RESEARCH ARTICLE

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Effects of Irinotecan (CPT-11), Ellagic Acid and Combination of both on HeLa Cells in 2D and 3D Culture

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Abstract

BACKGROUND/AIMS: Investigation of new potential treatments is important today to increase the power of cervical carcinoma treatment. Although chemotherapeutic drugs are widely used for clinical tumor treatment, their severe toxicity limits their therapeutic efficacy. Irinotecan (CPT-11) is an agent with therapeutic activity against various cancers including cervical cancer. Ellagic acid (EA) is a phenolic natural substance that can be found in various fruits and has chemopreventive and chemotherapeutic effects. In this work, we looked into EA as an alternative agent to improve the therapeutic effect of CPT-11 *in vitro*.

MATERIALS AND METHODS: The effects of the two agents and their combination on 2 and 3D HeLa cultures at different time periods were evaluated by real-time-polymerase chain reaction and intraclass correlation coefficient analyses. In cultures, MMP-2 and -9 were used to determine the basis of cancer cell metastasis, HIF1α and TGF-β1 were used to evaluate the mechanism of angiogenesis and epithelial-mesenchymal change, and Bax and Bcl-2 were used to evaluate the apoptotic mechanism.

RESULTS: In comparison to the DMSO (Dimethyl Sulfoxide) group, HeLa cells treated with EA and its combination showed considerable suppression of MMPs, TGF-β1, and HIF1α. In addition, the increase in proapoptotic Bax level with treatment and the suppression of antiapoptotic Bcl-2 expression were revealed as another important finding of EA and combined treatment.

CONCLUSION: We propose that EA would be a good candidate for additional preclinical studies for the management of human cervical cancer.

Keywords: Cervical cancer, CPT-11, ellagic acid, HeLa

INTRODUCTION

With a 13.3% incidence rate, cervical cancer is the third most prevalent disease in women worldwide and a significant public health issue. In terms of mortality, it ranks third with a rate of 73%.[1] Although postoperative radiation therapy and chemotherapy are used for treating cervical cancer, chemotherapeutic agents have many side effects. Therefore, research for new drugs with less side effects that selectively show greater cytotoxic effects on cancer cells at low doses continues.[2,3]

A semi-synthetic plant alkaloid derived from camptothecin is called irinotecan (CPT-11). Clinical trials in the 1990s and 2000s revealed that CPT-11 is therapeutically effective against several malignancies, including cervical carcinoma.[4] Toxic effects are seen in a significant

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number of patients using the CPT-11 drug, which is used to treat cancer patients. This makes it difficult for this drug to be used in chemotherapy.[5]

A naturally occurring phenolic substance called ellagic acid (EA) is present in berries such as red and black currants, red and black raspberries, blackberries, blueberries, and strawberries. Studies both *in vivo* and *in vitro* have established that it has chemopreventive and chemotherapeutic effects by inhibiting the proliferation of different cancer cells at low doses.[6] EA reduces tumor development, angiogenesis, and metastasis, although the exact biochemical pathways by which it does so are still poorly known.

Based on the aboveobservations, it is hypothesized in this study that human cervical cancer cells can be inhibited by EA and CPT-11, and the inhibition mechanism may play a role in the control of angiogenetic, proliferative, and metastatic signals. In this study, we developed an alternative treatment for cervical cancer. For this purpose, we used 2 and 3D HeLa cell cultures exposed to CPT-11, EA and its combination at different times in our study. In the study, MMP-2 and MMP-9 to reveal metastatic findings with real-time PCR and immunocytochemical analyzes, TGF-β1 and HIF1α to determine angiogenesis, and *Bax* and *Bcl-2* genes and proteins to evaluate apoptosis were used as targets.

MATERIALS AND METHODS

2D Cell Culture

Cervical cancer cell lines (HeLa) were purchased from ATCC (USA). A consent form was not required for this study as the cells were purchased commercially. Cervical cancer (HeLa) cells were cultured in RPMI-1640 media with 10% fetal bovine serum, 1% gentamicin, and 1% penicillin antibiotic added in 75 cm^2 flasks at 37 °C and 5% CO_2. Cells were passaged using trypsin as 80% of the flask surface was covered by cells.

3D Cell Culture

Cell lines were propagated spherically using the three-dimensional hanging drop method. For this, 96-well cell seeding plates without scaffolds were used. Suspended cells were dropped into the wells with the help of pipette into a volume of 40-45 µL. Then, cells were grown at 37 °C and in an incubator with 5% carbon dioxide. To the wells, 4 µL of fresh medium was introduced every day to ensure effective growth of cell lines globally in the hanging drop method. Cell groups were expected to take a spherical shape and reach a size of 300-500 µL at the end of approximately 4 days.

Cytotoxicity Analysis

The cytotoxic impact of metabolites on HeLa cells was assessed using XTT assay kit (Biological Industries, USA). For the analysis of 2D cultures, $8x10^3$ cells were seeded in each well of a 96-well plate. The cells were given drugs after incubation for 24 h. For 3D cultures, the drug was applied after the 3D structure had already been created. The solutions from XTT kit were applied to the cells after 48 h of incubation. Then, using a microplate reader (BIOTEK ELX808, USA), cell viability was evaluated at a wavelength of 450 nm. Briefly, IC_{50} values were determined by applying certain doses of CPT-11 (Irinocam-Irinotecan Hydrochloride Trihydrate, Kocak, Türkiye) and EA (EA, Cat. No: sc-202598A, Santa Cruz Biotech, Dallas, ABD) to HeLa cells. Real-time PCR and immunocytochemical experiments were performed at these concentrations.

Total RNA Isolation and qRT-PCR

Total RNA isolation was carried out using the WizPrep Total RNA Mini Kit (WizBio, Korea) in accordance with the instructions using cells that had been grown in 2D and 3D. RNA concentrations were determined using a Genova Nano Micro-volume Spectrophotometer (Jenway, UK). cDNA synthesis was performed using the HyperScript™ First strand Synthesis cDNA Synthesis Kit (GeneAll, Korea) for real-time PCR. A thermal cycler was used to perform the reaction. Real-time quantitative polymerase chain reaction (RT-qPCR) was then used to assess the change in gene expression. The Applied Biosystems™ 7500 Fast Real-Time PCR apparatus was used to perform the real-time qPCR reaction. The internal reference control and housekeeping gene beta-actin was used. The gene-specific primers used in the study are shown in Table 1. The formula $2^{-\Delta\Delta Ct}$ was used to determine the mRNA expression fold change.

Immunocytochemical Method

Samples that were previously seeded on glass coverslips coated with poly-D-lysine in 24-well dishes were used. The samples were divided into groups with different durations. When the cells in the culture medium reached a sufficient density and after the agent applications, the cells were fixed in the well with 3% paraformaldehyde. TBS + 2.5% triton X solution was filled to the wells. Then, with cold PBS, cells were cleaned and incubated with block solution (PBST +1% BSA). The block solution was removed from the wells and rabbit polyclonal primary antibodies MMP-2 (bs-41146R, Bioss), MMP-9 (bs-41146R, Bioss), TGF-β1 (bs-20411R, Bioss), HIF1α (bs-20399R, Bioss), Bax (PA5-86062, Thermo Fisher) and Bcl-2 (PA5-27094, Thermo Fisher) were used for immunolabeling of cells. Primary antibodies were added to different wells at 1:200 dilution and incubated at 4 °C overnight. The secondary antibody with biotin was applied to them for 10 min. After, the streptavidin peroxidase enzyme complex was activated for 10 min. Finally chromogen containing aminoethyl carbazole was added and visible immune reaction was achieved. Mayer's hematoxylin was used as a background marker. The stained samples were evaluated with a computer assisted imaging system. While the blue-violet parts in the images express the cell nuclei; red-pink areas mark immune-positive areas.

Statistical Analysis

The Shapiro-Wilk test was used to assess the distribution of the data. Post-hoc paired ANOVA test was used to evaluate normally distributed data. Independent data that did not show a normal distribution were evaluated with the Kruskal-Wallis test. If there was a statistically

Table 1. RT-PCR primer sequences

Gene	Primer sequences	
	Forward	Reverse
Beta actin	5'-CACCAACTGGGACGACAT-3'	5'-ACAGCCTGGATAGCAACG-3'
HIF1α	5'-AGACCTTCCTTAGCCGTCAC-3'	5'-GTCTCCACCCACACAAAACC-3'
TGF-β1	5'-TTGAGACTTTTCCGTTGCCG-3'	5'-CGAGGTCTGGGGAAAAGTCT-3'
MMP-2	5'-AATCCCACCAACCCTCAGAG-3'	5'-GTGCCCTCTTGAGACAGTCT-3'
MMP-9	5'-GAGTTCCCGGAGTGAGTTGA-3'	5'-AAAGGTGAGAAGAGAGGGCC-3'
Bax	5'-CATCATGGGCTGGACATTGG-3'	5'-CCTCAGCCCATCTTCTTCCA-3'
Bcl-2	5'-CTCCTTCATCGTCCCCTCTC-3'	5'-CGGCGGCAGATGAATTACAA-3'

RT-PCR: Real-time-polymerase chain reaction.

significant distinction between the groups, Bonferroni correction post-hoc pairwise comparisons were used to identify the different groups. Statistical analyzes were carried out using IBM SPSS Statistics 21 (IBM Corp. Armonk, NY). Statistics were considered significant at $p<0.05$.

RESULTS

Cytotoxicity Effects of CPT-11, Ellagic Acid and Combination of both (CPT-11 + EA) on HeLa Cells in 2D and 3D Culture

Because of XTT analysis, it was found that the cell viability in HeLa cell lines decreased as the concentration of CPT-11 and EA increased. The IC_{50} for CPT-11 in 2D cultures was 57.8 µM; the IC_{50} value for EA was determined as 45.4 µM (Figure 1A, B). In 3D cultures, the IC_{50} value for CPT-11 is 94 µM; the IC_{50} value for EA was determined as 120 µM (Figure 2A, B). When the two agents were applied in combination with IC_{50} doses, it was determined that the viability of the 2D and 3D cultures was statistically significantly decreased ($p<0.05$) (Figure 1C, 2C).

Alteration of MMP Expression on HeLa Cells in 2D and 3D Culture

When *MMP-2* gene expressions were evaluated in the 2D culture experiment, it was determined that the expression levels of EA, CPT-11 and EA + CPT-11 groups were statistically significantly decreased compared to the DMSO (Dimethyl Sulfoxide) group after 12 and 48 h of application (for 12 hours: p=0.021; 0.017; 0.031, respectively, for 48 hours: p<0.001; <0.001; <0.001, respectively). This decrease in the 24-hour application was insignificant (p>0.05) (Figure 3A). When *MMP-9* gene expressions were evaluated in the 2D culture experiment at 12, 24 and 48 hours, it was determined that the expression levels of EA, CPT-11 and EA + CPT-11 groups decreased compared to the DMSO group after 12, 24 and 48 h of applications. This decrease was found to be statistically significant in the 24-h and 48-h EA + CPT-11 groups (p=0.014; 0.005, respectively). This decrease was not statistically significant in the 12-hour application (p>0.05) (Figure 3B). Statistical data for all groups are presented in Table 2.

When *MMP-2* gene expressions were evaluated in 12, 24 and 48 hours applications in 3D culture experiment, it was seen that the expression levels of all groups decreased compared to the DMSO group. According to our findings, the expression levels of the EA, CPT-11, and EA + CPT-11 groups were found to be statistically significantly decreased compared with the DMSO group after 12 h of administration (p=0.002; 0.016; <0.001, respectively). Expression levels of the EA and EA + CPT-11 groups were statistically significantly decreased compared to the DMSO group in 24-hour application (p<0.001; <0.001, respectively). In the 48-hour application, only the expression level of the EA + CPT-11 group was found to be statistically significantly decreased compared with the DMSO group (p=0.010) (Figure 4A).

When *MMP-9* gene expressions were evaluated in 12, 24 and 48 hours applications in 3D culture experiment, it was seen that the expression levels of all groups decreased compared to the DMSO group. According to our findings, it was determined that the expression level of the EA + CPT-11 group was statistically significantly decreased compared with the DMSO group in only 12 h of application (p=0.003) (Figure 4B). Statistical data for all groups are presented in Table 3.

When protein expressions of MMPs in 2D and 3D cultures were evaluated by immunocytochemical analysis, it was seen that they were compatible with gene expression levels (Figure 5, 6). Briefly, MMP-2 and -9 levels were found to be highest in the DMSO group at 12, 24 and 48 h of treatment. In addition, it was determined that the expression of MMPs, which are metastasis markers, decreased in the treatment groups (EA and Irino) and especially in the combined group (EA + CPT-11) compared to the DMSO group.

Alteration of TGF-β1 Expression on HeLa Cells in 2D and 3D Cultures

When *TGF-β1* gene expressions were evaluated in the 2D culture experiment, it was seen that the expression levels of all groups decreased compared to the DMSO group. Our data indicate that after, and 48 h of treatment, TGF-β1 expression levels in the EA, CPT-11, and EA + CPT-11 groups were statistically substantially lower than in the

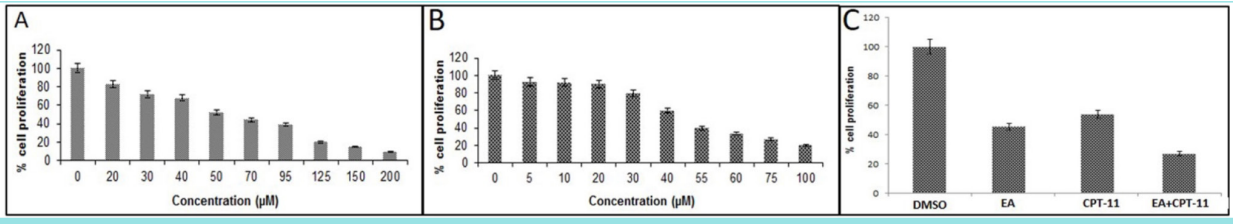

Figure 1. Cytotoxicity findings in 2D HeLa cultures, (A) CPT-11 (IC_{50}: 57.8 µM); (B) ellagic acid (IC_{50}: 45.4 µM); (C) toxicity of agents alone or in combination.

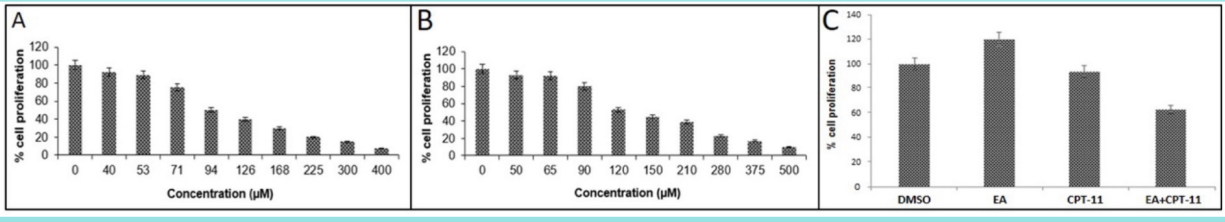

Figure 2. Cytotoxicity findings in 3D HeLa cultures, (A) CPT-11 (IC_{50}: 94 µM); (B) ellagic acid (IC_{50}: 120 µM); (C) toxicity of agents alone or in combination.

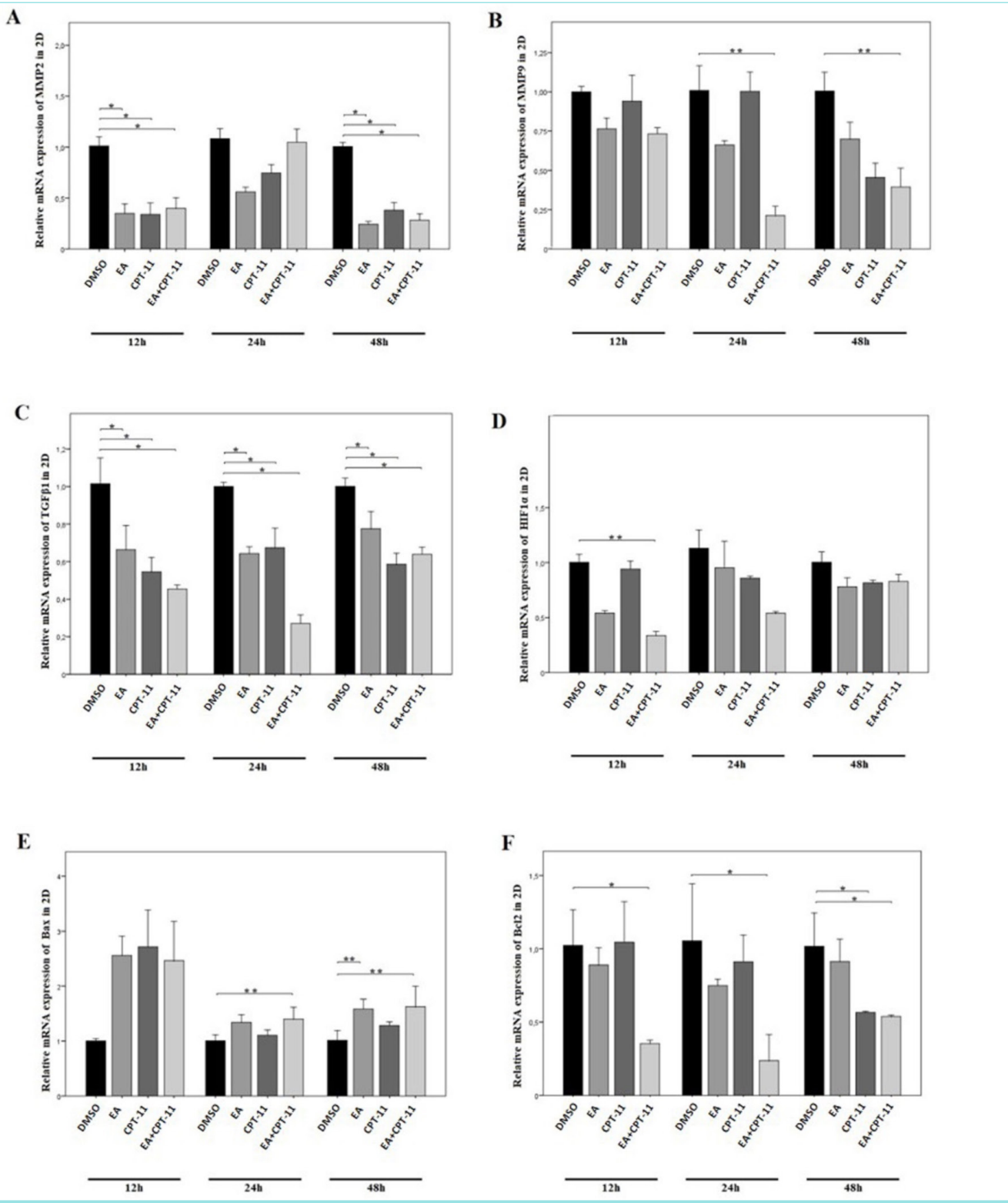

Figure 3. Relative gene expression of *MMP-2* (A), *MMP*-9 (B), *TGF-β*1 (C), *HIF1α* (D), *Bax* (E), and *Bcl-2* (F) of all groups at different times in 2D planar HeLa cell cultures. Data ($2^{-\Delta\Delta Ct}$ ratio to beta-actin mRNA) are expressed as the mean relative to the DMSO group (mean: 1). *: $p<0.05$, vs. The DMSO group (One-Way ANOVA with Bonferroni correction). **: $p<0.05$, vs. The DMSO group (Kruskal-Wallis test with Bonferroni correction pos-hoc test). All experiments were conducted four times.

DMSO group (for 12 hours: p=0.039; 0.005; 0.001, respectively, for 24 hours: p<0.001; <0.001; <0.001, respectively, for 48 hours: p=0.001; <0.001; <0.001, respectively) (Figure 3C). Statistical data for all groups are presented in Table 2.

When *TGF-β1* gene expressions were evaluated at 12, 24 and 48 h in the 3D culture experiment, it was seen that the expression levels of all groups decreased compared to the DMSO group. According to our data, the expression level of the EA + CPT-11 group was found to be statistically significantly decreased compared to the DMSO group in the 12 and 24 h applications (p=0.002; 0.045, respectively). In the 48-hour application, this reduction was statistically insignificant (p>0.05) (Figure 4C). Statistical data for all groups are presented in Table 3.

TGF-β1 protein expressions in 2D and 3D cultures was also found to be compatible with gene expression levels when evaluated by immunocytochemical analysis (Figure 7). Briefly, TGF-β1 expression levels were found to be highest in the DMSO group at 12, 24 and 48 h of administration. In addition, it was determined that TGF-β1 expressions, which are markers of angiogenesis and epithelial mesenchymal transition (EMT), were decreased in the treatment groups and especially in the combined group (EA + CPT-11) compared to the DMSO group.

Alteration of HIF1α Expressions on HeLa Cells in 2D and 3D Cultures

When *HIF1α* gene expressions were evaluated in the 2D culture experiment, it was seen that the expression levels of all groups decreased compared to the DMSO group. It was determined that the expression level of the EA + CPT-11 group was statistically significantly decreased compared with the DMSO group in only 12 h of application (p=0.008) (Figure 3D). Statistical data for all groups are presented in Table 2.

When *HIF1α* gene expressions were evaluated in the 3D culture experiment, it was seen that the expression levels of all groups decreased compared to the DMSO group. It was determined that the expression level of HIF1α in the EA + CPT-11 group was statistically lowered compared to the DMSO group in all applications at different times (p=0.031; 0.001; 0.001, respectively) (Figure 4D). Statistical data for all groups are presented in Table 3.

When HIF1α protein expressions in 2D and 3D cultures were evaluated by immunocytochemical analysis, it was found to be compatible with gene expression levels (Figure 8). Briefly, HIF1α expression levels were found to be decreased in all treatment groups compared to the DMSO group in applications at different times. This decrease in HIF1α expression, which promotes angiogenesis, was more pronounced in the combination group than in the DMSO group, especially in 3D cells.

Elevated Expression of Bax and Decreased Expression of Bcl-2 in Response to EA and CPT-11 + EA Treatment on HeLa Cells in 2D and 3D Culture

When the expression levels of the proapoptotic gene *Bax* were analyzed in 2D culture experiments, it was seen that the Bax levels of all groups increased compared to the DMSO group. In the 24 and 48 h applications, it was found that the Bax level in the EA + CPT-11 group was statistically important compared to the DMSO group (p=0.036; 0.045, respectively). In addition, it was determined that the expression level of the lean EA group increased statistically significantly compared with the DMSO group in the 48-hour application (p=0.023) (Figure 3E). Statistical data for all groups are presented in Table 2.

Table 2. Post-hoc pairwise comparisons for 2D cell culture

	Pairwise groups	Adjusted p-value					
		MMP-2*	MMP-9**	TGF-β1*	HIF1α**	Bax**	Bcl-2*
12 h	DMSO vs. EA	**0.021**	0.321	**0.039**	0.270	0.105	1.000
	DMSO vs. CPT-11	**0.017**	1.000	**0.005**	1.000	0.069	1.000
	DMSO vs. EA + CPT-11	**0.031**	0.069	**0.001**	**0.008**	0.155	**0.002**
24 h	DMSO vs. EA	0.087	0.380	**<0.001**	1.000	0.069	0.535
	DMSO vs. CPT-11	0.528	1.000	**<0.001**	1.000	1.000	1.000
	DMSO vs. EA + CPT-11	1.000	**0.014**	**<0.001**	0.105	**0.036**	**0.002**
48 h	DMSO vs. EA	**<0.001**	1.000	**0.001**	0.084	**0.023**	1.000
	DMSO vs. CPT-11	**<0.001**	0.056	**<0.001**	0.084	1.000	**0.003**
	DMSO vs. EA + CPT-11	**<0.001**	**0.005**	**<0.001**	0.322	**0.045**	**0.002**
DMSO	12 h vs. 24 h	1.000	1.000	1.000	1.000	1.000	1.000
	12 h vs. 48 h	1.000	1.000	1.000	1.000	1.000	1.000
	24 h vs. 48 h	1.000	1.000	1.000	1.000	1.000	1.000
EA	12 h vs. 24 h	0.689	0.084	1.000	**0.018**	**0.013**	0.352
	12 h vs. 48 h	1.000	0.992	0.809	0.150	0.187	1.000
	24 h vs. 48 h	0.225	1.000	0.589	1.000	0.980	0.219
CPT-11	12 h vs. 24 h	**0.047**	1.000	0.159	0.596	**0.007**	1.000
	12 h vs. 48 h	1.000	0.118	1.000	0.332	0.287	**0.019**
	24 h vs. 48 h	0.076	**0.024**	0.482	0.332	0.509	0.092
EA + CPT-11	12 h vs. 24 h	**<0.001**	**0.013**	**<0.001**	0.350	**0.032**	0.454
	12 h vs. 48 h	0.471	0.187	**<0.001**	**0.005**	0.350	0.097
	24 h vs. 48 h	**<0.001**	0.980	**<0.001**	0.350	0.980	**0.008**

*ANOVA test, **Mann-Whitney U test, bolded values remain significant after correction for multiple comparisons (p<0.05)

When Bax expression levels were analyzed in 3D culture experiments, it was seen that the expression levels of all groups increased compared to the DMSO group. In the 12- and 48-h applications, it was determined that the EA + CPT-11 group expression level was statistically higher than that of the DMSO group (p=0.006; 0.010, respectively). In addition, it was determined that the expression level of the lean CPT-11 group increased statistically significantly compared with the DMSO group in the 12-hour application (p=0.043) (Figure 4E). Statistical data for all groups are presented in Table 3.

When the expression levels of anti-apoptotic gene *Bcl-2* were analyzed in 2D culture experiments, it was seen that the expression levels of all

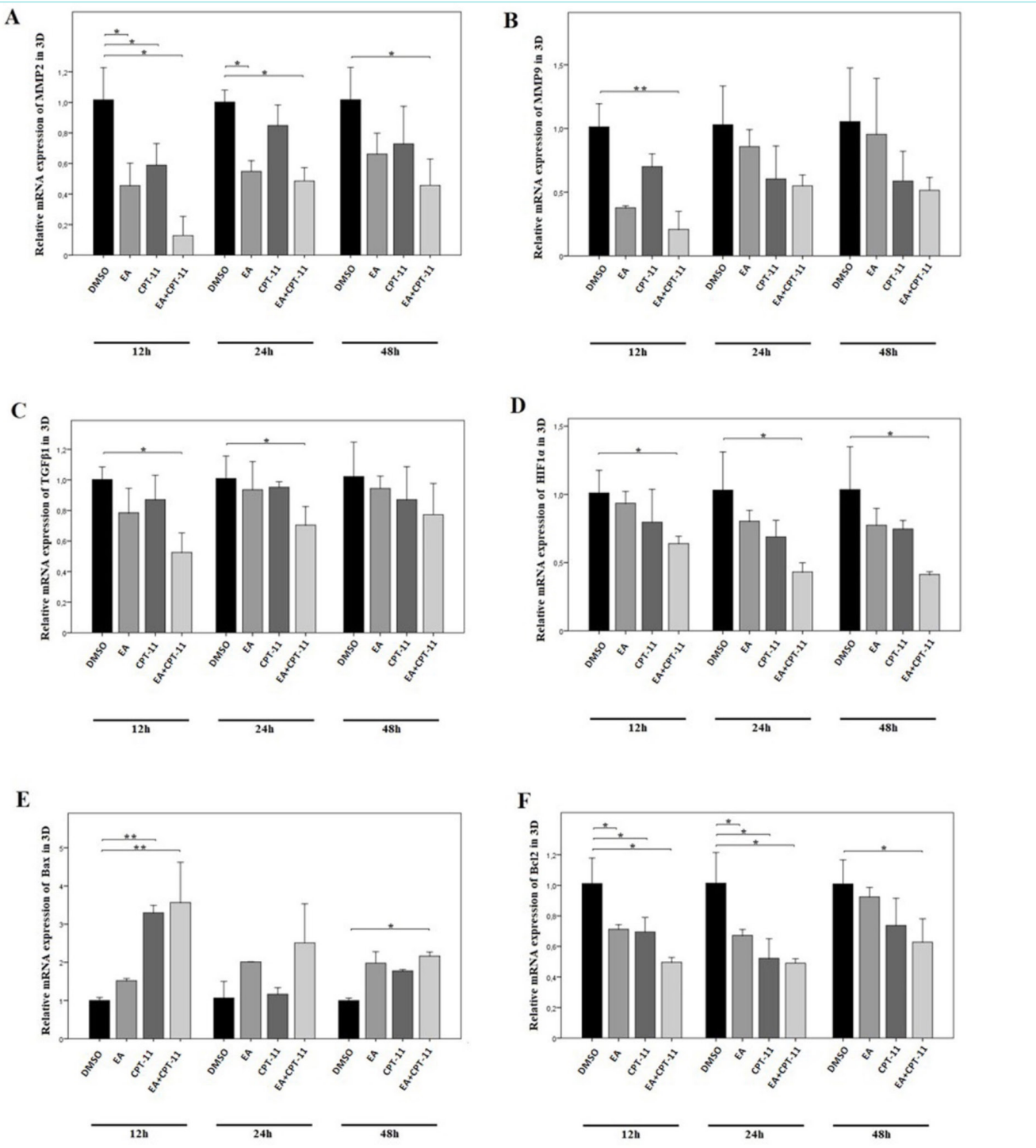

Figure 4. Relative gene expression of *MMP-2* (A), *MMP-9* (B), *TGF-β1* (C), *HIF1α* (D), *Bax* (E), and *Bcl-2* (F) of all groups at different times in 3D HeLa cell cultures. Data ($2^{-\Delta\Delta Ct}$ ratio to beta actin mRNA) are expressed as the mean relative to the DMSO group (mean: 1). *: p<0.05, vs. The DMSO group (One-Way ANOVA with Bonferroni correction). **: p<0.05, vs. The DMSO group (Kruskal-Wallis test with Bonferroni correction post-hoc test). All experiments were conducted four times.

Table 3. Post-hoc pairwise comparisons for 3D cell culture

	Pairwise groups	Adjusted p-value					
		MMP-2*	MMP-9**	TGF-β1*	HIF1α*	Bax**	Bcl-2*
12 h	DMSO vs. EA	**0.002**	0.128	0.259	1.000	1.000	**0.006**
	DMSO vs. CPT-11	**0.016**	1.000	1.000	0.440	**0.043**	**0.004**
	DMSO vs. EA + CPT-11	**<0.001**	**0.003**	**0.002**	**0.031**	**0.006**	**<0.001**
24 h	DMSO vs. EA	**<0.001**	1.000	1.000	0.413	0.101	**0.011**
	DMSO vs. CPT-11	0.254	0.270	1.000	0.066	1.000	**0.001**
	DMSO vs. EA + CPT-11	**<0.001**	0.128	**0.045**	**0.001**	0.101	**<0.001**
48 h	DMSO vs. EA	0.149	1.000	1.000	0.315	0.067	1.000
	DMSO vs. CPT-11	0.355	0.332	1.000	0.208	0.813	0.125
	DMSO vs. EA + CPT-11	**0.010**	0.084	0.535	**0.001**	**0.010**	**0.017**
DMSO	12 h vs. 24 h	1.000	1.000	1.000	1.000	1.000	1.000
	12 h vs. 48 h	1.000	1.000	1.000	1.000	1.000	1.000
	24 h vs. 48 h	1.000	1.000	1.000	1.000	1.000	1.000
EA	12 h vs. 24 h	0.931	0.072	0.548	0.272	0.225	0.743
	12 h vs. 48 h	0.122	**0.043**	0.484	0.139	0.053	**<0.001**
	24 h vs. 48 h	0.664	1.000	1.000	1.000	1.000	**<0.001**
CPT-11	12 h vs. 24 h	0.222	1.000	1.000	1.000	**0.005**	0.329
	12 h vs. 48 h	0.919	1.000	1.000	1.000	0.338	1.000
	24 h vs. 48 h	1.000	1.000	1.000	1.000	0.338	0.166
EA + CPT-11	12 h vs. 24 h	**0.013**	**0.043**	0.413	**0.001**	0.234	1.000
	12 h vs. 48 h	**0.021**	0.072	0.154	**<0.001**	0.720	0.217
	24 h vs. 48 h	1.000	1.000	1.000	1.000	1.000	0.186

*ANOVA test, **Mann-Whitney U test, bolded values remain significant after correction for multiple comparisons ($p<0.05$).

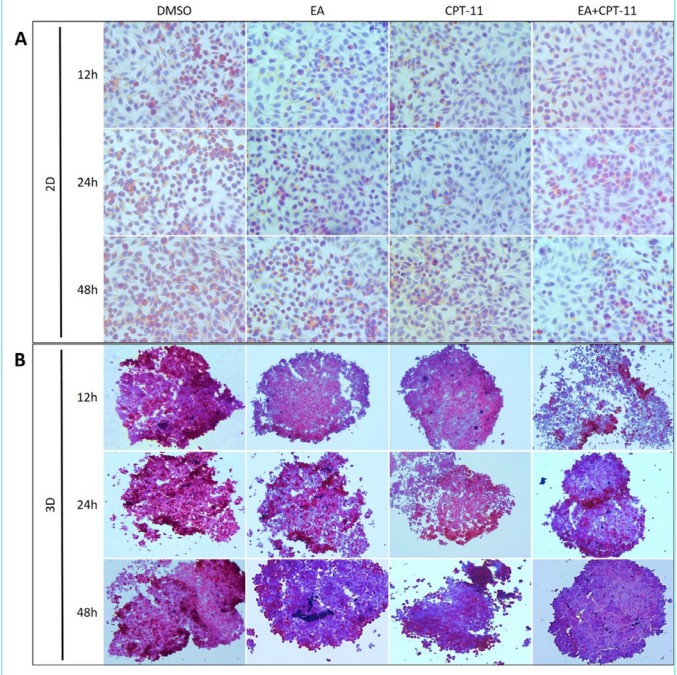

Figure 5. Representative images of MMP-2 immunoexpressions of HeLa cells in 2D planar culture (A) and 3D constructs (B) at 12, 24 and 48 h treatments (AEC & hematoxylin).

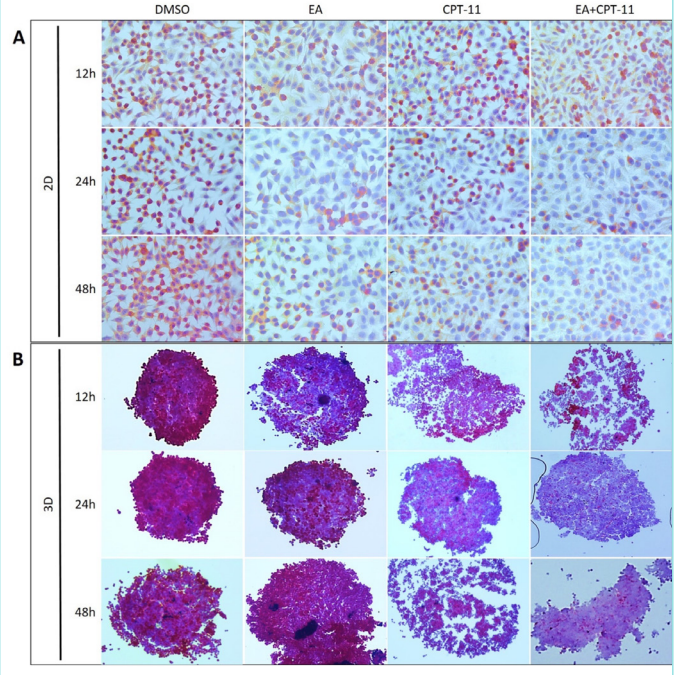

Figure 6. Representative images of MMP-9 immunoexpressions of HeLa cells in 2D planar culture (A) and 3D constructs (B) at 12, 24 and 48 h treatments (AEC & hematoxylin).

groups decreased compared to the DMSO group. The expression level of the EA + CPT-11 group was found to be statistically significant compared to the DMSO group in the 12, 24, and 48 h applications (p=0.002; 0.002; 0.002, respectively). In addition, it was determined that the expression level of the lean CPT-11 group was statistically significantly decreased compared with the DMSO group in the 48-hour application (p=0.003) (Figure 3F). Statistical data for all groups are presented in Table 2.

When Bcl-2 expression levels were analyzed in 3D culture experiments, it was seen that the expression levels of all groups decreased compared to the DMSO group. It was determined that the expression level of the EA, CPT-11, and EA + CPT-11 groups decreased statistically significantly in 12 and 24 h applications compared to the DMSO group (for 12 hours: p=0.006; 0.004; <0.001, respectively, for 24 hours: p=0.011; 0.001; <0.001, respectively). In addition, the expression level of the EA + CPT-11 group was found to be statistically significantly decreased compared with the DMSO group in the 48-hour application (p=0.017) (Figure 4F). Statistical data for all groups are presented in Table 3.

When Bax protein expressions in two- and three-dimensional cultures were evaluated by immunocytochemical analysis, they were found to be compatible with gene expression levels (Figure 9). Briefly, Bax expression levels were found to be increased in all groups compared with the DMSO group. Contrary to Bax protein, Bcl-2 protein expression was found to be highest in the DMSO group (Figure 10). Especially in the combined group (EA + CPT-11), the expression of anti-apoptotic gene *Bcl-2* was significantly decreased compared to the DMSO group.

DISCUSSION

Cancer is defined as a disease resulting from disruption of the balance of cell death and proliferation. Today, with the increase of carcinogenic agents, the incidence of cancer and the number of related deaths are also increasing.[7] The semi-synthetic camptothecin derivative CPT-11 is obtained from Camptotheca acuminate. This drug has a great antitumoral effect on many tumors such as lung, ovarian, colorectal, and cervical cancer. In addition, toxicology studies have shown that they have widespread toxic effects in the hematological system and lymphoid organs. Myelotoxicity, neutropenia, thrombocytopenia, and anemia are among the most serious toxic effects. Hematological side effects are dose-dependent. In a study investigating cell proliferation, cell migration, and cell cycle in HeLa cells, researchers found the lethal dose of CPT-11 to be 78.5 µg/mL.[8-10] In our study, we determined the IC_{50} dose of CPT-11 applied to 2D and 3D HeLa cells as 57.8 µM, and 94 µM, respectively.

Phytochemicals are useful in cancer prevention because they simultaneously target multiple pathways active in cancer progression. For improved cancer management and therapy, combining phytochemicals with anticancer medications may be a novel and very effective therapeutic approach. When taken alone, commercial medications can be more successful when combined with phytochemicals that are comparatively less harmful.[11,12] Previous research has shown that the phenolic component EA, which is generated from plants and is present in raspberries and other plant foods, has strong anticarcinogenic properties.[13,14] In a study, it was claimed that EA caused human cervical cancer Ca Ski cells to go into G0/G1 arrest and eventually die.[13] *In vivo* and *in vitro* studies, EA has been demonstrated to be a potent antioxidant with free radical scavenging ability, anti-aging, anti-proliferative, anti-fibrotic, anti-atherosclerotic, anti-cancer and anti-mutagenic properties.[15-17] In this study, we demonstrated the time-dependent effects of EA and its combination with irinotecan on HeLa cells. In a study examining the effect of different doses of EA (5, 25 and 50 µM) on the cell cycle, histogram findings demonstrated that EA mostly inhibited the cell cycle by dose-dependently arresting cells in

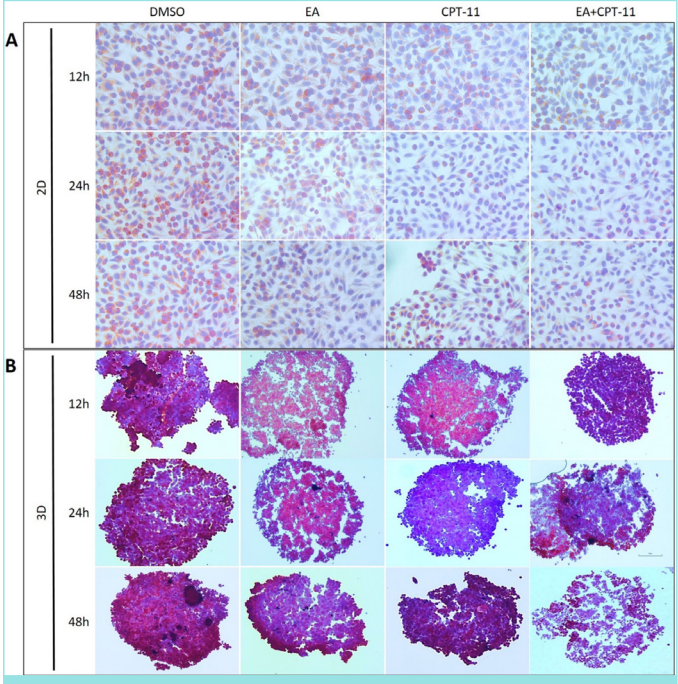

Figure 7. Representative images of TGF-β1 immunoexpressions of HeLa cells in 2D planar culture (A) and 3D constructs (B) at 12, 24 and 48 h treatments (AEC & hematoxylin).

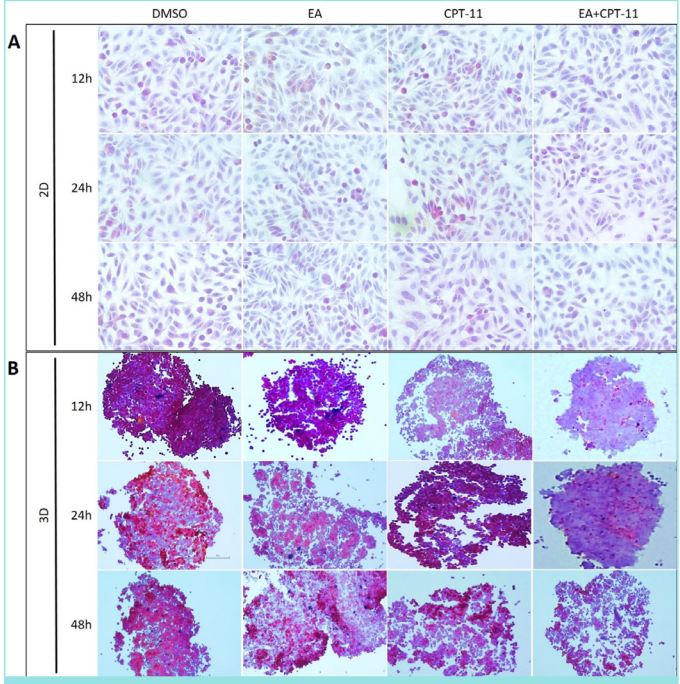

Figure 8. Representative images of HIF1α immunoexpressions of HeLa cells in 2D planar culture (A) and 3D constructs (B) at 12, 24 and 48 h treatments (AEC & hematoxylin).

the G0/G1 phase.[18] Li et al.[19] used a variety of human cervical cancer cell lines (HeLa, SiHa and C33A) in their study, they showed that EA applied to cell cultures at 10, 20 and 30 µM doses inhibited cell proliferation at a statistically significant level. In our study, we determined the IC_{50} dose of EA applied to 2-dimensional HeLa cells as 45.4 µM, and 120 µM, respectively.

Studies have demonstrated that EA suppresses angiogenesis-related markers in tumor tissues, including COX-2, HIF1α, VEGF, VEGFR, and IL-8, as well as inhibits the metastatic markers MMP-2 and MMP-9. In studies, it has been shown that tumor metastasis and development can be suppressed by the EMT mechanism in this way.[20] In a study, it was shown that EA significantly inhibited the migration of human ovarian carcinoma A2780 cells. In the same study, EA administered at doses of 10 and 15 µg/mL significantly downregulated the expression levels of MMP-2 and -9. In the *in vivo* part of the same study, it was stated that EA caused a significant decrease in the weight and volume of the tumor. The immunohistochemical findings of the study also showed that by reducing the expression of MMPs, EA had anticancer properties. In addition, the antitumor properties of EA were confirmed by serum ELISA analysis.[21]

A study with human and rat prostate cancer cell lines showed that EA treatment slightly reduced MMP-2 secretion but did not affect the MMP-9 expression.[22] In our study, it was determined that the levels of MMP-2 and -9 were highest in the DMSO group at 12, 24 and 48 h of treatment. In addition, it was determined that the expression of MMPs, which are markers of metastasis, decreased in the treatment groups and especially in the combined group (EA + CPT-11) compared to the DMSO group.

The characteristics of the tumor microenvironment, such as the synthesis of TGF-β, probably contribute to the induction of EMT even though it is a multistep process with complicated mechanisms.[23] *In vitro* results of a study revealed that partial inhibition of TGF-β1 must reduce the migration of cancer cells and induce apoptosis.[24] In a study, it was reported that EA induces cell cycle arrest in MCF-7 cells through TGF-β/Smad3 signaling.[25]

Recent findings have shown the role of HIF1α and TGF-β1 in the development in tumors.[23,26] Even though research suggests that HIF1α and TGF-β may cause EMT, further research is still needed to fully understand how HIF1α and TGF-β interact and how this interacts with EMT in cervical cancer. In our study, it was determined that TGF-β1 expression levels were highest in the DMSO group in 2D and 3D cultures in applications with varying durations. In addition, it was determined that TGF-β1 expressions, a marker of angiogenesis and EMT, was decreased in the treatment groups and especially in the combined group (EA + CPT-11) compared to the DMSO group.

Detecting intra-tumor oxygen tension, HIF1 promotes the activation of hypoxia-related reactions implicated in cancer progression, such as proliferation, metabolism, angiogenesis, invasion, metastasis, and treatment resistance.[23] The upregulation of HIF1α activity promotes tumor-associated angiogenesis and thus tumor cell survival and proliferation in solid tumors.[27,28] In a study, it was shown that EA decreased HIF1α in lung cancer cells and tumor tissues.[29] Numerous studies link elevated levels of HIF1α expression to the emergence and spread of various malignancies. Inhibiting HIF1α is therefore a promising anticancer therapy.[30,31] Since angiogenesis plays an important role in tumor growth,[32] we tried to determine the effects of EA, CPT-11, and their combination on angiogenesis by determining HIF1α expression levels in cancer cells by RT-PCR and intraclass correlation coefficient analyses. In our study, HIF1α expression levels were decreased in all treatment groups compared with the DMSO group in applications for varying durations. Although this decrease

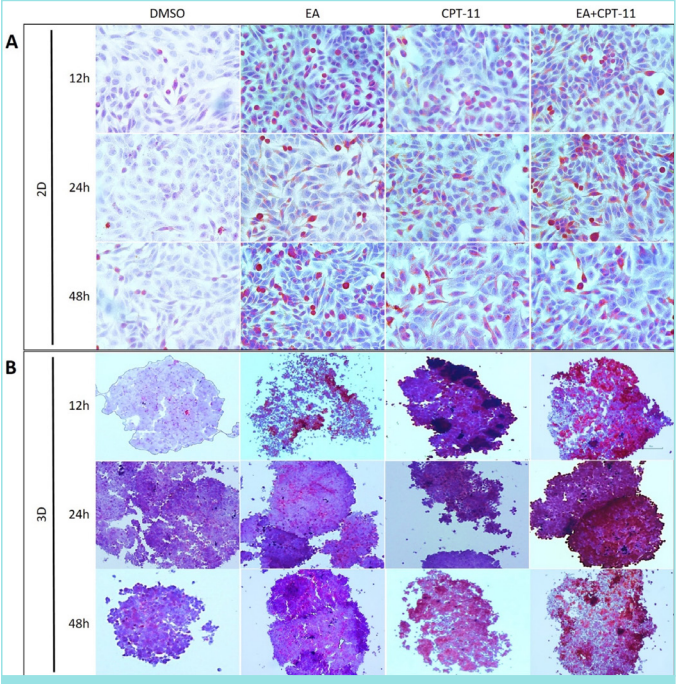

Figure 9. Representative images of Bax immunoexpressions of HeLa cells in 2D planar culture (A) and 3D constructs (B) at 12, 24 and 48 h treatments (AEC & hematoxylin).

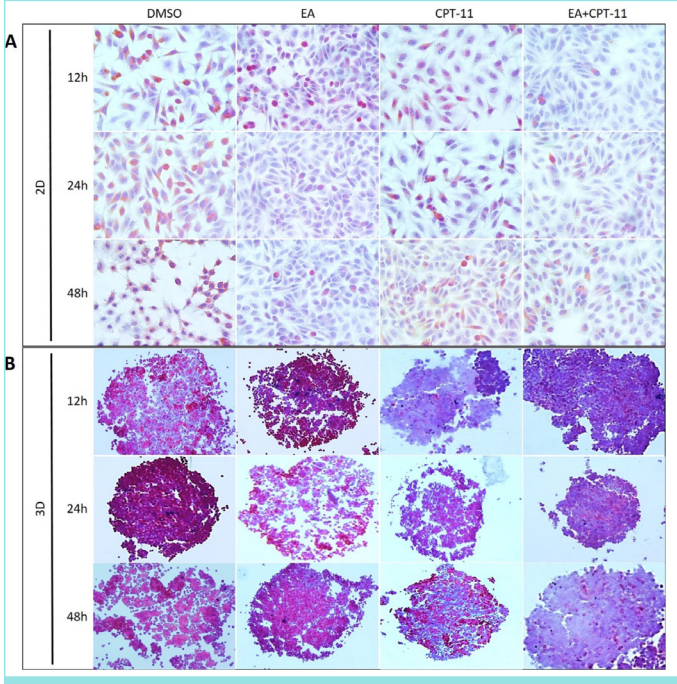

Figure 10. Representative images of Bcl-2 immunoexpressions of HeLa cells in 2D planar culture (A) and 3D constructs (B) at 12, 24 and 48 h treatments (AEC & hematoxylin).

was not significant in 2D cultures, this decrease in HIF1α expression level in 3D cultures was significantly higher in the combined group than in the DMSO group.

Promoting the synthesis of pro-apoptotic members of the Bcl-2 family and suppressing the expression of anti-apoptotic members are crucial in terms of increasing the effectiveness of chemotherapy and radiotherapy. It has been shown that Bax, one of the pro-apoptotic members of the Bcl-2 family, is suppressed in various cancers, and that promoting Bax expression also creates susceptibility to apoptosis in tumors.[33,34] According to studies, EA causes cancer tissue to express less antiapoptotic protein Bcl-2 and more proapoptotic protein Bax. Likewise, while EA increases the level of active caspase-3, it decreases the expression of proliferation markers such as Ki67 and PCNA. In this way, tumor development with apoptotic mechanisms can be suppressed by EA.[20] In the study of Li et al.[19], it was revealed by flow cytometric and cell cycle analyzes that EA stopped the cell cycle in G1 phase and induced cell apoptosis in HeLa cells. In one study, HeLa cells were treated with 25 μM dose of curcumin and EA for 24 hours. It has been demonstrated that the anticancer effects of this combination are superior to those of either medication alone. In the study, curcumin and EA restored p53, induce ROS generation and DNA damage. Immunocytochemical analysis in the study showed that EA and curcumin did not alter the expression of Bax. However, it was stated that the combination of curcumin and EA showed higher Bax expression in cells.[35] In our study, it was determined that Bax expression levels increased in all groups after 12, 24 and 48 h of treatment compared with the DMSO group. Contrary to Bax protein, Bcl-2 protein expression was found to be highest in the DMSO group. Especially in the combined group (E+ CPT-11), the expression of antiapoptotic gene Bcl-2 was significantly decreased compared to the DMSO group.

CONCLUSION

In the study, it was shown that the expressions of MMPs, TGF-β1 and HIF1α were significantly inhibited in HeLa cells treated with EA and treated with the combination of CPT-11. In addition, the increase in proapoptotic Bax level with the treatment and suppression of antiapoptotic Bcl-2 expression were revealed as another important finding of EA and combined treatment. From these results, we can conclude that EA can reduce the invasion of cervical cancer cell lines and increase apoptosis through modulation of MMP activity, TGF-β1 and HIF1α levels. Therefore, EA can be used as an adjuvant therapy for cervical cancer.

MAIN POINTS

- Cervical cancer is an important public health problem worldwide with a high incidence rate.
- Ellagic acid downregulated the expression of MMPs, TGF-β1 and HIF1α in HeLa cells.
- While ellagic acid increased the proapoptotic Bax expression level in HeLa cells, it suppressed the antiapoptotic Bcl-2 expression.
- These data indicate that ellagic acid would be a good candidate for additional preclinical studies for the treatment of human cervical cancer.

ETHICS

Ethics Committee Approval: Ethics committee form was not required.

Informed Consent: A consent form was not required for this study as the cells were purchased commercially.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: G.S.S., G.T.S., Design: G.S.S., G.T.S., S.Y.A., S.Y., Data Collection and/or Processing: G.S.S., G.T.S., S.Y.A., Ö.A.D., S.Y., Analysis and/or Interpretation: G.S.S., S.Y.A., Ö.A.D., S.Y., Literature Search: G.S.S., G.T.S., S.Y.A., Ö.A.D., S.Y., Writing: G.S.S., Ö.A.D.

DISCLOSURES

Conflict of Interest: No conflict of interest was declared by the authors.

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