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Quercetin and 5-Fluorouracil Synergistically Suppress ZEB1 and Restore Epithelial Integrity in Triple-Negative Breast Cancer

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ABSTRACT

Background: Triple-negative breast cancer (TNBC) remains a therapeutic challenge due to its metastatic features and resistance to conventional therapies. This study investigated the synergistic potential of quercetin (QC), a natural flavonoid, and 5-fluorouracil (5-FU) in targeting epithelial-mesenchymal transition (EMT) to inhibit proliferation and migration of the MDA-MB-231 TNBC cell line.

Methods: Cytotoxicity and selectivity were assessed using the MTT assay in MDA-MB-231 cells and normal MRC-5 fibroblasts. Synergy was quantified using combination index (CI) values. Scratch assay was assessed for migration inhibition, while quantitative RT-PCR analyzed EMT-related gene expression (*Vimentin*, *ZEB1*, *N-cadherin*, and *E-cadherin*). Western blotting confirmed protein expression of ZEB1 and E-cadherin following treatment with QC (50 µM), 5-FU (25 nM), or their combination.

Results: QC and 5-FU demonstrated concentration- and time-dependent cytotoxicity in MDA-MB-231 cells, with QC being highly selective for cancer cells. Toxicity in normal MRC-5 fibroblasts occurred only at 800 µM (25% viability). The combination of 50 µM of QC and 25 nM of 5-FU exhibited the strongest synergy (CI = 0.08), reducing viability by 75% and migration by 87.8%. QC alone downregulated *ZEB1*, *Vimentin*, and *N-cadherin*, while 5-FU further reduced these markers. Combined treatment enhanced suppression (*ZEB1*: 0.46-fold; *Vimentin*: 0.47-fold; *N-cadherin*: 0.68-fold) and significantly upregulated *E-cadherin* (3.75-fold), indicating EMT reversal. Western blotting confirmed decreased ZEB1 and increased E-cadherin by over four-fold with QC + 5-FU, indicating a shift to an epithelial phenotype.

Conclusion: Combining QC and 5-FU effectively inhibited TNBC proliferation and migration while minimizing toxicity to normal cells. This dual-action strategy offers a promising low-toxicity therapeutic approach for aggressive TNBC. ***DOI: 10.22034/IBJ.30.1.47***

Keywords: Fluorouracil, Epithelial-mesenchymal transition, Quercetin, Triple negative breast neoplasms, Zinc finger E-box binding homeobox 1

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1. INTRODUCTION

Breast cancer remains the most prevalent malignancy and the leading cause of cancer-related mortality among women, representing a significant health challenge for the female population globally[1]. Despite considerable advancements in treatment modalities, including radiation therapy, surgical interventions, endocrine therapy, chemotherapy, and targeted therapies, the

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incidence of metastasis and the emergence of drug resistance continue to hamper therapeutic efficacy[2]. These factors are critical contributors to treatment failure and elevated mortality rates, particularly in patients diagnosed with triple-negative breast cancer (TNBC)[3]. The MDA-MB-231 cell line, a widely utilized model for TNBC, exemplifies the aggressive nature of this subtype, which is often associated with a heightened propensity for metastasis, predominantly driven by the process of epithelial-mesenchymal transition (EMT)[4].

EMT is a biological process through which epithelial cells acquire mesenchymal characteristics, facilitating tumor invasion and migration[5]. This transition is marked by downregulating epithelial markers, such as occludin, E-cadherin, and claudin, and upregulating mesenchymal markers, including *ZEB1*, N-cadherin, and Vimentin[6]. The dysregulation of these markers is pivotal to the promotion of breast cancer cells. Consequently, targeting the pathways governing EMT represents a viable strategy for impeding the malignant progression of breast cancer[7]. Key transcription factors and proteins, particularly *ZEB1* and *N-cadherin,* play integral roles in this transition, underscoring the necessity for innovative therapeutic approaches that address these mechanisms[8].

5-fluorouracil (5-FU) is a cornerstone chemo-therapeutic agent employed in the treatment of various malignancies, including breast cancer[9,10]. However, its clinical efficacy is frequently compromised by the development of resistance and the emergence of aggressive cancer phenotypes[11]. Recent investigations have suggested that the co-administration of 5-FU with natural compounds, particularly flavonoids, may enhance its therapeutic effectiveness while mitigating its associated adverse effects[12-14]. Quercetin (QC), a prominent flavonoid, has attracted considerable attention for its potential anticancer properties, including its ability to inhibit EMT and reduce cancer cell proliferation[15-17].

The anticancer properties of QC are well-established, and 5-FU is recognized as a key chemotherapeutic agent. However, the potential synergistic effects of these compounds on EMT, a crucial process in cancer metastasis and drug resistance, remain largely unexamined, particularly in the context of TNBC. While some studies have indicated that QC can trigger apoptosis and inhibit cell migration, and that 5-FU serves as a standard chemotherapeutic agent, the combined effects of these two substances on EMT-related molecular markers have yet to be thoroughly investigated. The impact of co-treating with QC and 5-FU on important transcription factors like *ZEB1*, along with its downstream targets such as *E-cadherin*, *N-cadherin*, and *Vimentin,* is still not well understood. Furthermore, the optimal dosing regimens required to achieve synergistic effects on cellular motility and sensitivity in aggressive TNBC cell lines, like MDA-MB-231, have yet to be determined. This study highlights these important gaps by elucidating the molecular mechanisms involved in combination therapy. Gaining a better understanding of these mechanisms could provide a rationale for applying this combination to address EMT-driven metastasis and enhance treatment outcomes in TNBC.

2. MATERIALS AND METHODS

2.1. Cell culture

MDA-MB-231 breast cancer and MRC-5 human normal lung fibroblast cell lines were obtained from the Pasteur Institute of Iran, Tehran, Iran. Cells were cultured in Roswell Park Memorial Institute Medium supplemented with 4.5 g/L glucose, 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin. All reagents were sourced from Bio-Idea, Tehran, Iran.

2.2. Treatment preparation

5-FU (Ebewe Pharma Ges.m.b.H. Nfg. KG, Austria) was purchased at a concentration of 50 mg/mL and added directly to the culture medium.QC, obtained from Sigma-Aldrich, USA, was prepared in dimethyl sulfoxide (DMSO) to create a 100 mM solution, which was then diluted in the culture medium to ensure that the DMSO concentration remained below 0.1%.

2.3. Cell viability assay (MTT)

Cell viability was evaluated using the MTT assay. MRC-5 (normal lung fibroblast) and MDA-MB-231 (breast cancer) cells were seeded into 96-well plates at densities of 1×10^4 and 4×10^3 cells per well, respectively, and incubated in a humidified atmosphere containing 5% CO_2 at 37° C overnight. Cells were then treated with various concentrations of QC (12.5, 25, 50, 100, and 200 μM) and 5-FU (6.25, 12.5, 25, 100, 400, and 1,000 nM) for 24 h and 48 h. After treatment, MTT solution (0.5 mg/mL) was added to each well, and the plates were incubated in the dark for 4 h. The resulting formazan crystals were dissolved in DMSO, and absorbance was measured at 570 nm, with a reference wavelength of 630 nm used for background correction, using a microplate reader. Cell viability was calculated according to the following formula: cell viability (%) = [1-(absorbance of treated/ absorbance of control)] × 100. Cell cytotoxicity percentages were then used to determine IC_{50} values, and the selectivity index (SI) was calculated as the ratio of IC_{50} in normal cells to IC_{50} in cancer cells. An SI > 1 indicates higher selectivity toward cancer cells.

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Table 1. CI values determined for various combinations of QC and 5-FU

QC (µM)	5-FU (nM)	CI
12.5	6.25	0.26
25	6.25	0.21
50	6.25	0.24
100	6.25	0.32
12.5	12.5	0.14
25	12.5	0.11
50	12.5	0.13
100	12.5	0.20
12.5	25	0.15
25	25	0.11
50	25	0.08
100	25	0.09

CI >1 Antagonism; CI=1 Additive; CI <1 Synergistic effect between drug

2.4. Combination index (CI) determination

The synergistic interaction between QC and 5-FU was evaluated using CompuSyn software, which calculated the CI and Drug Reduction Index (DRI) values based on the Chou–Talalay method[18]. According to the standard interpretation criteria, CI < 0.9 indicates synergy (CI < 0.3, strong synergy), CI = 0.9–1.1 indicates an additive effect, and CI > 1.1 indicates antagonism. Table 1 shows the CI values for the different pairs of QC and 5-FU concentrations that were tested using a variable-ratio design. Based on our analyses, the combination of 50 µM QC and 25 nM 5-FU, which yielded the lowest CI value (0.08, indicating strong synergy), was selected as the final working concentration (Fig. S1). These concentrations were subsequently used in all experimental protocols, including migration, gene expression, and protein expression assays.

2.5. Wound healing assay

Cells were seeded in six-well plates, and, upon reaching approximately 90% confluence, a linear scratch was created in each well using a sterile 50 µL pipette tip. To ensure reproducibility, scratches were performed in triplicate for each condition, and care was taken to use the same tip orientation and angle to maintain a consistent wound width across all wells.To minimize proliferative effects, cell migration was assessed in the reduced-serum medium (1% FBS). After washing with PBS, images were captured immediately upon scratching (0 h) and again at 24 h and 48 h following treatment with QC and 5-FU. Images were consistently captured from the same representative field in each well to reduce variability. Cell migration was quantified using NIH ImageJ software, and the migration rate was calculated using the formula: Wound closure (%) = $[(A_0 - A_t)/A_0] \times 100$, where A_0 is the scratch area at 0 hour and At is the area at 24 or 48 hours.

2.6. Total RNA extraction and cDNA synthesis

Total RNA was extracted using a Hybrid-R RNA isolation kit (GeneAll Biotechnology, Seoul, South Korea) according to the manufacturer's instructions. The RNA concentration was measured using a NanoDrop Spectrophotometer, and the quality was assessed via agarose gel electrophoresis. RNA was eluted in DEPC-treated water and stored at -70°C. cDNA synthesis was performed in a 20 µL reaction using a commercial cDNA synthesis kit (Yekta Tajhiz Azma).

2.7. Quantitative RT-PCR

RT-PCR was used to evaluate the expression of *N-cadherin*, *E-cadherin*, *Vimentin*, and *ZEB1*. Specific primers listed in Table 2 for each gene were used in conjunction with a SYBR Green master mix, with *GAPDH* serving as the housekeeping gene. PCR products were verified by melting curve analysis and 2% agarose gel electrophoresis.

2.8. Western blot examination

After treatment, breast cancer cells were washed twice with cold PBS (pH 7.42) and lysed using RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with protease inhibitor cocktail. Protein concentrations were quantified by the BCA assay

Table 2. Sequences of primers used for quantitative RT-PCR

Gene	Primer sequences
E-cadherin	Forward: 5'-AGAACGCATTGCCACATACA-3' Reverse: 5'-GAGGATGGTGTAAGCGATGG-3'
Vimentin	Forward: 5'-CATTGAGATTGCCACCTAC-3' Reverse: 5'-CGTTGATAACCTGTCCATC-3'
ZEB1	Forward: 5'-TGAATCATCGCTACTCCTACTGT-3' Reverse: 5'-TTTCACTGTCTTCATCCTCTTCCC-3'
N-cadherin	Forward: 5'-ATTCGGGTAATCCTCCCAAATC-3' Reverse: 5'-CCCACAATCCTGTCCACATC-3'

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(Thermo Fisher Scientific). Equal amounts of protein (30 µg) were separated on 10% SDS-PAGE gels and transferred onto PVDF membranes (0.45 µm pore size; Millipore, Burlington, MA, USA). Membranes were blocked with 5% non-fat dry milk in TBST (0.1% Tween-20 in TBS) at room temperature for 1 h and then incubated with primary antibodies: anti-ZEB1 (1:1000, ~190 kDa; Cell Signaling Technology, Inc., Danvers, MA, USA, #3396), anti-E-Cadherin (1:1000, ~120 kDa; Cell Signaling Technology, Inc., Danvers, MA, USA, #3195), and anti-GAPDH (1:5000, ~37 kDa; Cell Signaling Technology, Inc., Danvers, MA, USA, #5174) at 4°C overnight. After washing, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-rabbit IgG-HRP, 1:10,000; Cell Signaling Technology, Inc., Danvers, MA, USA, #7074) at room temperature for 1 h[19,20]. Protein bands were visualized using an enhanced chemiluminescence detection kit (Abcam, Cambridge, UK) and quantified with ImageJ software (NIH, Bethesda, MD, USA).

2.9. Statistical analysis

All experiments were conducted in triplicate, and data are presented as mean ± SEM. Statistical analyses were performed using GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA). The Shapiro–Wilk test was used to evaluate data normality, and all datasets satisfied the assumptions required for parametric analysis. Differences among groups were analyzed using one-way ANOVA, followed by Tukey's post-hoc test for multiple comparisons. A p value < 0.05 was considered statistically significant.

3. RESULTS

3.1. Synergistic effect of 5-FU and QC on cell growth and viability

As depicted in Figure S1A, QC treatment (12.5–200 µM) significantly reduced MDA-MB-231 cell viability in a concentration- and time-dependent manner. At 24 h, viability progressively decreased, reaching ~65% at 200 µM, and further to ~50% at 48 h. Figure S1B illustrates 5-FU concentration-dependent efficacy (6.25–1000 nM). After 24 h, 1000 nM of 5-FU reduced viability to approximately 85%, while extending treatment to 48 h further decreased viability to ~75% at the same concentration. Notably, 5-FU exhibited anticancer activity at nanomolar concentrations, highlighting its potency compared to QC (which operates in the micromolar range). In contrast, Figure S1C shows that QC (50–800 µM) induced notable cytotoxicity in MRC-5 fibroblasts only at very high concentrations. After 48 h, cell viability decreased to ~25% at 800 µM (p < 0.001), underscoring QC preferential activity toward cancer cells. For comparison, 5-FU (2.5–200 µM) reduced MRC-5 viability to ~35% at 200 µM (Fig. S1D), suggesting a narrower safety margin relative to QC. The combination treatment of QC and 5-FU, as depicted in Figure S1, reveals a synergistic effect on reducing cell viability. Figure 1 demonstrates that the combined treatment at various concentrations of QC (12.5 µM to 100 µM) and 5-FU (6.25 nM to 25 nM) resulted in a more significant reduction in cell viability (ranging from 30% to 75%) compared to either treatment alone. This observation suggests that combining QC and 5-FU enhances the cytotoxic effect on MDA-MB-231 cells, potentially offering a more effective therapeutic strategy. To further evaluate the interaction between QC and 5-FU, CI values were calculated for each treatment combination. The combination of 50 µM QC and 25 nM 5-FU showed the lowest CI value (0.08; Table 1), which indicates a strong synergistic interaction. This particular combination was subsequently used in all subsequent experimental protocols.

3.2. Reduced breast cancer cell migration with QC and 5-FU combination

As shown in Figure 2, representative phase-contrast images of the wound area at 0, 24, and 48 hours visually demonstrate the progressive inhibition of cell migration across all treatment groups. The combination of QC and 5-FU significantly impaired the migration of MDA-MB-231 cells in the wound healing assay. Also, the control group showed extensive migration, with wound closure of 95.8 ± 1.2% at 24 hours. Treatment with 50 µM QC alone resulted in moderate inhibition, with wound closure of 72.5 ± 2.1% at 24 hours and 92.7 ± 1.5% at 48 hours. Treatment with 25 nM 5-FU alone led to closure of 61.4 ± 1.8% and 88.7 ± 1.7% at 24 and 48 hours, respectively. Notably, the combined treatment of QC and 5-FU potently inhibited cell migration, significantly reducing wound closure to 11.2 ± 0.9% at 24 hours and 34.2 ± 1.4% at 48 hours. This inhibition was significantly greater than that caused by QC alone (p < 0.0001 at both 24 h and 48 h) or 5-FU alone (p < 0.0001 at both time points).

3.3. Expression profiles of mesenchymal and epithelial genes in breast cancer

The expression of mesenchymal and epithelial genes, particularly *Vimentin*, *ZEB1, N-cadherin,* and *E-cadherin*, plays a crucial role in the metastatic progression of breast cancer cells. To elucidate the effects of QC and 5-FU on the expression of these genes, we conducted a detailed analysis of their expression levels, both individually and in combination. As presented in Figure 3, treatment with QC (50 µM) alone resulted in a minor and non-significant change in the expression of *ZEB1* and *Vimentin* (0.98- and 0.97-fold,

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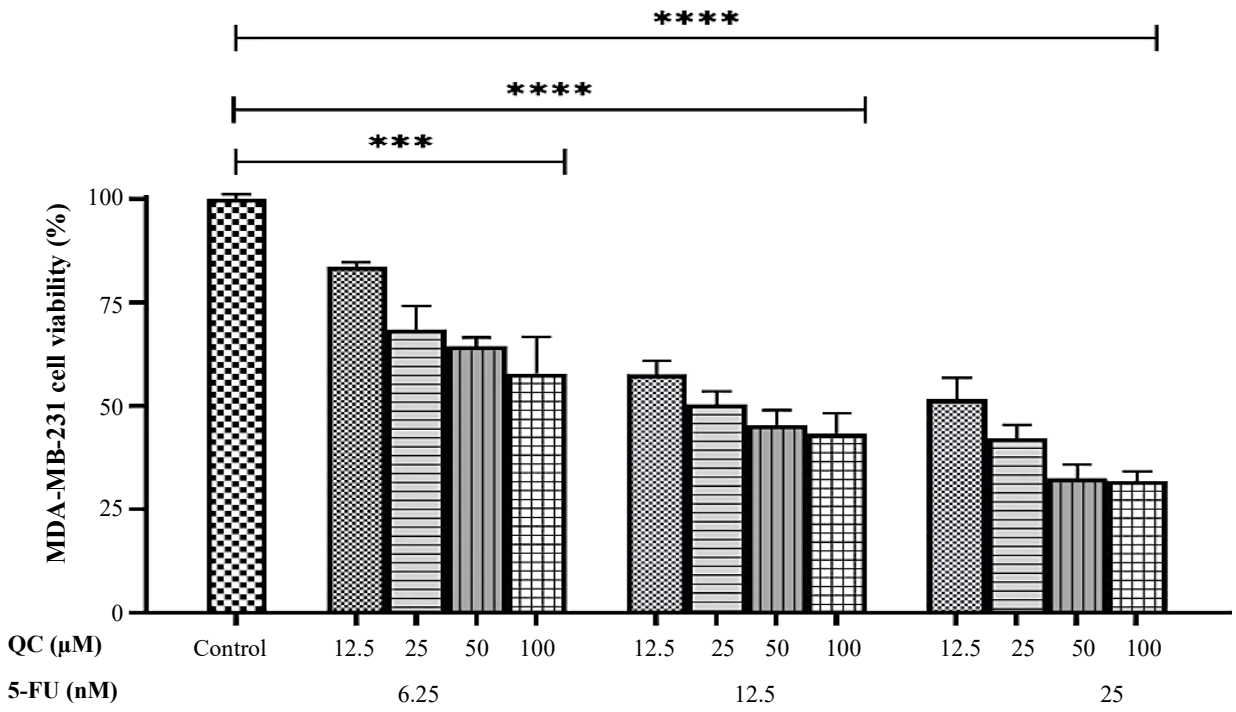

Fig. 1. Dose-response synergy of QC and 5-FU in MDA-MB-231 cells. The combination of QC (12.5–100 μM) with escalating 5-FU concentrations (6.25–25 μM) for 48 h was assessed via MTT assay. Data are presented as mean ± SEM from three independent experiments (n = 3). Statistical significance was determined by a one-way ANOVA followed by Dunnett's post-hoc test. $^{***}p < 0.001$ and $^{****}p < 0.0001$ indicate significant differences compared to the untreated control group.

$p > 0.05$, respectively). In contrast, 5-FU treatment (25 nM) significantly decreased *ZEB1* and *Vimentin* expression by 0.70-fold ($p < 0.05$) and 0.50-fold ($p < 0.01$), respectively. Notably, the combined administration of QC and 5-FU amplified this suppression, further reducing the expression of *ZEB1* and *Vimentin* (0.46- and 0.47-fold, $p < 0.01$, respectively). These results indicate that 5-FU, whether administered separately or in combination with QC, effectively downregulates key genes associated with the mesenchymal phenotype. Quantitative analyses (Fig. 4) demonstrated that the co-administration of QC and 5-FU markedly altered the expression of EMT markers in MDA-MB-231 cells. For *N-cadherin*, QC alone caused a 0.97-fold reduction, and 5-FU alone led to a 0.70-fold reduction. When combined, QC and 5-FU further decreased *N-cadherin* expression to 0.68-fold. In contrast, *E-cadherin* expression increased following treatment with both agents. QC alone elevated *E-cadherin* by 1.25-fold, while 5-FU led to a 2.75-fold increase. The combination of QC and 5-FU resulted in the most pronounced upregulation, with a 3.75-fold increase in *E-cadherin* expression. Overall, these findings indicate that QC and 5-FU differentially regulate *N-cadherin and E-cadherin*, suggesting potential synergistic effects in suppressing EMT and metastasis in breast cancer cells.

3.4. Expression protein profiles of mesenchymal and epithelial proteins in breast cancer

To evaluate the impact of QC and 5-FU, alone or in combination, on the expression of EMT-associated proteins, Western blot analysis was performed on MDA-MB-231 cells treated with QC (50 μM), 5-FU (25 nM), or their combination for 48 hours. As shown in Figure 5A, Quantification of ZEB1 and E-cadherin protein levels was normalized to GAPDH and expressed as fold change relative to the control. Treatment with 5-FU alone significantly reduced ZEB1 protein levels ($p < 0.01$), while QC alone had no statistically significant effect. However, combined treatment with QC and 5-FU led to a further and highly significant reduction in ZEB1 expression compared to 5-FU alone ($p < 0.001$, Fig. 5B), suggesting a synergistic inhibitory effect of the two compounds on the EMT-promoting factor ($p < 0.01$, combination vs. 5-FU). In contrast, E-cadherin protein levels were not changed in QC monotherapy, while 5-FU alone significantly enhanced E-cadherin expression level ($p < 0.05$). Notably, the combination of QC and 5-FU resulted in a

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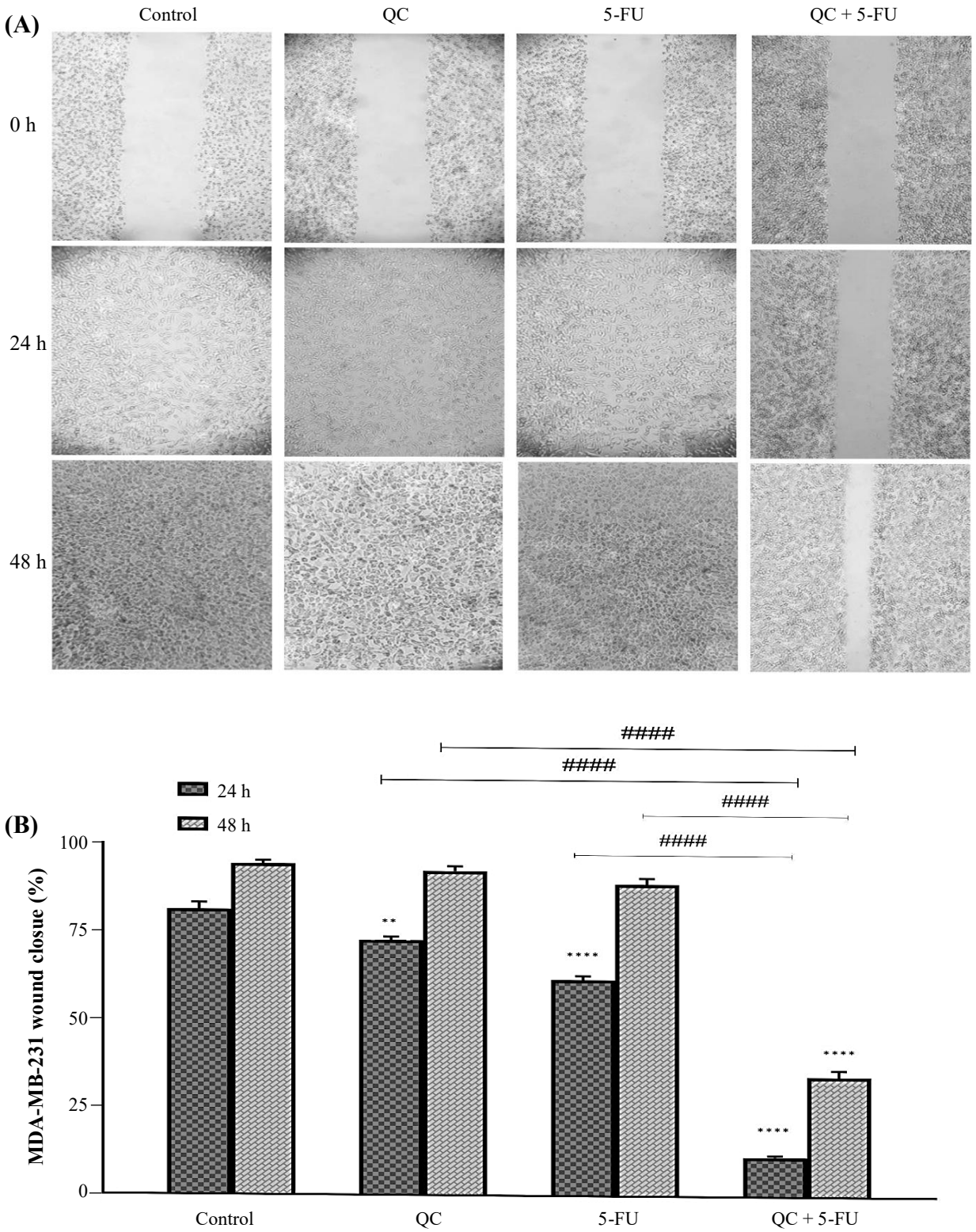

Fig. 2. Effect of QC and 5-FU on cell migration in MDA-MB-231 breast cancer cells. (A) Scratch assay images (magnification ×10), demonstrating the migration of MDA-MB-231 cells treated with 50 µM QC, 25 nM 5-FU, and the combination of both at 0, 24, and 48 hours. (B) Quantitative analysis of migration distance, showing a significant reduction in cell migration for the combination treatment compared to QC or 5-FU alone. Data are presented as mean ± SEM from three independent experiments (n = 3). Statistical significance was determined by one-way ANOVA followed by Tukey's post-hoc test. $^{**}p < 0.01$ and $^{****}p < 0.0001$ indicate significant differences compared to the untreated control group. $^{\#\#\#\#}p < 0.0001$ indicates a significant difference between the combination treatment (QC + 5-FU) group and the respective monotherapy groups (QC alone or 5-FU alone).

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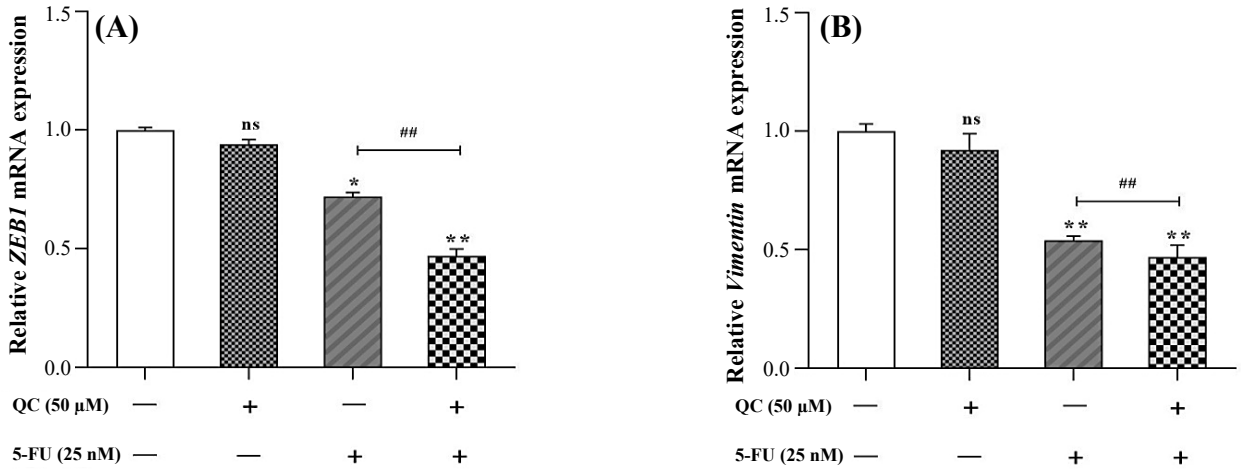

Fig. 3. Effects of QC and 5-FU on the expression of mesenchymal and epithelial genes in MDA-MB-231 breast cancer cells. (A) Quantitative analysis of *ZEB1* and (B) *Vimentin* expression levels following treatment with 50 µM QC, 25 nM 5-FU, and the combination of both agents at 48 h. Results are presented as percentage changes relative to untreated controls. Data are presented as mean ± SEM from three independent experiments (n = 3). Statistical significance was determined by one-way ANOVA followed by Tukey's post-hoc test. $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate significant differences compared to the untreated control group. $^{\#\#}p < 0.01$ indicates a significant difference between the combination treatment (QC + 5-FU) group and the respective monotherapy groups (QC alone or 5-FU alone); ns: not significant.

robust upregulation of E-cadherin protein, showing a greater than four-fold increase compared to control ($p < 0.001$) and a significant elevation relative to 5-FU alone ($p < 0.001$; Fig. 5C). These findings suggest that co-treatment potentiates the reversal of EMT and reinforces epithelial characteristics in aggressive breast cancer cells.

4. DISCUSSION

Breast cancer remains a leading cause of cancer-related mortality in women[19]. This is especially true for aggressive subtypes like TNBC, often modeled by the metastatic MDA-MB-231 cell line[20]. A key driver of this aggressiveness is the EMT, a process that enhances cell migration and invasion[21]. EMT is regulated by transcription factors like *ZEB1* and adhesion molecules

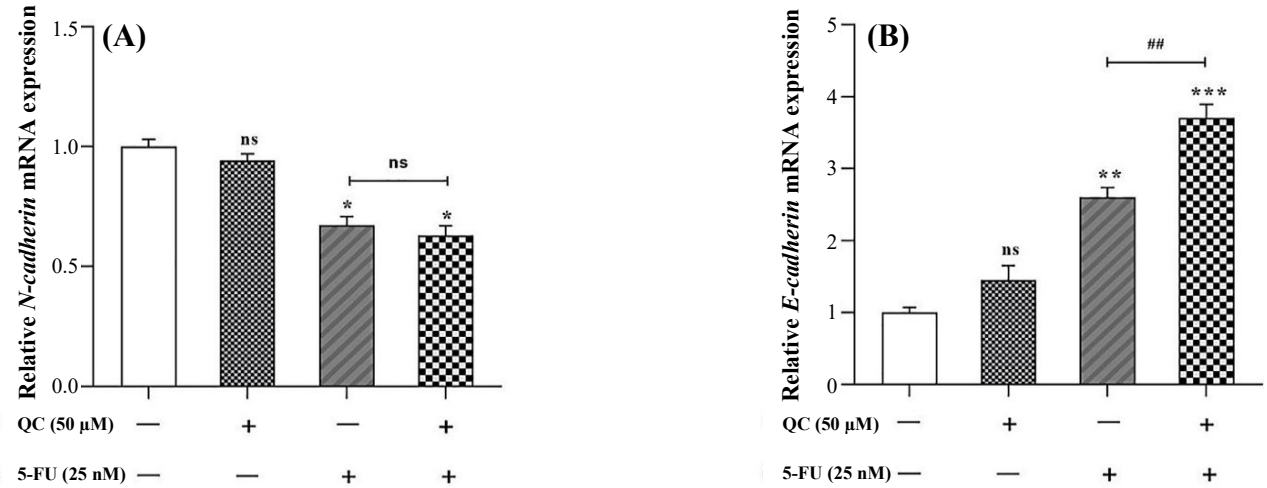

Fig. 4. Impact of QC and 5-FU on *N-cadherin* and *E-cadherin* gene expression in MDA-MB-231 breast cancer cells. Quantitative analysis of (A) *N-cadherin* and (B) *E-cadherin* expression levels following treatment with 50 µM QC, 25 nM 5-FU, and the combination of both agents. Data are presented as mean ± SEM from three independent experiments (n = 3). Statistical significance was determined by one-way ANOVA followed by Tukey's post-hoc test. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ indicate significant differences compared to the untreated control group. $^{\#\#}p < 0.01$ indicates a significant difference between the combination treatment (QC + 5-FU) group and the respective monotherapy groups (QC alone or 5-FU alone); ns: not significant.

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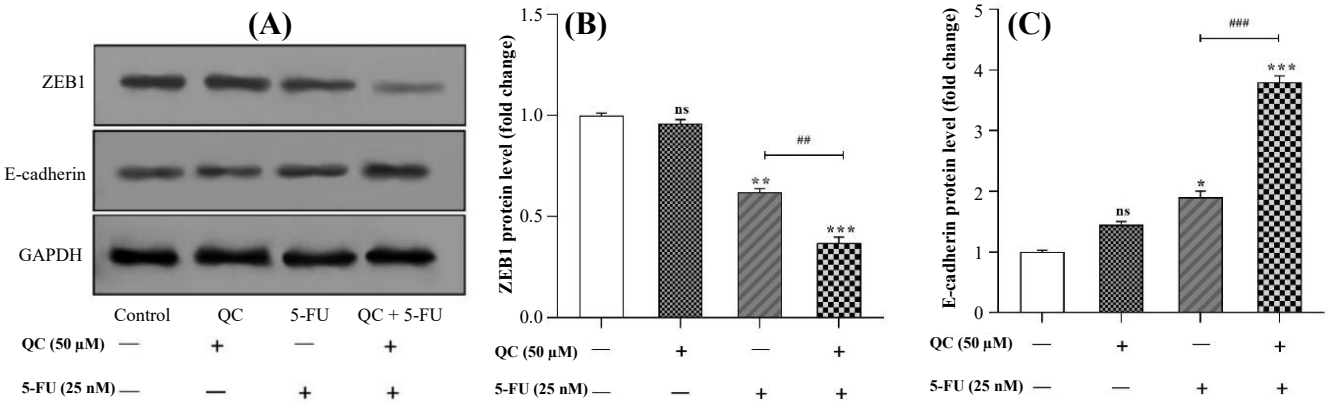

Fig. 5. Effects of QC (50 µM), 5-FU (25 nM), and their combination on EMT-related protein expression in MDA-MB-231 breast cancer cells after 48 hours of treatment. (A) Representative Western blot images showing protein expression levels of ZEB1, E-cadherin, and GAPDH (as loading control) across four treatment groups: control, QC alone, 5-FU alone, and combined QC + 5-FU; (B) quantification of ZEB1; (C) E-cadherin protein levels normalized to GAPDH and expressed as fold change relative to the control. Data are presented as mean ± SEM from three independent experiments (n = 3). Statistical significance was determined by one-way ANOVA followed by Tukey's post-hoc test. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ indicate significant differences compared to the untreated control group. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ indicate significant differences between the combination treatment (QC + 5-FU) group and the respective monotherapy groups (QC alone or 5-FU alone); ns: not significant.

such as N-cadherin, whose dysregulation promotes tumor progression and therapy resistance[22-24]. Although 5-FU is a common chemotherapy, its effectiveness is limited by drug resistance. Combining 5-FU with natural compounds like the flavonoid QC presents a promising strategy to enhance efficacy and reduce side effects[25,26]. QC exhibits multiple anti-cancer properties, including the inhibition of EMT and cell proliferation[27,28].

In our study, we evaluated the anticancer properties of QC both as a standalone agent and in combination with 5- FU, using MTT assays on MDA-MB-231 and MRC-5 cell lines. Our results demonstrated a significant concentration-dependent inhibitory effect on the proliferation of MDA-MB-231 cells treated with QC concentrations exceeding 50 µM and 5-FU concentrations exceeding 400 nM. This finding is consistent with a similar study by Mawalizadeh et al.[29], who reported a dose-dependent decrease in the cell viability of MCF-7 cells treated with 5-FU and QC. A notable observation in our study was the reduced cytotoxicity of QC in MRC-5 cells compared to 5-FU alone, indicating a selective preference for cancer cells. This selective cytotoxicity is promising as it suggests a lower risk of adverse effects in normal cells.

The strong synergy (CI = 0.08) allowed for a substantially reduced 5-FU concentration of 25 nM. This nanomolar concentration is much lower than the micromolar levels (≥500 nM) associated with toxicity in normal cells[30] and aligns with the lower clinically attainable range (~760 nM) of plasma concentrations from continuous infusion therapy[31,32]. This finding supports the potential of the combination therapy to widen the therapeutic index and reduce off-target effects. In contrast, the other study did not report any cytotoxicity of QC in normal cells and focused on the MCF-7 cell line[33].

In our study, combining 50 µM QC and 25 nM 5-FU yielded the lowest CI value, indicating strong synergy. This synergistic effect was observed with substantially lower concentrations of both agents compared to the combination of 100 µM of 5-FU and 446 µM of QC reported by Mawalizadeh et al., as the most effective doses against MCF-7 cells[29]. The Drug Reduction Index values in that study further supported the favorable nature of these combinations, indicating the potential for dose reduction in clinical applications[29]. The observed synergism could be attributed to the complementary modes of action of QC and 5-FU. QC is known for modulating multiple cellular pathways, including apoptosis, cell cycle regulation, and oxidative stress induction. In contrast, 5-FU primarily acts by inhibiting thymidylate synthase and disrupting DNA synthesis. The synergistic effect observed in the studies by Mawalizadeh et al. and Roshanazadeh et al. may arise from the combined impact of these mechanisms, leading to enhanced cancer cell death while sparing normal cells[27,29]. In summary, consistent with the findings of the comparative study[29], our results highlight the potential of QC as a valuable adjunct to 5-FU in breast cancer therapy.

In this study, we performed scratch assay on the breast

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cancer cell line MDA-MB-231 and treated the cells with 50 µM of QC and 25 nM of 5-FU, either individually or in combination, at 0, 24, and 48 h. Our results demonstrated that QC inhibited cancer cell migration, resulting in wound closure rates of 72.5% and 92.7% at 24 and 48 h, respectively. Similarly, 5-FU inhibited cancer cell migration, with wound closure rates of 61.4% and 88.72% for 24 and 48 h, respectively. The combined treatment of QC and 5-FU resulted in significantly greater inhibition of cell migration compared to treatments with either agent alone, reducing wound closure to 11.2% and 34.1% at 24 and 48 hours, respectively. These findings are consistent with those of Roshanazadeh et al., who also observed in that MDA-MB-231 cells treated with QC and 5-FU alone had wound closure rates of 71, 9%, and 92.5% at 24 hours, and 57.6% and 71.2% at 48 hours, respectively[27]. The combination of QC and 5-FU resulted in further inhibition of tumor cell migration, reducing wound closure to 29.3% and 36.1% for 24 and 48 hours, respectively. These results indicate that co-delivering QC with 5-FU enhances the effects of the chemotherapeutic agent on inhibiting MDA-MB-231 cell migration.

The observed inhibition of cell migration by QC and 5-FU, both individually and in combination, can be attributed to their distinct yet complementary mechanisms of action. QC modulates multiple cellular pathways involving in cell adhesion, migration, and invasion[34]. It can downregulate matrix metalloproteinase expression and inhibit the activation of signaling pathways, such as PI3K/AKT and MAPK, which are essential for cell migration and invasion. Meanwhile, 5-Fluorouracil primarily inhibits thymidylate synthase, disrupting DNA synthesis and cell proliferation. However, it can also affect cell migration and invasion by modulating the expression of related genes. The synergistic effect of QC and 5-FU suggests that these agents may target different aspects of the migratory process, thereby inhibiting cell migration more effectively. Collectively, our findings and other studies highlight the potential of combining QC with 5-FU as a therapeutic strategy to inhibit cancer cell migration. This combination could be particularly beneficial for treating aggressive breast cancer subtypes, such as TNBC, which is characterized by high metastatic potential. By targeting both cell proliferation and migration, this combination therapy can enhance the efficacy of the treatment and reduce the risk of metastasis. Furthermore, the observed selective cytotoxicity of QC toward cancer cells, as reported in our study, suggests that this combination may offer a therapeutic window with reduced toxicity to normal cells. This is a critical consideration for the development of safe and effective cancer therapies.

We investigated the effects of QC and 5-FU on the expression of mesenchymal and epithelial genes, specifically *Vimentin*, *ZEB1*, *N-cadherin*, and *E-cadherin*, which are crucial in the metastatic progression of breast cancer cells. Understanding the regulation of these genes is essential for grasping the mechanistic underpinnings of EMT. Changes in their expression profiles, such as the loss of *E-cadherin* and gain of *N-cadherin*, or the upregulation of *Vimentin* and the master regulator *ZEB1*, significantly influence cellular phenotypic plasticity and metastatic potential. Our findings demonstrated that both QC and 5-FU, individually and in combination, significantly modulated the expression of key EMT genes, including *ZEB1*, *Vimentin*, *N-cadherin*, and *E-cadherin.*

Consistent with the findings of Hatami et al., who reported a significant reduction in *ZEB1* and *Vimentin* expression upon QC treatment[35], our study reinforces the potential of QC as an effective modulator of mesenchymal gene expression. We observed that QC treatment reduced *ZEB1* and *Vimentin* levels by 0.98- and 0.97-fold, respectively, although this decrease was not statistically significant. However, our results indicate that 5-FU enhances these effects, particularly when combined with QC, leading to a more pronounced downregulation of these mesenchymal markers by 0.46- and 0.47-fold, respectively. The differential modulation of *N-cadherin* and *E-cadherin* expression observed in our study highlights the complexity of EMT regulation. The combined treatment of QC and 5-FU significantly decreased *N-cadherin* expression while markedly increasing *E-cadherin* expression by 3.75-fold. This shift towards an epithelial phenotype aligns with Hatami et al.'s findings, who reported similar trends in *E-cadherin* upregulation and *N-cadherin* downregulation, suggest a potential reversal of mesenchymal traits. The ability of QC and 5-FU to disrupt the EMT signaling pathways may underlie the observed reduction in the metastatic potential of breast cancer cells. Hatami et al. also showed that QC encapsulated in solid lipid nanoparticles had enhanced efficacy in reducing mesenchymal markers and increasing *E-cadherin* expression[35]. Overall, these findings underscore the therapeutic potential of combining QC and 5-FU to target EMT-related pathways in breast cancer treatment. Future research should explore the precise molecular mechanisms underlying these effects and assess the clinical applicability of such combination therapies.

ZEB1 is a central transcriptional regulator of EMT, repressing epithelial markers such as cadherin-1 (encoding E-cadherin*)* and promoting mesenchymal gene programs. In this study, we demonstrated that 5-

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FU monotherapy significantly reduced ZEB1 protein levels in MDA-MB-231 cells, consistent with earlier findings[36] that chemotherapeutic agents can inadvertently influence EMT signaling. Intriguingly, co-treatment with QC and 5-FU led to a markedly greater suppression of *ZEB1*, suggesting a synergistic effect that may reflect interference with compensatory pathways that maintain *ZEB1* expression under genotoxic stress. This hypothesis is supported by recent findings from Wang et al., who showed that *ZEB1* marks a plastic intermediate state during the evolution of neuroendocrine prostate cancer. In their study, *ZEB1*-positive cells exhibited stem-like features, open chromatin landscapes, and high metabolic plasticity. *ZEB1* directly activates glycolytic enzymes such as HK2 and LDHA, promoting lactate production and subsequent histone lactylation (notably H3K18la), which epigenetically reinforces lineage plasticity[37].

Given that QC is a known modulator of both metabolism and epigenetics, it is plausible that in our model, QC disrupts *ZEB1*-driven metabolic reprogramming. Indeed, QC has been reported to inhibit glycolysis and reduce lactate output, potentially impairing histone lactylation and limiting chromatin accessibility at *ZEB1* target loci. This mechanistic interplay may explain the limited efficacy of QC alone while showing strong synergy with 5-FU. Whereas 5-FU directly induces DNA damage, QC concurrently disrupts the metabolic and epigenetic support systems necessary for stabilization and function of *ZEB1*. Furthermore, the robust upregulation of E-cadherin, with an increase greater than four-fold under QC + 5-FU treatment, highlights a functional reversal of EMT. The loss of E-cadherin is a well-established hallmark of EMT, contributing to cellular detachment, invasive capacity, and therapeutic resistance. Restoration of E-cadherin thus serves as a reliable marker of epithelial reprogramming. Our study showed that QC monotherapy did not significantly alter E-cadherin expression at either the mRNA (1.25-fold; $p > 0.05$) or protein level. This observation suggests that at a concentration of 50 μM, QC alone is insufficient to drive E-cadherin restoration, underscoring the necessity of the synergistic combination with 5-FU to achieve the robust four-fold protein upregulation. While 5-FU alone moderately increased E-cadherin levels, likely through *ZEB1* downregulation, the markedly enhanced effect observed in the combination with QC demonstrated the potential of this therapeutic approach.

Emerging evidence from Ban and colleagues underscores the critical role of ribosome biogenesis (RiBi) in governing the dynamic balance between EMT and mesenchymal-epithelial transition. They found that inhibiting RiBi can "lock" cells in either an epithelial or mesenchymal state, making tumors more sensitive to chemotherapy[38]. Quercetin, a pleiotropic kinase inhibitor, suppresses the mTOR and ERK pathways, key regulators of RiBi and protein synthesis during phenotypic switching. Inhibiting these pathways may prevent the translation of critical EMT effectors, such as *ZEB1*, which helps maintain E-cadherin expression and reinforces epithelial identity. In the context of 5-FU treatment, QC may enhance epithelial stabilization not only by suppressing *ZEB1* expression but also by impairing the ribosomal and translational processes necessary for EMT plasticity. Altogether, these findings provide mechanistic insights into how metabolic, epigenetic, and translational modulation by QC can improve the therapeutic efficacy of 5-FU. This combinatorial strategy may offer a promising avenue for reversing EMT-associated TNBC-negative breast cancer.

5. CONCLUSION

This study establishes that the synergistic combination of QC and 5-FU significantly impairs both the survival and metastatic potential of TNBC cells. The primary discovery lies in the robust reversal of the EMT program, in which the combination therapy achieves a more potent suppression of malignant phenotypes than either agent alone. These results advance the field by demonstrating that natural flavonoids can act as powerful chemosensitizers, effectively broadening the therapeutic index of traditional chemotherapeutics. By concurrently targeting ZEB1-driven metabolic plasticity and restoring E-cadherin gene/protein expression, this strategy addresses the root causes of chemoresistance and invasive capacity. This dual-action mechanism provides a promising molecular foundation for developing low-toxicity regimens that prioritize selective cytotoxicity. Despite these promising insights, the investigation was constrained to in vitro models using established cell lines. Consequently, the observed synergistic effects may not fully reflect the physiological complexities or the tumor microenvironment present in clinical settings. Future research should prioritize in vivo validation to assess the pharmacokinetic profile and systemic safety of this combinatorial approach. Additionally, elucidating the precise epigenetic crosstalk between QC and 5-FU will be essential for refining clinical dosage regimens and optimizing therapeutic outcomes in aggressive breast cancer subtypes.

DECLARATION

Acknowledgments

Not applicable.

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Grammarly was used for language editing and grammar correction to improve readability. The authors reviewed and edited the content as needed and take full responsibility for the publication's content.

Ethical approval

All experimental procedures in this study were approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (ethical code: IR.AJUMS.MEDICINE.REC. 1401.076).

Consent to participate

Not applicable.

Consent for publication

All authors reviewed the results and approved the final version of the manuscript.

Authors' contributions

LN: methodology, formal analysis, investigation, data curation, writing original draft, visualization; MH: conceptualization, validation, resources, writing–review & editing, supervision; MA: methodology, formal analysis, data curation; SI: methodology, investigation, visualization; MR: conceptualization, validation, resources, writing–review & editing, supervision, project administration.

Data availability

All relevant data can be found within the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Supplementary information

The online version does contain supplementary material.

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