International Journal of Health Services Research and Policy
www.dergipark.org.tr/ijhsrp
IJHSRP
e-ISSN: 2602-3482

Research Article

INVESTIGATION OF THE EFFECTS OF JUGLONE AND CISPLATIN ON BREAST CANCER CELL LINES

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Abstract: *Breast cancer is one of the most commonly diagnosed malignancies worldwide, accounting for approximately 11% of all cancer-related deaths. Cisplatin induces DNA damage, thereby leading to apoptotic cell death, while juglone, a phytochemical compound, exhibits antioxidant and antiproliferative properties. BLACAT1, miR-155-5p, and CCR2 are non-coding RNAs implicated in breast cancer metastasis. This study aimed to investigate the effects of juglone and cisplatin on breast cancer cells by evaluating their impact on cell viability, gene expression, and invasive potential. MDA-MB-231 and MCF-7 breast cancer cell lines were treated with juglone and cisplatin. Cytotoxic effects were determined using the CCK-8 assay, while qPCR was employed to analyze changes in the expression levels of BLACAT1, miR-155-5p, and CCR2. The impact on cell invasion was assessed using the Transwell invasion assay. Juglone and cisplatin exhibited dose-dependent cytotoxicity in both cell lines. qPCR analysis revealed significant alterations in the expression levels of BLACAT1, miR-155-5p, and CCR2 following treatment. The Transwell invasion assay demonstrated that juglone and cisplatin affected the invasive potential of breast cancer cells, with notable differences observed between individual and combined treatments. Juglone and cisplatin modulate breast cancer cell viability, gene expression, and invasive behavior, with juglone demonstrating potential as a therapeutic agent, particularly for luminal-type breast cancer. However, the combined application did not enhance the therapeutic effect, suggesting a complex interaction between these agents.*

Keywords: *Breast Cancer, Cisplatin, Juglone, BLACAT1, miR-155-5p, CCR2.*

Received: February 19, 2025 *Accepted: June 3, 2025*

1. Introduction

Breast cancer is among the most prevalent types of cancer worldwide, accounting for approximately 12% of all cancer cases and 11% of cancer-related deaths, with a case-to-death ratio of 15% according to 2020 data [1], [2]. Chemotherapy, a frequently used method in breast cancer treatment, has shown increasingly promising results due to advancements in treatment approaches and faster access to scientific knowledge [3], [4].

Cisplatin (CAS No. 15663-27-1, MF: $Cl_2H_6N_2Pt$; NCF-119875) interferes with DNA repair mechanisms and induces programmed cell death in cancer cells by causing DNA damage. Cisplatin binds to the N7 position of purine bases, thereby blocking cell division and leading to apoptotic cell death. Another cellular effect of cisplatin is the generation of reactive oxygen species (ROS), which leads to oxidative stress. Oxidative stress is one of the most important mechanisms involved in cisplatin toxicity [5], [6].

In addition to chemotherapy, the use of phytochemicals -natural compounds used in complementary cancer therapies- has become increasingly common in recent years. Specifically,

phytochemicals are used to reduce the toxic side effects of chemotherapy and enhance its efficacy [7]. Juglone is one of the most notable examples of these phytochemicals. It is a naturally occurring naphthoquinone derivative found in the roots, bark, leaves, and fruits of walnut trees. It is also widely used in Chinese, Indian, and Korean traditional medicine. Juglone, which has been shown to have anticancer properties, continues to be the subject of extensive scientific research. Juglone exhibits antioxidant, antiproliferative, antitumor, anti-inflammatory, and antiviral effects on living cells [8].

The effects of juglone on cancer have been investigated in various cell types, including breast, prostate, pancreatic, glioma, ovarian, melanoma, HeLa, and endometrial cancer cells. These studies have concluded that juglone influences multiple cellular processes. Its anticancer effects include inhibition of tumor cell proliferation, induction of apoptosis and autophagy, suppression of angiogenesis, and inhibition of tumor cell migration and invasion. Juglone's role as a specific inhibitor of peptidyl-prolyl isomerase Pin1 has been identified as a key mechanism underlying its anticancer activity [8]. Studies investigating the effects of juglone on breast cancer cell lines have shown that increasing doses significantly affect cell viability, apoptosis, metastasis, and angiogenesis [9], [10], [11], [12].

The development of secondary tumors in organs or tissues distant from the primary tumor site is defined as metastasis. Non-coding RNAs (ncRNAs) also play important roles in metastasis, and this has been widely documented in the literature [13]. ncRNAs represent 98% of the transcriptome. Among these, lncRNAs and miRNAs are among the most recently identified RNA molecules, possessing distinct biological functions. They have been increasingly recognized as key regulators of various cellular control mechanisms [14]. They exhibit both oncogenic and tumor-suppressive properties in carcinogenesis [15].

BLACAT1 (Bladder Cancer Associated Transcript 1) is a long non-coding RNA (lncRNA) located on chromosome 1q32.1. Recent studies have shown that BLACAT1 expression is upregulated in various cancers, including bladder, breast, prostate, lung, glioma, cervical, thyroid, and hepatocellular carcinomas. Elevated BLACAT1 expression has been associated with shorter overall survival, advanced TNM stage, and increased lymph node metastasis [14].

miR-155-5p is a microRNA located on human chromosome 21q21.3, and it has been shown to play roles in inflammatory responses, immune regulation, hematological disorders, cardiovascular diseases, and tumorigenesis. miR-155-5p is implicated in cancer-related pathways, including increased cell proliferation, inhibition of differentiation, epithelial-mesenchymal transition (EMT), and modulation of DNA damage repair [16]. It is considered an oncogenic microRNA and is frequently overexpressed in breast cancer. Moreover, it has been associated with high-grade tumors, advanced disease, and lymph node metastasis. A study reported that high miR-155-5p expression was associated with TNBC and HER2-enriched subtypes, whereas lower expression was observed in luminal subtypes [17].

CCR2 is a chemokine receptor expressed in various immune cells and multiple cancer types. Upon binding to its ligand CCL2, CCR2 activates key signaling pathways involved in cell proliferation, migration, and survival, including the PI3K/AKT, MAPK/p38, and JAK/STAT pathways [18], [19]. The CCL2–CCR2 signaling axis has been implicated in the development and progression of vascurious malignancies, including breast, prostate, lung, hepatocellular, pancreatic, nasopharyngeal, and kidney cancers. Overexpression of CCR2 has also been linked to recurrence and metastasis in advanced cancers [18].

Studies focusing on BLACAT1, miR-155-5p, and CCR2 -key non-coding RNAs involved in breast cancer metastasis- may provide important insights into the mechanisms of metastasis and contribute to the development of novel therapeutic strategies. This study aimed to investigate the combined effects of juglone and cisplatin on breast cancer cell lines. The study also aimed to evaluate changes in the expression levels of BLACAT1, miR-155-5p, and CCR2, to explore their roles in breast

cancer progression, assess their potential as biomarkers, and elucidate their possible involvement in metastasis.

2. Materials and Methods

2.1. Cell lines and cell culture conditions

All cells were provided by Pamukkale University Medical Biology Department. MCF-7; ER+/PR+/HER2- invasive ductal carcinoma cell line and MDA-MB-231 triple negative breast cancer cell line were used in the study.

MCF-7 and MDA-MB-231 cell lines were cultured in RPMI 1640 (NutriCulture) + 10% FBS (Capricorn) + 1% penicillin/streptomycin (Capricorn) medium and both cell lines were incubated at 37°C incubator which was containing 5% CO2 and 95% humid air.

2.2. Cytotoxicity analysis (CCK-8 Test)

CCK-8 test was applied to determine the IC50 (The half maximal inhibitory concentration) value of cisplatin and Juglon. CCK-8 (Abbkine, Catalog no: KTA1020) test was applied to determine the IC50 (The half maximal inhibitory concentration) value of Cisplatin (Sigma Aldrich CAS-No: 15663-27-1) and Juglon (Sigma Aldrich, CAS-No: 481-39-0). For cisplatin, all 0.2 g of cisplatin was dissolved with 1.2 ml of DMSO, and a 50 mM stock solution was prepared. Then, 0.5, 1, 2.5, 5, 7.5, 10, 12.5, 25 and 50 µM/ml concentrations were prepared by diluting the stock solution with medium and applied to the cells for 24, 48 and 72 hours in 3 replicates. For juglone, all 1 g of juglone was dissolved with 10 ml of DMSO and 100 mM stock solution was prepared. 1, 2.5, 5, 10, 12.5, 25, 50, 75 and 100 µM/ml concentrations were prepared by diluting the stock solution with medium and applied to the cells for 24, 48 and 72 hours in 3 replicates. A separate DMSO control group was not established for both cisplatin and juglone as the concentration of DMSO in the media-diluted stock solution content was well below the toxic value (<0.1%). The applications were carried out by applying juglon to each plate 3x10 well and cisplatin to each plate 3x10 well on the cells planted in 96-well plates with 10,000 cells in each well.

2.3. Analysis of BLACAT1, miR-155-5p and CCR2 expression levels in cell lines

2.3.1 RNA isolation

RNA isolation from cell lines was performed with the help of the Norgen Total RNA isolation kit (Catalog No: 17200) working with the Trisol-based spin column method. In 6 well plates, 105 cells were planted, and RNA isolation was performed by applying the relevant kit protocol from the cells to which the substance was applied and control cell lines.

2.3.2 cDNA (Complementary DNA) synthesis

cDNA synthesis was performed from isolated RNAs with the help of BIO-RAD iScript™ cDNA Synthesis kit (Catalog No: 1706891) with reverse transcriptase enzyme. cDNA synthesis was performed with Qiagen RotorGene Real-Time PCR device in 500 µl PCR tubes in accordance with the appropriate kit procedure.

2.3.3 Expression analysis

After cDNA synthesis, quantitative Real-Time PCR was performed with the Jena Bioscience qPCR SybrMaster kit (Catalog No: PCR-372L) with SYBR® Green master mix and with the Qiagen RotorGene device at 95ºC for 2 minutes, 15 seconds at 95ºC and 1 minute at 55ºC using primers specific to the BLACAT1, miR-155 and CCR2 genes. Primer sequences are listed in Table 1.

Table 1. Primer sequences of the genes used.

Oligonucleotide	Base sequence (5´-3´)
CCR2 Forward	CATGGTGACAGAGACTCTTGGGA
CCR2 Reverse	GGCAATCCTACAGCCAAGAGCT
miR-155 Forward	TGCTAATCGTGATAGGGG
miR-155 Reverse	GAACATGTCTGCGTATCTC
BLACAT1 Forward	CCTGCTTGGAAACTAATGACC
BLACAT1 Reverse	AGGCTCAACTTCCCAGACTCA

2.4. Transwell Invasion Assay

For the Transwell invasion experiment, the substances were applied to the cells at a concentration and time determined, and then the invasion experiment was carried out by staining with crystal violet dye on 12 well plates using BioCoat Matrigel Invasion Chamber (Catalog No: 354480, Corning).

2.5. Statistical Analysis

The percentage of cell viability was calculated as follows:

$$\text{Cell viability (\%)} = (\text{OD of treatment}/\text{OD of control}) \times 100. \quad (1)$$

The IC50 was obtained from the dose-response curve using Microsoft Excel. Dose–response curves were generated by plotting cell viability (%) against compound concentrations (μM). The resulting data were subjected to linear regression analysis, and the relationship was described using the following linear equation:

$$\mathbf{y = a \cdot x + b} \quad (2)$$

Where:

y represents the percentage of cell viability,

x denotes the compound concentration (μM),

a is the slope of the line,

b is the y-intercept.

The half-maximal inhibitory concentration (IC50) was calculated as the concentration at which cell viability is reduced to 50%. This corresponds to setting y = 50 in the regression equation and solving for x as follows:

$$x = \frac{50 - b}{a} \quad (3)$$

IC50 values were determined individually for each time point using the parameters obtained from the linear regression equations.

Expression fold changes were determined by RT^2 lncRNA PCR data analysis (Qiagen) on a web-based basis with the $2^{-\Delta\Delta CT}$ method. This web-based analysis is based on the Student's-t test principle. All experiments were performed in triplicate, and a *p*-value of < 0.05 was considered statistically significant.

3. Results

IC50 values were calculated as 6.24 μM/ml at 72nd hour for MCF-7 and 7.64 μM/ml at 72nd hour for MDA-MB-231 in cisplatin administration. In Juglon application, it was calculated as 7.43 μM/ml at 48th hour for MCF-7 and 8.61 μM/ml at 48th hour for MDA-MB-231 [Table 2].

Table 2. IC50 values of cell lines for cisplatin and juglone treatment.

	IC50 Values (µM/ml)	
	Cisplatin (72nd hour)	**Juglone (48th hour)**
MCF-7	6.24	7.43
MDA-MB-231	7.64	8.61

Expression changes of BLACAT1, CCR2 and miR155 were analyzed as a result of juglon, cisplatin and the combined administration of two substances in MCF-7 and MDA-MB-231 cell lines. As a result of the application of juglon in the MCF-7 cell line, the expression of BLACAT1 decreased 5.98 times, while a 2.09 -fold decrease was observed in CCR2. In addition, the expression of miR155 increased 2.13 times. In the administration of cisplatin, no significant change was observed in the expression of BLACAT1, the expression of CCR2 increased 4.55 times, and the expression of miR155 decreased 3.19 times. In the combined application, while the expressions of miR155 and CCR2 did not change, the expression of BLACAT1 decreased by 2.11 times [Figure 1].

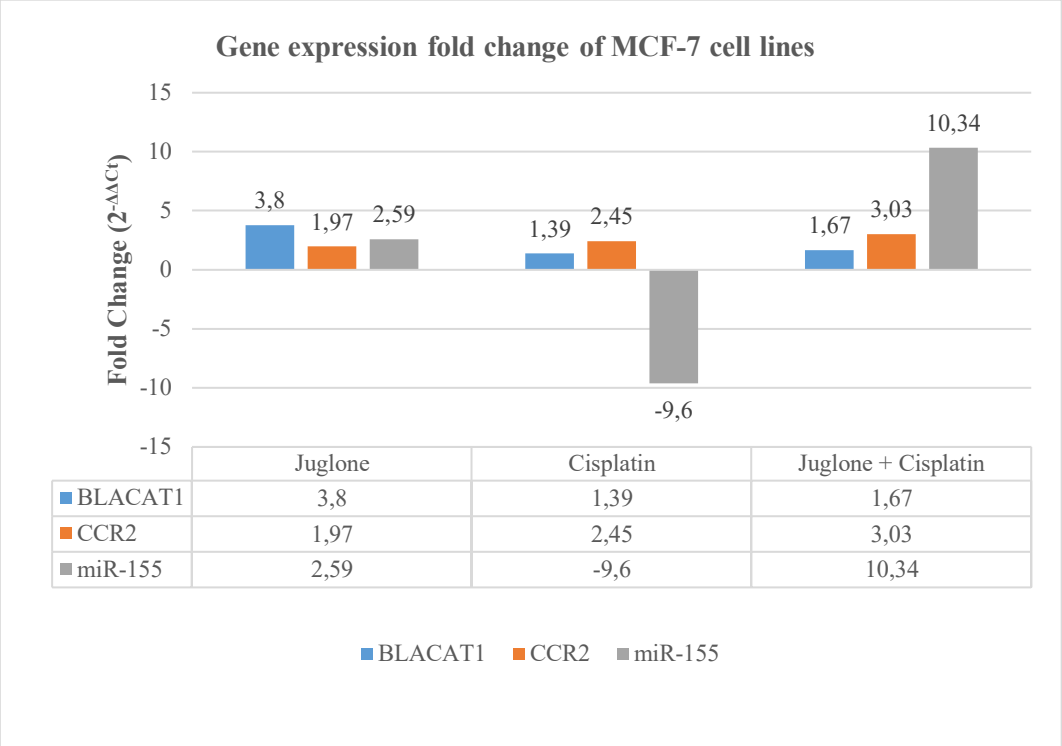

Figure 1. Expression fold changes of MCF-7 cell lines according to the control group.

As a result of juglone application in the MDA-MB-231 cell line, the expression of BLACAT1 increased 3.8-fold and the expression of miR155 increased 2.59-fold, and no significant change was observed in the expression of CCR2. While the expression of BLACAT1 did not change in cisplatin administration, the expression of CCR2 increased by 2.45 times, and that of miR-155 decreased by 9.6 times. In the combined administration, the expression of BLACAT1 did not change, CCR2 increased 3.03 times, and miR-155 increased 10.34 times [Figure 2.].

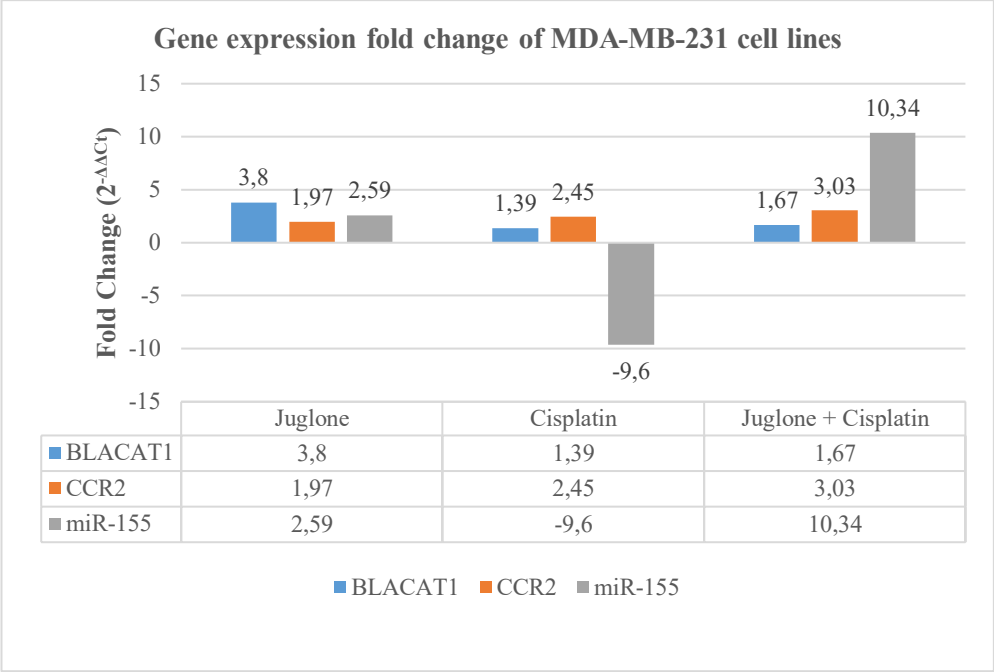

Figure 2. Expression fold changes of MDA-MB-231 cell lines according to control group.

In the invasion test results, the application of juglone and cisplatin in the MCF-7 cell line decreased invasion compared to the control group, and the combined application was less effective on invasion than the single application and the control group [Figure 3]. Juglone administration in the MDA-MB-231 cell line reduced invasion more than cisplatin. In the combined application, it affected the invasion less compared to the single application and the control group [Figure 4].

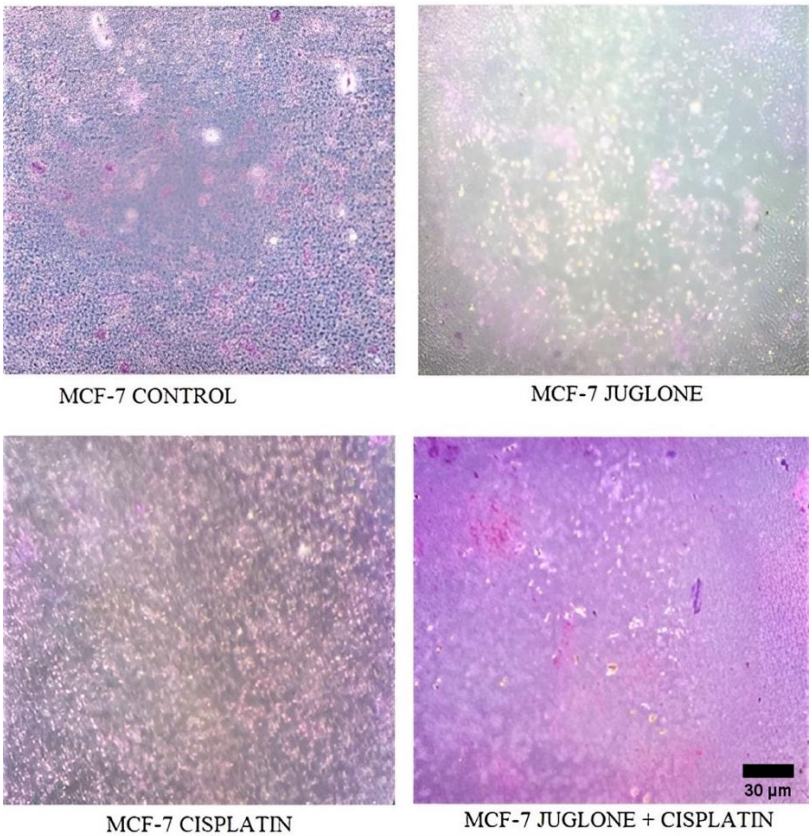

Figure 3. Transwell invasion assay images of MCF-7 (40x).

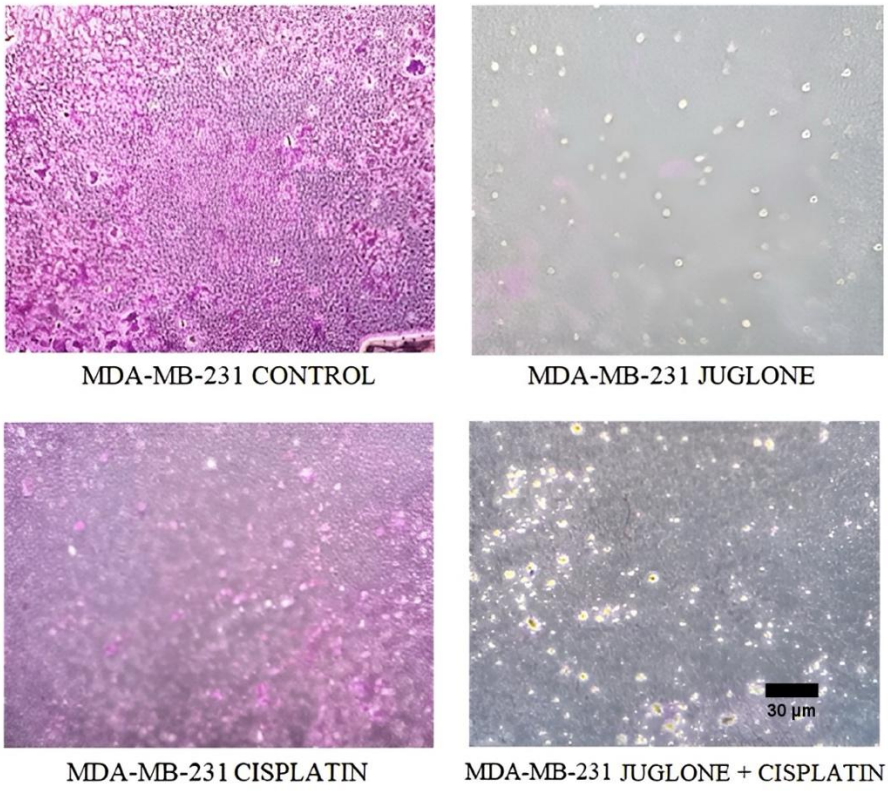

Figure 4. Transwell invasion assay images of MDA-MB-231 (40x).

4. Discussion

Chendan Zou et al. demonstrated that juglone suppresses tumor cell mobility in both breast and colorectal cancers by modulating EMT markers—upregulating E-cadherin and downregulating vimentin and N-cadherin [20]. Consistent with their findings, our study also showed that juglone reduced the invasive and metastatic capacities of both MCF-7 and MDA-MB-231 breast cancer cells.

A study by Xiaopeng Hu et al. reported significant overexpression of BLACAT1 in breast cancer tissues and cell lines, correlating with advanced TNM stage and poor prognosis [21]. Knockdown of BLACAT1 was shown to inhibit cell proliferation and metastasis. In our study, reduced BLACAT1 expression in MCF-7 cells following juglone treatment was accompanied by decreased invasion and metastatic potential, supporting these findings.

Prior research by Jinhang Hu et al. demonstrated that high CCR2 expression is linked to improved survival in breast cancer and that the 3′ UTR of CCR2 suppresses EMT and metastasis in vitro and in vivo [22]. Our results align with these findings, showing that reduced CCR2 expression was associated with enhanced invasion and metastasis, particularly in MCF-7 cells.

The same study by Hu et al. further revealed that BLACAT1 promotes CCR2 expression post-transcriptionally by sponging miR-150-5p. Luciferase reporter assays confirmed that miR-150-5p directly targets the 3′ UTR of CCR2 mRNA. This regulatory axis contributes to breast cancer progression by enhancing CCR2-mediated signaling [21].

Approximately 90% of breast cancer-related deaths are attributed to metastasis, with epithelial–mesenchymal transition (EMT) playing a key role in this process. During EMT, tumor cells upregulate mesenchymal markers and downregulate epithelial markers, enhancing their invasive potential. Liu et al. demonstrated that inhibition of miR-155 in MCF-7 cells decreased the expression of mesenchymal

markers (FN and α-SMA) and increased epithelial markers (E-cadherin and CK18), thereby suppressing EMT and reducing proliferation and survival [22], [23]. In line with these findings, our results showed that cisplatin treatment significantly reduced miR-155 expression and correspondingly decreased the invasive capacity of both MCF-7 and MDA-MB-231 cells.

Our data indicate that juglone alone effectively reduced the invasion and metastasis potential of MCF-7 cells, whereas cisplatin primarily downregulated miR-155 expression without substantially impacting invasive behavior. Interestingly, the combined application of juglone and cisplatin appeared to neutralize or even counteract each other's effects. This antagonistic interaction may be attributed to the antioxidant nature of juglone, which could counteract the reactive oxygen species (ROS) generated by cisplatin—one of its primary mechanisms of cytotoxicity.

In MDA-MB-231 cells, juglone modulated the expression of BLACAT1 and CCR2 as expected, while cisplatin primarily influenced miR-155 expression. However, their combined administration again resulted in mutual antagonism. These findings suggest that neither agent, alone or in combination, produced a therapeutically significant effect in triple-negative breast cancer cells.

In contrast, in luminal-type MCF-7 cells, both agents exhibited significant effects when applied individually. Notably, juglone demonstrated potential as a therapeutic candidate for luminal breast cancer, warranting further investigation. However, contrary to expectations, their combined use did not result in enhanced therapeutic benefit.

5. Study Limitations

This study has certain limitations that should be acknowledged. The MCF-10A healthy cell line could not be included in the study, which limited our ability to compare malignant and non-malignant responses to the treatments.

In addition, although gene expression changes of BLACAT1, miR-155-5p, and CCR2 were observed, no further mechanistic assays (e.g., gene knockdown, overexpression, or luciferase reporter assays) were performed to validate the causal relationship between these molecules and the observed phenotypic effects. This limits our ability to confirm whether the expression alterations directly contribute to changes in cell behavior.

Furthermore, all findings are based on in vitro experiments. While these provide valuable preliminary insights, they cannot fully replicate the tumor microenvironment or systemic drug responses. Therefore, in vivo studies are needed to validate the therapeutic relevance of juglone and cisplatin, individually and in combination.

Lastly, the combined administration of juglone and cisplatin did not yield a synergistic effect and, in some instances, exhibited antagonistic interactions. This may stem from juglone's antioxidant properties counteracting cisplatin-induced oxidative stress, one of its primary cytotoxic mechanisms. Dose optimization studies are warranted to identify potential synergistic concentrations. Despite these limitations, this study offers foundational insights and may serve as a guide for future mechanistic and translational breast cancer research.

6. Conclusions

In conclusion, this study demonstrated that juglone and cisplatin individually modulate gene expression and invasive behavior in breast cancer cell lines, particularly in luminal-type MCF-7 cells. Juglone showed promising anticancer activity and may serve as a potential therapeutic agent. However, their combined administration did not enhance therapeutic efficacy and, in some cases, exhibited antagonistic interactions. These findings highlight the importance of evaluating drug interactions at the

molecular level and support further in vitro and in vivo investigations, especially regarding juglone's role in luminal breast cancer treatment.

Ethical statement:

This study does not require ethical approval.

Acknowledgment:

We would like to thank Pamukkale University Scientific Research Projects Coordination Unit for funding this study.

Conflict of interest:

The authors have no competing interests to declare that are relevant to the content of this article**.**

Funding:

This research was funded by Pamukkale University Scientific Research Projects Coordination Unit (project numbers: 2022SABE015).

Authors' contributions:

Concept and Design: The concept and design of the study were developed by B. E. U, S. Ş and S. Ç.

Experimental Work: The experimental studies and data acquisition were carried out by S. Ç.

Data Analysis and Interpretation: Data analysis and interpretation were performed by B. E. U. and S. Ç.

Manuscript Writing: The initial draft of the manuscript was written by S. Ç.

Manuscript Revision and Approval: The manuscript was revised and approved by all authors prior to submission.

Generative AI statement:

The authors declare that no Gen AI was used in the creation of this manuscript.

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