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QUERCETIN AND RALOXIFENE EFFECT ON BREAST CANCER CELL VIABILITY, MIGRATION,NITRIC OXIDE SECRETION AND APOPTOTIC GENES EXPRESSION

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ABSTRACT – *Objective:* Breast cancer (BC) is one of the most commonly diagnosed malignancies among females all over the world. The use of natural and complementary compounds is a new option in chemotherapy. The aim of the present study was to investigate the synergic effect of Quercetin (QUR) and Raloxifene (RAL) on BC cell lines *in vitro*.

Materials and Methods: The cell lines (MCF-7 and MDA-MB-231) were treated with QUR (0, 25 50, 100, 150, 200 µM), and RAL (1 µM) alone, and in combination. Cell viability was evaluated using the MTT assay. Ferric reducing antioxidant power (FRAP) and Griess method were used to measure total antioxidant capacity (TAC) and NO level of biological samples respectively. Changes in the expression of apoptotic-related genes were detected using real-time PCR.

Results: QUR (100, 150 and 200 µM) decreased cell viability significantly in MDA231 and MCF7 cells (p<0.01). Furthermore, Ral (1 µM) showed a significant decrease in both cell types (p<0.01). The synergistic effect of QUR (150) and RAL was also greater in MDA231 cells. NO levels in QUR, Ral, and synergic groups increased significantly in both cell lines (p<0.001). In treated groups, QUR and RAL significantly decreased cell migration, MMP2 and MMP9 expression, and increased apoptotic genes expression significantly (p<0.001). QUR increased TAC in both BC cell lines (p<0.00) while it was decreased by RAL. Synergic groups increased TAC in BC cells significantly (p<0.001).

Conclusions: QUR and RAL show synergistic anti-cancer effects on cell viability, NO production, cell migration, and apoptotic genes. QUR as a supplement can potentiate the anti-cancer effects of RAL in BC.

KEYWORDS: Breast cancer, Quercetin, Raloxifene, Apoptosis, Migration.

INTRODUCTION

Breast cancer (BC) is one of the most diagnosed malignancies and causes of mortality among females. It has high annual global prevalence and mortality rates (1.7 million new cases and about 0.5 million malignancy death cases) and involves environmental and genetic factors[1,2]. The most common treatment options for this multi-factorial disease comprise chemotherapy, radiotherapy, and surgery[3,4].

Raloxifene (RAL), a chemotherapeutic agent and selective estrogen receptor modulator (SERM), is frequently used to treat BC in postmenopausal women and applied to treat and prevent osteoporosis and reduce the risk of BC[5]. RAL has been shown to inhibit cell growth and induce apoptosis in several BC cell lines, namely SkBr3 (isolated by the Memorial Sloan–Kettering Cancer Center in 1970), MCF-7 (isolated from a 69-year-old white woman in 1970), and MDA-MB-231 (a highly aggressive, invasive, and poorly differentiated triple-negative BC (TNBC) cell line lacking an estrogen receptor)[6]. RAL can affect hormonal mechanisms and stimulate agonist and antagonist effects on estrogen receptors in various tissues. In breast tissue, RAL has an antagonist effect on estrogen receptors, thereby blocking estrogen binding and reducing BC risk in postmenopausal women[7]. Because of the drug resistance, toxicities against healthy tissues, and side effects of chemotherapeutic agents, the use of natural compounds as a complementary treatment has garnered much attention in recent decades[8]. Quercetin (QUR) (3, 3, 4, 5, 7-pentahydroxyflavone) is a natural flavonoid polyphenol found in many plants, fruits, and seeds that has various pharmacological properties, including anti-diabetic, antioxidant, and anti-inflammatory activities[9-11]. Some investigations have further indicated that QUR may possess anti-proliferative, anti-metastatic, and anti-angiogenic functions against cancer cells[11].

Nitric oxide (NO) is a colorless, water-soluble free radical and temporary endogenous gas. NO levels play significant roles in tumor-related processes such as apoptosis, angiogenesis, cell cycle regulation, metastasis, migration, and invasion[12]. Better clinical outcomes are achieved when combination therapies comprising a chemotherapy compound with phytochemicals are used. Previous study has reported that the combination of resveratrol and RAL influenced the overexpression of apoptotic genes, subsequently inducing cell death[13]. As no study to date has investigated the effects of the combination of QUR and RAL on BC cell inhibition and apoptosis, the present study evaluated the synergic impact of QUR and RAL on inhibiting the MDA-MB231 and MCF-7 cell lines by suppressing viability and migration and stimulating apoptosis and NO secretion.

PATIENTS AND METHODS

In the current *in vitro* study, RAL (Pfizer, Berlin, Steinheim , Germany), QUR (3, 3, 4, 7-pentahydroxyflavone) (Sigma-Aldrich, Berlin, Steinheim, Germany), and MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (Roche, Mannheim, Baden-Württemberg, Germany) were purchased, and BC cell lines MCF7 (estrogen receptor positive) and MDA-MB-231 (estrogen receptor negative) were procured from the National Cell Bank (Tehran, Iran). Dulbecco's modified Eagle's medium (DMEM-F12) culture medium and fetal bovine serum (FBS) (Gibco, Berlin, Brandenburg, Germany) and antibiotic (AppliChem, Darmstadt, Hesse, Germany) were also purchased.

Cell Culture

MCF7 and MDA-MB-231 cells were cultured in DMEM-F12 medium supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 µg/mL) and incubated at 37°C with 5% CO^2 and 95% humidity. The medium was changed every two days until cells reached 70% confluency.

MTT assay

The effect of QUR on cell viability was examined and its optimum dose for use in treatment ascertained through MTT assay. 10^5 cells were cultured in each well of a 96-well culture plate and treated with different concentrations of QUR (0, 25 50, 100, 150, and 200 µM) for 24, 48, and 72 hr. IC50 values for QUR (150 µM) were determined, and the IC50 value for RAL (1 µM) was taken from our previous study. Then, cells were treated for 72 hr with IC50 concentrations of QUR and RAL combined[13]. After treatment, the culture media were discarded, and 100 µL of the MTT solution (0.5 mg/mL) was added to each well. The cell plate was incubated at 37°C for 3–4 hr in the dark. Then, the MTT solution was removed, and the produced formazan crystals were dissolved by adding 100 µL of DMSO. The optical density (OD) of all samples was measured using a microplate reader (STAT FAX2100, Woodland Hills, CA, USA) at 570 nm. Cell viability was measured according to the formula (optical density of test /optical density of negative control 570, 630) ×100[14].

NO assay

NO concentration was measured using the Griess colorimetric method. The MCF7 and MDA-MB-231 cells (10^5/well) were treated with QUR, RAL, and their combinations for 72 hr. Next, the supernatants were collected, deproteinized by adding 6 mg zinc sulfate/400 µL supernatant and centrifuged at 12000 rpm for 12 min. Then, 100 µL of the deproteinized sample was mixed with 100 µL of vanadium chloride, followed by adding 50 µl sulfonamides and 50 µL naphthyl ethylenediamine dihydrochloride (NEED) and incubating for 30 min. Absorbance was measured at 540 and 630 nm by an ELISA reader (Stat fax 100, Woodland Hills, CA, USA) and normalized against standard concentrations of sodium nitrate (0, 6.25, 12.5, 25, 50, 100, and 200 µL) (n=3)[15].

FRAP assay

The ferric reducing antioxidant Power (FRAP) method was used to measure total antioxidant capacity (TAC). In this method, colorless ferric ion (Fe^{3+})-TPTZ complex is reduced to a violent blue Fe^{2+}- TPTZ, indicating reduced properties. The working FRAP reagent was prepared by mixing 2.5 ml TPTZ (2, 4, 6-tripyridyl-s-triazine 40 mM dissolved in 40 mM HCl) and 2.5 ml ferric chloride (20 mM in water) with 25 ml acetate buffer (300 mM, pH=3.6). The mixture was heated to 37°C for 10 minutes before use. A total of 200 µL of supernatants were added to 1.5 ml of working FRAP solution and placed in a water bath (37°C) for 30 min. Absorption at 593 nm was measured and recorded by spectrophotometer (Jenway, Edinburgh, England). Standard solutions of FeSO4.7H2O were used at concentrations of 0, 125, 250, 500, and 1000 µM (n=3)[16].

Migration (scratch) assay

MCF7 and MDA 231 cells were cultured in 24-well plates and grown to 80% confluency. Then, a scratch was created using a sterile 1000-µL pipette tip, and wells were rinsed with media to remove cell debris. The cells were treated with QUR (150 µM), RAL (1 µM), and a combination of QUR and RAL for 72 hr. Images were captured with an inverted microscope at 20× magnification.

Real-time polymerase chain reaction

The expression of apoptotic and metastatic genes was evaluated by treating MCF7 and MDA231 cell lines with IC50 concentrations of QUR and RAL alone and in combination for 72 hr. The total RNA was isolated using the TRIzol reagent (Life Biolab, Hanseatic, Hamburg, Germany) according to the manufacturer's protocol. The quantity and quality of purified RNA were measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific™, Waltham, MA, USA). Then, 1 µg of the total RNA was used to synthesize cDNA by a Transcription First-Strand cDNA synthesis kit (Biofact, Daejeon, Seoul, South Korea) based on the manufacturer's instruction. Quantitative real-time PCR (qRT-PCR) was performed using SYBR Green, high Rox real-time master mix (Biofact, Daejeon, Seoul, South Korea) on the Applied Biosystems Step One Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). Cycle conditions were as follows: 95°C for 10 (denaturation) minutes, followed by 40 cycles (95°C for 15 seconds and 60°C for 60 seconds) [17]. The data were collected and analyzed using the $2^{-\Delta\Delta Ct}$ method. The GAPDH (glyceraldehyde3-phosphate dehydrogenase) gene was considered as an internal control. The primers for RT-PCR amplification of different genes are shown in Table 1.

Statistical analysis

All data were reported as mean values ± standard deviation for at least three independent experiments. The normality of data was determined by the Kolmogorov-Smirnov test ($p<0.05$), and differences among data were statistically analyzed using one-way ANOVA followed by Tukey's test as post hoc ($p<0.05$ was considered significant). The charts were designed with Graph Pad Prism software package version 9 (Graph Pad Prism Software Inc., San Diego, CA, USA).

Table 1. Primers and expected length of products: forward and reverse sequence.

Gene	Primer sequences	Product Length
p53	F: 5'-TAACAGTTCTGCATGGGCGGC-3	121 bp
	R: 5'-AGGACAGGCACAAACACGCACC-3	
Bax	F: 5'-CCTGTGCACCAAGGTGCCGGAACT-3'	99 bp
	R: 5'-CCACCCTGGTCTTGGATCCAGCCC-3'	
Bcl2	F: 5'-TTGTGGCCTTCTTTGAGTTCGGTG-3'	114 bp
	R: 5'-GGTGCCGGTTCAGGTACTCAGTCA-3'	
MMP2	F: 5'-TACAGGATCATTGGCTACACACC-3'	119 bp
	R: 5'-GGTCACATCGCTCCAGACT-3'	
MMP9	F: 5'-TGTACCGCTATGGTTACACTCG-3'	128 bp
	R: 5'-GGCAGGGACAGTTGCTTCT-3'	
GAPDH	F: 5´-GTCTCCTCTGACTTCAACAGCG-3'	120 bp
	R: 5'- ACCACCCTGTTGCTGTAGCCAA-3	

RESULTS

Cell viability

The MTT assay results regarding cell viability are shown in Figure 1. Low concentrations of QUR had no toxicities against MCF7 cells after 24 hr treatment, but at 100, 150, and 200 µM concentrations, cell viability decreased significantly (p<0.05). This inhibitory effect on cell proliferation was dose-dependent at 48 and 72 hr, and the reduction was more significant at 72 hr (p<0.001). At a concentration of 150 µM QUR at 72 hr, the viability rate was reduced by 50%. Therefore, this optimal dose was considered for further experiments (Figure 1). QUR treatment showed no toxic effects but a significant reduction in MDA231 cell viability at 24 hr. Nonetheless, at 48 and 72 hr and concentrations of 100, 150, and 200 µM, the survival rate was significantly decreased in a dose- and time-dependent manner (p<0.01). According to the results, the survival rate was reduced by 50% at a concentration of 150 µM QUR in 72 hr. Therefore, the optimal dose of 150 µM was considered for further experiments (Figure 1). QUR and RAL alone and in combination reduced the viability of MCF7 and MDA231 cells after 72 hr treatment. A higher reduction in the combined treatment indicated synergistic effects (Figure 2). A significant difference in MCF7 cells was seen between the groups receiving QUR alone and those receiving the RAL and QUR combination (p<0.001). In MDA231 cells, this significant difference was seen in both QUR and RAL treatment groups and the combined group (p<0.001).

TAC assay

QUR significantly increased TAC in MDA231 and MCF7 cells (p<0.001); however, the RAL treatment group showed a significant decrease in both cell lines. The synergistic effect of QUR and RAL on MDA231 cells was more remarkable, and the antioxidant capacity in the QUR and RAL combination treatment groups was significantly increased (p<0.001). In other words, QUR enhanced TAC in cancer cells even in cytotoxic conditions (Figure 3).

Effects of QUR and RAL on NO levels

The results indicated that treatment with QUR, RAL, and a combination of the two significantly reduced NO levels in MCF7 and MDA 231 cells (p<0.001). The remarkable increase seen in NO secretion in the combination group showed the synergistic effects of QUR and RAL (Figure 4).

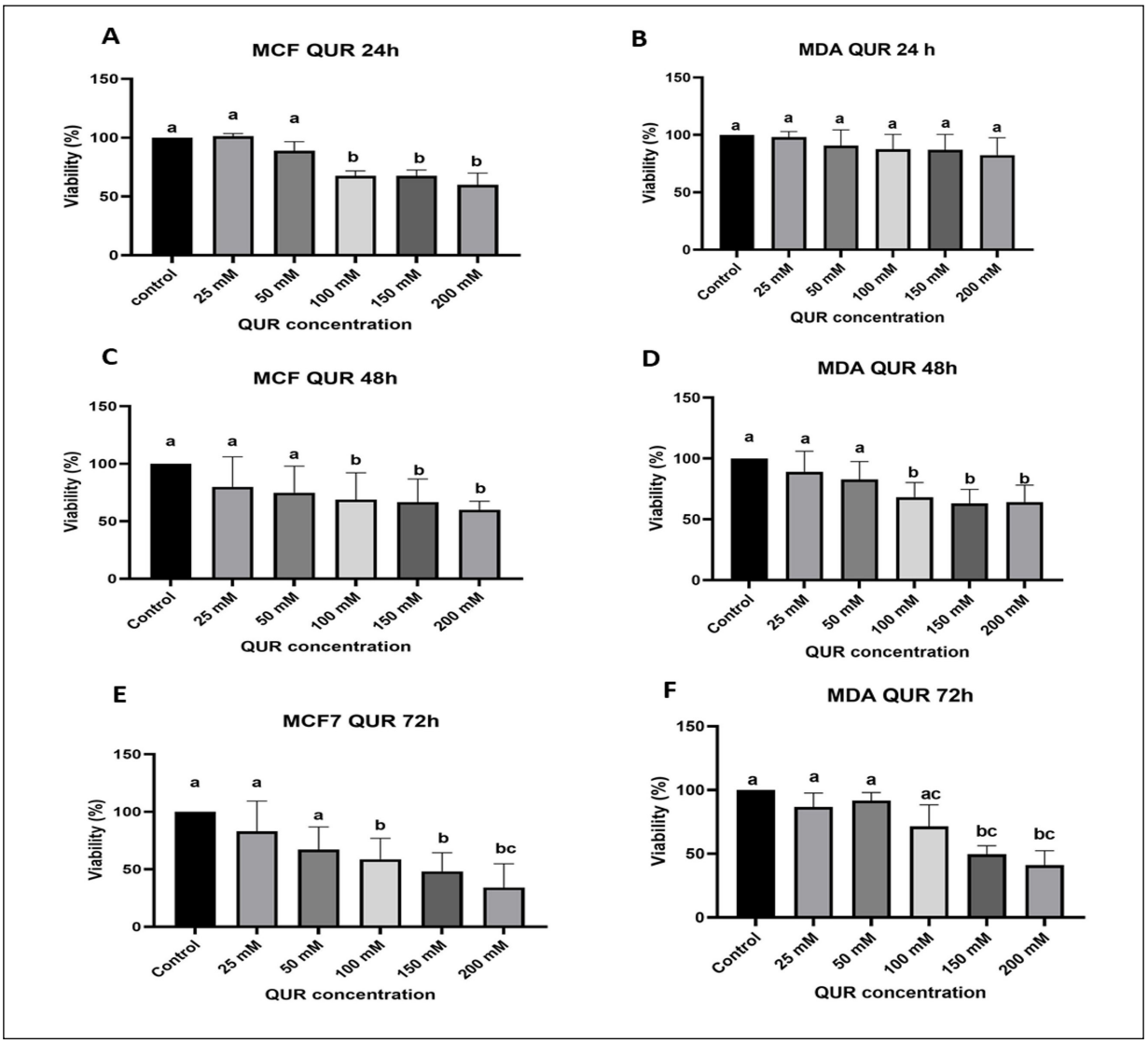

Figure 1. The effect of QUR on the cell viability of MCF-7 and MDA-MB-231 cells. Cells were treated with different concentrations of QUR for 24 (A, B), 48 (C, D), 72 (E, F) hr. Cell viability was determined using the MTT assay. Data are expressed means ± SD compared (GraphPad Prism 9 Software Inc, San Diego, CA, USA). Different letters mean significant differences between groups.

Apoptotic gene expression

Real-time PCR data showed that Bax expression increased significantly in MCF-7 and MDA-MB231 cells treated with QUR, RAL, and their combination (p<0.001), with a higher increase seen in MDA-MB-231 cells treated with RAL than in MCF-7 cells (Figure 5A, B). Bcl-2 expression declined significantly in MDA-MB-231 and MCF-7 cells treated with RAL and the combined group, respectively (p<0.01) (Figure 5C, D). P53 was significantly overexpressed in MCF-7 and MDA-MB-231 cells treated with QUR, RAL, and their combination, with higher levels being seen in MDA-MB-231 cells (p<0.001) (Figure 5E, F).

Cell migration

Scratch assay results indicated that the cell migration rate was reduced in cells treated with QUR and RAL alone and in combination. As shown in Figure 6, the migration of MCF-7 and MDA-MB231 cells decreased upon treatment with QUR, RAL, and their combination compared to the control (untreated) group, where only a limited number of cells migrated (Figure 6).

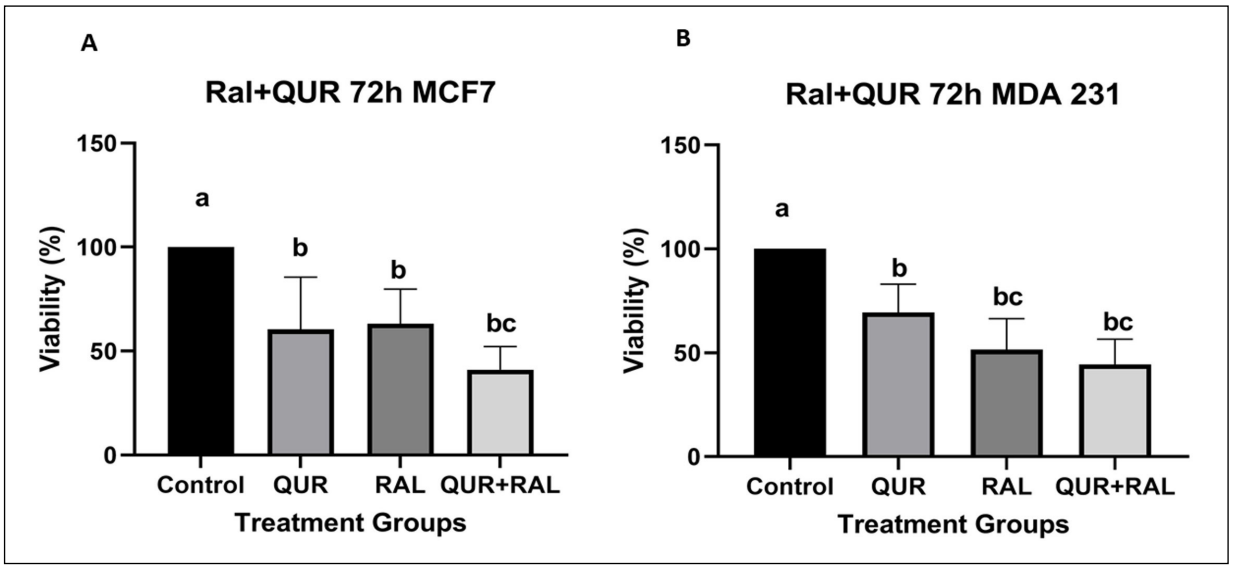

Figure 2. The synergic effect of RAL (1µM) and QUR (150 µM) on the cell viability of MCF-7 (A) and MDA231 (B) cells. Cell viability was determined using the MTT assay described in the method's section. The data are expressed in terms of the percent of control cells as the means ±SD (GraphPad Prism 9 Software Inc, San Diego, CA, USA). Different letters mean significant differences between groups.

DISCUSSION

The results of the present study showed that QUR (a powerful natural antioxidant) and RAL (a member of the SERM family) alone and in combination reduced the viability of MCF7 and MDA231 cells in a time- and dose-dependent manner, and this effect was more significant in the combined group, confirming their synergic effect. QUR showed an inhibitory effect on MCF7 cells at higher doses

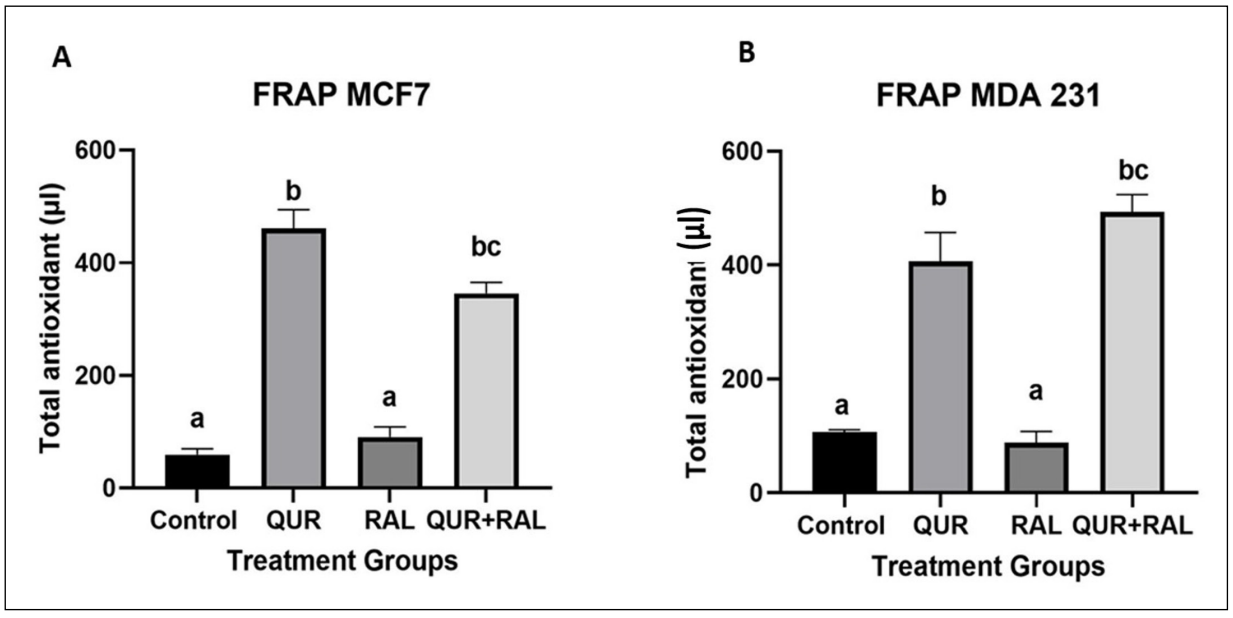

Figure 3. The effect of QUR, RAL, and their synergic on TAC level by MCF-7 (A) and MDA231 (B) cells was measured by FRAP reaction. The data are expressed in terms of the percent of control cells as the means ±SD (GraphPad Prism 9 Software Inc, San Diego, CA, USA). Different letters mean significant differences between groups. The level of NO in the QUR and RAL groups showed a substantial decrease in MCF7 and MDA 231 cells. In addition, the NO level decreased significantly in the combination groups (p<0.001) and showed the synergistic effects of these two compounds in both cells.

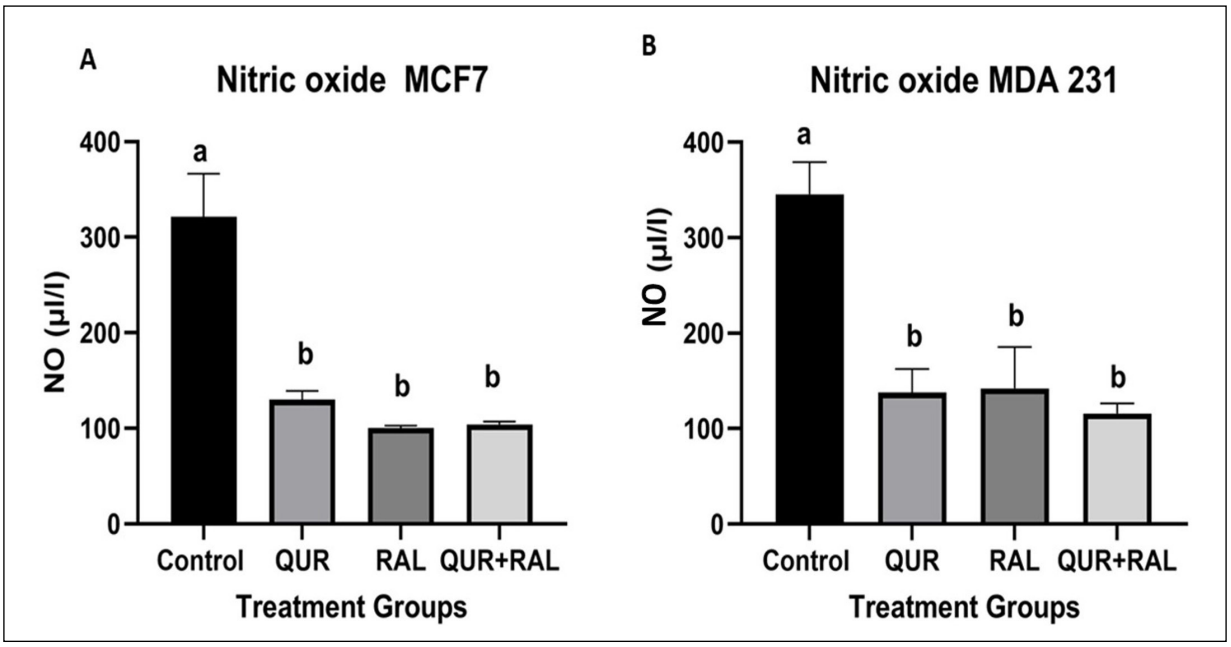

Figure 4. The effect of QUR, RAL, and QUR+RAL on NO production was measured by Griess reaction in MCF-7 (A) and MDA231 (B) cells. Control wells were treated with the equivalent amount of a medium alone. The data are expressed in terms of the percent of control cells as the means ± SD (GraphPad Prism 9 Software Inc, San Diego, CA, USA). Different letters mean significant differences between groups.

(100, 150, and 200 µM) but not lower doses (25 and 50 µM). Although it had no significant inhibitory effect on MDA231 cells at 24 h, QUR inhibited cells at 48 h and 72 h. A combination of QUR (150 µM) and RAL (1 µM)[13] showed a powerful significant inhibitory effect on both cell lines. Similar synergistic effects were reported in our previous study, in which RAL (1 µM) and resveratrol (RSV) (20 µM) were shown to inhibit MCF7 and MDA231 cells. In the present study, however, co treatment with RAL and QUR showed a more potent effect than RAL or RSV alone. The results showed that QUR and RAL, both alone and in combination, induced apoptosis by increasing Bax expression and decreasing Bcl2 expression. In confirmation of the present study, Mirzapour et al[13] showed that RAL induced cell death in MCF7 and MDA 231 cell lines. In the present study, p53 was over expressed in the treatment groups, consistent with the study of Mohammadi et al[18] in which p53 mRNA levels were significantly increased in Saos-2 cancer cells treated with QUR. As shown in the migration test, treatment with QUR and RAL reduced BC cell migration and metastasis and evidenced their synergistic effects. The reduced migration was associated with decreased MMP2 and MMP9 expression. Consistent with our results, MMP2 and MMP9 mRNA expression was significantly down regulated in human osteosarcoma cells (HOS) treated with QUR compared with control cells, followed by attenuated tumor lung metastasis[19]. Furthermore, Balakrishnan et al[20] reported that QUR significantly reduced MMP-2 and MMP-9 protein expression and inhibited MCF-7 and MDA-MB-231 cells migration and invasion. Ağardan et al[21] indicated that RAL-loaded liposomes containing DM-β-CD could inhibit MMP-2. The developed formulations can be helpful for developing further treatment alternatives and new strategies for cancer therapy. In the current study, the upregulation of the Bax and downregulation of Bcl2 in treatment groups was in accordance with the results of Alhakamy et al[22] confirming Bax increment and Bcl2 reduction in MCF-7cell lines. The current results further showed that QUR, RAL, and their combination reduced NO levels in the treatment cells. As NO levels are a critical factor in the angiogenesis of cancer cells, such treatments may inhibit the growth and subsequent angiogenesis of cancer cells by reducing NO levels. Li et al[23] reported that QUR promoted PVT1 expression and reduced NO levels, thus regulating inflammation, oxidative stress, apoptosis, and mitochondrial structure and function in H9C2 cells.

Data in the current study showed that QUR increased TAC significantly in both cell lines, but RAL had no potent effect on it. Nonetheless, the synergic treatment of QUR and RAL increased TAC in both cell lines significantly. It is noteworthy that QUR significantly increased TAC in MCF7 and MDA231 cells,

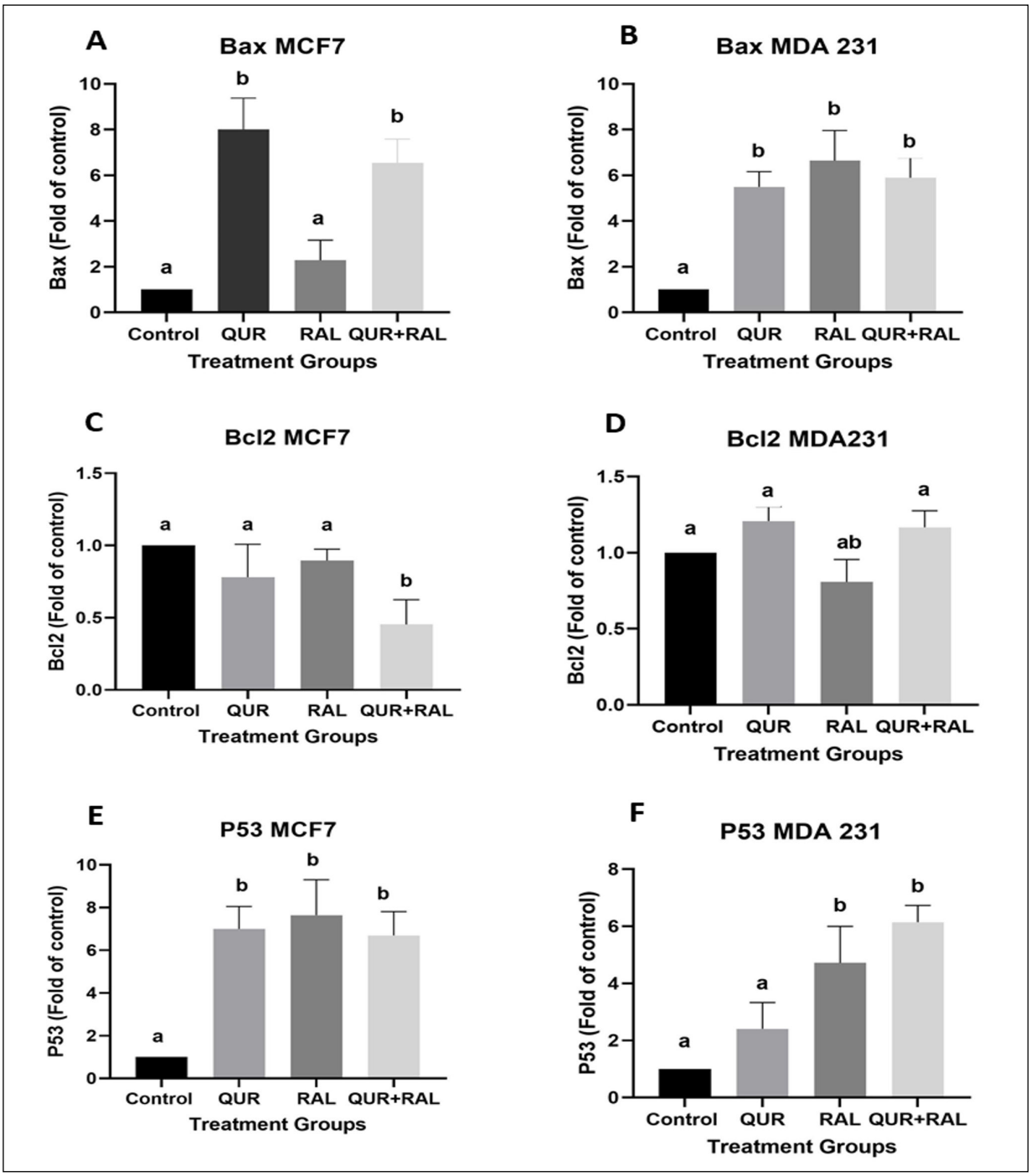

Figure 5. Effects of QUR and RAL on the levels of apoptotic genes in MCF7 (A, C, E) and MDA231 (B, D, F). Quantitative real-time RT-PCR analysis of apoptotic gene expression was performed after exposure to QUR and RAL and their combination for 72 hr. The mRNA expression of genes was normalized using GAPDH mRNA as an internal standard. The results are shown as the mean ± SD of three independent experiments. (GraphPad Prism 9 Software Inc, San Diego, CA, USA).

even in cytotoxic conditions in BC cells. Tsao et al[24] reported that oral QUR supplementation significantly increased TAC in post-exercise glucose-induced insulin-healthy participants. Contrary to the current study, MostafaviPour et al[25] indicated that the endogenous production of ROS decreased in a dose-dependent manner following the treatment of BC cells with Vitamin C and QUR.

Our finding of increased antioxidant activity for QUR conflicts with the generally accepted property of antioxidants in cancer cells. QUR can act as both anti- and pro-oxidant, depending on its concentra-

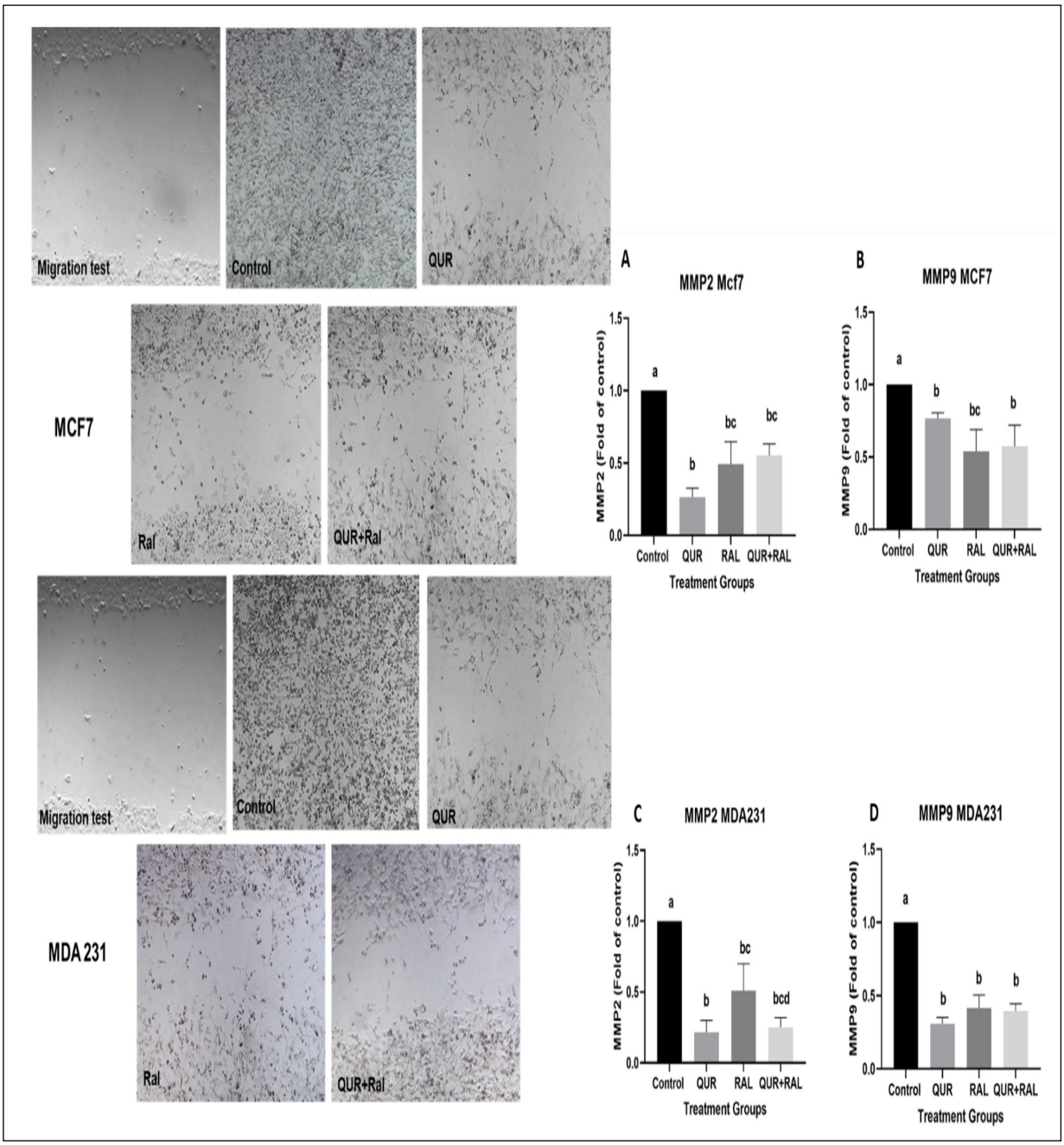

Figure 6. MCF7 and MDA-MB 231 cell line migration test by scratch creation after 72 hr; cells were treated with QUR (150 µM), RAL (1 µM), and a combination of QUR and RAL, magnification 20X. Effects of QUR and RAL on the expression of migration genes in MCF7(A, B) and MDA231 (C, D). Quantitative real-time RT-PCR analysis of migration gene expression was performed after exposure to QUR and RAL and a combination of QUR and RAL for 72 hr. The mRNA expression of genes was normalized using GAPDH mRNA as an internal standard. The results are shown as the mean ±SD of three independent experiments (GraphPad Prism 9 Software Inc, San Diego, CA, USA).

tion and source of free radicals in the cell. The use of combination therapies is one of the most common strategies for achieving better clinical outcomes. Previous study has reported that resveratrol and RAL combined increases the number of apoptotic genes, followed by cell death[13]. RAL has an antagonistic impact on estrogen receptors and blocks estrogen binding, which reduces BC risk in postmenopausal women. Because of the chemotherapeutic agents' resistance, toxicity, and side effects, using natural compounds as complementary therapies along with conventional chemotherapy is becoming much more attractive in cancer research and development[26].

CONCLUSIONS

The synergic effect of QUR and RAL showed potent anticancer properties on the BC cell lines (MCF-7 and MDA-MB-231) by inhibiting cell migration and invasion, down-regulating Bcl2 gene, and upregulating P53, MMP2, and MMP9, inducing apoptosis. QUR as a supplement can potentiate the anticancer effects of RAL in BC. QUR also showed a more potential effect on MCF-7 cell viability compared to MDA-MB-231.

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Ethics approval and consent to participate:
The manuscript was reviewed and approved with the Ethics code: IR.KUMS.REC.1399.696.

Availability of data and materials:
Data are available on reasonable request from the corresponding author

Conflicts of interest:
The authors report no potential conflicts of interest.

Authors contribution:
M. Kh. and Z. R: Project administration, manuscript writing and editing; M. M. Kh. and F. K.: conducted experiments and analyzed data; M. B. consulted in real-time. All authors read and approved the final manuscript.

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All authors studied the authorship agreement of the journal, and they reviewed and approved the manuscript. The authors express their appreciation to Kermanshah University of Medical Sciences for their support of the present study [Grant No: 990706].

REFERENCES

1. Bozorgi A, Bozorgi M, Khazaei M. Immunotherapy and immunoengineering for breast cancer; a comprehensive insight into CAR-T cell therapy advancements, challenges and prospects. Cell Oncol 2022; 13402: 755-777.
2. Behravan H, Hartikainen JM, Tengstrom M, Kosma VM, Mannermaa A. Predicting breast cancer risk using interacting genetic and demographic factors and machine learning. Sci Rep 2020; 10: 1-16.
3. Yue Xi, Pengfei Xu. Global colorectal cancer burden in 2020 and projections to 2040. Transl Oncol 2021; 10: 1936-1952.
4. Wang L, Cao J, Li C, Wang X, Zhao Y, Li T, Du Y, Tao Z, Peng W, Wang B, Zhang J, Zhang S, Wang Z, Hu X. Efficacy and safety of mitoxantrone hydrochloride liposome injection in Chinese patients with advanced breast cancer: a randomized, open-label active-controlled, single-center, phase II clinical trial. Invest New Drugs 2022; 40: 330-339.
5. Gaspar HA, Gerring Z, Hübel C, Middeldorp CM, Derks EM, Breen G. Using genetic drug-target networks to develop new drug hypotheses for major depressive disorder. Transl Psychiatry 2019; 9: 117-126.
6. Koroth J, Nirgude S, Tiwari S, Gopalakrishnan V, Mahadeva R, Kumar S, Karki SS, Choudhary. Investigation of anticancer and migrastatic properties of novel curcumin derivatives on breast and ovarian cancer cell lines. BMC Complement Altern Med 2019; 19: 273-289.
7. Hernando C, Ortega-Morillo B, Tapia M, Moragon S, Martinez MT, Eroles P, Garrido-Cano I, Adam-Artigues A, Lluch A, Bermejo B, Cejalvo JM. Oral Selective Estrogen Receptor Degraders (SERDs) as a Novel Breast Cancer Therapy: Present and Future from a Clinical Perspective. Int J Mol Sci 2021; 22: 7812-7833.
8. Lee HW, Kang WY, Jung W, Gwon MR, Cho K, Lee B, Seong SJ, Yoon YR. Pharmacokinetic Drug Interaction Between Raloxifene and Cholecalciferol in Healthy Volunteers. Clin Pharmacol Drug Dev 2022; 11: 623-631.
9. Kaysaw Tuy, Lucas Rickenbacker, Hjelmeland AB. Reactive oxygen species produced by altered tumor metabolism impacts cancer stem cell maintenance Redox. Biology 2021; 44: 101953-101965.
10. Bozorgi A, Khazaei S, Khademi A, Khazaei M. Natural and herbal compounds targeting breast cancer, a review based on cancer stem cells. IJBMS 2020; 23: 970-983.
11. Kasiri N, Rahmati M, Ahmadi L, Eskandari N, Motedayyen H. Therapeutic potential of quercetin on human breast cancer in different dimensions. Inflammopharmacology 2020 ; 28: 39-62.
12. Mirzaei F. Khazaei M. Role of nitric oxide in biological systems: a systematic review JMUMS 2017; 27: 333-345.
13. Mirzapur P, Khazaei MR, Moradi MT, Khazaei M. Apoptosis induction in human breast cancer cell lines by synergic effect of raloxifene and resveratrol through increasing proapoptotic genes. Life Sci 2018; 205: 45-53.
14. Khaleseh F, Hemati Azandaryani A, Fathian Kolahkaj F, Khazaei M, Derakhshandeh K. Enhancement of in vitro antitumor activity of epirubicin in HER2+ breast cancer cells using immunoliposome formulation. IET Nanobiotechnol 2021; 15: 257-265.
15. Khazaei MR, Gravandi E, Ghanbari E, Niromand E, Khazaei M. Trifolium pratense extract increases testosterone and improves sperm characteristics and anti-oxidant status in diabetic rats. Biotec Histochem 2022; 97: 576-583.

16. Khazaei F, Ghanbari E, Khazaei M. Improved hormonal and oxidative changes by Royal Jelly in the rat model of PCOS: An experimental study. Int J Reprod Biomed 2021; 19: 515-524.
17. Akbaribazm M, Khazaei MR, Khazaei F, Khazaei M. Doxorubicin and Trifolium pratense L.(Red clover) extract synergistically inhibits brain and lung metastases in 4T1 tumor-bearing BALB/c mice. Food Sci Nutr 2020; 8: 5557-5570.
18. Mohammadi E, Alemi F, Maleki M, Malakoti F, Farsad-Akhtar N, Yousefi B. Quercetin and Methotrexate in Combination have Anticancer Activity in Osteosarcoma Cells and Repress Oncogenic MicroRNA-223. Drug Res (Stuttg) 2022; 72: 226-233.
19. Lan H, Hong W, Fan P, Qian D, Zhu J, Bai B. Quercetin inhibits cell migration and invasion in human osteosarcoma cells. Cell Physiol Biochem 2017; 43: 553-567.
20. Balakrishnan S, Bhat F, Raja Singh P, Mukherjee S, Elumalai P, Das S, Patra CR, Arunakaran J . Gold nanoparticle–conjugated quercetin inhibits epithelial–mesenchymal transition, angiogenesis and invasiveness via EGFR/VEGFR-2-mediated pathway in breast cancer. Cell Prolif 2016; 49: 678-697.
21. Agardan NB, Degim Z, Yılmaz S, Altıntas L, Topal T. The effectiveness of raloxifene-loaded liposomes and cochleates in breast cancer therapy. AAPS Pharm Sci Tech 2016; 17: 968-977.
22. Alhakamy NA, Fahmy UA, Eldin SMB, Ahmed OAA, Aldawsari HM, Okbazghi SZ, Alfaleh MA, Abdulaal WH, Alamoudi AJ, Mady FM. Scorpion Venom-Functionalized Quercetin Phytosomes for Breast Cancer Management: In Vitro Response Surface Optimization and Anticancer Activity against MCF-7 Cells. Polymers (Basel) 2021; 14: 93-114.
23. Li F, Liu J, Tang S, Yan J, Chen H, Li D, Yan X. Quercetin regulates inflammation, oxidative stress, apoptosis, and mitochondrial structure and function in H9C2 cells by promoting PVT1 expression. Acta Histochem 2021; 123: 151819-151827.
24. Tsao JP, Bernard JR, Hsu HC, Hsu CL, Liao SF, Cheng IS. Short-Term Oral Quercetin Supplementation Improves Post-exercise Insulin Sensitivity, Anti-oxidant Capacity and Enhances Subsequent Cycling Time to Exhaustion in Healthy Adults: A Pilot Study. Front Nutr 2022; 9: 875319-875330.
25. Mostafavi-Pour Z, Ramezani F, Keshavarzi F, Samadi N. The role of quercetin and vitamin C in Nrf2-dependent oxidative stress production in breast cancer cells. Oncol Lett 2017; 13: 1965-1973.
26. Taurin S, Nimick M, Larsen L, Rosengren RJ. A novel curcumin derivative increases the cytotoxicity of raloxifene in estrogen receptor-negative breast cancer cell lines. Int J Oncol 2016; 48: 385-398.