Quercetin Potentiates the Chemosensitivity of Human Breast Cancer Cells to 5-Fluorouracil

Fatemeh Mawalizadeh
Ahvaz Jundishapur University of Medical Sciences
Ghorban Mohammadzadeh
Ahvaz Jundishapur University of Medical Sciences
azam khedri
Ahvaz Jundishapur University of Medical Sciences
mojtaba rashidi (rashidi-mo@ajums.ac.ir)
Ahvaz Jundishapur University of Medical Sciences https://orcid.org/0000-0003-4311-6137

Research Article

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Abstract

Background: breast cancer is one of the leading causes of cancer mortality worldwide. 5-fluorouracil (5-FU) is one of the chemotherapy drugs to treat breast cancer, but it is associated with several side effects. Combination therapy is a way to increase the effectiveness of chemo drugs and decrease their usage dose. Quercetin (Quer) is one of the natural polyphenols with anti-cancer properties. This study investigated the apoptotic effect of 5-FU in combination with Quer compared with 5-FU alone on MCF7 breast cancer cells.

Method and Results: Different single and combined concentrations of 5-FU and Quer were applied to MCF 7 cells for 48 hours. Cell viability, apoptosis, gene expression of Bax and Bcl2, and colony number were assessed using MTT assay, flow cytometry, quantitative real-time PCR, and Colony formation assay, respectively. The combination of 5-FU and Quer compared to 5-FU alone improved apoptosis by increasing and decreasing the gene expression of Bax and Bcl2, respectively, and decreased colony formation in MCF7 cells.

Conclusion: Quer potentiates the sensitivity of breast cancer to 5-FU so that this combination may be proposed as a treatment for breast cancer. Therefore, this combination can be suggested for future in vivo studies.

1. Introduction

Cancer is a disease featured with unlimited growth and proliferation of the cells in which the cells do not obey the normal rule of cell division [1]. Breast cancer is the most prevalent cancer in women and a top cause of mortality in developed and developing countries and represents 36.7% of all female cancers [2]. One of the main treatments to cure breast cancer is chemotherapy, and 5-Fluorouracil (5-FU) is a common chemotherapeutic drug in treating breast and prostate cancers, which exerts apoptotic effects by modulating anti- and pro-apoptotic proteins [3-5]. Although the chemo-drugs effectively kill cancer cells, they damage normal cells as well, so they cause several side effects, including nausea, vomiting, mucositis, increased risk of infection and bleeding, and anemia.

On the other hand, the resistance of some cancer cells against chemo-drugs is proven. The drug combination is an effective method to deal with drug resistance and reduce the damage to normal cells by reducing the dose of chemo-drugs. As one way to achieve this, currently, researchers have focused on combinations of chemo-drugs with herbal compounds, including polyphenols, to cure different types of cancer cells [6, 7]. Previous studies have shown that a combination of chemo-drugs with herbal polyphenols potentiates the anti-carcinogenic effect of these drugs and decreases their side effects. It also reduces the resistance of cancer cells to these drugs compared with using the drugs alone [8]. Quercetin (Quer) is one of these polyphenols categorized in the flavonoid group, found in different vegetables and fruits, especially onion, kale, apple, and broccoli. Several beneficial properties of Quer, such as antioxidative, anti-inflammatory, antiviral, and anti-neoplastic effects, have been proven in

several in vitro and in vivo researches [9-11]. As a tyrosine kinase inhibitor, Quer was used in the first phase of clinical trials for hematological malignancies without serious side effects [12]. It has been revealed that Quer exerts its apoptotic effect through Bax and Bcl2 modulation [13-16]. Synergistic effects of Quer with classical and new drugs to cure breast cancer have been shown by several studies [2, 17-20]. Combination therapy of Quer with resveratrol and catechin in *breast cancer xenograft model* reduces proliferation of cancer cells compared to using each compound alone [18]. This effect has also observed in the combination of Quer and doxorubicin so that Quer enhances the doxorubicin anti-proliferative effect and reduces drug toxicity to normal cells [19-21]. The present study is an attempt to examine enhancing the apoptotic effect of 5-FU in combination with Que in MCF7, a human mammary breast cancer cell line.

2. Materials And Methods

2.2 Cell Culture

The MCF-7 (human breast cancer cells) and MRC-5 (normal human lung fibroblast) were supplied by the national cell bank of Pasteur institute of Iran. They were cultured in DMEM High Glucose medium containing 10% fetal bovine serum plus 100 U/mL of Penicillin and 100 mg/mL of Streptomycin in T25 flasks and kept in an incubator (5% CO2, 37°C).

2.3 MTT assay

MTT assay was done to determine the cytotoxic effect of 5-FU and Quer on the MCF-7 cells. Briefly, 4×10^3 MCF7 cells were seeded in each well of 96 well-plates. After overnight incubation, the old media were discharged, and 200 µL of medium containing different concentrations of the 5-FU (Venus Remedies Limited, India) (100- 300 µM) and Quer (Sigma, USA) (150-750 µM, Soluble in DMSO with a final concentration of 0.1%) were added. Incubation was done in 5% CO2 at 37°C for 48h. Afterward, MTT solution (*0.5 mg/ml*) was added and incubated in the dark at 37°C for 4h. The media were replaced by 150 µL DMSO for each well (Merck, Darmstadt, Germany), and plates were shacked for 15 minutes. The absorbance of the wells was read at 570nm using a microplate reader (BMG Labtech, Germany)[17].

2.4 Evaluation of synergistic effects of 5-FU and Quer

The lowest dose of 5-FU (100µM) was combined with IC50 and two below IC50 concentrations of Que (446,300 and 150 µM) to evaluate the synergistic effect. Following treatment with three combination states for 48h, cell viability was assessed; at the next step, according to these results, the combination indexes (CI) were determined for each combination state using CompuSyn software and applying as an indicator for synergism. Accordingly, CI below, equal, and higher than 1 represents synergism, additive, and antagonism effects, respectively [22]. Dose reduction index (DRI) value is another index for evaluating synergism, which indicates fold-reduction of each drug's dose in combination use compared to its use as a separate agent. The DRI value > 1 is favorable because it denotes a reduction in toxicity despite retention in efficacy[17].

2.5 Flow cytometry analysis

4×10^5 MCF-7 cells were seeded in the wells of six-well plate and treated with 100 µM 5-FU and 446 µM Quer individually or in combination for 48 hours. After the treatment, the percentage of normal and apoptotic cells was examined by utilizing the Annexin V-FITC/propidium iodide assay kit (IQ Products, Groningen, Netherlands), according to the manufacturer's protocol. Briefly, trypsinized cells were rinsed with calcium buffer and resuspended in 100 µl calcium buffer containing 5 µl Annexin V FITC cell and incubated at 4°C for 20min. Afterward, the Annexin V- FITC was discharged, and the cells were incubated in 500 µl calcium buffer with 5 µl propidium iodide at 4°C for 10min and analyzed with a flow cytometer (Becton Dickinson, San Jose, CA, USA). The status of the cell populations was determined according to the annexin V / PI staining pattern. The population of cells with FITC and PI negative patterns were considered as living cells, FITC positive and PI negative cells as early apoptotic cells, FITC and PI-positive cells as late apoptotic cells, and FITC negative and PI-positive cells as necrotic cells. FlowJo v10 software was used for data analysis[23].

2.6 Evaluation of gene expression by real-time PCR

Following treatments with Quer, 5-FU, and their combination, total RNA was isolated from the cells using an RNA extraction kit (Yekta Tajhiz Azma Company, Iran) according to the manufacturer's instructions. Then, cDNA synthesis was performed using the Yekta Tajhiz Azma cDNA kit according to the instructions. Real-time PCR was performed using RealQ Plus 2x Master Mix Green "low Rox" kit (Ampliqon, Denmark) and QuantStudio™ 3 Real-Time PCR System (ABI Applied Biosystems). The reactions were heated to 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. GAPDH was preferred as the internal reference. The sequence of primers are as follow: Bax: forward *5′-AAACTGGTGCTCAAGGCCC-3′*, reverse: *5′- AAAGTAGGAGAGGAGGCCGT-3′*; Bcl2: forward *5′-CCCGCGACTCCTGATTCATT- 3′*, reverse *5′-AGTCTACTTCCTCTGTGATGTTGT-3′*; *GAPDH*: forward *5′-GACAGTCAGCCGCATCTTCT-3′*, reverse *5′-GCCCAATACGACCAAATCCGT-3′* [16].

2.7 Colony formation assay

3×10^3 MCF7 cells were seeded in each well of 6-well plates for 24h in high glucose DMEM medium with 10% fetal bovine *serum* (FBS) and 1% Penicillin /Streptomycin. Then, MCF7 cells were treated with 100 µM 5-FU, 446 µM Quer, and their combination for 48 hours. After that, the media were replaced by complete media without drugs and incubated at 37°C for 10 days to evaluate the formation of colonies. The culture media were replaced every two days. Subsequently, culture media were removed, and the wells were rinsed by PBS and fixed by 3.8% formaldehyde. Colonies were stained with 0.5% crystal violet for 60 minutes. At the next step, each well was washed with tap water and dried at ambient temperature. ImageJ software (version 1.49v) was used to count the number of colonies[16].

2.8 Statistical Analysis

The experiments were repeated three times, and data were shown as Mean±SEM. SPSS 22 software (Chicago, USA) was used for statistical analyses. One-way ANOVA and LSD post hoc tests were used to compare the groups. IC50 values were calculated using Curve Expert 1.3. P-value<0.05 is considered statistically significant. Combination index (CI) and DRI were obtained using CompuSyn software (Combo SynInc, City, State, USA).

3. Results

3.1 Effect of 5-FU and Quer on cell proliferation inhibition

Figure 1 illustrates the cell viability of the MCF7 cells treated with 5-FU and Quer for 48 hours at different concentrations. As shown in Figures 1A and B, 5-FU and Quer decreased the MCF7 viability depending on the dosage. The IC50 values of 5-FU and Quer were 229 μM and 446 μM, respectively. The combination effect of 100 μM 5-FU with 446,300 and 150 μM Quer on MCF7 cells was shown in Figure 1C. All three combinations of 5-FU and Quer compared to 5-FU alone showed a significant decrease in MCF7 cell viability, and the highest decrease in cell viability was in 100 μM 5-FU plus 446 μM Quer. The growth inhibition rate of 100 μM of 5-FU in MCF7 was 2% whereas, following the treatment of 100 μM 5-FU plus 446 μM Quer in MCF7, it reached 71%. According to CI values (Table 1), the three combinations show synergistic effects, and the best of them belongs to the combination of 100 μM 5-FU plus 446 μM Quer; therefore, this combination was selected for the rest of the experiments. The DRI value above 1 (DRI > 1) is favorable and shows a fold change in drug dose reduction. Here, for all the combinations, this value was higher than 1 (Table 1), which indicates that the combinations of 5-FU plus Quer were favorable for luminal breast cancer cells and the best DRI values belong to a combination of 100 μM 5-FU and 446 μM Quer.

3.2 Effect of 5-FU and Quer on apoptosis

As the pharmacodynamic endpoint of cancer therapy, the apoptosis assay was performed to investigate the Quer enhancing effect on 5-FU induced- apoptosis. Figure 2A shows the apoptotic effects of the 5-FU and Quer treatment alone or in a combination in MCF7 cells. Percentages of total apoptosis (early apoptosis plus late apoptosis) following *treatment* with 5-FU, Quer, and their combination were 31%, 30%, and 44%, respectively. Comparing to the 5-FU treatment, 5-FU/ Quer combination significantly ($P < 0.05$) improved apoptosis rate by 1.3 fold (Figure 2B).

3.3 Effect of 5-FU and Quer on Bax and Bcl2 gene expression

Bax and Bcl2 are two key regulators of mitochondrial pathway-induced apoptosis, whose expression in apoptosis increases and decreases, respectively. Therefore, the effects of Que and 5-FU on Bax and Bcl2 gene expression were evaluated alone and in combination. As shown in Figure 3, Bax's relative gene expression following treatment with Que, 5-FU, and Que plus 5-FU was 1.37, 1.84, and 2.48 fold, respectively, and this increase was significant in 5-FU and its combination but not in Que treatment. Bcl2 gene expression significantly decreased in Que, 5-FU, and Que plus 5-FU by 0.76, 0.56, and 0.3 fold,

respectively. According to these results, the Bax / Bcl2 ratio in Que, 5-FU, and Que plus 5-FU treated cells were 1.37, 3.3, and 8.3 fold, respectively. The combination of 5-FU with Que changed the expression of both genes significantly compared with 5-FU alone, which indicates that Que potentiated the 5-FU effect on these genes expression and subsequently apoptosis.

3.4 Effect of 5-FU and Quer on colony formation

Figure 5 shows the MCF7 formed colonies following treatments after 10 days. The percentage of colony formation for 5-FU, Quer, and the combination of 5-FU with Quer was 64, 96, and 45%, respectively. Colony formation significantly decreased in combination state compared with 5-FU alone by 1.4 fold (Figure 4B). As illustrated in Figure 4A, following the 5-FU/ Quer combination treatment, the number of colonies was less than treatment with 5-FU alone.

4. Discussion

Breast cancer is the most common cancer and the major global cause of mortality in women. Despite advancements in cancer therapy, it remains a leading cause of cancer- death worldwide [24]. Although chemotherapy is one of the main cancer treatments, it has significant limitations, including drug resistance and cytotoxicity on normal cells [25]. Even though 5-FU is a chemotherapeutic agent, which is commonly utilized to treat different types of solid tumors[26, 27], the response rate of treatment with 5-FU is still low. The main problem of long-term treatment with 5-FU is its side effects and the resistance of tumor cells to this drug [26]. Hence, there is a need for developing more effective and less cytotoxic chemotherapeutic strategies [27]. In this context, combination therapy can be a potentially viable option. Combining therapy aims to obtain synergistic therapeutic effects, reduce the dose and toxicity, and decrease induction of drug resistance [28]. Combination therapies of flavonoids and conventional chemotherapeutic drugs have been proposed in several studies to enhance therapeutic response in cancer cells and lower the side effects in normal cells [29]. One of the most common flavonoids is Quer which is found in some vegetables and fruits. Several studies have evaluated the anti-cancer activity of Quer and shown its inhibitory effect on tumor growth of various cancer cell lines, including breast, colorectal, head and neck, stomach, lung, melanoma, ovarian, ovarian, and leukemia [30-34]. In this study, the effect of combined 5-FU and Quer on growth inhibition and apoptosis was evaluated in MCF7 breast cancer cell line. To the best of our knowledge, this is the first study to explore the effects of 5-FU and Quer combination in breast cancer. Our results showed that Quer synergistically potentiates the effect of 5-FU on growth inhibition and apoptosis of MCF7 breast cancer cell line.

It was shown that the combination of 5-FU and Quer has a more cytotoxicity effect than individual drugs in MCF7 cells (Figures 1c). The growth inhibition rate of 100 µM 5-FU in MCF7 was 2%, whereas it reached 71%, following treatment with 100 µM 5-FU plus 446 µM Quer. The CI and DRI were calculated using CompuSyn software. The CI represents the quantitative measure of the degree of drug interaction in terms of synergistic, additive, or antagonistic effect at a particular dose [35]. CompuSyn analysis of 5-

FU plus Quer combinations in MCF 7 revealed that CI values of all the combinations were <1, which are indicative of the synergistic effect of the combined 5-FU/Quer, and the best synergistic effect was belong to 100 μM 5-FU and 446 μM Quer combination (Table 1). The DRI values indicate a reduction of the dose of each drug in combined use compared to their use alone. The DRI value > 1 is favorable because it denotes a reduction in toxicity despite the retention of its efficacy [35]. The DRI values of the 5-FU/Quer combination were >1 (~1.7–3.3) (Table 1), so up to 3.3 fold reduction in 5-FU applied dose can be achieved by the combination state. These results are in agreement with the previous studies, which have reported the synergistic effects of the combination of 5-FU with Kaempferol, CHC (α-cyano-4-hydroxycinnamic acid), DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid), Quer, and Tannic acid in colon and cholangiocarcinoma cell lines[36-38].

Our findings showed that the apoptotic effect of the 5-FU/Quer combination (44% apoptotic cells) was significantly more than 5-FU (31% apoptotic cells) and Quer (30% apoptotic cells) alone (Figures 2), so Quer potentiates the 5-FU apoptotic effects on MCF7. The results of this part are in line with the previous studies, which showed the synergistic effects of curcumin and 5-FU in the induction of apoptosis in MCF7 [39]. In accordance with our findings, previous researches have shown that the combination of 5-FU with Quer and melatonin potentiates its effect on growth inhibition and apoptosis in liver and colon cancer cells compared with 5-FU individual treatment [40, 41].

The Bax and Bcl2 gene expression evaluation showed that treatment with Quer, 5-FU, and their combination up- and down-regulate Bax and Bcl2 gene expression, respectively. It indicates that the apoptotic effect of the treatments occurred through modulating these genes, which consistent with flow cytometry results. On the other hand, the combination state significantly increased and decreased the Bax and Bcl2 gene expression, respectively, compared to the 5-FU alone treatment. It shows that Que developed the apoptotic effect of 5-FU by enhancing its effect on the gene expression of Bax and Bcl2. The results are consistent with previous studies showing that the apoptotic effect of Quer and 5-FU occurred by modulating the Bax and Bcl2 gene or protein [4, 5, 13-16].

The colony formation assay results showed that colony numbers decreased by 4%, 36%, and 55% when cells were treated with Quer,5-FU, and their combination, respectively (Figures 4). So the combination state significantly decreased colony numbers by 19% compared to using 5-FU alone, which showed increased effectiveness of 5-FU in combination application. Our results are in agreement with other studies that have shown that the combination of 5-FU with methylglyoxal (MG), Huaier extract, β-Elemene, Metformin, and Glabridin significantly decreased the colony numbers compared to 5-FU in breast, cholangiocarcinoma, colon, and gastric cancer cells [39, 42-44].

Conclusion

In conclusion, it was shown that Quer combined with 5-FU enhanced the apoptotic effects of the chemotherapy agent on breast cancer cells. So at the first step, this combination can be proposed as a new therapeutic strategy, but further animal studies and clinical trials are needed.

Declarations

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Authors' contributions:

Dr. Mojtaba Rashidi: made substantial contributions to the conception and design of the work

Dr. Ghorban Mohammadzadeh: drafted the work and revised it

Dr. Azam Khedri: analysis, interpretation of data

Fatemeh Mawalizadeh: the acquisition, analysis, interpretation of data

Research involving human and/or animal rights: This article does not contain any studies with human participants or animals performed by any of the authors.

Ethics approval code: IR.AJUMS.MEDICINE.REC.1398.029

Consent to participate : all of the Authors declare Consent to participate

Consent for publication: all of the Authors declare Consent for the publication

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Tables

Table.1 Combination index (CI) and Dose reduction index (DRI) values of 5-FU combined with Quer at different concentrations

5-FU(μM)	Quer(μM)		DRI	
		CI	5-FU	Quer
100	150	0.91	2	2
100	300	0.96	2.6	1.7
100	446	0.87	3.3	1.76

Figures

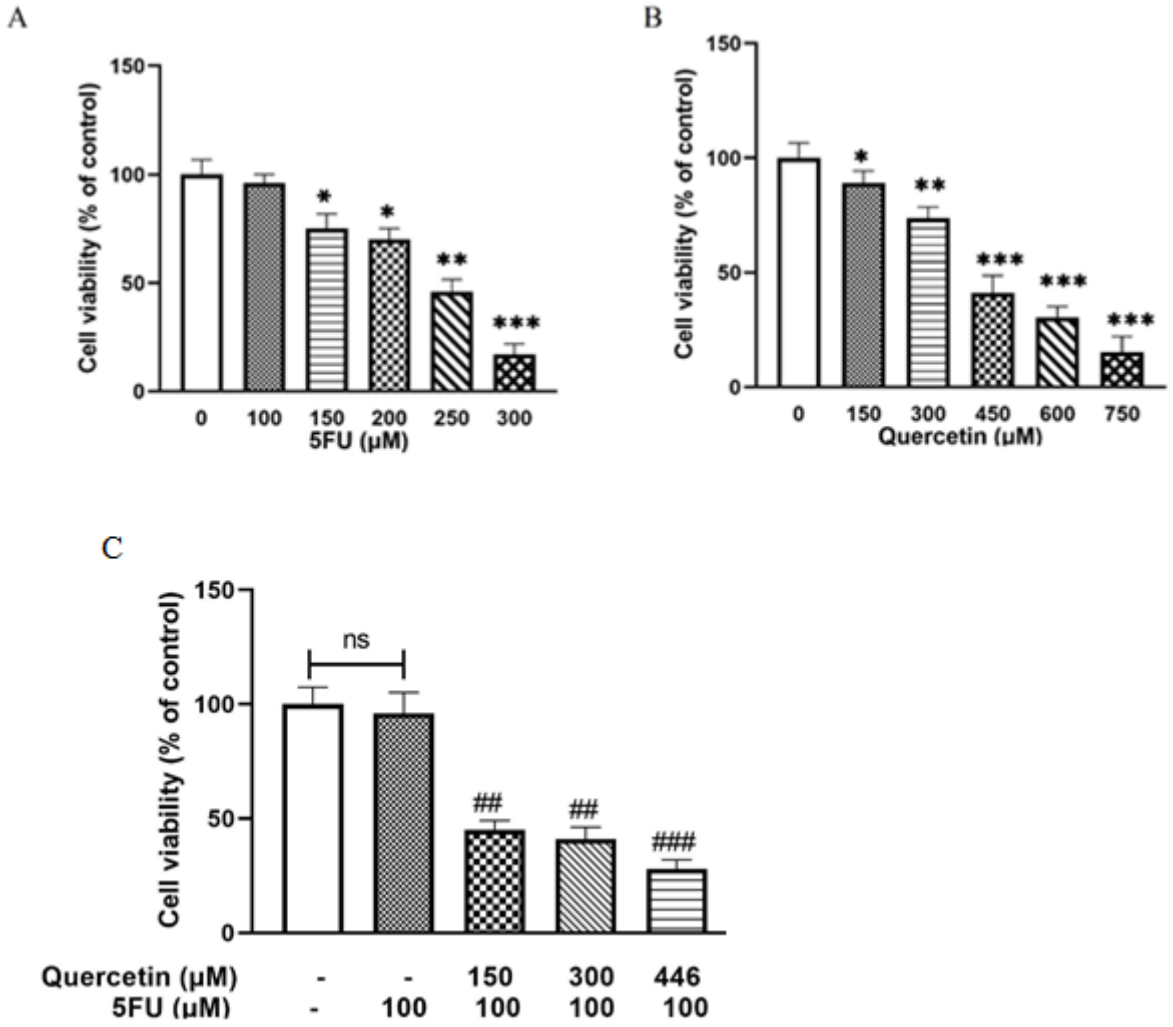
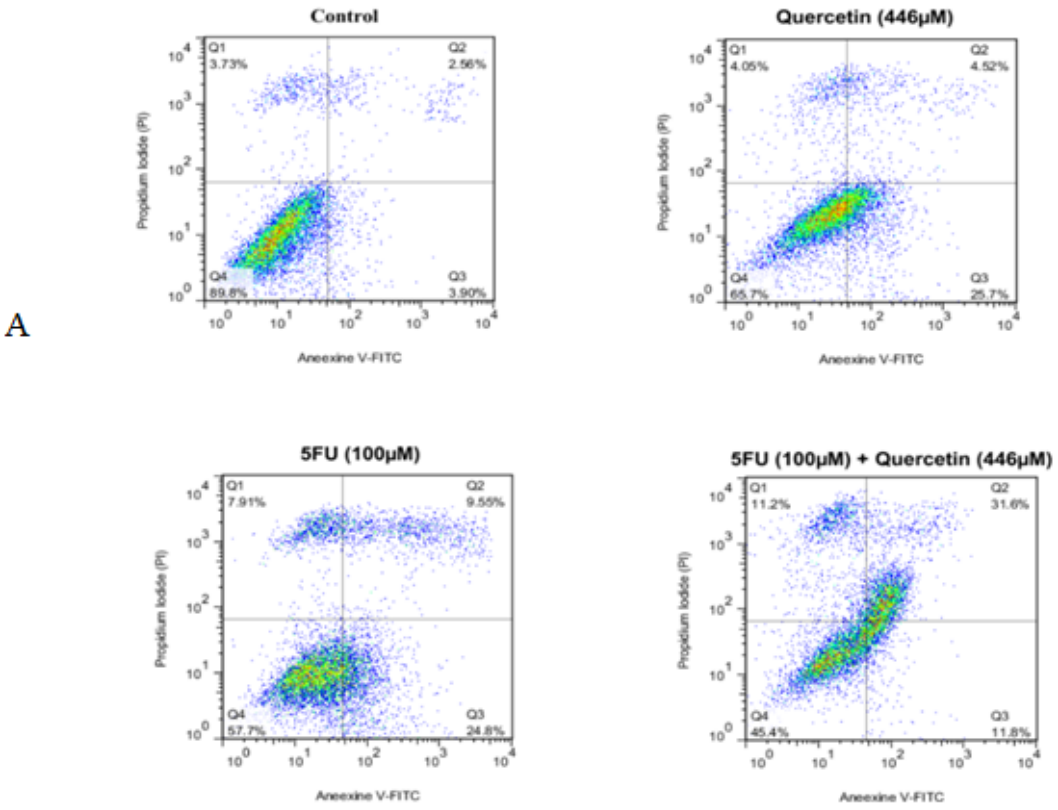


Figure 1

alone and combined effects of Quercetin and 5-FU on cell viability in MCF7 cells after 48h. MTT assay was done to assess cell viability. (A) MCF7 cells treated with different concentrations of 5-FU. (B) MCF7 cells treated with different concentrations of Quercetin. (c) MCF7 cells were exposed to alone and combined concentrations of 5-FU and Quercetin. The results are given as mean± SEM of three independent experiments. *p<0.05, **p<0.01, ***p<0.001 significant compared with the control. #p<0.05, ##p<0.01, and ###p<0.001 significant from 5-FU-alone treated cells



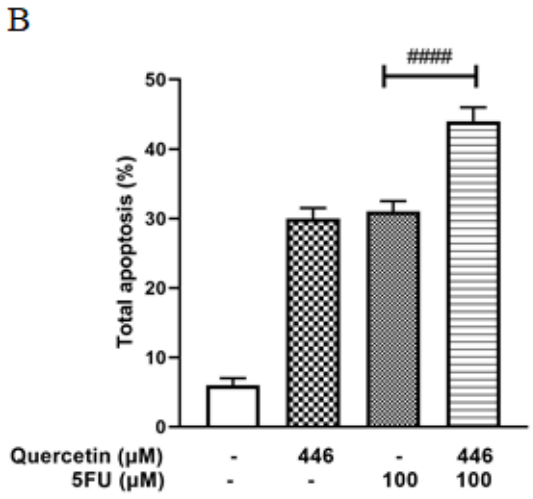


Figure 2

the effect of 5-FU and Quercetin on apoptosis in MCF7 cells for 48h. flow cytometry was performed to evaluate apoptosis. (A) Dot-plots of flow cytometry analysis demonstrate the apoptotic status of MCF7 cells. (B) Total percentage of apoptosis in MCF7 cells treated with 5-FU (100 µM) and Quercetin (446 µM) alone or in a combination. The results are presented as mean± SEM of three independent experiments. ###P<0.001 significant compared with 5-FU alone

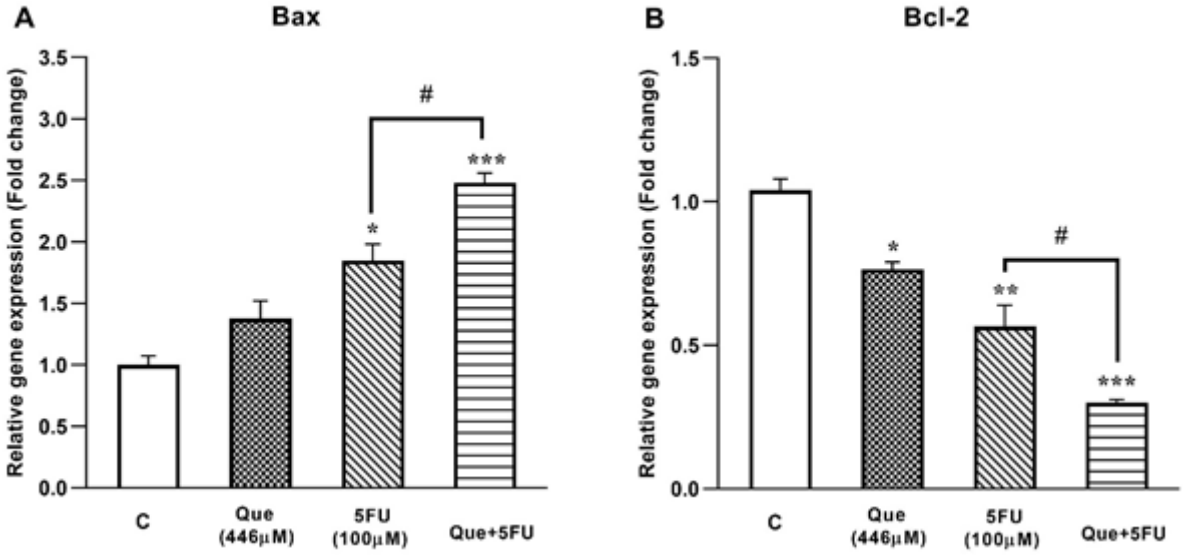


Figure 3

Bax and Bcl2 gene expression in MCF7 cell line. the quantitative real-time PCR assay was done to assess gene expression. (A) Relative Bax mRNA expression following Que, 5-FU, and their combination treatment for 24h. (B) Relative Bcl2 mRNA expression following Que, 5-FU, and their combination treatment for 24h. Results are the mean ± SEM of two independent experiments and expressed as fold changes in mRNA expression. GAPDH was used as the reference gene. *p <0.05, **P < 0.01, and ***P < 0.001 vs. control untreated group; #p<0.05 vs. 5-FU-alone treated group

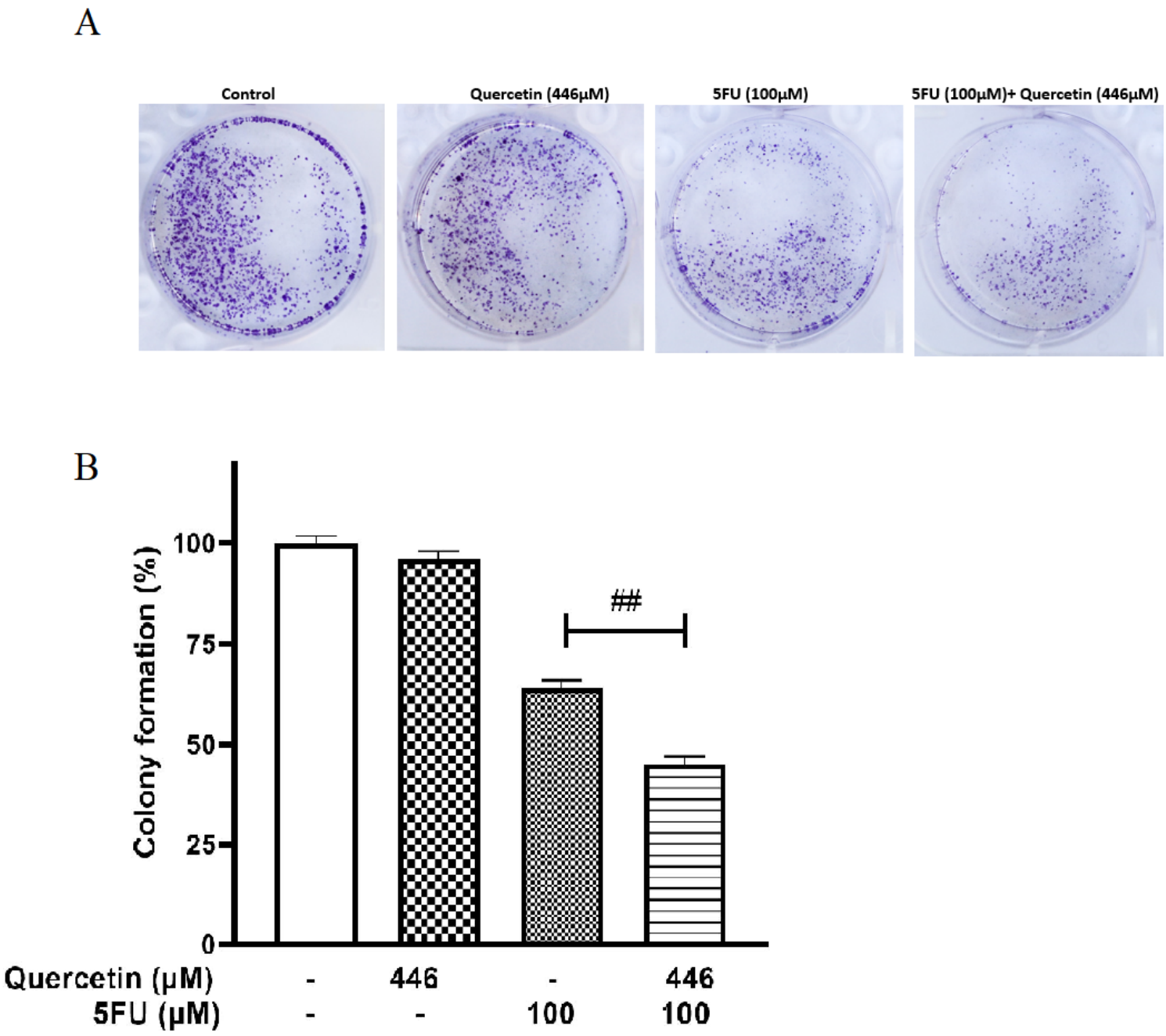


Figure 4

the effects of 5-FU and Quercetin on colony formation in MCF7 cells for 48h. colony formation assay was done to assess cellular clonogenic potential following treatments. (A) Colony formation image following treatment with 5-FU (100 µM), Quer (446 µM), and a combination of them. (B) Histogram plots of colony formation percentage. Results are presented as mean± SEM of three independent experiments. The combination compared with 5-FU alone ##p<0.001

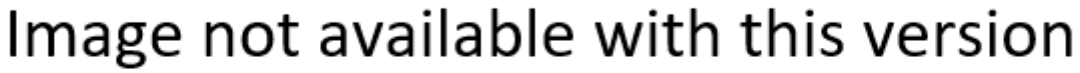


Figure 5

the MCF7 formed colonies treatments after 10 days. The percentage of colony formation for 5-FU, Quer, and the combination of 5-FU with Quer was 64, 96, and 45%, respectively.