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Quercetin synergistically potentiates the anti-metastatic effect of 5-fluorouracil on the MDA-MB-231 breast cancer cell line

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ABSTRACT

Objective(s): Breast cancer (BC) cells' ability to metastasize to other tissues increases mortality. The Matrix metalloproteinases 2 and 9 (MMP-2 and MMP-9) facilitate cancer cell migration. 5-fluorouracil is a frequently applied chemotherapeutic agent in cancer treatment with destructive side effects on normal tissues. Hence, researchers have focused on finding a way to reduce the dose of chemotherapeutic drugs. Quercetin, a natural polyphenolic compound, has inhibitory effects on proliferation and migration of tumor cells. This study evaluated the effect of the combination of Quercetin and 5-fluorouracil on migration of the MDA-MB-231 breast cancer cell line.

Materials and Methods: The effect of Quercetin, 5-fluorouracil , and their combination on MDA-MB-231 breast cancer cell proliferation was investigated through MTT assay. Inhibition of tumor cell migration was examined by wound healing assay. Finally, the effect of treatments on gene expression of MMP-2 and MMP-9 was evaluated by quantitative real-time PCR.

Results: The IC_{50} values for Quercetin and 5-fluorouracil after 48 hr treatment were 295 µM and 525 µM, respectively. The combination index (CI) for Quercetin and 5-fluorouracil was <1, indicating synergy between them. The combination of Quercetin plus 5-fluorouracil resulted in a significant reduction in migration rate and MMP-2 and MMP-9 gene expressions of MDA-MB-231 cancer cells compared with the individual application of 5-FU.

Conclusion: Quercetin enhances the suppressory effect of 5-fluorouracil on migration of BC cells. The combination of Quercetin and 5-fluorouracil can be an attractive field for future studies.

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Introduction

Breast cancer (BC) is the most prevalent cancer among women accounting for over 600000 cancer deaths in 2018 across the globe (1). Fifteen to 20 % of all BC cases are Triple-negative breast cancer (TNBC). Compared with other breast cancer subtypes, TNBC has an earlier relapse after treatment and a greater probability of metastasis (2, 3).

The metastasis of cancer cells all over the body is the main cause of cancer mortality (4-7). This process's main event is the degradation of the extracellular matrix (ECM) components by proteolytic enzymes such as MMPs(8-12). It has been observed that MMP-2 and MMP-9, also known as gelatinases, have frequently contributed to the metastatic ability of BC cells (11, 13).

Chemotherapy is used as the main treatment for breast cancer (14). However, due to the destructive effects of these drugs on normal cells, the patients have to endure some severe side effects of this treatment (15-18). Therefore, due to their extensive biological activity and low toxicity, natural substances are considered potential alternative treatments for cancers, including BC (19-23). Besides, many studies have focused on combining natural compounds with chemotherapeutic agents to decrease chemotherapy's adverse effects (20, 21, 24-26).

5-fluorouracil (5-FU) is a uracil nucleotide in which fluorine replaces the hydrogen atom at position 5 and acts as an antimetabolite. 5-FU's active form can inhibit DNA synthesis and obstruct tumor growth; 5-FU also disrupts some cellular RNAs' structure and gene expression (27). The inhibitory effect of fluorouracil alone and combined with natural compounds such as resveratrol and EGCG has been shown in various studies on the migration of various cancer cells such as colorectal and gastric cancers (28-32).

Quercetin (Que) is a natural flavonoid that is mainly found in vegetables and fruits such as onions, apples, and broccoli. Que's therapeutic effect has been shown in multiple diseases, including cardiovascular disease, neurodegenerative disease, and cancer (33-35). It has been widely reported that Que can inhibit migration of various cancer cell lines such as skin, colorectal, and ovarian cancers (36-40). However, the effects of Que's combination with chemotherapeutic agents such as 5-FU against the migration of highly metastatic BC cells are yet to be clear.

In this study, we evaluated the effects of Que alone and in combination with the widely used chemotherapeutic agent 5-FU on the migration of MDA-MB-231 breast cancer cell line, as a highly invasive BC cell line, and on the expression of MMP-2 and MMP-9 genes *in vitro*.

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Materials and Methods

Cell culture

MDA-MB-231 breast cancer and MRC-5 human normal lung fibroblast cell lines were obtained from the Pasteur Institute (Tehran, Iran). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 4.5 g/l glucose, 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin (all reagents obtained from Bio-Idea, Tehran, Iran).

Preparation of treatments

5-FU was purchased from Ebewe Pharma (Unterach, Austria) at 50 mg/ml. To gain the desired concentration, the right amount of drug was added directly to supplement the medium. Que was obtained from Sigma–Aldrich (St. Louis, Missouri, United States). Preparation of Que solutions was performed readily before use at 100 mM through dilution in dimethylsulfoxide (DMSO, Bio-Idea), then the solutions were diluted in the cell culture medium. The concentration of DMSO in the cell culture medium was constantly < 0.1%. Thus it has not affected any cell functions (results not shown).

Cell viability assay (MTT)

The viability of cancerous and normal cells was assessed using the MTT assay. MDA-MB231 and MRC-5 cells were seeded in 96-well plates (4×10^3 and 10^4 cells per well, respectively) and incubated at 37 ˚C in 5% CO2 and kept overnight. Then treatment with various concentrations of Que (25, 50, 200, 350, 500, and 650 µM) and 5-FU (1.5, 6.25, 25, 100, 400, and 1600 µM) was performed and incubated for 24 and 48 hr. The medium containing drugs was then removed, and MTT solution (0.5 mg/ ml) was added to the cells. plates were incubated in the dark for 4 hr. Then, the MTT solution was replaced by 150 µl of DMSO (Merck, Darmstadt, Germany) and left 20 min on the shaker. The optical density (OD) of each well at 570 nm was assessed using a microplate reader (BioTek ELx800 Winooski, Vermont, United States). The cell viability percentage was calculated as the ratio of absorbance of treated and untreated control cells. IC_{50} was obtained from the proliferation curves produced by Graphpad Prism 8 computer software (La Jolla, CA). The selectivity index (SI) value was calculated for Que and 5-FU using IC_{50} values obtained from MTT assay results for MDA-MB-231 and MRC5 cell lines using the following formula, SI=IC_{50} of normal cell line/ IC_{50} of cancer cell line. SI values >1 indicate the drug's selective effect on cancer cells and lower adverse effects on normal cells.

CI and DRI determination

Combination index (CI) and Dose reduction index (DRI) determination were performed using CompuSyn (Chou and Martin, 2005, Compusyn Inc, USA) to evaluate the synergistic relation between Que and 5-FU. CI=1 represents additive effects, while CI >1 and CI <1 mean antagonism and synergy, respectively. Also, DRI was used to indicate the synergy between drugs and fold change in the reduction of drug dose. DRI >1 is favorable and indicates synergy between drugs.

Migration assay

Cells were grown to form a single layer with about 80% confluency in 6-well plates. Wounds were made in the cultures using the sterile tip of a 200 µl pipette. Cells were then washed using PBS to remove cell debris and non-attached cells. Fresh medium with specific concentrations of Que and 5-FU were added to the cultures. At 0, 24, and 48 hr, wounded areas were photographed in random microscopic zones. The distances of cell migration were obtained through pixel count using the NIH image J software package (National Institutes of Health, Bethesda, USA) through the following formula:

Migration rate = $[(T_0 - T_{48})/T0] \times 100$.

Total RNA extraction and cDNA synthesis

10^6 cells were used for RNA extraction. Total RNA was isolated using a Hybrid-R RNA isolation kit (GeneAll, Songpa-gu, Seoul, South Korea) in accordance with the manufacturer's instructions. RNA purity and integrity were confirmed with A260/A280 ratio (~ 1.8-2.0) and agarose gel electrophoresis, respectively. The isolated RNA was eluted in 50 µl DEPC treated water and stored at -70 ˚C. According to the manufacturer's instructions, reverse transcription was performed in a 20 µl reaction mixture using the cDNA synthesis kit (Yekta Tajhiz Azuma Tehran, Iran).

Quantitative Real-time PCR

The MMP-2 and -9 gene expression levels were evaluated through Real-time PCR using Syber green kit (Yekta Tajhiz Azuma Tehran, Iran). HPRT was preferred as the internal reference. The sequences of primers are as follow:

MMP-2 F: 5'-CCCAGCCAGAAGCGGAAA-3'
R: 5'- CGAACAGATGCCACAATAAAGC-3',
MMP-9 F: 5'-CCTTTGGACACGCACGAC-3'
R: 5'-CCACCTGGTTCAACTCACTC-3', HPRT
F: 5' GACCAGTCAACAGGGGACAT 3' R: 5'
CCTGACCAAGGAAAGCAAAG 3'.
The reaction conditions were as follows: 94 ˚C for 3 min; 94 ˚C for 40 sec, 59 ˚C for 30 sec, 72 ˚C for 30 sec and 30 cycles; and 72 ˚C for 5 min.

Statistical analysis

All experiments were performed in three separate tests, and the data were presented as mean±SEM. Statistical analysis was done using IBM SPSS 26 software (Chicago, USA). The results of the experimental groups were compared using one-way ANOVA and LSD post-hoc. A *P*-value less than 0.05 was considered significant.

Results

Effects of Que and 5-FU on cell proliferation

We performed an MTT assay to confirm Que's ability as an anticancer agent alone and combined with 5-FU. MDA-MB-231 and MRC5 cells were treated with different concentrations of Que and 5-FU for 24 and 48 hr. As revealed in Figures 1A and B, after treatment with Que concentrations above 200 µM and 5-FU concentrations above 25 µM, tumor cells› viability was decreased significantly in a dose-dependent manner. Figures 1C and D represent the effects of Que and 5-FU on proliferation of MRC5 cells for 24 and 48 hr. As shown in Figure 1C, Que concentrations above 200 significantly reduced the viability of normal cells in a dose-dependent manner. As shown in Figure 1D, 5-FU concentrations

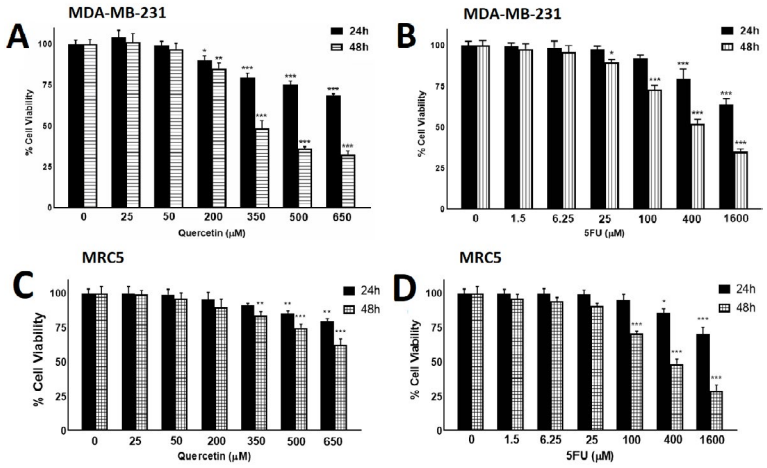

Figure 1. The effects of Quercetin and 5-fluorouracil on growth inhibition of MDA-MB-231and MRC5 cell line
(A) MDA-MB-231 cells were treated with different concentrations of Quercetin for 24 and 48 hr. cell viability was assessed using MTT assay. (B) MDA-MB-231 cells were treated with different concentrations of 5-fluorouracil for 24 and 48 hr. cell viability was assessed using MTT assay. (C) MRC5 human lung fibroblasts as normal cells were treated with different concentrations of Quercetin for 24 and 48 hr. cell viability was assessed using MTT assay. (D) MRC5 cells were treated with different concentrations of 5-fluorouracil for 24 and 48 hr. cell viability was assessed using MTT assay. Results are presented as mean± SEM of at least three independent experiments **P*<0.05; ***P*<0.01; ****P*<0.001 significant from control untreated cells

Table 1. Selectivity index (SI) of Quercetin and 5-fluorouracil for MDA-MB-231 and MRC5 Cell lines

Cell line	IC_{50} (μM)	
	5-FU	Que
MDA-MB-231	525	295
MRC5	348	>1000
SI [a]	0.65	>3.39

[a] Selectivity index

above 25 μM significantly inhibited the growth of MRC5 cells. IC_{50} values of Que and 5-FU for MDA-MB-231 and MRC5 cell lines were shown in Table 1. According to these IC_{50} values, Que has a high selectivity index (SI) (>3.39), and in an opposing manner, 5-FU has a low selectivity index (0.65). In SI analysis, SI values lower than 1 represent more adverse effects on normal cells, and SI values above 2 mean highly selective inhibitory effects on tumor cells compared with normal cells. In the next step, six combination states were produced by three concentrations of 5-FU (1.5, 6.25, and 25 μM), which have no significant effect on MDA-MB-231 growth inhibition and two below IC_{50} concentration of Que (50 and 200 μM). After treatment with the combination of Que and 5-FU, the viability of cancer cells was reduced significantly compared with treatment with Que or 5-FU alone (Figure 2). To further assess the synergy between Que and 5-FU, the CI value was determined for each combination state. As presented in Table 2, all of the CI values were <1, indicating a synergistic effect between Que and 5-FU. The least CI value (0.33) was for the combination of 50 μM Que and 25 μM 5-FU. Hence, these concentrations were chosen for the rest of the experiments. Also, the DRI value was calculated for the combination of Que and 5-FU to determine the reduction in the applied dose of 5-FU. All DRI values were >1, which is considered desirable (results not shown). To show the effect of the selected combination state on growth inhibition of normal cells, treatment with the combination of 50 μM of Que and 25 μM of 5-FU was performed. As presented in Figure 3, the combination treatment did not significantly increase the cytotoxicity of 5-FU on normal cells compared with 25 μM of 5-FU alone.

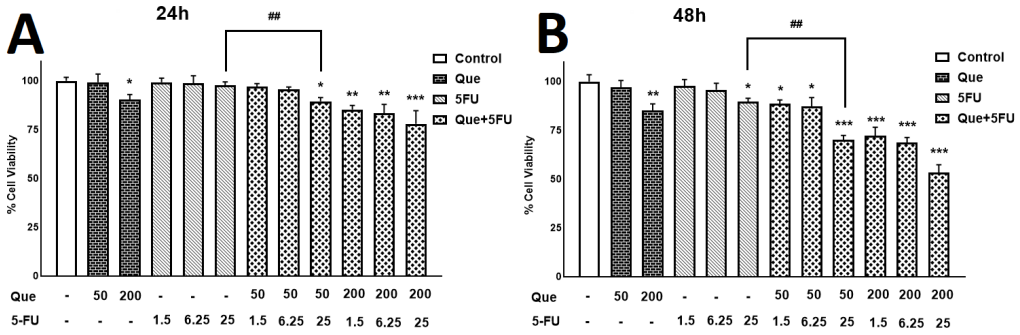

Figure 2. The effect of combinations of Quercetin and 5-fluorouracil on growth inhibition of MDA-MB-231
(A) Viability of MDA-MB-231 cells after treatment with Quercetin (50 and 200μM) combined with 5-fluorouracil (1.5, 6.25, and 25μM) for 24 hr was measured using MTT assay. (B) Viability of MDA-MB-231 cells after treatment with Quercetin (50 or 200μM) combined with 5-fluorouracil (1.5, 6.25, or 25μM) for 48 hr was measured using MTT assay. Results are presented as mean± SEM of at least three independent experiments **P*<0.05; ***P*<0.01; ****P*<0.001 significant from control untreated cells, and ##*P*<0.01 significant from 5-FU-alone treated cells

Table 2. CI [b] and DRI [c] values determined for various combinations of Quercetin and 5-fluorouracil. CI >1 Antagonism; CI=1 Additive; CI <1 Synergistic effect between drugs

5-FU (μM)	Quer (μM)	CI
1.5	50	0.45
6.25	50	0.53
25	50	0.33
1.5	200	0.74
6.25	200	0.7
25	200	0.47

[b] Combination index; [c] Dose-reduction index

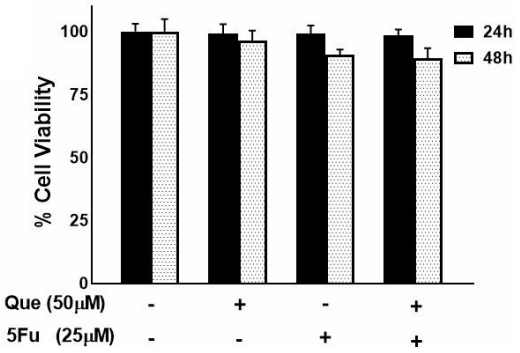

Figure 3. Effects of Quercetin and 5-fluorouracil combination on the proliferation of MRC5 cell line
MRC5 cells were treated with the combination of selected concentrations of Quercetin (50μM) and 5-fluorouracil (25 μM). Viability was determined by MTT assay. Results are presented as mean±SEM of at least three independent experiments

Effects of Que and 5-FU on the migration of breast cancer cells

To evaluate the migration rate of tumor cells treated with Que and 5-FU, a wound-healing assay was performed. MDA-MB-231 cells were treated with Que and 5-FU alone and combined for 24 and 48 hr. As shown in Figure 4, Que inhibited cancer cells' migration, leaving the wound closure of 71.9% and 92.5% for 24 and 48 hr, respectively. So did 5-FU with the wound closure of 57.6% and 71.2% for 24 and 48 hr, respectively. Also, the combination of Que and 5-FU resulted in further inhibition of tumor cell migration, reducing the wound closure to 29.3% and 36.1% for 24 and 48 hr, respectively. These results show that Que co-delivered with 5-FU could enhance the chemotherapeutic agent's effects on the inhibition of MDA-MB-231 cell migration.

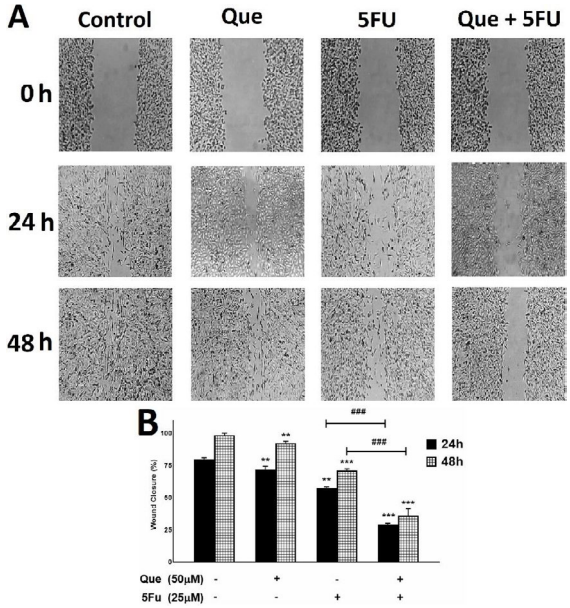

Figure 4. wound healing assay of MDA-MB-231 breast cancer cell line treated with 5-fluorouracil and Quercetin
Quercetin and 5-fluorouracil inhibited migration of tumor cells alone and in combination. (A) image of MDA-MB-231 cells migration following treatment with Quercetin (50 μM), 5-fluorouracil (25 μM), and their combination for 48h. (B) quantitative analysis of the anti-migrative effect of Quercetin (50 μM), 5-fluorouracil (25 μM), and their combination for 48 hr. Results are presented as mean± SEM of at least three independent experiments **P<0.01; *** P<0.001 significant from control untreated cells ; and ### P<0.001 significant from 5-FU-alone treated cells

Effects of Que and 5-FU on the expression of MMP-2 and MMP-9 genes

MMP-2 and MMP-9 have key roles in breast cancer cells' metastasis; therefore, the effects of Que and 5-FU

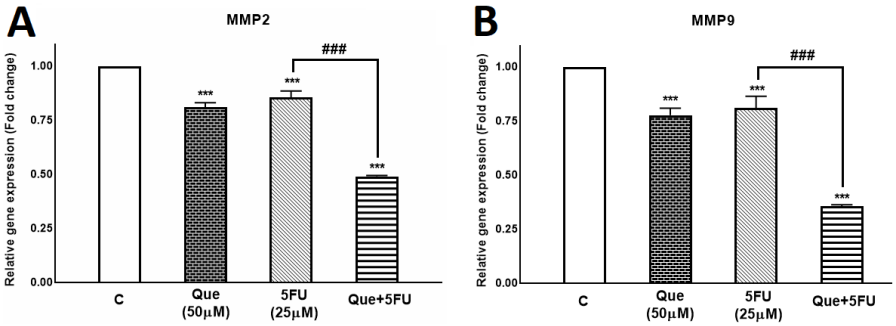

Figure 5. Quercetin enhanced the effect of 5-fluorouracil on the Matrix metalloproteinase-2 and Matrix metalloproteinase-9 gene expression in the MDA-MB-231 breast cancer cell line
Quercetin and 5-fluorouracil decreased gene expression of Matrix metalloproteinase-2 and Matrix metalloproteinase-9 alone and in combination. (A) Expression of Matrix metalloproteinase-2 gene was evaluated in MDA-MB-231 untreated control, treated with Quercetin(50 μM), 5-fluorouracil (25 μM), and the combination of Quercetin plus 5-fluorouracil using quantitative Real-time PCR. (B) Expression of Matrix metalloproteinase-9 gene was evaluated in MDA-MB-231 untreated control, treated with Quercetin(50 μM), 5-fluorouracil (25 μM), and the combination of Quercetin plus 5-fluorouracil using quantitative Real-time PCR. . Results are presented as mean± SEM of at least three independent experiments ***P<0.001 significant from control untreated cells and ### P<0.001 significant from 5-FU-alone treated cells

on MMP-2/-9 gene expression were assessed alone and in combination. As shown in Figure 5, 5-FU reduced the expression of MMP-2/-9 genes to 0.85 and 0.8 fold, respectively. Also, Que decreased expression of MMP-2 and MMP-9 genes to 0.8 and 0.77 fold, respectively. However, the combination of Que and 5-FU reduced the expression of the MMP-2 gene to 0.48 fold and the expression of the MMP-9 gene to 0.35 fold. According to these results, Que and 5-FU alone reduced the expression of MMP-2 /-9 genes significantly, and the combination of Que and 5-FU reduced the expression of these genes more significantly compared with each drug alone.

Discussion

BC is one of the deadliest cancers worldwide (41). Cancer cell metastasis is a major factor in increasing cancer mortality (5). Hence, one of the major challenges is finding ways to suppress this capacity in cancer cells. MMP-2 and MMP-9 enzymes are two major factors in the occurrence of cell migration by facilitating their exit from the tissue through ECM degradation. Increased expression of these enzymes in various cancer cells has been frequently shown and resulted in increased invasion properties (42). 5-FU is a frequently used chemotherapeutic agent. In its initial use, 5-FU effectively inhibits the proliferation of cancer cells. However, long-term application of 5-FU causes resistance in cancer cells and reduces the inhibitory effect of this drug along with destructive effects on normal tissues (15, 43). Therefore, finding a way to decrease the dose of chemotherapeutic agents while conserving their therapeutic effects has become a hot topic of research. Que has been shown to inhibit viability and reduce migration in different cancer cell lines, such as colorectal and HeLa cells. Studies have also shown that Que can increase the sensitivity of various cancer cell lines such as colorectal, esophageal, and Hela cells to 5-FU (44-47). In this research, we studied the effects of a combination of 5-FU and Que on the inhibition of growth and migration of the MDA-MB-231 breast cancer cell line. Our results indicate a synergistic effect between Que and 5-FU in inhibiting breast cancer cells' proliferation and migration.

To investigate Que and 5-FU's synergy in inhibiting cancer cell growth, we performed a cell proliferation assay. Our results showed that both Que and 5-FU could individually reduce the proliferation of tumor cells. However, the cytotoxic effect of the combination of Que and 5-FU on BC cells is significantly more than that of individual drugs. In other words, Que enhances the inhibitory effect of 5-FU on the proliferation of BC cells. CI and DRI values were calculated to further examine the synergy between Que and 5-FU. According to our results, CI values for all combinations between Que and 5-FU were <1, which further confirms Que and 5-FU's synergistic effect. The lowest CI value (0.33) was related to 50 µM of Que and 25 µM of 5-FU, indicating the highest synergy level at this combination between Que and 5-FU. To confirm the non-toxic effect of this combination, we examined the effects of the mentioned combination on the growth of MRC5 human normal lung fibroblast cells, and results showed that this combination did not significantly reduce normal cell viability compared with each drug alone. The DRI value indicates the reduction in drug dose in the combined state compared with individual drugs' implication while maintaining the inhibitory effect. Therefore, DRI values >1 reflect the synergy between drugs that results in a reduced dose of the chemotherapeutic agent. According to our results, the DRI values for 5-FU in combination with Que were >1. Therefore, the dose of 5-FU in combination with Que is reduced while maintaining its inhibitory effect on the proliferation of cancer cells. These results are in line with previous works reporting the synergistic effects between 5-FU and other polyphenolic compounds such as resveratrol, EGCG, and Que in colorectal, OSCC, and esophageal cancers (30, 44, 48).

We performed a wound healing assay to evaluate the effects of Que, 5-FU, and their combination on BC cells' migration. Our results indicated that the rate of BC cell migration is significantly reduced after treatment with Que and 5-FU by 6% and 27%, respectively. However, migration of tumor cells treated with the combination of Que and 5-FU showed a significant reduction (62%) compared with Que and 5-FU alone, which is consistent with the results of our proliferation assay. Decreased tumor cell migration by 5-FU may be due to its effect on the structure and gene expression of cellular RNAs involved in tumor cell migration. Also, a significant reduction in tumor cell migration after treatment with the combination of Que and 5-FU can be due to Que's ability to increase the sensitivity of tumor cells to 5-FU, which leads to a more significant inhibitory effect of 5-FU on their migration. These results are in line with the previous works on the inhibitory effects of 5-FU in combination with other polyphenol compounds such as resveratrol, kaempferol, and EGCG on colorectal and OSCC cancer cells migration (30, 48, 49).

Increased expression of gelatinase enzymes in metastatic tumor cells has been shown in various studies (50-53). Therefore, we assessed the effects of Que, 5-FU, and their combination on the expression of MMP-2 and MMP-9 genes. Our results showed that expression of MMP-9 and MMP-2 genes treated with Que and 5-FU decreased significantly. However, these genes' expression showed a significant decrease after treatment with Que and 5-FU's combination compared with the treatment with Que and 5-FU individually. These results are in agreement with the results of the migration assay. The results obtained in this experiment are consistent with the results of previous studies regarding the effect of the combination of 5-FU and natural compounds on the expression of mmp2 and mmp9 genes in tumor cells (54-58). Our results indicate that 5-FU, Que, and their combination can affect tumor cells' migration at the gene expression level. However, Que and 5-FU's combination has a more significant effect on the gene expression of gelatinase enzymes than Que and 5-FU alone, which suggests the synergistic effect between them.

Conclusion

Our results showed that not only Que can reduce the growth and migration of BC cells, it can also synergize with 5-FU as a chemosensitizing agent that results in the reduction of 5-FU dose and also further reduces the proliferation and migration capacity of BC cells. These

findings introduce Que as a new therapeutic agent in the treatment of breast cancer. However, further *in vitro* and *in vivo* studies are needed to clarify the continuation of the route.

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Statement of Ethics

No human or animal experiments were performed in this research.

Authors' Contributions

Title of manuscript: Quercetin synergistically potentiates the anti-metastatic effect of 5- Fluorouracil on MDA-MB-231 breast cancer cell line

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Study conception or design: M R and MR R; Data analyzing and draft manuscript preparation: H BR and MR R; Critical revision of the paper: H BR; Supervision of the research:M R; Final approval of the version to be published: M R, H BR, and MR R.

Conflicts of Interest

We have no conflicts of interest to declare.

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