RESEARCH ARTICLE

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Quercetin Effects on Cell Cycle Arrest and Apoptosis and Doxorubicin Activity in T47D Cancer Stem Cells

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Abstract

Backgrounds: Targeting breast cancer stem cells with the CD44+/CD24- phenotype is critical for complete eradication of cancer cells due to its Self-renewal, differentiation, and therapeutic resistance ability. Quercetin is a popular flavonoid with lower adverse effects and has anti-tumor properties. Therefore, we assessed the anticancer activity of Quercetin and Doxorubicin alone and in combination in the T47D cells of human breast cancer and their isolated Cancer stem cells (CSCs). **Materials and Methods:** The human breast cancer cell line T47D was used for this experiment. T47D CSCs were isolated by magnetic bead sorting using the MACS system. The anticancer activity of Quercetin and Doxorubicin alone and in combination were evaluated using MTT cytotoxicity assay and cell cycle distribution and apoptosis induction by flow cytometry analysis. **Results:** We have shown that almost 1% of T47D cell populations are made up of CD44+/CD24- cells, which considered as cancer stem cells. Quercetin and Doxorubicin alone or in combination inhibited cell proliferation and induced apoptosis in breast cancer T47D cells and in lower extent in CD44+/CD24- cells. Quercetin significantly strengthened Doxorubicin's cytotoxicity and apoptosis induction in both cell populations. Quercetin and Doxorubicin and their combination induced G2/M arrest in the T47D cells and to a lesser extent in isolated CSCs. A value of $p < 0.05$ was considered as indicating a statistically significant difference. **Conclusion:** These outcomes suggested that CSCs are a minor population of cancer cells, which play a significant role in drug resistance by being quiescent, slow cycling and resistance to apoptosis. Furthermore, our data showed that adding Quercetin to Doxorubicin is an effective approach for the treatment of both CSCs and bulk tumor cells.

Keywords: Breast cancer cells- cancer stem cells- quercetin- doxorubicin- apoptosis- cell cycle- drug resistance

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Introduction

Breast cancer is the most common malignancy and the second cause of cancer deaths among women. There is an increasing occurrence of breast cancer in women worldwide (Barroso et al., 2020; Li et al., 2016). Establishment of novel strategies to improve the cure rate requires an understanding of the molecular biology of breast cancer cells. Recent discoveries have shown the existence of breast cancer stem cells (CSCs), which play a major role in carcinogenesis, metastases and resistance to various methods of treatments, including radiotherapy and chemotherapy (Butti et al., 2019). The CSC model indicates that malignancies are caused by a scarce fraction of cancer cells that have the ability to initiate, advance and sustain tumor growth (Butti et al., 2019).

Stem cells, which are a minor and distinguished cell population, have two main exclusivities: self-renewal and producing of differentiated cells (Ji et al., 2019). If the regulation of these capabilities of stem cells is impaired, normal stem cells can mutate and convert to cancer stem cells (Papaccio et al., 2017). CSCs are a small percentage of tumor cells and are relatively quiet with a slow rate of cycling, capable of tumor development and resistant to most chemotherapeutic agents by expressing anti-apoptotic and drug-resistant genes. CSCs should be specifically targeted in order to remove a tumor and prevent relapses (Taurin and Alkhalifa, 2020; Trosko, 2021).

CSCs can be identified by the expression of specific cell surface markers. High CD44 expression and no or low CD24 expression is the surface phenotypic index

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for identifying the breast CSCs (Klonisch et al., 2008). These CD44+/CD24-/low cells can be differentiated into mature differentiated cancer cells and are associated with the enhanced possibility of metastases and invasion in human breast cancer. Transplantation of these CSCs into NOD/SCID mice can cause carcinogenesis (Ji et al., 2019).

Many dietary phytochemicals, in particular the natural flavonoids, have been recognized to have chemopreventive and chemotherapeutic activities in many types of tumors (Kashyap et al., 2019; Erdogan et al., 2018; Selvakumar et al., 2020; Lamoral-Theys et al., 2010; Shakeri et al., 2020; Sotoodeh Jahromi et al., 2021). Quercetin (Quer), 3, 3′, 4′, 5, 7-pentahydroxylflavone, is a flavone-3ol-class of flavonoid, which exists in most edible vegetables and fruits, including tea, red grapes, onion, apples, broccoli and berries. It exhibits antioxidative, anti-tumor, anti-inflammatory, vasodilating, anti-mutagenic and chemopreventive effects, and has been proposed to be a potential anti-cancer agent with the advantage of low adverse effects (Hashemzaei et al., 2017). This anti-cancer activity of quercetin was described in several human cancer cell lines, including HL60 leukemia, glioma, breast, osteosarcoma, colon, cervical, oral cavity, prostate, myeloid leukemia and hepatoma cancer cells (Chen et al., 2010; Tang et al., 2020; Atashpour et al., 2015; Torello et al., 2021).

Quercetin's anti-tumor effects have been attributed by different mechanisms including anti-oxidative activity, anti-proliferative activity, inhibition of activating carcinogens enzymes, amendment of signal transduction pathways, apoptosis induction, cell cycle arrest in G1 or G2 phase, inhibition of angiogenesis by downregulating the expression of oncogenes and mutant p53 or up-regulating cell cycle control proteins (p21WAF1 and p27KIP1) 9Zhao et al., 2019; Murakami et al., 2008; Haghi et al ., 2017). Quercetin can also induce apoptosis through altering the pro-apoptotic (Bax) and antiapoptotic (Bcl-2) gene expression, releasing cytochrome c and activating caspase-9 and caspase-3 (Ong et al., 2004; Alam et al., 2021). These effects of quercetin are mainly seen in cancer cell lines but not in normal cells (Erdogan et al.,2018; Clemente-Soto et al ., 2019).

Interestingly, quercetin can function as a pump inhibitor by reducing the expression and competitively inhibiting MRP1, BCRP, P-gp and the metabolizing enzyme CYP3A4 and it could improve the bioavailability of drugs (Chen et al., 2010). Therefore, quercetin has the ability to reverse multi-drug resistance in several cancer models and the co-administration of quercetin with chemotherapeutic agents may show enhanced anticancer effect and this effect can be seen in the nontoxic concentration of quercetin (Chen et al., 2010). Doxorubicin (Dox) is commonly used in the treatment of diverse human cancers, including breast cancer, as part of a chemotherapy regimen. Dox can cause DNA damage, growth arrest and cell death through DNA intercalation, topoisomerase II inhibition and free radical formation (Carvalho et al., 2009). Hence it could be suggested that co-administration of quercetin with doxorubicin may alter the activity and toxicity of doxorubicin in the treatment of cancer (Du et al., 2010).

Therefore, due to the critical role of CSCs and complete eradication of cancer cells needs to target this small fraction of tumor cells, the aim of this study was to evaluate the effects of quercetin and doxorubicin alone and in combination on cellular proliferation, cell-cycle progress and apoptosis against breast cancer T47D cells and CD44+/CD24- CSCs.

Materials and Methods

Materials

Roswell Park Memorial Institute (RPMI-1640) Medium and Fetal bovine serum (FBS) were purchased from Biosera (UK). Pen-strep and trypsin- EDTA were purchased from Gibco (UK). MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide), propidium iodide (PI), Annexin V-FITC and Quercetin were obtained from sigma (Germany). DAPI (4, 6-diamidine-2-phenylindole) and Nonidet P40 were purchased from Roche (Germany). Doxorubicin was purchased from Ebewe Pharma (Austria). CD24 and CD44 isolation kit, LD separator column, CD24 and CD44 antibodies were obtained from MiltenyiBiotec GmbH.

Cells and Cell Culture

The human breast cancer cell line T47D (purchased from National Cell Bank of Iran, Pasteur Institute of Iran, Tehran, Iran), were grown in RPMI-1640 medium supplemented with 10% heat-inactivated (50°C, 30 minutes) fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 µg/mL) at 5% CO_2 and 95% air in a humidified 37°C incubator.

Drug preparation

Quercetin was initially solubilized in dimethylsulfoxide (DMSO) at a concentration of 10 mM, stored at 4°C and protected from the light. Then, different concentrations of Quercetin (Quer) were freshly prepared in complete culture medium before use and added to the cells in different experiments. In all experiments, DMSO concentration never exceeded 1% that has no effect on T47D cells. Doxorubicin (Dox) (20 mg/10ml) was diluted in complete culture medium freshly before use and added to the cells at different concentrations.

Magnetic cell sorting (MACS) and flow cytometry

CSCs were isolated by magnetic bead sorting using the MACS system (Miltenyi Biotech). Cells were centrifuged at 300g for 10 min, triturated with a fire-narrowed Pasteur pipette, and resuspended in 0.35 ml PBS with 0.5% BSA and 2 mM EDTA. Cells were then incubated with a monoclonal CD24 antibody labeled with MicroBeads (Miltenyi Biotech) for 15 min at 4°C. CD24- cells were enriched using a QuadroMACS magnet and LS columns (Miltenyi Biotech). Then, the CD24- cells were incubated with CD44 microbead for 15 min at 4°C and CD44+ cells were isolated like CD24- cells. All MACS procedures were performed according to the manufacturer's instructions (He et al., 2011; Sheng et al., 2013). The purity of isolated CD44+/CD24- cells was evaluated by flow cytometry with specific CD44-FITC and CD24-PE antibody.

MTT Cytotoxicity Assay

The proliferation of T47D cells and isolated CSCs was determined using the colorimetric MTT assay. Briefly, 5000 cells per well were seeded in 96-well plates. After 48h, culture media was removed and the cells were treated with Dox and Quer alone and in combination at different concentrations and time points. Then, MTT solution (4 mg/ml in phosphate-buffered saline (PBS)) was added to each well. After 3h incubation at 37°C at 5% CO_2, DMSO was added to each well to dissolve the formazan crystals, and the absorbance of each well was read at 540 nm using a microplate reader (Sunrise, Tecan, Switzerland). The results are presented as a percentage to the control RPMI. Drug concentrations that inhibited cell proliferation to 50% of the control RPMI (IC_{50}) were determined from at least three independent experiments in quadruplicate format for each treatment (Bazargan et al., 2008).

Cell cycle analysis

DAPI staining was used to determine the distribution of cells in different phases of the cell cycle by flow cytometry analysis. Briefly, cells were seeded into 6-well plates at a density of 2.5×10^5 cells/well. The cells were treated with IC_{50} of quercetin, Dox and the combination (which was previously determined). $5x10^5$ Treated and untreated cells/ml were trypsinized, resuspended in DAPI staining solution at 4oC for 30 min in the dark and analyzed by Partec flow cytometer using UV light at FL4 then data analysis was done using FloMax software. The percentage of cells at the different phases was specified and statistical analysis of data from flow cytometric experiments was performed (Czerwonka et al., 2020).

Apoptosis analysis

Apoptotic cells were determined using Annexin V-FITC and PI dual staining and flow cytometry analysis. Briefly, cells were seeded into 6-well plates at a density of 2.5×105 cells/well. The cells were treated with IC_{50} of Quercetin and Dox alone and in combination for 48 h. Cells were collected by trypsinization and stained with Annexin V-FITC and PI for 15min at 4◦C in dark. Finally, stained cells were analyzed for the percentage of apoptotic cells by Partec flow cytometer and FloMax software (Atashpour et al., 2015).

Statistical analysis

All experiments were repeated at least three times and data were presented as the mean ± SE. Data sets were analyzed by one-way analysis of variance (ANOVA) followed by Tukey post hoc test. A value of $p < 0.05$ was considered as indicating a statistically significant difference.

Results

Isolation of CD44+/CD24- cancer stem cells of T47D cells by MACs

T47D breast cancer stem cells were identified and isolated by using the surface marker CD44, which is highly expressed by T47D CSCs and CD24 that has no/low expression in T47D CSCs (Koch et al., 2020). Flowcytometry analysis showed the presence of a scarce (˷1%) CSC population (Figure 1a). After isolation of CSCs by MACS, we assessed the purity of isolated CSCs by using a specific antibody against CD24 and CD44 with flow cytometry that showed 94% purity (Figure 1b).

Effects of different treatments on Cell Viability

Cell viability is a measure of the proportion of living, healthy cells within a population. Cell viability assays are used to determine the overall status of cells, to optimize culture or experimental conditions, and to measure cell survival after treatment with compounds. The effect of different treatments on cell viability/proliferation in T47D cell line was determined by MTT assay. Cell proliferation was significantly decreased following treatment with

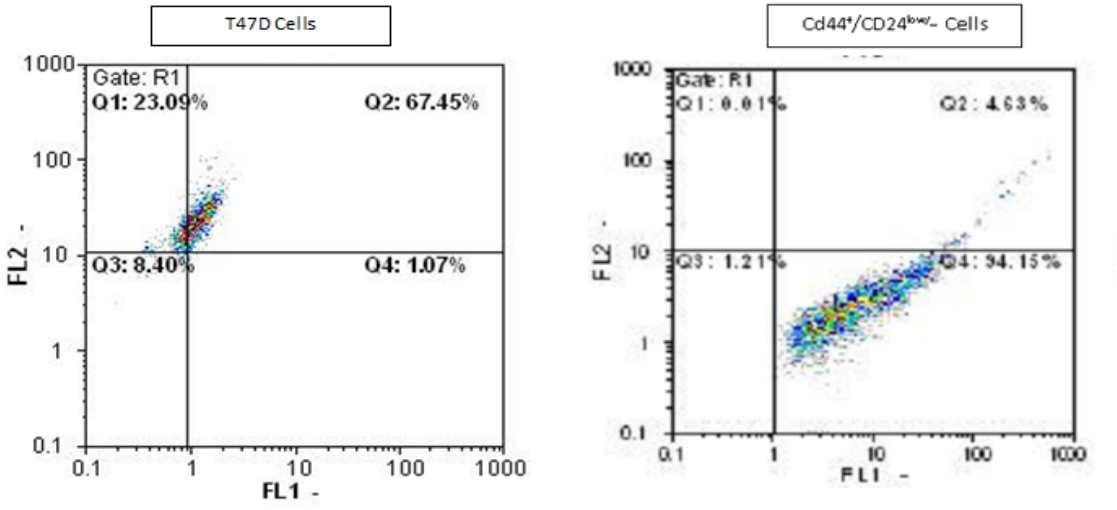

Figure 1. Isolation of CD44+/CD24- CSCs from T47D Cancer Cells by MACs. The CD44+/CD24- CSCs of T47D Cancer Cells were Isolated by CD24 and CD44 Magnetic beads Using the Miltenyi's MACS System. The parental T47D cancer cells (A) and its isolated CD44+/CD24- CSCs (B) were subjected to immunostaining with anti-CD44-FITC and anti-CD24-PE antibodies and analyzed by flow cytometry to determine the percentage of CD44+/CD24- cells within each population of T47D cells.

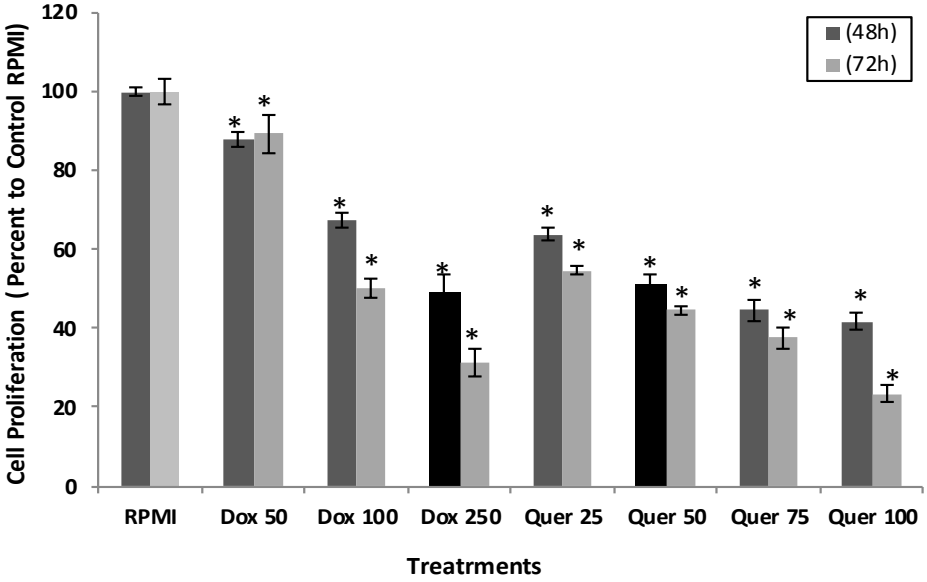

Figure 2. Cytotoxic Effects of Quercetin and Doxorubicin on T47D Cells. The T47D cells were treated with different concentrations of Doxorubicin and Quercetin alone to determine cell proliferation using MTT assay. The results were expressed as mean±SE of three independent experiments in quadruplicates layout for each concentration. *denotes p<0.001 for significant difference between treatments in comparison to control RPMI.

Doxorubicin or Quercetin in a concentration- and time-dependent manner in T47D cell lines (Figure 2). The IC_{50} for Dox and Quercetin were determined to be 250nM and 50µM after 48h treatment of T47D cells, respectively (P value < 0.05).

Effects of Quercetin on cytotoxicity of Dox in T47D cancer cells

The effect of Quercetin on the cytotoxicity of Doxorubicin in T47D cancer cells was established using MTT assay. Co-treatment of T47D cells with Quercetin and Doxorubicin resulted in significant sensitization of these cells to Dox chemotherapy (Figure 3). Quercetin at low concentration (25µM) significantly reduced the IC_{50} of Dox from 250nM to 50nM in T47D cancer cells (P value < 0.05).

Cytotoxic effects of treatments on isolated CD44+/CD24- CSCs of T47D cancer cells

The cytotoxic effect of treatments on CD44+/CD24- T47D CSCs was assessed by using IC_{50} concentration of the treatment drugs and MTT assay. The CSC population of T47D cells showed resistance to different treatments compared to parental T47D cells. The IC_{50} of Dox and

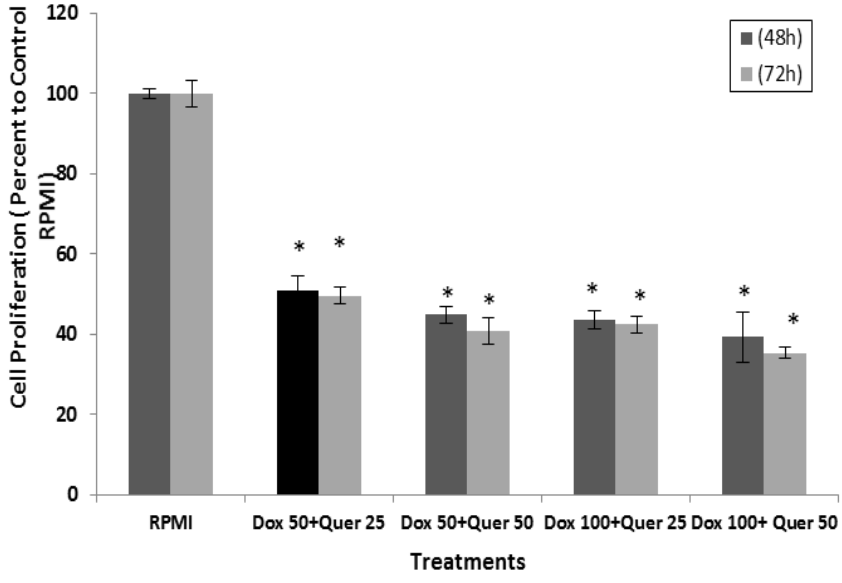

Figure 3. Effects of Quercetin on Doxorubicin Cytotoxicity in T47D Cells. The T47D cells were co-treated with different concentrations of Quercetin and Doxorubicin to determine cell proliferation using MTT assay. The results were expressed as mean±SE of three independent experiments in quadruplicates layout for each concentration. *denotes p<0.001 for significant difference between treatments in comparison to control RPMI.

Quercetin alone that determined on parental T47D cancer cells showed 23 and 28 percent reductions in cell proliferation in CD44+/CD24- CSCs, respectively (P value < 0.05). Importantly, the combination of Quercetin and Doxorubicin at low concentration was more effective in inhibiting the CSCs proliferation (36 percent) in comparison to Quercetin and Dox alone at their IC_{50} (Figure 4). Results of MTT assay showed that the IC_{50} of Dox and Quercetin alone had significantly lower cytotoxic effects on the isolated CD44+/CD24- CSCs in comparison with the parental T47D cancer cells.

Effects of treatments on cell cycle distribution of T47D cells and its CD44+/CD24- CSCs

In order to evaluate whether the cytotoxic effect of Quercetin and Doxorubicin is due to cell cycle arrest, effects of Doxorubicin and Quercetin alone and in combination on cell cycle distribution were analyzed following DAPI staining and flowcytometric method. In T47D cell line, approximately, 62.04% of control RPMI cells were in G0/G1 phase, 10.4% in S phase and 27.4% in G2/M phase (Figure 5-A). Flow cytometry analysis showed that Doxorubicin treatment at IC_{50} induced an accumulation of T47D cells in G2/M phase (66.26%) compared to the control cells. Quercetin-treated cells exhibited accumulation of the cells in G2/M phase and the G2/M cells accumulation increased from 27% in control RPMI to 54.7% for 50µM Quercetin in T47D cells. Importantly, similar level of G2/M arrest (64.1%) was observed in T47D cells treated with combination of Doxorubicin and Quercetin at much lower concentrations than their IC_{50} (Figure 5-A) (P value < 0.05).

Interestingly, the pattern of cell cycle distribution of the isolated CD44+/CD24- CSCs showed a significantly higher percentage of cells at G0/G1 (79%) compared to parental T47D cells (62%) in control (RPMI) condition (Figure 5A and B). This further indicated that CSCs were more in the quiescent phase and therefore, less responsive to Dox treatment at IC_{50}, which resulted in significantly less accumulation of CSCs in G2/M phase (43.69%) in comparison to parental T47D cells (66.26%). Similar results for G2/M arrest (41.77%) were observed following Quercetin treatment at IC_{50} or combination of Dox and Quercetin (54.48%) at much lower concentrations than their IC_{50} (Figure 5B) (P value < 0.05).

Effects of treatments on apoptosis induction in T47D cells and its CD44+/CD24- CSCs

To evaluate whether the treatment inhibited cell proliferation was associated with the induction of apoptosis, T47D cells and isolated CD44+/CD24- CSCs were treated with IC_{50} of Dox and Quercetin alone and in combination and the percentage of apoptotic cells was assessed using the Annexin V-FITC and PI dual staining by flow cytometry. Results of flow cytometry analysis showed the percentage of necrotic (Q1: AnnexinV-FITC_/PI+), late apoptotic (Q2: AnnexinV-FITC+/PI+), viable (Q3: AnnexinV-FITC_/PI_), and early apoptotic (Q4: AnnexinV-FITC+/PI_) cells. Apoptotic cells were counted as late(Q2) and early (Q4) apoptotic cells; the sum of

Table 1. Apoptosis Induction in T47D Cell Line and Isolated CD44+/CD24- CSCs. The T47D Cells and Its Isolated CD44+/CD24- CSCs were Treated with Doxorubicin and Quercetin alone and in Combination for 48h to Evaluate Induction of Apoptosis Using Annexin V-FITC/PI double-staining by flow cytometry. The percentage of early and late apoptotic cells were presented for each treatment group. Data are presented as means ± SE of three independent experiments. *denotes p<0.05 and ** denotes p<0.001 for significant difference between treatments in comparison to control RPMI.

Group	T47D cells	T47D cells	T47D cells	T47D cells	Isolated CD44+/ CD24-/low cells	Isolated CD44+/ CD24-/low cells	Isolated CD44+/ CD24-/low cells	Isolated CD44+/ CD24-/low cells
	Q1%	Q2%	Q3%	Q4%	Q1%	Q2%	Q3%	Q4%
RPMI	1.43± 0.25	0.31±0.21	97.8±1.1	0.45±0.2	1.33±0.1	0.07±0.32	98.46±0.53	0.14±0.53
Doxorubicin 250nM	2.01±01**	19.4±0.5**	45±0.9**	33.59±1.1**	6±0.3**	9.26±0.26**	73.31±1.5**	11.43±1.5**
Quercetin 50µM	1.7±0.15	25.2±0.54**	41.3±0.1**	31.8±0.2**	2.2±0.2**	9.74±0.25**	70.6±1.5**	16.46±1.23**
Doxorubicin 50nM + Quercetin 25µM	5.2±0.7**	8.7±0.62**	21.89±0.85**	64.33±0.57**	0.76±0.65*	9.42±0.51**	68.14±2**	21.68±1.5**

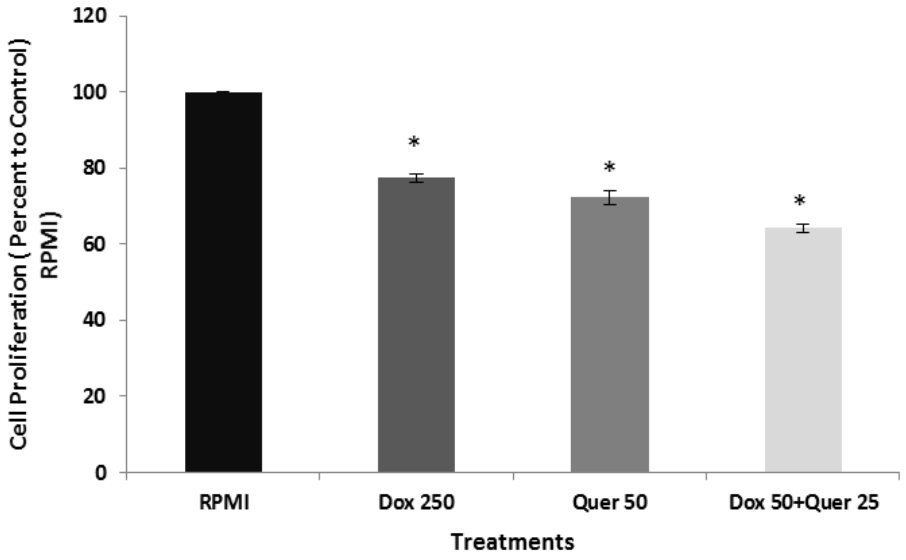

Figure 4. Cytotoxic Effects of Quercetin and Doxorubicin on Isolated CD44+/CD24- CSCs. The CD44+/CD24- CSCs were treated with IC_{50} of Doxorubicin and Quercetin alone and in Combination for 48h to determine cell proliferation using MTT assay. The results were expressed as mean±SE of three independent experiments in quadruplicates layout for each concentration. *denotes p<0.001 for significant difference between treatments in comparison to control RPMI

the percentage of early (Q4) and late (Q2) apoptotic cells in T47D cells treated with IC_{50} of Dox (53.11%) and Quercetin (57.02%) alone and in combination (73.31%) were significantly greater than that of the control (RPMI) treated (0.76%) cells (Figure 6A). In the isolated CD44+/CD24- CSCs, Dox (20.7%) and Quercetin (26.2%) alone and in combination (31%) induced apoptosis but at a significantly lower percentage than that observed in the parental T47D cells (P value < 0.05) (Figure 6B). As shown in figure 6B, quercetin and combination of quercetin with doxorubicin can increase the number of apoptotic cells more effectively than doxorubicin. The apoptosis data confirmed that the CSCs are more resistant to the treatment than parent T47D cells.

Discussion

Breast cancer is the most common malignant tumor in women and, in contrast to early detection, is the second most common cause of cancer death in women (BarrosoSousa et al., 2020). A new model of tumor biology assumes that cancer growth is triggered by stem-like cells within the tumor. Cancer stem cells have the ability of self-renewal and pluripotent differentiation, and show high ability in the regeneration of new tumor masses (Trosko, 2021). Cancer stem cells play an important role in the development, metastasis, and recovery of cancer, and cancer recurrence is a major cause of cancer treatment failure. CSC is more resistant to radiotherapy and chemotherapy (Ji et al., 2019; Cho and Kim, 2020).

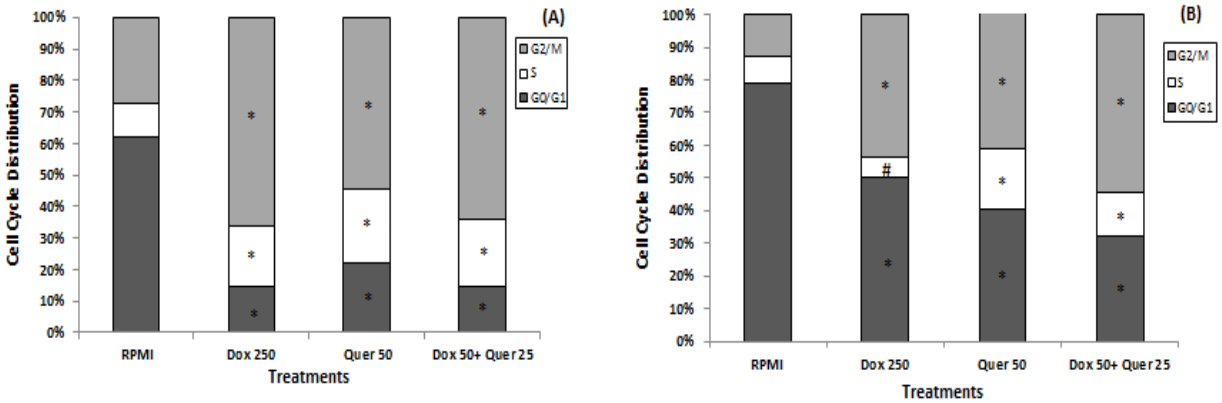

Figure 5. Cell Cycle Alteration in T47D Cell Line and Isolated CD44+/CD24- CSCs. The T47D cells (A) and its isolated CD44+/CD24- CSCs (B) were treated with Doxorubicin and Quercetin alone and in combination for 48h to determine cell cycle distribution pattern using DAPI staining by flow cytometry analysis. Data are presented as the mean±SE of three independent experiments. * denotes p<0.05 for significant difference between treatments in comparison to control RPMI.

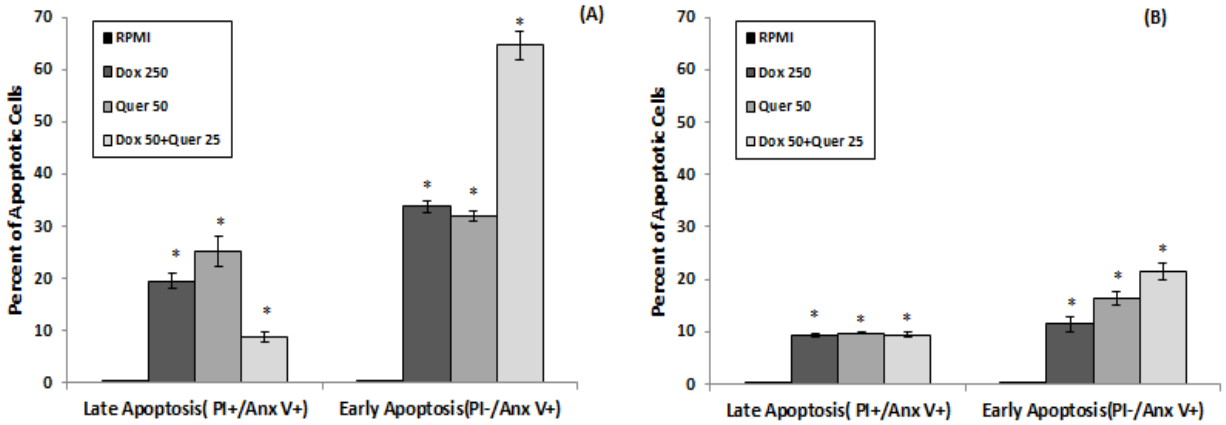

Figure 6. Apoptosis Induction in T47D Cell Line and Isolated CD44+/CD24- CSCs. The T47D cells (A) and its isolated CD44+/CD24- CSCs (B) were treated with Doxorubicin and Quercetin alone and in combination for 48h to evaluate induction of apoptosis using Annexin V-FITC/PI double-staining by flow cytometry. The percentage of early and late apoptotic cells were presented for each treatment group. Data are presented as means ± SE of three independent experiments. *denotes $p<0.001$ for significant difference between treatments in comparison to control RPMI.

Flavonoids are phytochemicals found in large amounts in edible vegetables, especially tea, yellow onions, cocoa, leeks, soybeans, and blueberries. Flavonoids have antioxidant properties and are traditionally used to treat and prevent cancer (Selvakumar al., 2020).

Over the past few decades, many studies have demonstrated the antiproliferative and cytotoxic effects of flavonoids, including quercetin, on various cancer cells (White et al., 2012; Lee et al., 2015). The inhibitory properties of flavonoids on cancer cells depend on various mechanisms of action, such as induction of apoptosis, cell cycle arrest, changes in the expression level of Bcl2, Bax, P53, and caspase families, and regulation of tumor suppressor gene and oncogene expression (Ezzati et al., 2020).

In this report, the effects of quercetin and doxorubicin on viability, cell cycle and apoptosis of human breast Cancer T47D and isolated CSCs were analyzed and compared. Dox is an important anthracycline chemotherapeutic agent and its mechanisms of action include DNA and protein destruction through DNA intercalation, topoisomerase II inhibition and free radical emergence, which cause cell death or growth arrest (Carvalho et al., 2009).

The cell surface marker CD44+/CD24_ is considered as CSC marker for breast cancer (Klonisch et al., 2008; Hadnagy et al., 2006). We found that CD44+/CD24- cells accounted for approximately 1% of the T47D cells population. Evidence demonstrated that the breast cancer stem cells with the phenotype of CD44+/CD24− are a very small subpopulation of breast cancer cells (1%) but have a high self-renewal capacity, strong tumor initiation potential, are insensitive and immune to traditional chemotherapy, and may cause carcinogenesis after transplantation into NOD/SCID mice. This typicality makes CSCs major targets for anticancer treatment (Klonisch et al., 2008; Taurin and Alkhalifa, 2020; Trosko, 2021).

Quercetin reduced the viability of T47D in a concentration and time-dependent manner with IC_{50} of approximately 50µM quercetin for 48 h in T47D cells. The IC_{50} of Doxorubicin was 250nM for 48 h in T47D. When they used in combination, lower concentrations of Dox and quercetin were needed to induce death in 50% of the cell population, which shows quercetin can increase the susceptibility of cancer cells to the cyctotoxicity effect of Dox. Therefore, Quercetin and Dox may have synergistic effects that enhance the anticancer effects as well as reduce the side effects of Dox chemotherapy on normal cells. Our results illustrate that Quercetin can enhance the efficacy of Dox chemotherapy as well as reducing its side effects on normal cells by lowering the IC_{50} of Dox. Rostamzadeh Khameneh et al., also demonstrated that natural agents like curcumin could act as chemosensitizer agents along with Doxorubicin and increase Doxorubicin efficacy (Rostamzadeh Khamenehet al., 2019). However, the results of this study showed that IC_{50} of Dox and quercetin significantly had lower cytotoxic effect on CD44+/CD24- cells. Our results were consistent with other reports of resistance to CSC to chemotherapy (Ji et al., 2019; Papaccio et al., 2017; Atashpour el at., 2015). The mechanisms involved in chemotherapy resistance are not fully understood; very likely due to enhanced expression of anti-apoptotic proteins and thus resistance to apoptotic stimuli by CSCs (Chen et al., 2010), CSCs are quiescent or slowly replicate and therefore more resistant to apoptosis (Signore et al., 2013) and CSCs express high levels of different members of ATP-binding cassette (ABC) transporters, which are responsible for cancer stem cell resistance (Cho and Kim, 2020).

We found that the percentage of CD44+/CD24−cells at G0/G1 phase was notably higher than that of T47D parent cells, which confirmed that CSCs are slow cycling and quiescent. Most common chemotherapeutic agents are targeted at rapidly dividing cells within the tumor, but likely have minor impact on the slowly cycling CSCs and, thus, long-term and permanent cures may not occur (Ji et

al., 2019). The inhibition of cell growth by quercetin may be due to cell cycle arrest. Thus, we examined cell cycle distributions in quercetin-, Dox, and Dox+Quercetin-treated T47D Cells. Cell cycle analysis revealed that quercetin induced G2/M phase arrest in T47D cells. The accumulation of cells at G2/M by quercetin has previously been described in various cancer cells; This G2/M phase arrest was explained by increased expression of cyclin B and reduced levels of the cyclin D, E2F1, cyclin E, and E2F2 in quercetin-treated histiocytic lymphoma U937 cells (Atashpour et al., 2015; Lee et al.,2006). Moreover, T47D cells treated with Dox and combination of Dox with quercetin were associated with G2/M delayed cell cycle.

Furthermore, Dox and Quercetin induced G2/M arrest in isolated CSCs of T47D; however, the share of accumulated cells at G2/M phase in CSCs was significantly lower than that in T47D parent cells. In CSCs, the key population is at G0/G1 phase (quiescent phase); therefore, traditional chemotherapeutic drugs that eradicate differentiated tumor cells have lower effects on CSCs.

Apoptosis is a type of programmed cell death that plays a fundamental role in the regulating homeostasis and the physiological regulation of development. The deficit of apoptosis plays a key role in tumorigenesis, progression and chemoresistance. A possible approach to treat cancer is to activate apoptosis in tumor cells (Erdogan et al., 2018; Haghi et al., 2017). Quercetin reduced the number of viable cells and induced G2/M phase arrest; we proposed that the cytotoxic effects of quercetin could also be associated with apoptosis. We showed that the IC_{50} of doxorubicin and quercetin alone and in combination cause up to 70% apoptosis in T47D cells, the percentage of apoptotic cells induced by the combination of doxorubicin and quercetin was higher than the either of the treatments alone. We concluded that treatment-induced loss of T47D cell viability was associated with the induction of apoptosis. Similarly, the induction of apoptosis and the accumulation of cells at G2/M by quercetin and Dox have previously been described in various cancer cells such as K562, HK1, CNE2, HT29, and LNCaP cells (Atashpour et al., 2015; Ong et al., 2004; Alam et al., 2021; Grzanka et al., 2005). Extremely high levels of p53 and p53 negative regulator, Mdm2, were also detected in Dox-treated MCF7 cancer cells (Balmer et al., 2014).

Another mechanism that can classify CSC resistance to chemotherapy is resistance to apoptosis. Apoptosis analysis showed that CSCs were resistance to Dox-, quercetin- and Dox+quercetin-induced apoptosis and the percentage of apoptotic cancer stem cells in all treatment groups was significantly lower than that of the relative group of parental T47D cells. The resistance of CSCs to apoptosis can be explained by several mechanisms. Higher expression of anti-apoptotic proteins is one of the major pathways (Madjd et al., 2009). On the other hand, it has been shown that CSCs express higher BCRP levels that protect cells against drug damage through the process of efflux pumping process and decrease drug accumulation within cancer cells and thus reduce their apoptotic effects (Ji et al., 2019). The activation of the Wnt / β-catenin signaling pathway in CSCs has also been shown to inhibit apoptosis (Kruyt et al., 2010).

We concluded that CSCs are small cancer cell populations that play a significant role in resistance to chemotherapy, metastasis, and cancer recurrence. Accumulation of CSCs at G0/G1 phase, being quiescent and resistance to apoptosis partly explain the CSCs chemoresistance. In T47D cells and to a lower degree in CSCs, Quercetin induces growth inhibition, cell cycle arrest and apoptosis. Our research provides evidence that quercetin is an extremely potent chemotherapeutic and chemopreventive agent and can sensitize T47D and tumor stem cells to Dox treatment. An effective treatment to prevent the recurrence of cancer requires finding the ideal drug regimen that not only eliminate differentiated cancer cells, but can also quickly, selectively, and most importantly, target and kill CCS. The strategy of combining quercetin with conventional chemotherapeutic agents such as doxorubicin could have clinical benefits in increasing efficacy and reducing toxicity in cancer patients. Further studies are needed to study the chemoresistance of CCS and other potential molecular mechanisms of the antitumor action of quercetin.

Author Contribution Statement

The authors confirm contribution to the paper as follows: study conception and design: Dr.Azizi, Dr. Ostad, Dr. Ghahremani and Dr. Atashpour; data collection: Dr. Fouladdel, Dr. Barzegar, Dr. Komeili Movahhed and Dr. Atashpour; analysis and interpretation of results: Dr. azizi, Dr. Ostad, Dr. Ghahremani and Dr. Atashpour; draft manuscript preparation: Dr. Modaresi and Dr. Atashpour. All authors reviewed the results and approved the final version of the manuscript.

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