetic estrogen in the 1890s. Howewer, currently, BPA is mainly used as a component of many consumer products, including plastic bottles, food and tin beverage packaging inner linings, thermal paper, medical devices, dental materials, and so on [3]. The BPA may penetrate the substances in contact with the temperature. Exposure to BPA is mostly by food intake (about 90%). The use of BPA threatens human health and the natural enviroment. BPA affects fertility and pregnancy by acting as xenoestrogens. It can cause carcinogenic effects, can cause prostate[4,5–7] and breast cancer [4,8–10].

Phenolic compounds are chemical structures found in all the plants as secondary metabolites and are thought to be used by plants to protect themselves againts certain pests [11]. These compounds, which are the products of secondary metabolism in plants, are among the most common chemicals found in the plants. Phenolic compounds can be found in different parts of the plant and may have different effects on the plants. For example, it is effective on color formation in some plants and can be effective on taste in some compounds. Several thousand molecules (i.e., aromatic rings wih several hydroxyl groups) have been identified in plants with polyphenol structure. Hundreds are also found in edible plants. These compounds can be classified into different groups accordings to the function of the number of phenol rings. Each of the phenol rings contains structural elements attached to the phenol rings. These diffrences result from diffrent phenolic products, such as phenolic acid, flavonoids, stilbenes, and lignans. The common structure of the two aromatic rings (A and B) shared by the flavonoids and the three carbon oxygenated heterocyclic rings which hold them together other C atoms. Flavonoids can be divided into six groups according to the functions of heterocyclic structure: flavonols, flavones, isoflavones, flavovones, anthocyanidins, and flavanols (catechins and proanthocyanidins) [11]. There are many research studies on the effects of phenolic compounds on health. Phenolic compounds have been shown to have positive effects on the prevention of the development of many diseases, such as cancer [12,13], cardiovascular diseases [13–17], and diabetes mellitus [18–20] (image 1).

Gallic acid (GA) (3,4,5-trihydroxybenzoic acid) is a phenolic compound. This phenolic acid, which has many different derivates, is one of the main components of polyphenolic acids. It is found in many plants and fruits as secondary metabolites. Gallic acid and its derivatives are found in almost all the kinds of plants in nature; such as shell, wood, fruit, roots, and seeds [21]. GA is known to have bioactivity, such as antioxidants [22–24], antimicrobials [24,25], anti-inflammatory [26–29], and anti-cancer [30–33].

Materials and Methods

Cell culture

Cells were treated with 5% carbon dioxide at 37°C. Cell as malignant (DU-145 human prostate cancer cell) and benign (gingiva: human fibroblast cell) cell line was used. RPMI-1640 (10% fetal bovine serum, 1% penicillin-streptomycin, and 1% 1-glutamine) was used as the culture medium and the cells were passaged every 2 days. During the passage of the cells, 3 ml of trypsin-EDTA was used to remove the cells from the adhered surface and then incubated for 3 minutes in the incubator. The cell seperated from the surface were placed in a 15-ml conical tube and centrifuged at 2,250 rpm for 6 minutes. Then, trypsin-EDTA was removed from the culture medium and 1 ml of medium was added onto the cell pellet. Nearly, 5 ml of medium were added to the flask. On top of this, 200 ml cells were collected

from the falcon tube containing the cells and added to the flask.

MTT test

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) test was used to measure the cytotoxicity of the substances we applied. DU-145 cells were plated in 96-well cell culture plates at 4.0×10^3 DU-145 and 6.0×10^3 human gingival fibroblast cell-1 (HGF-1) in each well. After 24 hours of incubation, plaques was determined to reach the 60%–70% density and they were separated to apply the chemical substance. Eight different chemical exposure groups were formed by separating 12 wells for each [Control, Fulvestrant (FV), GA, GA + FV, BPA, BPA + GA, BPA + FV, and BPA + GA + FV]. First, FV applied, GA was added after 4 hours of incubation and BPA added after approximately 16 hours of incubation. We waited 72 hours after the first substance application and then MTT (5 mg/ml) was placed in each well and incubated for 4 hours. After incubation, plaques were removed and 100 ml of DMSO were placed in each well. Plaques were incubated at room temperature for 15 minutes and absorbance values at 560 nm were read in the standard plaque reader. The percentage of cytotoxicity was calculated using the following formula:

$$\text{Cytotoxicity} = 100 \times [(\text{Absorbance}_{\text{group}} - \text{Absorbance}_{\text{control}})/\text{Absorbance}_{\text{control}}]$$

Real time polymerase chain reaction (real time PCR)

In order to investigate mRNA expression of enzymes, cells were placed in standard cell culture plates (Corning, NY) containing six wells. For DU-145, 30×10^3 density cells were planted and 50×10^3 cells were planted for HGF-1. After 24-hour incubation, different combinations were prepared and chemicals were applied (Control, FV, GA, GA + FV, BPA, BPA + GA, BPA + FV, and BPA + Gallik acid + FV). First, FV was applied to cells, GA was added after 4 hours of incubation and BPA added after approximately 16 hours of incubation. Forty eight hours after the first substance application, the cells were treated with trypsin and collected and total RNA extraction was performed with TRI-Reagent (Sigma-Aldrich, Taufkirchen, Germany). Isolated RNA quantities were calculated by optical reading at 260 and 280 nm with standard microplate reader (Epoch-Take3, BioTek instruments, Winooski, VT, USA). Isolated RNA samples were dissolved in RNA-ase free distilled water and stored at −80°C. Nearly, 900 ng of the obtained RNA samples were taken and 200 units of Moloney Murine Leukemia virus reverse transcriptase (M-MuLV Reverase, Bioron, Ludwigshafen, Germany) and 0.5 µM random primer (Bioron, Ludwigshafen, Germany) were added. The total volume was completed to 20 µl. The cDNA was generated using the PCR program at 45°C for 50 minutes and 67°C for 10 minutes. Five microliters of the resulting cDNA were used for RT-PCR: RT-PCR was performed (Exicycler 96, Bioneer, Daejeon, Republic of Korea) by adding 15 µl sybr-green qPCR master mix (Brilliant II, Agilent, Santa Clara, CA), 200 µM DNTP and 200 nM appropriate primers (Table 1).

After 10 minutes at 95°C, the RT-PCR protocol was administered at 95°C for 15 seconds and 60°C for 1 minute with 45 cycles. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference (house-keeping gene).

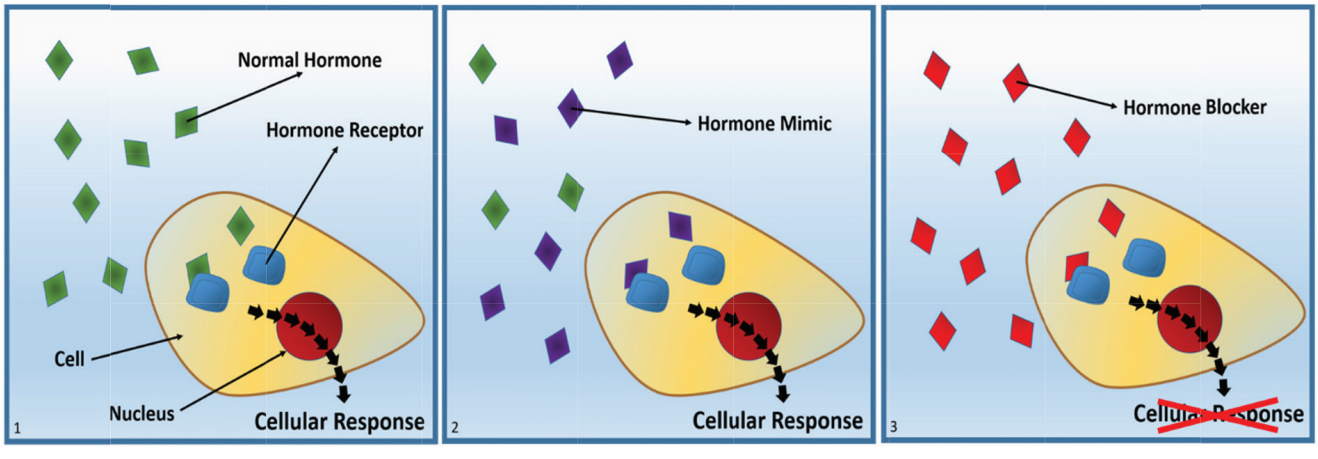

Image 1. Endocrine disruptors increase and decrease normal hormone levels after being absorbed (left), mimics the normal hormones of the body (middle), or changes the production of natural hormones (right).

Table 1. Sequence and list of primers used in RT-PCR analysis.

Gene	Primers	Sequence	Reference
GAPDH	Sense Antisense	5′-CCACCATGGAGAAGGCTGGG-3′ 5′-ATCACGCCACAGTTTCCCGG-3′	[34]
CYP19	Sense Antisense	5′-TTGGAAATGGTCAACCCGAT-3′ 5′-CAGGAATCTGCCGTGGGAGA-3′	[35]
QR	Sense Antisense	5′-AGAAGAGCACTGATCGTACTGG-3′ 5′-CGTAATTGTAAGCAAACTCTCCTATG-3′	[36]
CYP3A4	Sense Antisense	5′-GGGAAGCAGAGACAGGCAAG-3′ 5′-GAGCGTTTCATTCACCACCA-3′	[37]
GST	Sense Antisense	5′-CCAGAACCAGGGAGGCAAGA-3′ 5′-GAGGCGCCCCACATATGCT-3′	[38]
CYP11A	Sense Antisense	5′-GAGATGGCACGCAACCTGAAG-3′ 5′-CTTAGTGTCTCCTTGATGCTGGC-3′	[39]
PARP1	Sense Antisense	5′-AGCGAGAGCATCCCCAAGG-3′ 5′-TCAAACATGGGCGACTGCAC-3′	[40]
MKI67	Sense Antisense	5′-ATTGAACCTGCGGAAGAGCTGA-3′ 5′-GGAGCGCAGGGATATTCCCTTA-3′	[40]
COMT	Sense Antisense	5′-ATTGACACCTACTGCGAGCA-3′ 5′-CCACATTCCTCCAAGAGAAGC-3′	[41]

Statistical analysis

The samples used in PCR measurements were studied in two replicates. Statistical evaluation was evaluated using SPSS for Windows software and non-parametric Mann–Whitney U test was used where appropriate. Data are presented as mean ± standard deviation. p <0.05 was considered as statistically significant.

Results

In our study, DU-145 (malignant prostate cancer cell) and HGF-1 (human gingival fibroblast cell) cell lines were inserted into 96-well plates and BPA, BPA + GA, BPA + FV combinations were added. With these combinations, we investigated the enzymes which affect BPA, and the results of BPA when administered with phenolic compound and estrogen receptor blocker.

According to the results of MTT analysis, GA and BPA (separately and together) were found to have toxic effects on cells, this effect was found to be approximately the same level on both malignant and benign cells. It has been found that there is no selective cytotoxicity (p > 0.05). The MTT results also show that the cytotoxic effect when BPA is applied is statistically reduced in case of GA application (p < 0.05). FV slightly reduced the cytotoxic effect when it is included in the combination in all the cases, however, no significant reduction in any group; therefore, it can be argued that the cytotoxic effects of BPA and GA are not predominantly estrogen alpha and estrogen beta receptor mediated (Fig. 1).

The CYP11A enzyme can be considered the first step in the synthesis of steroid hormones. This enzyme plays a role in the transformation from cholesterol to pregnenolone. GA and BPA applied cells, changes in mRNA expression of the CYP11A enzyme were examined (Fig 2). It was observed that CYP11A mRNA expression in malignant cells was slightly higher than HGF1 cells and the applied substances had no specific effects on HGF-1 cells. While the effects of GA treated DU-145 cells are similar to HGF-1, it is observed that BPA strongly induced CYP11A enzyme mRNA expression (Fig. 3).

CYP19 enzyme which is known as aromatase enzyme is a key enzyme for estrogen metabolism and it provides estrogen (estrone and estradiole) from androgenes (androstenedione and testesterone). Following the application of GA, the CYP19 enzyme mRNA expression increase occured at HGF-1 cells, while DU-145 cells CYP19 mRNA expression was decreased. There was no standart effect on GA and BPA treated cells following administration of FV. Following the application of FV to GA and BPA treated cells: FV slightly increases the effect of GA related effect in HGF-1 cells while slightly decreasing the effectiveness of BPA (Fig. 4).

CYP1A1, CYP1B1, and CYP3A4 are act as a part at second hydroxilation of estrogen metabolism. In our study, mRNA levels of all the three enzymes were investigated but CYP3A4, which was found in the liver and intestinal tract, could be detected in

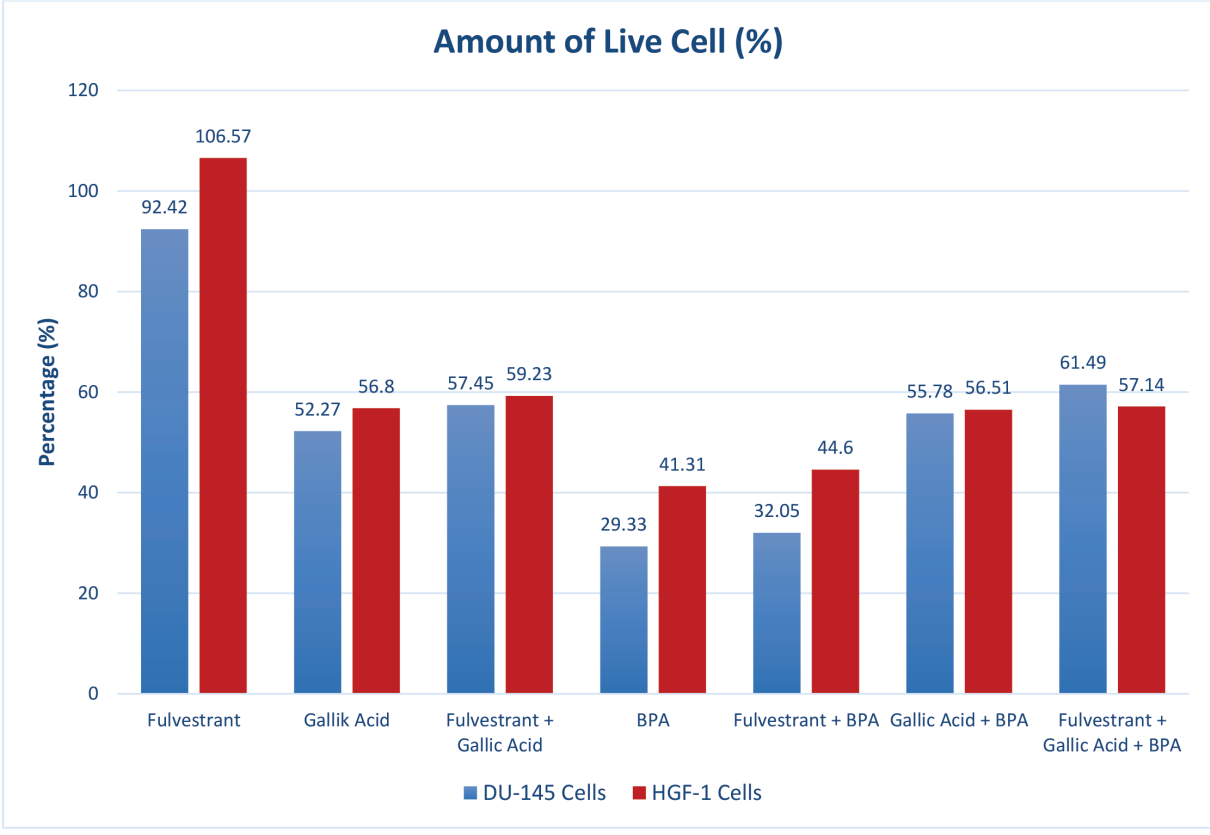

Figure 1. The MTT test results of the toxic/proliferative effect of DU-145 and HGF-1 cells on the chemicals used in the study. Percent cell quantity values were calculated according to the amount of cells in the "control" group.

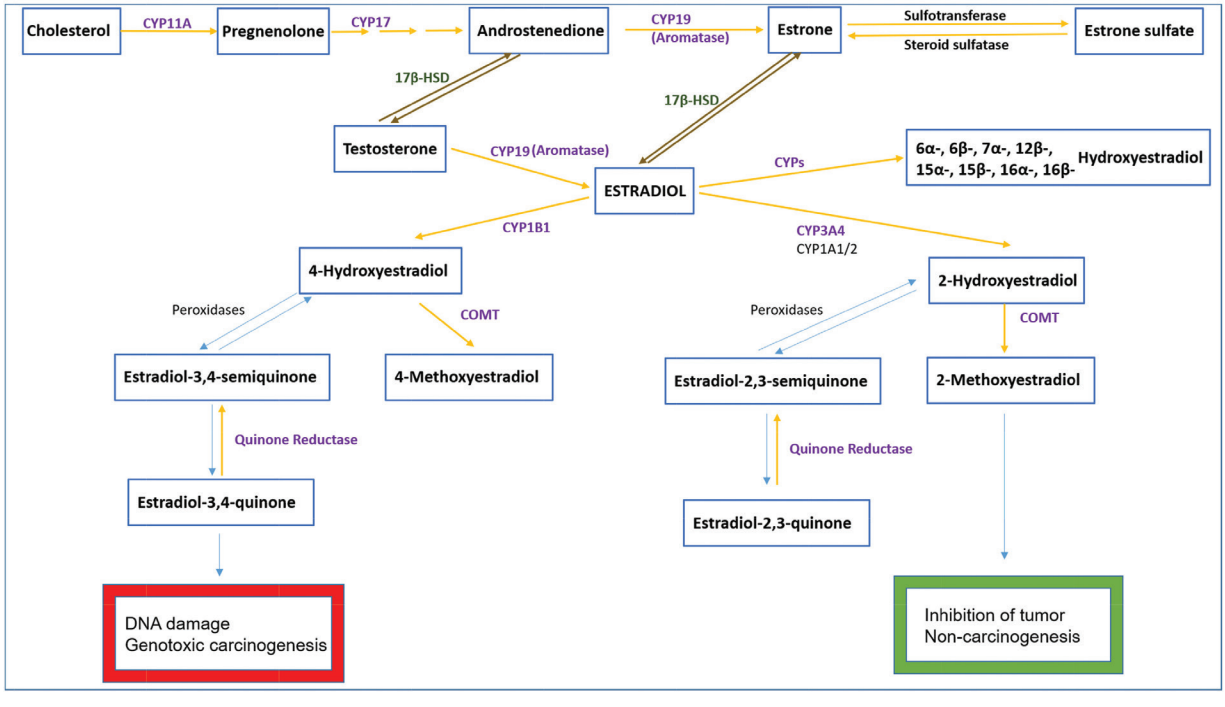

Figure 2. Basic stages of estrogen metabolism [42].

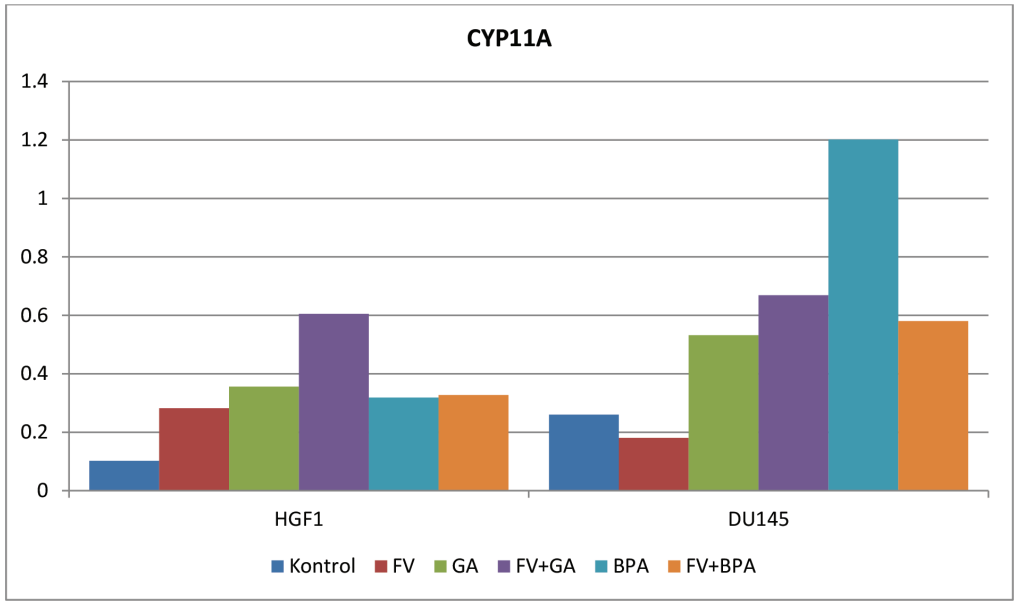

Figure 3. CYP11A mRNA levels in Fulvestrant (FV), Gallic Acid (GA), and Bisphenol A (BPA) applied cells.

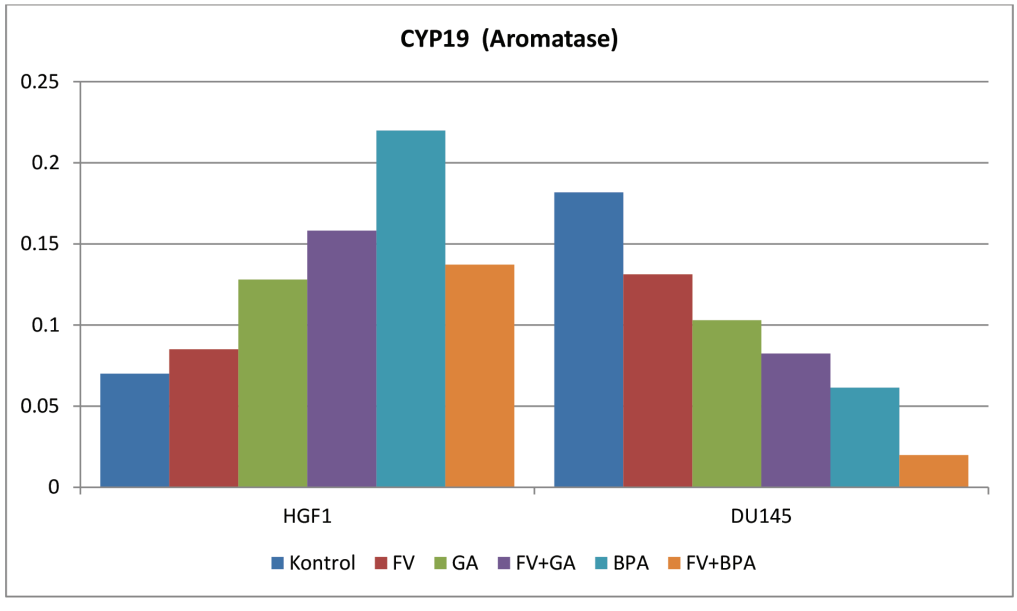

Figure 4. CYP19 mRNA levels in Fulvestrant (FV), Gallic Acid (GA), and Bisphenol A (BPA) applied cells.

each sample. Therefore, only the result of CYP3A4 were included in our study. It was concluded that CYP3A4 mRNA expression in DU-145 cells was higher than HGF-1 cells. When we applied GA and BPA separately to DU-145 and HGF-1 cells, we observed that CYP3A4 mRNA expression increased in both cell types (Fig. 5).

Catechol-O-methyltransferase (COMT) enzyme plays a role in the metabolism of estrogen as well as catecholamines. The changes in mRNA expression were investigated in the synthesis mechanism of steroid hormones, 2-Methoxyestradiol formation from 2-hydroxyestradiol, and 4-Methoxyestradiol in 4-hydroxyestradiol. In GA- and BPA-treated cells, it was observed that the COMT mRNA expression in HGF-1 cells was slightly higher than that of DU-145 cells. When FV was applied to HGF-1 cells, the expression of COMT mRNA decreased while an increase in DU-145 cells was observed. When BPA was applied to the cells, it was found that COMT

mRNA expression in both cell types decreased similarly to the control group. COMT mRNA expression was reduced when FV was administered with BPA (Fig. 6).

NAD(P)H: quinone reductase (QR) is one of the phase II enzymes involved in estrogen metabolism [43]. This enzyme induces the conversion of catechol estrogen quinones to catechol estrogens. Catechol estrogen quinones are causes the formation of "DNA adducts," which ocur during estrogen metabolism and which are suggested to play a major role in the development of cancer. Therefore, significantly reduces the potential for carcinogenicity of xenobiotics/carcinogens [43]. In our study, it was observed that QR the mRNA expression was very high in GA- and BPA-treated DU-145 cells compared to GA- and BPA-treated HGF-1 cells. When FV and GA were applied separately to the cells, it was found that the expression of QR mRNA was almost completely eliminated in both HGF-1 cells and DU-145

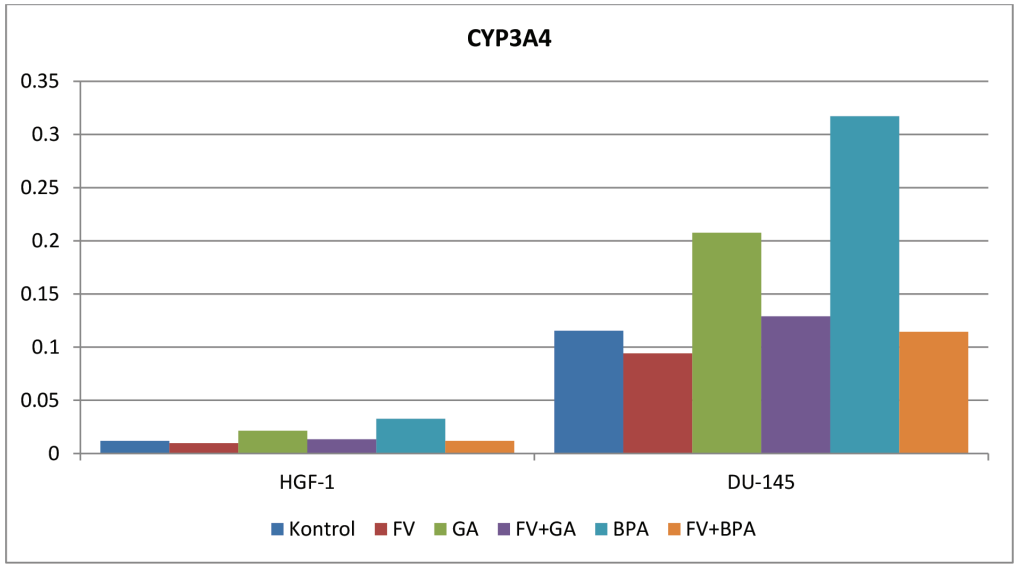

Figure 5. CYP3A4 mRNA levels in Fulvestrant (FV), Gallic Acid (GA), and Bisphenol A (BPA) applied cells.

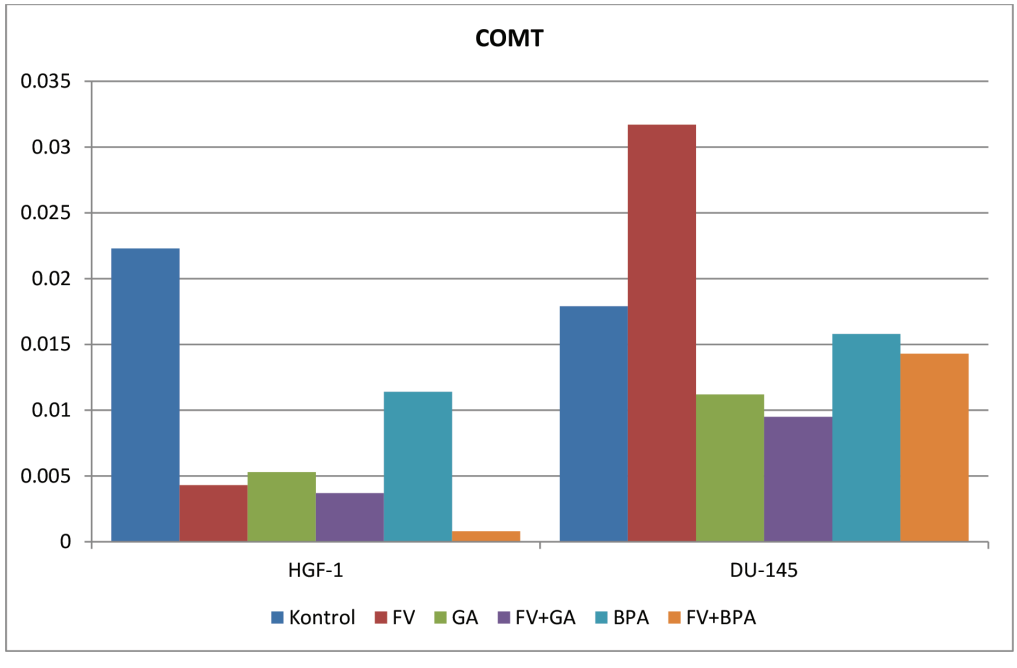

Figure 6. COMT mRNA levels in Fulvestrant (FV), Gallic Acid (GA), and Bisphenol A (BPA) applied cells.

cells. BPA administration to HGF-1 cells resulted in a decrease in QR mRNA expression, but an increase in DU-145 cells compared to the control group (Fig. 7).

The glutathione S-transferase (GST) enzyme has similar function to the QR enzyme. GST enzyme's DNA adduct reducing effect during estrogen and xenobiotic metabolism is not as high as QR enzyme. However, GST shows strong detoxifying activity in a diffrent way than the QR enzyme and is one of the phase II enzymes involved in the prevention of cancer formation [43,44]. It was observed that the GST mRNA expression in DU-145 cells was significantly higher than HGF-1 cells. Therefore, the applied chemicals did not cause significant changes in GST mRNA levels which are already in extremely low levels in HGF-1 cells. GST mRNA expression was significantly increased when FV was administered to the DU-145 cells (Fig. 8).

MKI67 is an important indicator for cellular proliferation. Diffrent levels of increase in MKI67 mRNA expression were observed in HGF-1 cells treated with BPA. In the control group of DU-145 cells was found that mRNA expression of this enzyme was high and that mRNA expression increased in all of the chemicals we applied (Fig. 9).

Poly (ADP-ribose) polymerase (PARP), an enzyme involved in DNA repair mechanism and apoptosis, was examined in GA- and BPA-treated cells. PARP mRNA expression in DU-145 cells was significantly higher than HGF-1. PARP expression was significantly increased in GA-treated HGF-1 cells, whereas PARP expression was significantly decreased in GA-treated DU-145 cells. Based on this result, it can be argued that GA has a positive effect on DNA repair mechanism by increasing PARP expression in benign cells and decreasing replication in malignant cells. A significant increase in PARP mRNA expression has been observed in BPA treated DU-145 cells, wich may be considered to aid the carcinogenic effect of BPA. The decrease in BPA induced mRNA expression by the administration of FV and FV + GA may indicate that BPA is mediated by estrogen receptors and a significant decrease in the effectiveness of GA with detoxifying and anticancer effects (Fig. 10).

The plus (+) and cons (−) in the Tables 2 and 3 show how much the applied substances increase and decrease the amount of cells compared to the control group.

As seen in Tables 2 and 3, the effects on BPA-exposed cells were changed as a result of the application of GA and FV. The effects of the FV on the results show that the chemicals examined also show their effects through estrogen receptors. GA is increasing detoxification by activating the GST enzyme and decreasing DNA adducts with its positive effects on QR, demonstrates that it can show protective properties against not only BPA but similar chemical carcinogens. In addition, GA increases the expression of PARP in benign cells and it has

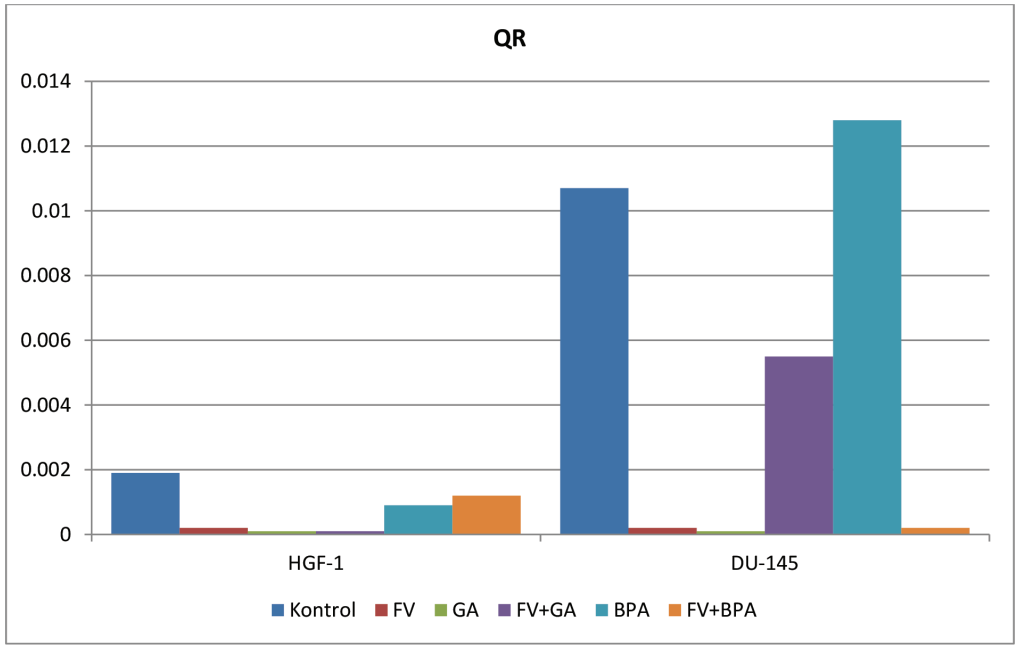

Figure 7. QR mRNA levels in Fulvestrant (FV), Gallic Acid (GA), and Bisphenol A (BPA) applied cells.

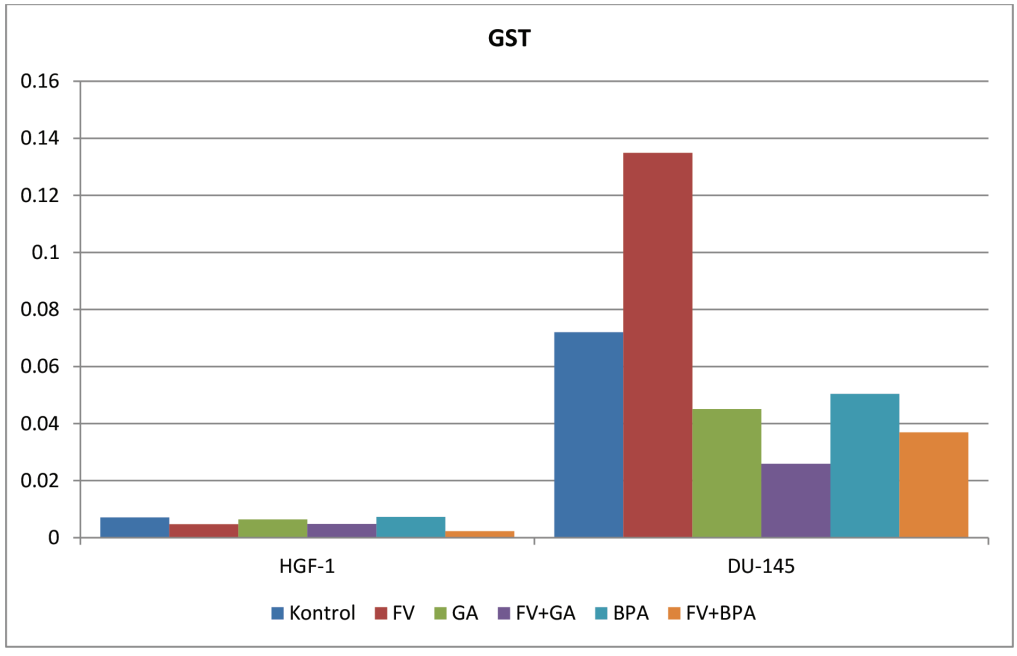

Figure 8. GST mRNA levels in Fulvestrant (FV), Gallic Acid (GA), and Bisphenol A (BPA) applied cells.

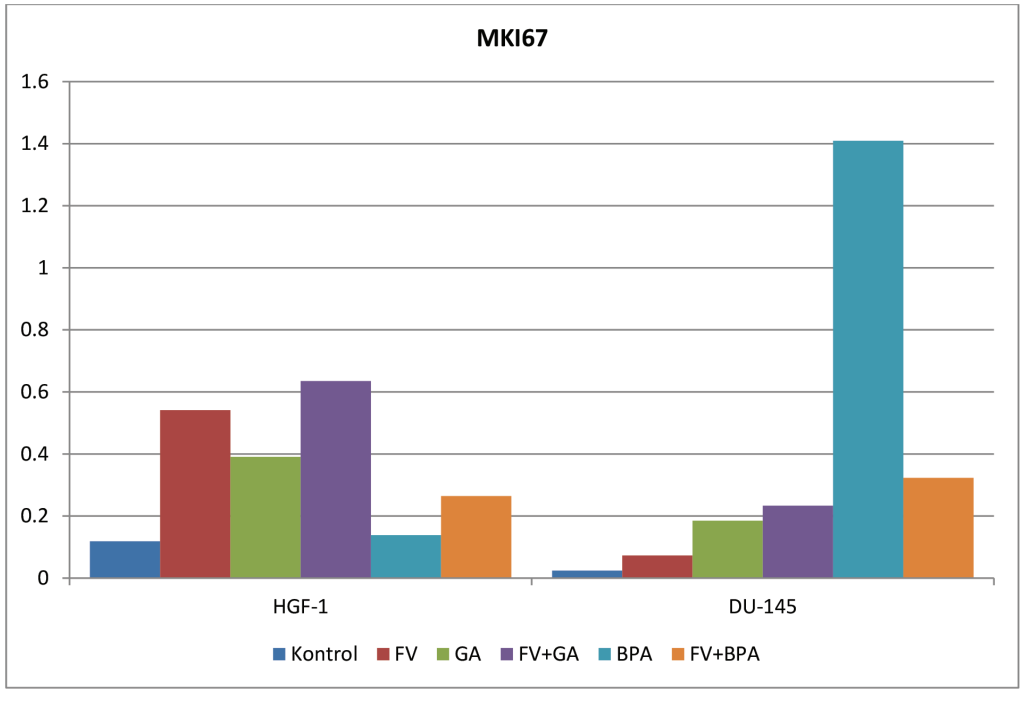

Figure 9. MKI67 mRNA levels in Fulvestrant (FV), Gallic Acid (GA), and Bisphenol A (BPA) applied cells.

positive effects on polymerization, which is one of the effective steps in DNA repair. In order to support the results mentioned here and to determine the status of estrogen receptors in the process, it is planned to carry out studies using Western Blot technique.

Discussion

Endocrine disrupting agents are harmful to our body. They show their harmful effects by imitating some hormones, such as estrogen, androgen, and thyroid hormones in our body [1,2]. Endocrine disruptors can lead to malignant tumors, birth defects,

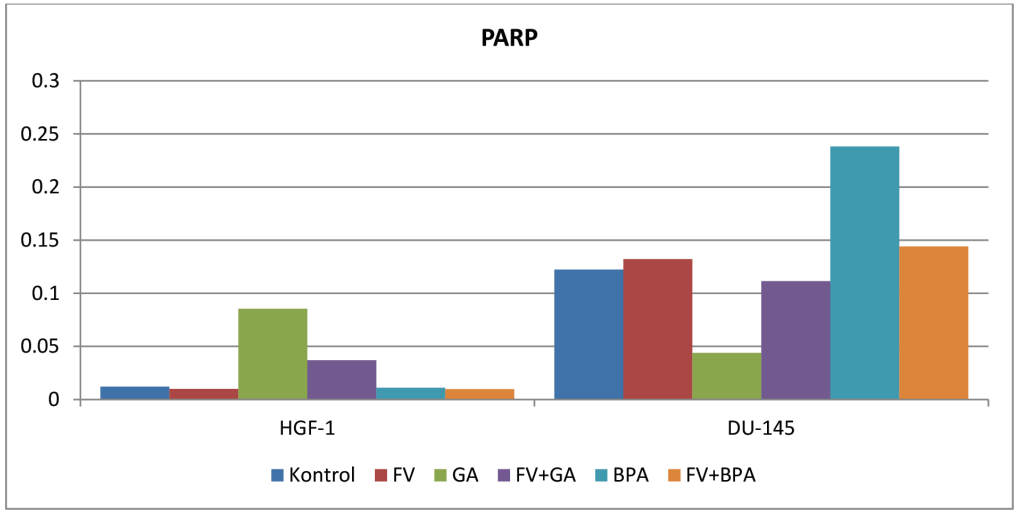

Figure 10. PARP mRNA levels in Fulvestrant (FV), Gallic Acid (GA), and Bisphenol A (BPA) applied cells.

Table 2. The effect of the applied substances on CYP11A, CYP19, CYP3A4, and COMT mRNA expression in HGF-1 and DU-145 cells.

	CYP11A		CYP19 (Aromatase)		CYP3A4		COMT	
	HGF-1	DU-145	HGF-1	DU-145	HGF-1	DU-145	HGF-1	DU-145
GA	+	+	+	-	0	+	--	-
FV+GA	++	++	+	-	0	0	--	-
BPA	+	+++	++	--	+	++	-	-
FV+BPA	+	+	+	---	0	0	--	-

Table 3. The effect of the applied substances on QR, GST, MKI67, and PARP mRNA expression in HGF-1 and DU-145 cells.

	QR		GST		MKI67		PARP	
	HGF-1	DU-145	HGF-1	DU-145	HGF-1	DU-145	HGF-1	DU-145
GA	--	---	0	-	+	+	++	-
FV+GA	--	-	0	-	++	+	+	0
BPA	-	+	0	-	0	+++	0	+
FV+BPA	-	---	0	-	+	+	0	0

and other developmental disorders [1,2]. Endocrine disruptors are found in the content of most products around us, such as disinfectants, food additives, plastic products, and cleaning agents. BPA is one of the endocrine agents. BPA is an effective agent on the endocrine system with carcinogenic effects and may lead to prostate [4,5–7] and breast cancer [4,8–10].

Phenolic compounds are chemical substances found in all the plants and that plants are considered to protect themselves against certain pests [11]. Phenolic compounds are found as secondary metabolites in plants. Phenolic compounds are also the most abundant in the plants.

There are many research studies on the effects of phenolic compounds on health. Phenolic compounds have been shown to have positive effects on the prevention of many diseases, such as cancer [12,13], cardiovascular diseases [13–17], and diabetes mellitus [18–20].

GA is a phenolic compound. GA is known to have bioactivity such as antioxidants [22–24], antimicrobials [24,25], anti-inflammatory [26–29], and anti-cancer [30–33].

We are exposed to 24,000 endocrine disrupting chemicals through bottled water. Endocrine disrupting agents have estrogen-mimetic effects. These substances constitute a significant part of their estrogen-mimetic effects due to BPA [42]. It is suggested that toxic effects of the cell are formed by the metabolism of estrogen by BPA. However, there is no detailed study on whether other enzymes involved in estrogen metabolism play a role in BPA-related effects. In this paper, endocrine-mediated cancers, such as prostate cancer, breast cancer, ovarian cancer, as well as developmental disorders, such as early puberty were investigated. In addition, methods of eliminating their harmful effects were investigated. For this purpose, GA, which is one of the prototypes of polyphenolic compounds taken with plant foods, was used. Changes in the effects of BPA were investigated by real-time PCR method in the cells with GA and the effects of endocrine disrupting effects were investigated.

In the study, it was determined that BPA had a serious cytotoxic effect on DU-145 and HGF-1 cell lines, and statistically significant decrease in case of GA application. However, in all the cases when the FV was added, it was found that the cytotoxic effect was reduced in small amounts. However, no significant reduction was observed in any group. Therefore, it can be argued that BPA and GA-dependent cytotoxic effects are not predominantly estrogen alpha and estrogen beta receptor mediated.

In this study, the effects of GA and BPA on CYP11A, CYP19 (Aromatase), CYP3A4, COMT, QR, GST, MKI67, and PARP enzymes in different stages of estrogen metabolism were investigated. Increasing detoxification by activating the GST enzyme, reducing the positive effects on QR, and reducing DNA inserts suggest that it can show protective properties against not all BPA but similar chemical carcinogens. In addition, increasing the PARP expression of GA in benign cells has positive effects on polymerization, one of the effective steps in DNA repair.

Conclusion

Further studies are needed to support the results stated in the paper and to fully demonstrate the status of estrogen receptors in the process.

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Conflict of interest

The authors declare that they have no conflict of interest.

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