Medical Oncology (2026) 43:162
https://doi.org/10.1007/s12032-026-03270-1

ORIGINAL ARTICLE

Evaluation of the anticancer effects of ellagic acid and cisplatin in cisplatin-sensitive and -resistant MDA-MB-231 breast cancer cells

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Received: 25 September 2025 / Accepted: 14 January 2026 / Published online: 3 March 2026

Abstract

Ellagic acid (EA) is a natural polyphenol noted for its antiproliferative and pro-apoptotic effects. This study investigates the impact of EA, alone or combined with cisplatin (CIS), on the expression of angiogenesis, apoptosis, metastasis, and chemoresistance-related genes and proteins in cisplatin-sensitive and -resistant MDA-MB-231 breast cancer cells. Cell viability was evaluated by cytotoxicity assay, while gene and protein expression levels were analyzed via qPCR and immunocytochemistry. Molecular docking was used to assess EA's binding affinity to target proteins. The IC_{50} values of EA and CIS were 29 µM and 38.2 µM in cisplatin-sensitive MDA-MB-231 cells, and 49.5 µM and 80.2 µM in cisplatin-resistant cells, respectively. In both cell types, EA significantly decreased the expression of the ABCB1 and VEGF genes, especially at 24 and 48 h. EA alone and combined with CIS suppressed MMP2 and MMP9 expression across both cell types. Additionally, EA and CIS + EA treatments suppressed Bcl-2 expression and upregulated Bax expression. Immunocytochemical results aligned with gene expression data, demonstrating reduced protein levels. Molecular docking demonstrated strong binding of EA to Bax and MMP9. These results indicate that EA exerts notable anticancer activity by targeting genes associated with drug resistance, angiogenesis, apoptosis, and metastasis. The findings highlight EA's therapeutic potential in breast cancer treatment, alone or with CIS. Further detailed in vivo and clinical studies are needed to confirm these promising results.

Keywords Ellagic acid · Cisplatin · Breast cancer · Chemoresistance

Abbreviations

(ABCB1/P-gp)	ATP Binding Cassette B1
(CIS)	Cisplatin
(DMSO)	Dimethyl sulfoxide
(EA)	Ellagic acid
(ECM)	Extracellular matrix
(HER2)	Human epidermal growth factor receptor 2
(MMP2)	Matrix metalloproteinases 2
(MMP9)	Matrix metalloproteinases 9
(MDR)	Multidrug resistance
(VEGF)	Vascular endothelial growth factor

Introduction

Breast cancer is one of the most common types of cancer among women and represents a significant global public health issue [1]. As of 2020, approximately 2.3 million women worldwide were diagnosed with breast cancer, and more than 500,000 women died as a result of the disease [1]. Breast cancer is considered a heterogeneous disease and is classified into various subtypes based on biomarkers such as hormone receptors and human epidermal growth factor receptor 2 (HER2) [2]. The effectiveness of current breast cancer treatments remains significantly limited due to factors such as drug toxicity, resistance profiles, and the lack

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of reliable and prognostic biomarkers [3]. In this context, the identification of novel biomarkers and the discovery of potential therapeutic agents are of great importance for the early diagnosis and effective treatment of breast cancer.

Cisplatin (CIS), one of the chemotherapeutic treatment options, is widely used in the treatment of various malignancies, including breast, testicular, ovarian, cervical, and head and neck cancers [4]. CIS exerts its cytotoxic effect by inhibiting replication with cisplatin-DNA adducts and induction of apoptosis [5]. Tumor initiation and progression is a multistep process that can be activated by various factors. This process is shaped by the regulation of transcription factors, anti-apoptotic proteins, protein kinases, growth factors, and cellular signaling pathways [6].

The adverse effects of chemotherapy medications are one of the main obstacles to modern cancer treatments. These side effects significantly lower patients' quality of life and provide major challenges throughout therapy [7]. Therefore, alternative therapeutic approaches aimed at minimizing the adverse effects of chemotherapeutic drugs have gained increasing importance. In recent years, the use of plant-based and naturally derived compounds with anticancer properties has emerged as a promising strategy to improve treatment outcomes [8]. Numerous natural antioxidants, including alkaloids, polyphenols, and flavonoids, have been shown to have chemotherapeutic effects in vitro and in vivo and are therefore regarded as possible therapeutic agents [9].

Ellagic acid (EA; 2,3,7,8-tetrahydroxy-chromeno[5,4,3-cde]chromene-5,10-dione) is a naturally occurring polyphenol found in pomegranates, blackberries, raspberries, and various other fruits and plants [10, 11]. EA has been shown in numerous studies to have the ability to reduce tumor cell proliferation, trigger apoptosis, and prevent tumor growth, angiogenesis, metastasis, and drug resistance [12, 13]. Breast, pancreatic, colon, and prostate cancer are among the cancer types for which EA has been shown to have anticancerogenic effects [14–17].

One of the most important factors that adversely affects the efficacy of cancer chemotherapy is multidrug resistance (MDR) [18]. The efflux of chemotherapeutic drugs from cancer cells to the extracellular space through ATP-binding cassette (ABC) transporters is one of the main mechanisms of multidrug resistance (MDR) [19].

ABC transporters are membrane proteins that regulate the transport of various molecules across the cell membrane, and among this family, ATP Binding Cassette B1 (ABCB1/P-gp) is recognized as the main efflux pump responsible for drug resistance in cancer cells [20]. Overexpression of ABCB1 leads to the development of drug resistance in cancer cells [20]. Furthermore, vascular endothelial growth factor (VEGF) is a crucial stimulator of tumor angiogenesis, a basic mechanism behind tumor growth and metastasis [21]. Similarly, matrix metalloproteinases (MMPs) are zinc-dependent enzymes involved in the degradation of the extracellular matrix (ECM), and among them, matrix metalloproteinase 2 (MMP2) and matrix metalloproteinase 9 (MMP9) are critically important for tumor invasion and metastasis development [22].

The purpose of this study is to compare the effects of CIS and EA applied separately and in combination on the expression levels of genes linked to drug resistance, angiogenesis, invasion, and apoptosis, including ABCB1, VEGF, MMP2, MMP9, Bax, and Bcl-2, in MDA-MB-231 breast cancer cells that are sensitive to and resistant to cisplatin.

Materials and methods

Cell culture

Cisplatin-sensitive and cisplatin-resistant MDA-MB-231 cells were cultured under standard cell culture conditions. Cisplatin-sensitive cells were maintained in appropriate culture medium (RPMI-1640) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin in a humidified incubator at 37 °C with 5% CO_2. Cisplatin-resistant cells were derived from the drug sensitive MDA-MB-231 line through long-term, stepwise exposure to increasing concentrations of cisplatin and were continuously maintained in medium containing a low maintenance dose of cisplatin to preserve the resistant phenotype [23].

Cytotoxicity analysis

The cytotoxicity of the molecules was assessed using an XTT-based cytotoxicity assay kit. Cells were seeded into 96-well plates at a density of 5,000 cells per well. After cell attachment, EA and CIS were applied individually and in combination to the wells in serial dilutions. Following 24 to 72 h of treatment, XTT reagent was added, and the plates were incubated at 37 °C for 2 to 5 h. Following incubation, the formazan dye in each well was measured using a microplate reader. Cell death was measured to determine the IC50 value [24].

Total RNA isolation and evaluation of gene expression by qRT-PCR

Total RNA was isolated from cisplatin-sensitive and cisplatin-resistant MDA-MB-231 breast cancer cells using the WizPrep Total RNA Mini Kit (WizBio, Korea), in accordance with the manufacturer's protocol. RNA concentrations were determined using an Optizen NanoQ

spectrophotometer (Optizen, Korea). cDNA synthesis was performed using the HyperScript™ First Strand Synthesis cDNA Kit (GeneAll, Korea) according to the manufacturer's instructions. Changes in gene expression were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) using the Applied Biosystems™ 7500 Fast Real-Time PCR system. The beta-actin housekeeping gene was used as an internal reference control. Gene-specific primers used in the study are listed in Table 1. mRNA expression changes were calculated using the 2^−ΔΔCt.

Immunocytochemical assay

Cisplatin-sensitive and -resistant MDA-MB-231 cells in culture were immunocytochemically stained using the Streptavidin-Biotin-Peroxidase method. Immunocytochemical analyses were performed in 24-well plates. Briefly, cells exposed to the optimal concentrations of the drugs for periods ranging from 12 to 48 h were fixed with 4% paraformaldehyde. After washing with PBS, the cells were incubated overnight at 4 °C with primary antibodies: rabbit polyclonal ABCB1 antibody (bs-0563R, Bioss Sci), rabbit polyclonal VEGF antibody (P802, Thermo Sci), rabbit polyclonal MMP2 antibody (bs-0412R, Bioss Sci), and rabbit polyclonal MMP9 antibody (bs-41146R, Bioss Sci). The cells were treated with an HRP-conjugated secondary antibody kit specific to the primary antibodies (TP-125-BN, Thermo Scientific). To visualize the reaction, aminoethyl carbazole (AEC) chromogen (TA125-HA, Thermo Scientific) was used. Hematoxylin was applied as a counterstain. The stained preparations were photographed under a computer-assisted microscope. To present the immunocytochemical analyses in a descriptive manner, counts were performed. Six different random fields were selected from each group (at 200x magnification). Total cell counts were performed in these fields, and the percentage of positive cells was determined.

Table 1 Real time PCR primer sequences

Gene	Forward	Reverse
ABCB1	5'-TCAGCTGTTGTCTTTGGTGC-3'	5'-GGTCGGGTGGGATAGTTGAA-3'
VEGF	5'-GAAGAAGCAGCCCATGACAG-3'	5'-CTCACACACACACAACCAGG-3'
MMP2	5'-AATCCCACCAACCCTCAGAG-3'	5'-GTGCCCTCTTGAGACAGTCT-3'
MMP9	5'-GAGTTCCCGGAGTGAGTTGA-3'	5'-AAAGGTGAGAAGAGAGGGCC-3'
Bax	5'-CATCATGGGCTGGACATTGG-3'	5'-CCTCAGCCCATCTTCTTCCA-3'
Bcl-2	5'-CTCCTTCATCGTCCCCTCTC-3'	5'-CGGCGGCAGATGAATTACAA-3'
Beta Actin	5'-CACCAACTGGGACGACAT-3'	5'-ACAGCCTGGATAGCAACG-3'

Molecular docking analyses

AutoDock Vina is a newly optimized molecular docking and virtual screening software. During the local optimization process, it employs a sophisticated gradient optimization method to enhance the accuracy and speed of molecular docking. For molecular docking analysis, the proteins ABCB1, VEGF, MMP2, MMP9, Bax, and Bcl-2 were selected. Affinity represents the binding ability of a ligand to its receptor; the greater the absolute value of the affinity (which should be negative), the stronger the binding interaction. Surpassing a certain absolute threshold indicates a high binding strength of the complexes [25]. In this project, AutoDock and SeamDock (https://bioserv.rpbs.univ-paris-diderot.fr/services/SeamDock/) were used in combination and repeatedly to perform the docking studies [26, 27].

Statistical analysis

The normality of continuous variables was assessed both graphically and using the Shapiro-Wilk test. Data were described using mean ± standard deviation, median, minimum, and maximum values. For comparison of skewed (non-parametric) data, the Kruskal-Wallis non-parametric analysis of variance was employed. When a significant difference was detected, post-hoc pairwise comparisons were performed using the Bonferroni-corrected Mann-Whitney U test to identify the differing groups. For the comparison of variables that met the assumptions of parametric tests, one-way analysis of variance (ANOVA) was used. When a significant difference was observed in the ANOVA, Bonferroni post-hoc pairwise comparisons were conducted to determine the source of the difference. Statistical analysis and computations were performed using IBM SPSS Statistics version 21 (IBM Corp., Armonk, NY, USA). A p-value of ≤ 0.05 was considered indicative of statistical significance.

Results

Cytotoxicity analysis

In this study, we investigated the inhibitory effects of CIS and EA on cancer cell proliferation in cisplatin-sensitive and -resistant MDA-MB-231 breast cancer cell lines. The cells were treated with increasing concentrations of CIS and EA. Both EA and CIS decreased the growth of sensitive and resistant cell lines, according to cell viability studies. Cell growth curves were used to calculate IC_{50} values. The IC_{50} values in the cisplatin-sensitive MDA-MB-231 cell line were 29 µM for EA and 38.2 µM for CIS (Fig. 1A). The IC_{50} values in the cisplatin-resistant MDA-MB-231 cells were

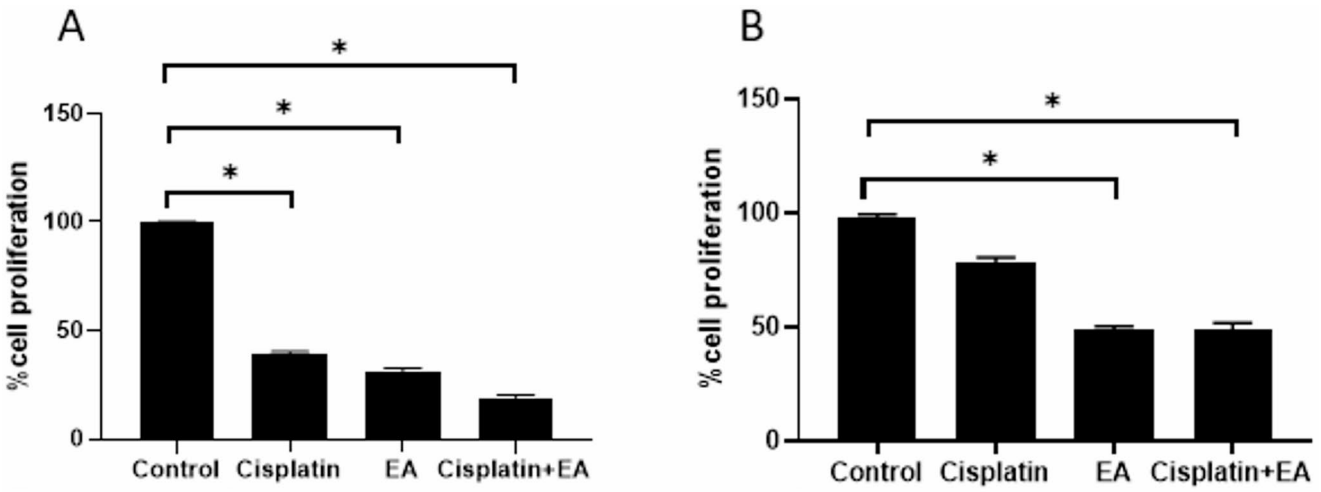


Fig. 1 (**A**) Cytotoxicity analysis results of cisplatin-sensitive MDA-MB-231 breast cancer cells (48 h). (**B**) Cytotoxicity analysis results of cisplatin-resistant MDA-MB-231 breast cancer cells (48 h)

determined to be 49.5 µM for EA and 80.2 µM for CIS, respectively (Fig. 1B). Co-administration of CIS and EA in combinatorial treatments effectively ($p<0.05$) suppressed cell growth in cisplatin-sensitive MDA-MB-231 cell lines (Fig. 1A). Even though the combined therapy in the cisplatin-resistant MDA-MB-231 cell lines led to a statistically significant decrease in cell viability when compared to the control group, the level of inhibition was similar to that seen with EA treatment alone (Fig. 1B).

Alterations in ABCB1 gene and protein expression in cisplatin-sensitive and resistant MDA-MB-231 cells

After 24 h of treatment, the EA-treated group significantly reduced the expression of the ABCB1 gene in cisplatin-sensitive MDA-MB-231 breast cancer cells as compared to the dimethyl sulfoxide (DMSO) control group ($p=0.018$). When the DMSO group was compared to the CIS and CIS + EA groups after 48 h of treatment, the ABCB1 gene expression in both treatment groups was significantly lower ($p=0.036$ and $p=0.045$, respectively) (Fig. 2A).

In cisplatin-resistant MDA-MB-231 breast cancer cells, a significant decrease in ABCB1 gene expression was detected in the EA-treated group compared to the DMSO group after 48 h of treatment ($p=0.013$) (Fig. 2B). Immunocytochemical analysis showed that the levels of ABCB1 protein expression were consistent with the mRNA expression results (Fig. 2C and D).

In the cisplatin-sensitive group, ABCB1 immunopositivity percentages were 12.83 ± 3.06, 18.33 ± 2.16, and 17.17 ± 2.86 in the DMSO group (at 12, 24, and 48 h, respectively), 10.67 ± 2.16, 3.00 ± 1.79, and 10.67 ± 2.16 in the EA group (at 12, 24, and 48 h, respectively), 6.83 ± 1.47, 8.17 ± 1.47, and 5.83 ± 2.32 in the CIS group (at 12, 24, and 48 h, respectively), and 10.50 ± 1.87, 11.33 ± 1.75, and 3.17 ± 1.72 in the CIS + EA group (at 12, 24, and 48 h, respectively).

In the cisplatin-resistant group, ABCB1 immunopositivity percentages were 11.50 ± 1.87, 13.50 ± 1.87, and 11.67 ± 2.16 in the DMSO group (at 12, 24, and 48 h, respectively), 14.17 ± 2.32, 13.50 ± 2.43, and 8.17 ± 1.47 in the EA group (at 12, 24, and 48 h, respectively), 7.83 ± 2.32, 15.17 ± 1.72, and 11.17 ± 2.14 in the CIS group (at 12, 24, and 48 h, respectively), and 8.83 ± 2.32, 12.67 ± 2.16 and 11.83 ± 2.48 in the CIS + EA group (at 12, 24, and 48 h, respectively).

Alterations in VEGF gene and protein expression in cisplatin-sensitive and resistant MDA-MB-231 cells

After 24 h of treatment, there was a significant decrease in VEGF gene expression in the CIS-treated group relative to the DMSO control group in cisplatin-sensitive MDA-MB-231 breast cancer cells ($p=0.005$). When the DMSO and EA groups were compared at 48 h, the EA-treated group's VEGF gene expression was significantly lower ($p=0.045$) (Fig. 3A).

In cisplatin-resistant MDA-MB-231 breast cancer cells, VEGF gene expression was significantly reduced in the EA and CIS + EA treatment groups compared to the DMSO control group after 24 h of treatment ($p=0.045$ and $p=0.023$, respectively) (Fig. 3B). VEGF protein expression levels were found by immunocytochemical staining to be in agreement with the mRNA expression findings. Notably, the

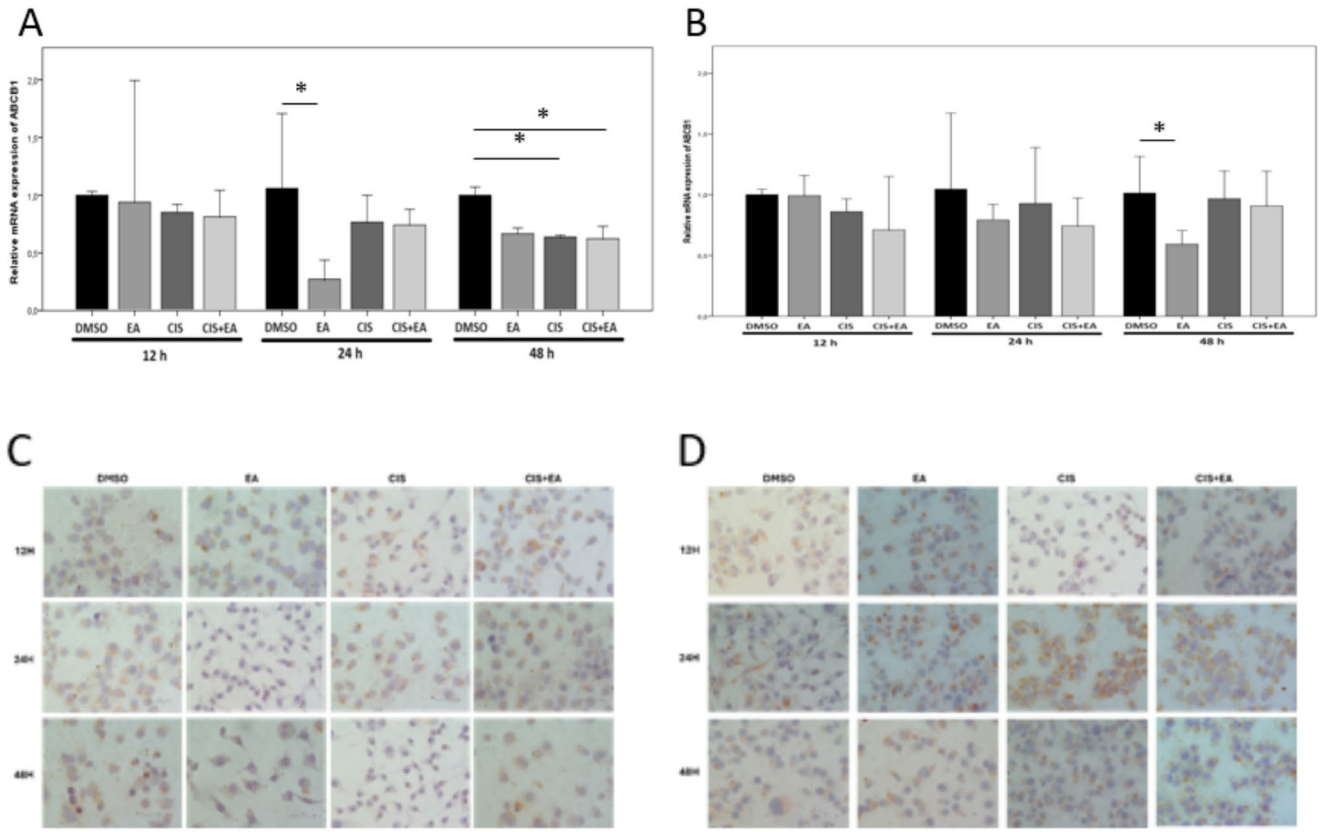


Fig. 2 (A) Relative ABCB1 gene expression in cisplatin-sensitive MDA-MB-231 cells at 12, 24, and 48 h. Data (2–ΔΔCt ratio to beta-actin) are shown as the mean relative to the DMSO control group (mean = 1).*$p < 0.05$;Kruskal-Wallis test with Bonferroni correction.**(B)** Relative ABCB1 gene expression in cisplatin-resistant MDA-MB-231 cells at 12, 24, and 48 h. Data (2–ΔΔCt ratio to beta-actin) are shown as the mean relative to the DMSO control group (mean = 1). *$p < 0.05$; One-Way ANOVA with Bonferroni correction. **(C)** ABCB1 immunoexpression in MDA-MB-231 cisplatin-sensitive and **(D)** MDA-MB-231 cisplatin-resistant cells after 12, 24, and 48 h of treatment, stained with AEC and hematoxylin

DMSO groups showed elevated VEGF protein expression. Additionally, EA treatment was associated with a marked reduction in VEGF staining (Fig. 3C and D).

In the cisplatin-sensitive group, VEGF immunopositivity percentages were 70.67 ± 2.16, 73.83 ± 3.49 and 72.83 ± 3.31 in the DMSO group (at 12, 24, and 48 h, respectively), 50.00 ± 2.61, 40.50 ± 1.87 and 21.00 ± 2.37 in the EA group (at 12, 24, and 48 h, respectively), 52.17 ± 2.32, 25.67 ± 2.34 and 38.33 ± 2.80 in the CIS group (at 12, 24, and 48 h, respectively), and 65.17 ± 2.32, 39.50 ± 4.23 and 55.50 ± 2.74 in the CIS + EA group (at 12, 24, and 48 h, respectively).

In the cisplatin-resistant group, VEGF immunopositivity percentages were 40.50 ± 1.87, 38.17 ± 2.32 and 50.00 ± 3.29 in the DMSO group (at 12, 24, and 48 h, respectively), 18.67 ± 2.16, 32.50 ± 2.74 and 6.50 ± 1.87 in the EA group (at 12, 24, and 48 h, respectively), 19.83 ± 2.32, 28.33 ± 2.16 and 9.33 ± 2.16 in the CIS group (at 12, 24, and 48 h, respectively), and 16.33 ± 2.80, 7.50 ± 1.87 and 8.83 ± 2.32 in the CIS + EA group (at 12, 24, and 48 h, respectively).

Alterations in MMP2 and MMP9 gene and protein expression in cisplatin-sensitive and resistant MDA-MB-231 cells

In cisplatin-sensitive MDA-MB-231 breast cancer cells, after 12 h of treatment, a significant decrease in MMP2 and MMP9 gene expression was observed in the EA-treated group compared to the DMSO control group ($p = 0.002$ and $p = 0.007$, respectively). MMP2 gene expression was still considerably lower in the EA group after 24 h ($p = 0.018$). MMP2 and MMP9 gene expression was significantly lower in the CIS-treated group than in the DMSO-treated group at 48 h ($p = 0.007$ and $p = 0.002$, respectively) (Fig. 4A and C).

In cisplatin-resistant MDA-MB-231 breast cancer cells, a significant decrease in MMP2 gene expression was observed in the CIS-treated group compared to the DMSO control group after 12 h of treatment ($p = 0.002$). At 24 and 48 h, MMP2 and MMP9 gene expressions were significantly reduced in the CIS + EA treatment groups compared

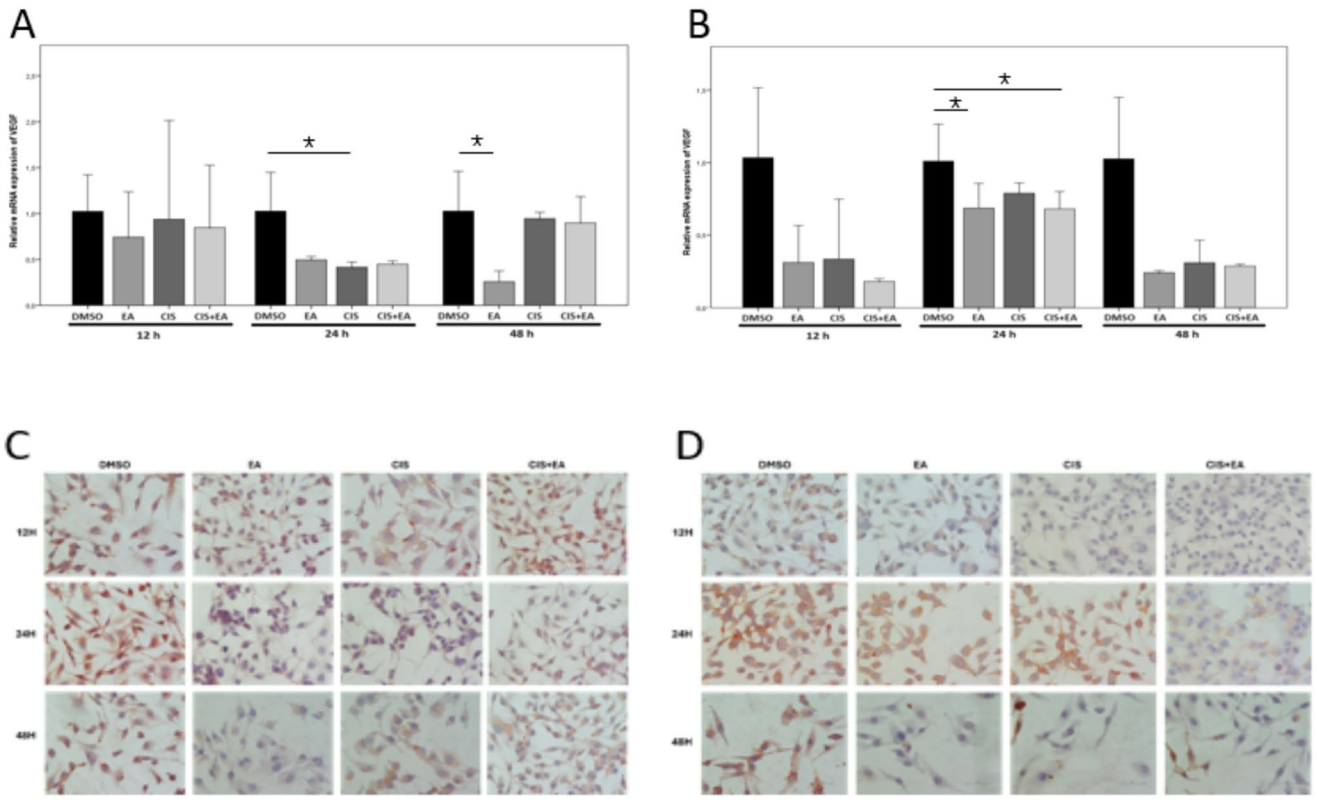


Fig. 3 **(A)** Relative VEGF gene expression levels in cisplatin-sensitive MDA-MB-231 cells, and **(B)** in cisplatin-resistant MDA-MB-231 cells at 12, 24 and 48 h time points. Data (2–ΔΔCt ratio to beta-actin mRNA) are expressed as the mean relative to the DMSO group (mean: 1). *: $p<0.05$; Kruskal-Wallis test with Bonferroni correction pos-hoc test. All experiments were conducted four times. **(C)** VEGF immunoexpression in MDA-MB-231 cisplatin-sensitive and **(D)** MDA-MB-231 cisplatin-resistant cells after 12, 24, and 48 h of treatment, stained with AEC and hematoxylin

to DMSO (for 24 h: $p=0.011$ and $p=0.002$, respectively; for 48 h: $p=0.004$ and $p=0.007$, respectively) (Fig. 4B and D).

Immunocytochemical staining of MMP2 and MMP9 expression levels was consistent with the mRNA expression results. Notably, high levels of MMP2 and MMP9 expression were observed in the DMSO control groups. Furthermore, staining intensity was reduced following EA treatment (Fig. 4E, F, G and H).

In the cisplatin-sensitive group, MMP2 immunopositivity percentages were 77.67±3.08, 75.83±2.32 and 70.17±3.06 in the DMSO group (at 12, 24, and 48 h, respectively), 51.17±3.06, 40.67±2.80 and 53.50±2.88 in the EA group (at 12, 24, and 48 h, respectively), 62.00±2.90, 55.67±2.80 and 58,33±2.58 in the CIS group (at 12, 24, and 48 h, respectively), and 66.00±3.22, 45.50±4.32 and 59.17±3.76 in the CIS+EA group (at 12, 24, and 48 h, respectively). In the cisplatin-resistant group, MMP2 immunopositivity percentages were 41.17±2.86, 50.67±3.14 and 48.67±2.16 in the DMSO group (at 12, 24, and 48 h, respectively), 32.67±2.16, 26.83±2.32 and 20.33±3.39 in the EA group (at 12, 24, and 48 h, respectively), 30.50±1.87, 26.50±1.87 and 23.00±2.37 in the CIS group (at 12, 24, and 48 h, respectively), and 30.50±3.83, 24.00±2.61 and 17.17±2.48 in the CIS+EA group (at 12, 24, and 48 h, respectively).

In the cisplatin-sensitive group, MMP9 immunopositivity percentages were 78.50±3.27, 78.17±3.06 and 79.00±2.61 in the DMSO group (at 12, 24, and 48 h, respectively), 33.17±2.64, 11.50±1.87 and 21.83±2.48 in the EA group (at 12, 24, and 48 h, respectively), 38.33±2.16, 19.00±2.61 and 20.67±2.16 in the CIS group (at 12, 24, and 48 h, respectively), and 32.33±1,86, 14,83±3,19 and 26,50±1,87 in the CIS+EA group (at 12, 24, and 48 h, respectively). In the cisplatin-resistant group, MMP9 immunopositivity percentages were 60.17±1.47, 58.33±2.16 and 46.50±3.39 in the DMSO group (at 12, 24, and 48 h, respectively), 51.50±2.43, 30.83±2.32 and 40.17±2.86 in the EA group (at 12, 24, and 48 h, respectively), 49.67±2.16, 34.17±2.64 and 43.83±2.32 in the CIS group (at 12, 24, and 48 h, respectively), and 22.00±2.90, 22.00±2.61 and 24.00±2.61 in the CIS+EA group (at 12, 24, and 48 h, respectively).

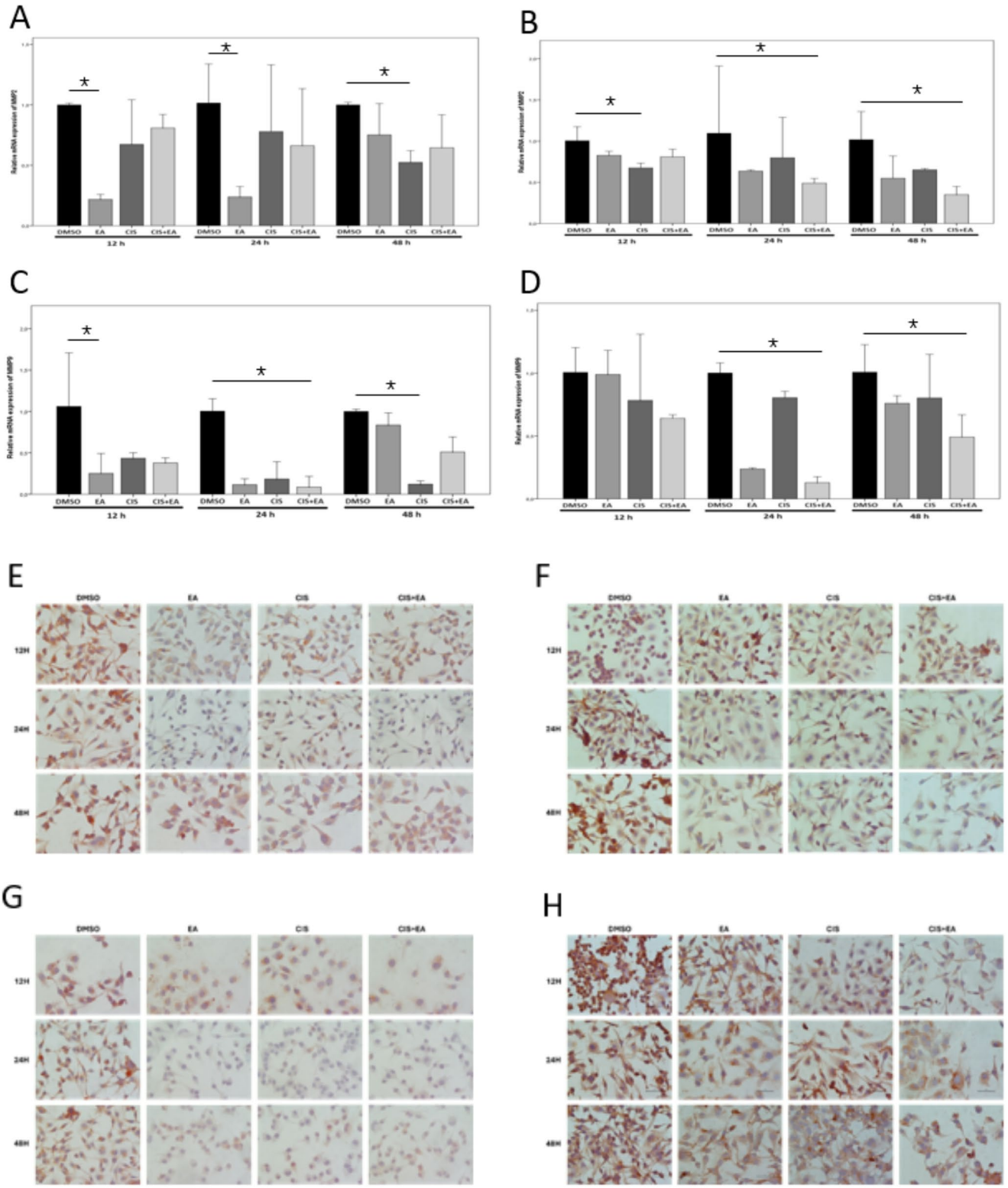


Fig. 4 **(A)** Relative MMP2 gene expression levels in cisplatin-sensitive MDA-MB-231 cells, and **(B)** in cisplatin-resistant MDA-MB-231 cells at 12, 24 and 48 h time points. **(C)** Relative MMP9 gene expression levels in cisplatin-sensitive MDA-MB-231 cells, and **(D)** in cisplatin-resistant MDA-MB-231 cells at 12, 24 and 48 h time points. Data (2–ΔΔCt ratio to beta-actin mRNA) are expressed as the mean relative to the DMSO group (mean: 1). *: $p < 0.05$; Kruskal-Wallis test with Bonferroni correction pos-hoc test. All experiments were conducted four times. **(E)** MMP2 immunoexpression in MDA-MB-231 cisplatin-sensitive and **(F)** MDA-MB-231 cisplatin-resistant cells after 12, 24, and 48 h of treatment, stained with AEC and hematoxylin. **(G)** MMP9 immunoexpression in MDA-MB-231 cisplatin-sensitive and **(H)** MDA-MB-231 cisplatin-resistant cells after 12, 24, and 48 h of treatment, stained with AEC and hematoxylin

Alterations in Bcl-2 and Bax gene and protein expression in cisplatin-sensitive and resistant MDA-MB-231 cells

In cisplatin-sensitive MDA-MB-231 breast cancer cells, Bcl-2 gene expression was significantly decreased in the EA-treated group compared to the DMSO control group at 12 and 48 h of treatment ($p<0.001$ and $p=0.027$, respectively). At 12 h, a significant reduction in Bcl-2 gene expression was also observed in the CIS + EA group compared to DMSO ($p=0.002$). Furthermore, the CIS + EA group exhibited significantly higher Bax gene expression at 12 and 48 h in comparison to DMSO group ($p=0.002$ and $p=0.007$, respectively). At 24 h, Bcl-2 gene expression was significantly lower in all treatment groups ($p<0.001$) when DMSO group was compared to EA, CIS, and CIS + EA groups (Fig. 5A and C).

After 12 h of treatment, the Bcl-2 gene expression in cisplatin-resistant MDA-MB-231 breast cancer cells was significantly lower in the CIS and CIS + EA treatment groups than in the DMSO group ($p<0.001$). At 24 h, the CIS group's Bax gene expression was considerably higher compared to that of the DMSO group ($p=0.018$). In comparison to the DMSO group, the EA, CIS, and CIS + EA groups showed significantly lower Bcl-2 gene expression after 48 h ($p=0.001$, $p=0.012$, and $p=0.003$, respectively) (Fig. 5B and D).

Molecular docking analysis results

EA demonstrates a strong affinity for the chosen target proteins, according to molecular docking analysis (Table 2). Notably, 16 hydrogen bonds were observed in the interaction with the Bax protein, with a binding energy of −7.7 kcal/mol. Furthermore, it was found that the binding energy between EA and the MMP9 protein was −8.4 kcal/mol. EA may significantly suppress genes that are crucial to the progression of cancer, especially when combined with medicines like CIS, which are used in both our experimental and clinical settings.

Discussion

In this study, the effects of EA, CIS, and their combination (CIS + EA) treatments were evaluated on the expression levels of ABCB1, VEGF, MMP2, MMP9, Bcl-2, and Bax genes, which are associated with biological processes such as angiogenesis, apoptosis, invasion, and drug resistance, in

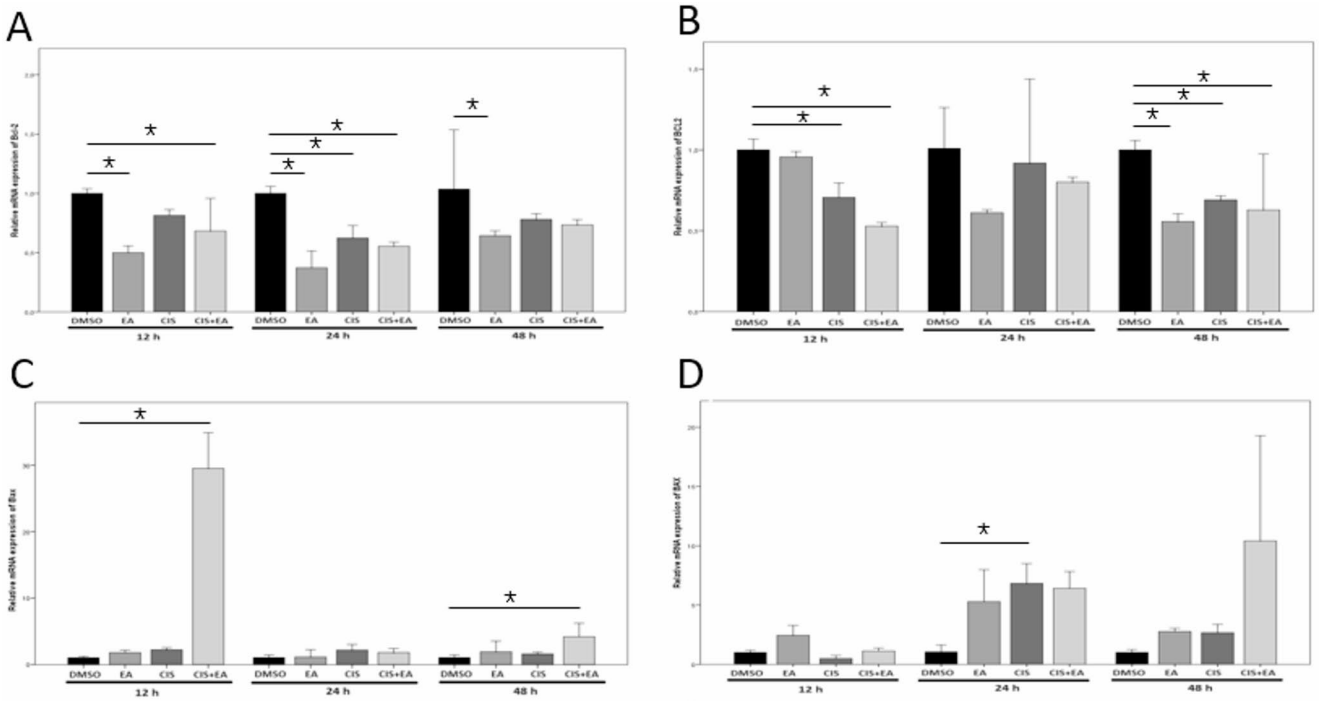


Fig. 5 **(A)** Relative Bcl-2 gene expression levels in cisplatin-sensitive MDA-MB-231 cells, and **(B)** in cisplatin-resistant MDA-MB-231 cells at 12, 24 and 48 h time points. Data (2–ΔΔCt ratio to beta-actin mRNA) are expressed as the mean relative to the DMSO group (mean: 1). *: $p<0.05$; One-Way ANOVA with Bonferroni correction test. **(C)** Relative Bax gene expression levels in cisplatin-sensitive MDA-MB-231 cells, and **(D)** in cisplatin-resistant MDA-MB-231 cells at 12, 24 and 48 h time points. Data (2–ΔΔCt ratio to beta-actin mRNA) are expressed as the mean relative to the DMSO group (mean: 1). *: $p<0.05$; Kruskal-Wallis test with Bonferroni correction pos-hoc test. All experiments were conducted four times

Table 2 Binding position, energy value and number of hydrogen bonds of EA to proteins

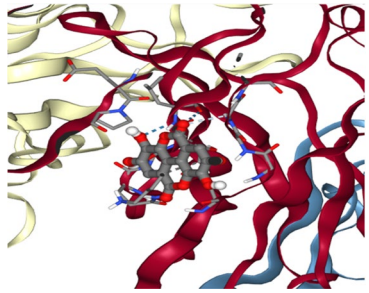
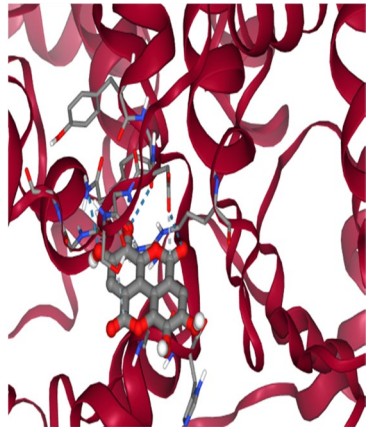
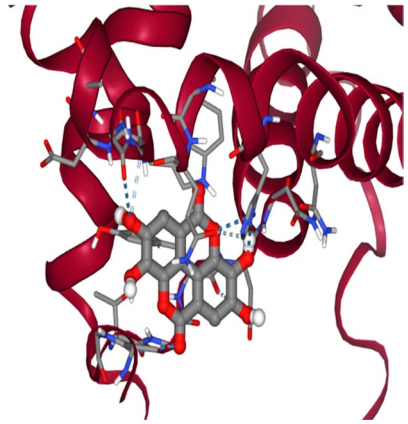
Protein	Energy binding (kcal/mol)	Number of Hydrogen bond		Interaction
VEGF (PDB ID: 1BJ1)	−7.2	3		
		Ligand atom	**Receptor**	
		O6	G116(H) O	
		O8	L118(H) O	
		O4	L118(H) N	
ABCB1 (PDB ID: 6C0V)	−7.4	7		
		Ligand atom	**Receptor**	
		O4	F267(A) O	
		O6	F267(A) O	
		O4	G1134(A) O	
		O8	D1135(A) OD1	
		O4	R1192(A) NH2	
		O4	N1136(A) N	
		O6	N1136(A) ND2	
Bcl-2 (PDB ID: 1GJH)	−6.8	5		
		Ligand atom	**Receptor**	
		O7	A4(A) O	
		O6	D10(A) OD1	
		O4	H186(A) ND1	
		O5	G193(A)	
		O2	H186(A) ND1	

Table 2 (continued)

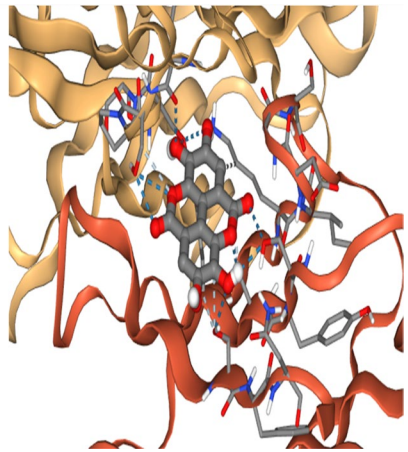
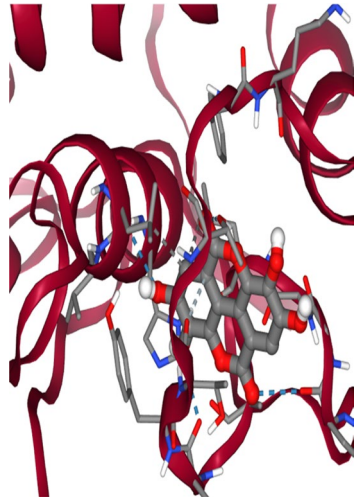
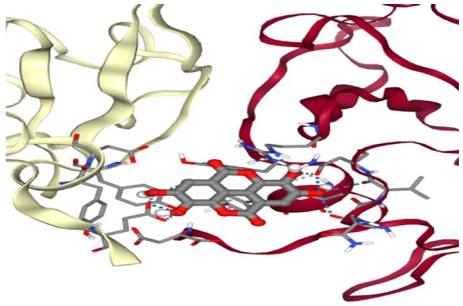
Protein	Energy binding (kcal/mol)	Number of Hydrogen bond		Interaction
Bax	−7.7	16		
(PDB ID: 2G5B)		**Ligand atom**	**Receptor**	
		O3	S190(C) OG	
		O7	S190(C) OG	
		O3	S207(C) OG	
		O4	Q6(E) O	
		O8	S7(E) OG	
		O4	S9(E) OG	
		O6	S9(E) OG	
		O1	T192(C) OG1	
		O2	S7(E) OG	
		O7	S190(C) OG	
		O8	S7(E) OG	
		O3	S207(C) OG	
		O3	S190(C) OG	
		O4	S9(E) OG	
		O6	K148(C) NZ	
		O6	S9(E) OG	
MMP2	−7.5	5		
(PDB ID: 1QIB)		**Ligand atom**	**Receptor**	
		O5	L197(A) O	
		O7	P215(A) O	
		O3	I222(A) O	
		O8	T229(A) OG1	
		O2	T229(A) OG1	

Table 2 (continued)

Protein	Energy binding (kcal/mol)	Number of Hydrogen bond		Interaction
MMP9 (PDB ID: 1ITV)	−8.4	9		
		Ligand atom	**Receptor**	
		O6	C4(A) O	
		O6	V6(A) O	
		O6	N7(A) OD1	
		O4	N7(A) OD1	
		O3	E194(B) OE1	
		O5	E194(B) OE1	
		O3	E194(B) OE2	
		O1	Y187(A) OH	
		O4	R165(A) NE	

both cisplatin-sensitive and cisplatin-resistant forms of the MDA-MB-231 breast cancer cell line.

According to reports, EA possesses anticancer and antioxidant qualities that prevent angiogenesis, migration, and metastasis in a variety of cancer types, such as colorectal, bladder, pancreatic, and breast malignancies [12, 14, 15, 28, 29]. ABC transporters are the main active transporters that mediate drug resistance by efluxing anticancer drugs from cells [20]. Drug resistance is a result of the overexpression of the membrane protein ABCB1 in many cancer cells [20].

Singh et al. demonstrated that quercetin, quercetin-3-glucoside, narcissoside, and EA inhibit the ATPase activity of ABCB1 [30]. Cetin et al. (2019) reported that the combined treatment of EA and bevacizumab significantly reduced ABCB1 at 72 h [31]. In this study, a significant decrease in ABCB1 mRNA expression was observed at 24 h following EA treatment in cisplatin-sensitive MDA-MB-231 cells, and at 48 h after CIS and CIS + EA treatments. Likewise, a similar reduction was observed in cisplatin-resistant MDA-MB-231 cells following 48 h of EA treatment, which was further supported by immunocytochemical analysis. These results indicate that EA may contribute additively to enhancing the sensitivity of cancer cells to chemotherapeutic agents while simultaneously affecting cellular survival and drug efflux mechanisms. Consequently, alterations in ABCB1 gene expression suggest that EA may possess the ability to inhibit this protein, potentially providing a complementary benefit in the treatment of drug-resistant malignancies.

Wang et al. showed that EA treatment dramatically reduces VEGF-induced angiogenesis processes in breast cancer cells, especially via inhibiting the migration and proliferation of endothelial cells [32]. In endothelial cells, they also determined that EA suppresses the MAPK and PI3K/Akt signaling pathways in addition to VEGFR-2 tyrosine kinase activity [32]. Furthermore, Ceci et al. discovered that EA reduces the invasiveness of bladder cancer cells by regulating VEGF-A levels [12]. Vanella et al. observed a marked decrease in VEGF protein expression in prostate cancer cells treated with EA compared to untreated controls [33].

In our study, consistent with the previously mentioned findings, VEGF mRNA expression was significantly decreased by CIS treatment at 24 h and by EA treatment at 48 h in cisplatin-sensitive MDA-MB-231 cells. In cisplatin-resistant cells, both EA and CIS + EA treatments induced a similar decrease in VEGF expression at 24 h. The mRNA results were corroborated by immunocytochemical analysis, which showed that the DMSO group had strong VEGF protein expression while the staining intensity decreased after EA treatment. In this study, it was observed that CIS and EA treatments reduced VEGF mRNA and protein levels at different time points. This finding suggests that EA, either alone or in combination with CIS, exerts additive

inhibitory effects on angiogenic processes in resistant cancer cells, thereby potentially contributing to reduced tumor vascularization.

The literature reports that EA and other natural compounds exhibit greater efficacy in inducing apoptosis and suppressing cell migration in breast cancer cells when used in combination with other agents, compared to single-agent treatments [34, 41]. Chen et al. demonstrated that EA induces apoptosis in MCF-7 breast cancer cells in a dose-dependent manner when treated with varying concentrations of EA [35]. Another study reported that EA treatment induced apoptosis in 23.3% of MCF-7 cells and 27.9% of MDA-MB-231 cells, respectively [14]. Ahire et al. showed that EA induced apoptosis in MCF-7 cells by upregulating the expression of the pro-apoptotic protein Bax and downregulating the expression of the anti-apoptotic protein Bcl-2 when combined with γ-radiation [36]. Furthermore, another study found that EA significantly increased the expression of p53, Bax, and caspase-3 while downregulating the expression of Bcl-2 in MCF-7 cells, resulting in a severe apoptotic effect [37].

Endometrial cancer cells treated with EA showed a time-dependent decrease of growth of almost 50% at 48 and 72 h. Furthermore, EA markedly reduced the treated cells' ability to migrate in comparison to the control group, according to migration assays. Furthermore, the number of invasive cells in the EA-treated group was reduced by more than 50% relative to controls. These findings were accompanied by a downregulation of MMP9 expression in the EA-treated cells [38]. In pancreatic cancer cells, EA treatment was shown to induce apoptosis and decrease proliferation in a dose-dependent manner [39].

The results of this study, EA treatment decreased Bcl-2 mRNA expression in cisplatin-sensitive cells after 12 h, whereas the EA+CIS combination enhanced Bax expression and decreased Bcl-2 mRNA expression. EA, CIS, and CIS+EA treatments all markedly decreased Bcl-2 expression after 24 h. At 48 h EA treatment was found to decrease Bcl-2 mRNA expression, while CIS+EA treatment increased Bax expression. Similar to the above, CIS and CIS+EA treatments reduced Bcl-2 expression in cisplatin-resistant cells after 12 h, moreover CIS treatment upregulated Bax expression at 24 h. At 48 h, Bax expression dramatically elevated in the CIS+EA group, whereas Bcl-2 expression decreased in all treatment groups.

With regard to apoptosis, it was determined that EA and CIS treatments, whether applied individually or in combination, exert additive effects on Bax upregulation and Bcl-2 downregulation. This shift represents a mechanistic indicator of the induction of programmed cell death. Suppression of Bcl-2 may weaken the cell's anti-apoptotic defense mechanisms, whereas upregulation of Bax can activate mitochondrial apoptotic pathways, leading to cytochrome c release and subsequent caspase activation. Collectively, these events may promote apoptotic signaling and trigger cancer cell death. These findings suggest that EA may possess the potential to inhibit cell survival processes and to direct cancer cells toward apoptosis by downregulating Bcl-2 expression and upregulating Bax expression.

It was found that EA triggered apoptosis in PANC-1 and AsPC-1 pancreatic cancer cells, which was mediated by caspase-3 and caspase-9. Both MMP2 and MMP9 expression decreased in a time- and dose-dependent manner in EA-treated PANC-1 and AsPC-1 pancreatic cancer cells, according to the same study's analysis of the mRNA and protein levels of MMP2 and MMP9 [15]. Similarly, Liu et al. determined that the EA group's protein expression levels of MMP2 and MMP9 were significantly decreased in a dose-dependent manner when they compared EA-treated ovarian cancer cells with the control group [40]. Saribas et al. (2023) found that MMP2 gene expression dramatically downregulated in all treatment groups as compared to the DMSO group After 12 and 48 h of treatment. They treatment HeLa cervical cancer cells with EA, irinotecan, and the EA + irinotecan combination and was found that the EA, irinotecan, and EA + irinotecan groups had decreased MMP9 expression levels at 12, 24, and 48 h compared to the DMSO group. MMP2 and MMP9 protein expression levels were also lower in these treatment groups than in the control group [41]. Another study showed that when EA was administered to prostate cancer cells, MMP2 expression decreased but MMP9 expression remained the same as compared to the control group [17].

In our investigation, cisplatin-sensitive cells treated with EA showed a marked downregulation of MMP2 and MMP9 mRNA expression at 12 h. The EA group showed a significant suppression of MMP2 expression at 24 h, whereas the CIS+EA group showed a significant reduction in MMP9 expression. Both genes' expression significantly decreased after 48 h of CIS treatment.

Similarly, MMP2 expression was inhibited by CIS treatment in cisplatin-resistant cells at 12 h, and the expression of both genes was significantly downregulated at 24 and 48 h by the CIS+EA combination, indicating that the observed effects of the combination are consistent with additive inhibitory activity. The mRNA data was supported by immunocytochemical analyses, which revealed that the DMSO control group had higher MMP2 and MMP9 expression, which was significantly lower after EA treatment. MMP2 and MMP9 are critical enzymes that enable cancer cells to degrade the extracellular matrix, thereby facilitating invasion and metastasis. In our study, the suppression of these genes' expression following EA treatment suggests that EA may impair the tissue penetration capacity and

metastatic potential of cancer cells, and when combined with CIS, these effects appear to be additive rather than synergistic.

Based on the obtained findings, EA appears to potentially modulate multiple and interconnected signaling networks simultaneously in both cisplatin-sensitive and cisplatin-resistant MDA-MB-231 cells. Within this context, the downregulation of ABCB1 by EA may increase intracellular drug accumulation, thereby potentially enhancing cellular sensitivity to chemotherapeutic agents, while the resulting cellular stress could trigger apoptotic responses. Indeed, the observed increase in Bax expression along with the decrease in Bcl-2 expression suggests that EA may shift the balance towards activation of intrinsic apoptotic pathways. Furthermore, the suppression of VEGF expression by EA may weaken angiogenic signaling, which could contribute to a reduction in tumor vascularization. Similarly, the observed decrease in MMP2 and MMP9 levels may be associated with inhibition of extracellular matrix remodeling, potentially limiting invasion and metastasis.

Taken together, these observations suggest that EA does not modulate pathways related to drug resistance, apoptosis, invasion, and angiogenesis independently, but rather may act within an integrated network in which these processes mutually reinforce one another. This study proposes that EA could exert a multi-targeted and coordinated effect on these biological processes, and that the suggested network warrants further validation through detailed mechanistic and in vivo studies.

This study has several limitations. Experiments were conducted exclusively in vitro using cisplatin-sensitive and -resistant MDA-MB-231 breast cancer cell lines; therefore, further investigations using additional breast cancer cell lines are required to more comprehensively assess the effects of EA. Moreover, EA exhibits low bioavailability and stability, and its limited solubility constrains the direct translational potential of these findings to clinical applications. This also suggests that the efficacy of EA in in vivo models may differ from the in vitro results, with effects observed under laboratory conditions potentially manifesting at lower or variable levels in living organisms. As this study represents a preliminary investigation, upstream signaling pathways (e.g., PI3K/AKT, NF-κB) were not analyzed; therefore, detailed pathway analyses are necessary to gain a more comprehensive understanding of the molecular mechanisms underlying EA's activity. Taken together, these limitations underscore the need for in vivo and advanced studies to fully elucidate the anticancer effects of EA and to obtain reliable data that can inform potential clinical applications.

Conclusion

In conclusion, the findings of this study suggest that EA may exert multi-targeted and time-dependent anticancer effects in both cisplatin-sensitive and cisplatin-resistant MDA-MB-231 breast cancer cells. EA was shown to modulate key mechanisms associated with drug resistance, apoptosis, invasion, and angiogenesis by regulating the expression of ABCB1, VEGF, MMP2, MMP9, Bcl-2, and Bax. The combination of EA with cisplatin further enhanced these effects, suggesting potential additional benefits in overcoming chemotherapy resistance. Overall, these results indicate that EA may act within an integrated signaling network that simultaneously affects multiple hallmarks of cancer. However, since this study was conducted exclusively in vitro using a single cell line, further preclinical and mechanistic studies, including in vivo experiments, are needed to fully elucidate the anticancer potential of EA and its possible application in clinical settings.

Author contributions Conception or design: G.T.S; Acquisition, analysis, or interpretation of data: G.T.S, G.S.S, S.Y.A, S.Y; Drafting the work or revising: G.T.S, S.Y.A; Final approval of the manuscript: G.T.S, G.S.S, S.Y.A, S.Y.

Funding Open access funding provided by the Scientific and Technological Research Council of Türkiye (TÜBİTAK). Please verify relation to: Kirsehir Ahi Evran University. This study was supported by the Scientific Research Projects Coordination Unit of Kırşehir Ahi Evran University under the project number TIP.A4.19.001.

Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Competing interests The authors declare no competing interests.

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

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