Türk Tarım ve Doğa Bilimleri Dergisi 12(1): 136–144, 2025
https://doi.org/10.30910/turkjans.1597186

TÜRK
TARIM ve DOĞA BİLİMLERİ
DERGİSİ

TURKISH
JOURNAL of AGRICULTURAL
and NATURAL SCIENCES

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Araştırma Makalesi

Synergistic Anticancer Activity of Quercetin Combined with Luteolin in MCF-7 Breast Cancer Cells via Induction of Apoptosis

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Geliş Tarihi: 06.12.2024 Düzeltme Geliş Tarihi: 20.12.2024 Kabul Tarihi: 20.12.2024

ABSTRACT

Cancer is a major public health problem with its increasing incidence and mortality. The striking role of phytochemicals in the prevention and treatment of cancer is undeniable in studies. In this study, the effects of the combination of quercetin (QUR) and luteolin (LTN) on the MCF-7 human breast cancer cell line were investigated *in vitro*. WST-1 (Water-Soluble Tetrazolium 1) cell cytotoxicity assay was used to determine cytotoxic activity. In the analysis of the interaction and synergism between QUR and LTN, combination index (CI) was calculated using CompuSyn software. The effects on colony survival and cell migration were determined by clonogenic assay and wound scratch assay, respectively. For the determination of apoptosis, Acridine orange/ethidium bromide (AO/EB) dual staining, and *Caspase-3* ELISA (Enzyme-Linked ImmunoSorbent Assay) analysis, and genomic analyses revealing *p53* (tumor protein *p53*) and *Bcl-2* (B-cell lymphoma 2) expression levels were performed. As a result of the analyses, it was seen that especially QUR plus LTN treatment exhibited a great cytotoxic activity in cells. It was determined that the combination treatment suppressed colony survival and significantly inhibited cell migration. Quantitative analysis results show that QUR+LTN treatment triggers cellular apoptosis by upregulating *Caspase-3* and *p53*, and downregulating *Bcl-2*. Supporting these findings with further *in vitro* and *in vivo* analyses may contribute significantly to revealing the promising efficacy of combined phytochemical treatment approaches on cancer.

Key words: Cancer, apoptosis, quercetin, luteolin, MCF-7

MCF-7 Meme Kanseri Hücrelerinde Luteolin ile Birlikte Kuersetinin Apoptozun İndüksiyonu Yoluyla Sinerjistik Antikanser Aktivitesi

ÖZ

Kanser, artan insidansı ve mortalitesi ile büyük bir halk sağlığı sorunudur. Kanserin önlenmesi ve tedavisinde fitokimyasalların çarpıcı rolünün yadsınamazlığı yapılan çalışmalarla ortaya koyulmaktadır. Bu çalışmada kuersetin (KUR) ve luteolin (LTN) kombinasyonunun MCF-7 insan meme kanseri hücre hattı üzerindeki etkileri *in vitro* olarak incelendi. Sitotoksik aktivitenin belirlenmesinde WST-1 (Suda Çözünür Tetrazolium 1) hücre sitotoksisitesi analizi kullanıldı. KUR ile LTN arasındaki etkileşimimin ve sinerjizmin analizinde CompuSyn yazılımı kullanılarak kombinasyın indeksi (KI) hesaplandı. Koloni sağkalımı ve hücre göçü üzerindeki etkiler ise sırasıyla klonojenik analiz ve yara iyileşmesi analizi ile belirlendi. Apoptozun belirlenmesi için, Akridin oranj/etidyum bromür (AO/EB) ikili boyama ve *Kaspaz-3* ELISA (Enzim Bağlantılı İmmünosorbent Testi) analizi ile *p53* (tümör protein *p53*) ve *Bcl-2* (B hücreli lenfoma 2) ekspresyon düzeylerini ortaya koyan genomik analizler gerçekleştirildi. Analizler sonucunda özellikle QUR+LTN tedavisinin hücrelerde büyük bir sitotoksik aktivite sergilediği görüldü. Kombinasyon tedavisinin koloni sağkalımını baskıladığı ve hücre göçünü de önemli ölçüde inhibe ettiği belirlendi. Kantitatif analiz sonuçları QUR+LTN tedavisinin *Kaspaz-3* ve *p53*'ü upregüle ederek, *Bcl-2*'yi ise downregüle ederek hücresel apoptozu tetiklediğini göstermektedir. Bu bulguların ileriki dönem *in vitro* ve *in vivo* analizlerle desteklenmesi, kombine fitokimyasal tedavi yaklaşımlarının kanser üzerindeki umut verici etkinliğinin ortaya konulmasına önemli katkılar sunabilir.

Anahtar kelimeler: Kanser, apoptoz, kuersetin, luteolin, MCF-7.

INTRODUCTION

Cancer, the second leading cause of death worldwide after cardiovascular diseases, poses an increasing burden on health systems and economic resources. It is of great importance to develop new treatment approaches that are low-cost and, more importantly, highly effective and accompanied by minimal side effects against this disease, which was reported to cause approximately 20 million new cases and 10 million deaths in 2022 (Bray et al., 2024). In addition to traditional methods such as surgery, radiotherapy and chemotherapy, complementary and alternative approaches, including herbal products and compounds, are also prominent in cancer treatment. Many disadvantages of traditional treatments, such as not being able to reduce mortality rates, reducing quality of life, causing serious side effects, high rates of disease recurrence and development of drug resistance, indicate the need for alternative approaches in cancer treatment (Rizeq et al., 2020; George et al., 2021).

Dietary factors, including nutrients, have been reported to play important roles in the development of different types of cancer and are directly related to nearly 35% of global cancer deaths (Manson, 2003). The pharmacological importance and health benefits of diets rich in polyphenolic compounds found in most fruits and vegetables are of interest. Dietary polyphenols are represented by many important plant derived compounds, especially flavonoids, phenolic acids, lignans and stilbenes (George et al., 2021). There is growing interest in screening phytochemicals, which are natural compounds in plants, to find those that are effective, safe, and have minimal toxic effects on cancer. The fact that mono- and combined use is effective in many metabolic processes such as angiogenesis, apoptosis, and metastasis reveals that phytochemicals have high pharmacological potential (Talib et al., 2022). There are many studies showing that phytochemicals increase their anticancer effects when used together with chemotherapeutic agents, which minimizes the emergence of drug resistance and side effects (Yap et al., 2013; Erdogan et al., 2022; Morales-Durán et al., 2024). The combination of two or more therapeutic agents targeting cellular pathways involved in carcinogenesis processes may offer more effective strategies compared to monotherapeutic approaches. Since traditional monotherapies generally target active proliferating cells without being selective, they affect healthy cells along with cancer cells, while cancer stem cells cannot be completely eliminated, which allows tumor recurrence and invasion (Mokhtari et al., 2017). In addition, exposure to the same agent as monotherapy causes cancer cells to develop alternative escape routes, increasing the susceptibility to drug resistance (Khdair et al., 2010). Phytochemicals used in combination treatments can reduce the toxic effects of chemotherapeutics, as well as exhibit synergistic effects that affect cancer cells more than normal cells by affecting different pathways (Mokhtari et al., 2017). Combination with phytochemicals reduces the incidence of drug resistance, as chemotherapeutics are more effective when used at lower doses than normal (Albain et al., 2008). Considering all these, it becomes interesting to reveal the anticancer effects of phytochemicals, especially when used in combination with each other. Quercetin (QUR), 3,3′,4′,5,7-pentahydroxyflavone, is a plant phytochemical that is widely found in many fruits and vegetables and belongs to the flavonoid class of polyphenolic compounds. In addition to the antioxidant, anti-inflammatory, and antimicrobial effects (Lamson & Brignall, 2000) of QUR, its anticancer activity has been reported especially on apoptosis, angiogenesis, and cell cycle (Kashyap et al., 2016). Luteolin (LTN), 3′,4′,5,7-tetrahydroxyflavone, is a flavonoid that can be found in fruits and vegetables such as broccoli, green pepper, parsley, carrot, and pumpkin (Miean & Mohamed, 2001). There are many studies showing that LTN has various biological activities such as antioxidant, anti-inflammatory, anti-microbial (Nabavi et al., 2015), and anticancer effects (Ganai et al., 2021).

The aim of this study was to elucidate the molecular mechanism of action of the combination of QUR and LTN on MCF-7 (Michigan Cancer Foundation-7) breast cancer cells and to investigate their synergistic effects.

MATERIALS AND METHODS

Cell line, cell culture, and reagents

Human breast cancer MCF-7 cell line were used in this study. The cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) with L-glutamine containing 10% FBS (Fetal bovine serum), and 1% penicillin/streptomycin, and maintained in a humidified incubator at 5% CO_2, 37 °C. QUR and LTN, acridine orange (AO), ethidium bromide (EB) from Sigma Aldrich Co.; DMEM, penicillin/streptomycin, and FBS from Lonza Co.; pure link RNA mini kit from Life Technologies Co.; WST-1 cytotoxicity test kit from Boster Co., *Caspase-3* colorimetric assay kit from BioVision Co.; 2 x qPCRBIO SyGreen mix Lo-ROX kit from PCR Biosystems Co., and high capacity cDNA reverse transcriptase kit from Thermo Fisher Scientific Inc. were used in studies.

Cytotoxicity assay and combination index

Cytotoxicity analyses were performed using WST-1 cell viability assay according to the manufacturer's protocol. 1×10^4 MCF-7 cells/well were seeded into a 96-well plate and after overnight incubation, increasing doses of mono- and combo- QUR and LTN (10, 20, 40, and 80 µM) treatments were performed for 24 h. After the treatments were completed, the wells were aspirated, fresh medium was placed into the wells, and 10 µl of WST-1 reagent was added. The absorbances were measured at 450 nm wavelength after 2 h, and percent cytotoxicity and IC_{50} (half maximal inhibitory concentration) values were calculated. Isobologram analysis allows the determination of synergism between two compounds or drugs in combined treatment. The interaction between QUR and LTN was assessed by the combination index (CI) with the isobologram and median effect equation method using CompuSyn software. CI<1, =1 and >1 indicate synergism, additive effect and antagonism, respectively (Chou, 2006).

Colony formation assay

0.5×10^3 MCF-7 cells seeded in 6 well plates were treated with 5 µM QUR, 5 µM LTN, 15 µM QUR, 15 µM LTN, 5 µM QUR + 5 µM LTN, and 15 µM QUR + 15 µM LTN treatments for 24 h the next day. After 12 days of culture in fresh medium, the cells were fixed with methanol/acetic acid mixture, and stained with crystal violet.

Wound scratch assay

Fully confluent monolayer cells seeded in 6-well plates were scraped using a sterile pipette to create a wound scratch. After initiating 40 µM QUR and 40 µM LTN mono- and combo-treatments, images of cells in the scratch area were captured at 10x magnification under an inverted microscope at 0, and 24 h.

Cell apoptosis assay

AO/EB dual staining was performed to detect apoptosis in cells morphologically. After 24 h of treatments, cells were stained with AO/EB mixture. Finally, apoptotic and normal cells were counted under a fluorescent-attached inverter microscope, and the percentage of apoptosis was calculated.

Caspase activity assay

Caspase-3 activity was measured using a colorimetric activity test kit (BioVision Co.). The test is based on the cleavage of the chromogenic substrate DEVD-pNA by *Caspase-3*. Cells were lysed in cold lysis buffer for 30 min and centrifuged. 5 µL of 4 mM DEVD-pNA substrate was added, and after 2 h of incubation under culture conditions, measurements were made with an ELISA reader at 405 nm. *Caspase-3* amounts were calculated as fold change.

RNA extraction and qRT-PCR

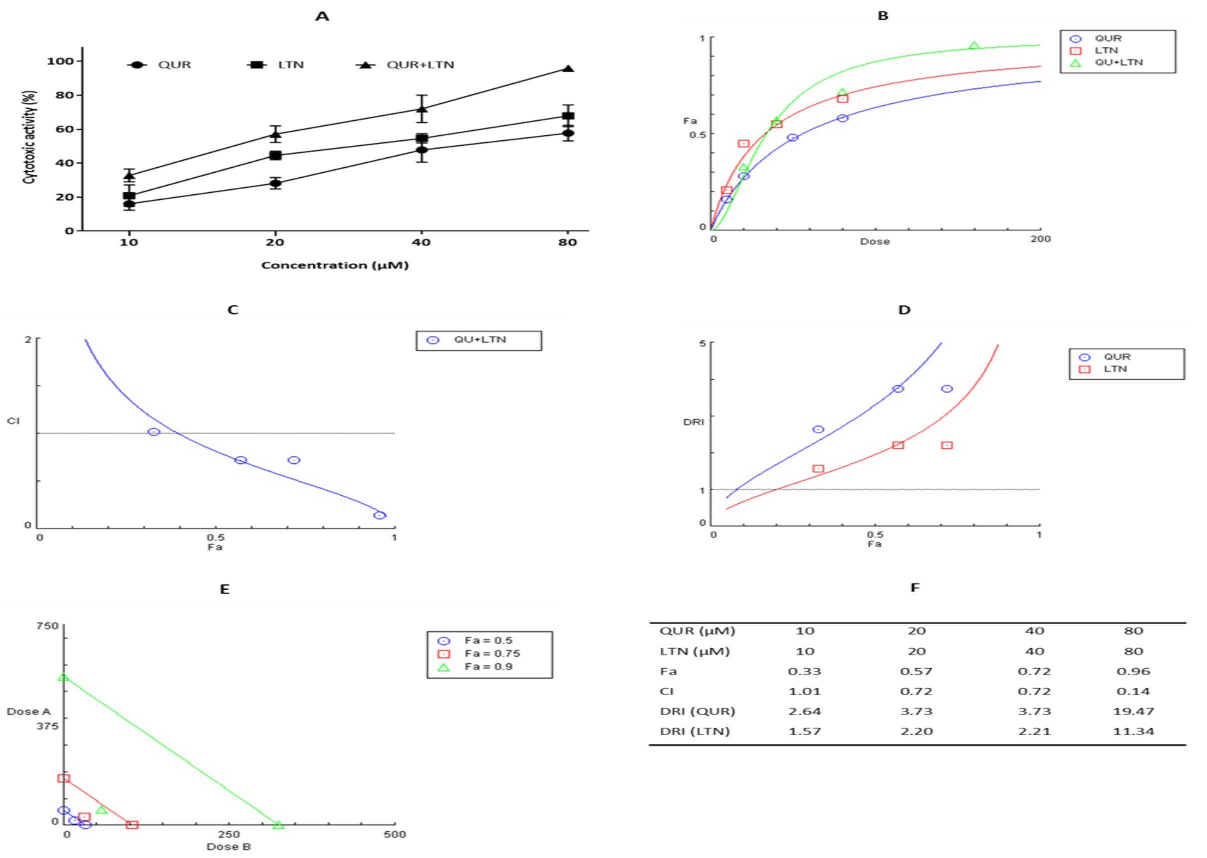
Total RNA was extracted from cells using the Pure Link RNA Mini Kit. Then, cDNA synthesis with the High Capacity cDNA Reverse Transcriptase kit and quantification with the SYBR Green kit were performed using a real-time PCR device (Rotor-Gene Q, Qiagen), according to the manufacturer's protocols. *p53* (forward primer: 5'-gtccaacaacaccagctcct-3'; reverse primer: 5'-cctcattcagctctcggaac-3'), *Bcl-2* (forward primer: 5'-gtgaactgggggaggattgt-3'; reverse primer: 5'-ggagaaatcaaacagaggcc-3'), and β-*actin* (forward primer: 5'-ctcttccagccttccttcct-3'; reverse primer: 5'-agcactgtgttggcgtacag-3') primers were used in studies (Erdogan et al., 2022), and mRNA expression levels were determined by the comparative threshold cycle (Ct) method ($2^{-\Delta Ct}$) (Livak & Schmittgen, 2001) for relative quantification. β-*actin* was used for normalization. The relative amounts of genes are shown as the fold change in their expression.

Statistical analysis

Statistical analysis of quantitative data from all experiments were performed using the one-way ANOVA and Tukey's multiple comparison tests with GraphPad Prism software. Data are presented as the mean ± SD of three experiments, and $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The serious side effects of traditional approaches to cancer treatment and the great burden they create on the healthcare system have recently led to increased interest in phytochemicals, that have the potential to regulate multiple cellular mechanisms with fewer side effects. The fact that phytochemicals inhibit the proliferation, invasion and migration of cancer cells, as well as induce their death and apoptosis, makes these compounds strikingly alternative for cancer treatment (Rizeq et al., 2020). In this study, the cytotoxic and apoptotic effects of monotherapeutic and combotherapeutic use of QUR and LTN in MCF-7 cells were investigated. The results of the cytotoxicity analysis are given in Figure 1. As seen in Figure 1.A, while QUR and LTN monotherapy exhibited concentration-dependent cytotoxic activity, this activity was seen to be more pronounced in combotherapy. It was observed that MCF-7 breast cancer cells treated with 80 µM QUR plus 80 µM LTN inhibited their viability by around 96%. IC_{50} values for QUR and LTN were determined as 55.5 ± 7.2 µM and 32.8 ± 2.9 µM, respectively. In order to evaluate the interaction between QUR and LTN, isobologram analyses were performed using CompuSyn program. Figure 1.B and 1.C show the dose-effect plots and combination index graphs, respectively. The combination indexes (CI) of the four tested combinations (10 µM, 20 µM, 40 µM, and 80 µM QUR plus LTN) were found to be 1.01, 0.72, 0.72, and 0.14, respectively. Since CI <1 indicates synergism (Chou, 2006), three of the four tested combinations (20 µM, 40 µM, and 80 µM QUR plus LTN) appear to have synergistic interactions. In addition, these three combinations had DRI (dose reduction index)>1, which means favourable combinations (Figure 1.D). The isobologram graph (Figure 1.E) and CompuSyn analysis data (Figure 1.F) also provide interesting results in terms of demonstrating the synergistic effect of QUR+LTN treatment.



QUR (µM)	10	20	40	80
LTN (µM)	10	20	40	80
Fa	0.33	0.57	0.72	0.96
CI	1.01	0.72	0.72	0.14
DRI (QUR)	2.64	3.73	3.73	19.47
DRI (LTN)	1.57	2.20	2.21	11.34

Figure 1. Cytotoxic activity of QUR and LTN mono- and combo- treatments on MCF-7 cells at 10-20-40-80 µM concentrations. **A.** The findings obtained from the WST-1 cell viability analysis were evaluated and % cytotoxic activities were determined. Cytotoxicity data analyzed with the CompuSyn program were used to determine whether there was synergism between QUR and LTN. **B.** Dose-effect plots of QUR, LTN and QUR + LTN (Fa, fraction affected). **C.** Combination index (CI) graph. CI <1 indicates synergism. **D.** Graph showing DRI values of QUR and LTN. DRI >1 indicates favourable combinations. **E.** Isobologram graph showing the concentrations required for 90% (Fa 0.9), 75% (Fa 0.75) and 50% (Fa 0.5) inhibition for each agent. **F.** CompuSyn analysis data. Data show the results of three replicate experiments. QUR and QU;quercetin, LTN;luteolin, Fa;Fraction affected.

The efficacy of QUR and LTN on survival and proliferation in MCF-7 cell line by clonogenic assay was estimated. In clonogenic assay, 5 and 15 µM concentrations of both compounds were used in mono- and combo-treatment groups. As clearly seen in Figure 2, 5 µM monotherapies ($P<0.05$) caused a significant decrease in the colony number of MCF-7 cells compared to untreated control group cells, while this decrease was more significant in combined treatment compared to both control group ($P<0.05$) and QUR treated group ($P<0.05$). Likewise, the findings show that 15 µM treatment decreased the colony number in QUR ($P<0.05$), LTN ($P<0.01$), and combined ($P<0.01$) treatment compared to the control cells. In addition, it was observed that MCF-7 cells treated with QUR + LTN showed higher inhibition of the tendency to form colonies compared to MCF-7 cells treated with monotherapies ($P<0.05$). Moreover, combined therapy was shown to be more effective than monotherapy by colony survival experiments, which was consistent with cytotoxicity analysis.

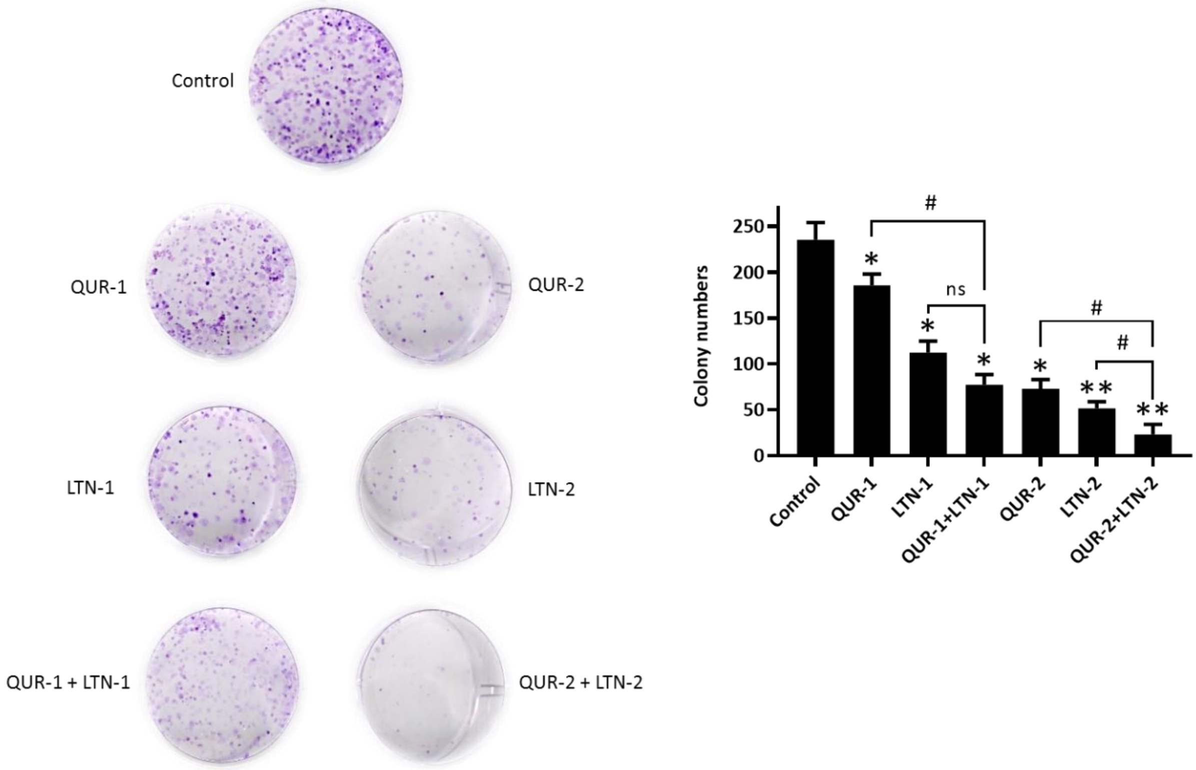


Figure 2. Clonogenic assay findings. The effects of different concentrations of QUR and LTN mono- and combo-treatments on colony survival in MCF-7 cells were observed. Colony numbers were determined and expressed graphically. QUR-1; 5 µM quercetin, LTN-1; 5 µM luteolin, QUR-2; 15 µM quercetin, LTN-2; 15 µM luteolin. represent the results of three replicate experiments as mean+SD. *$P<0.05$, ** $P<0.01$; compared to control. # $P<0.05$, ns:not significant; compared to QUR-1/2 or LTN-1/2 treatments.

In vitro wound healing assay was performed to evaluate the anti-metastatic abilities of monotherapeutic and combination treatments of QUR and LTN on MCF-7 cells. The width of the wound scratch areas captured in microscope images at 0 and 24 h after treatment was evaluated. As seen in Figure 3, both monotherapies ($P<0.05$), and combination treatment ($P<0.01$) significantly suppressed cell migration compared to the control group. It was also observed that the wound scratch in the combination treatment group remained significantly wider at 24 h compared to the monotherapy groups ($P<0.05$). This result shows that the combined treatment suppressed cell migration more than monotherapies.

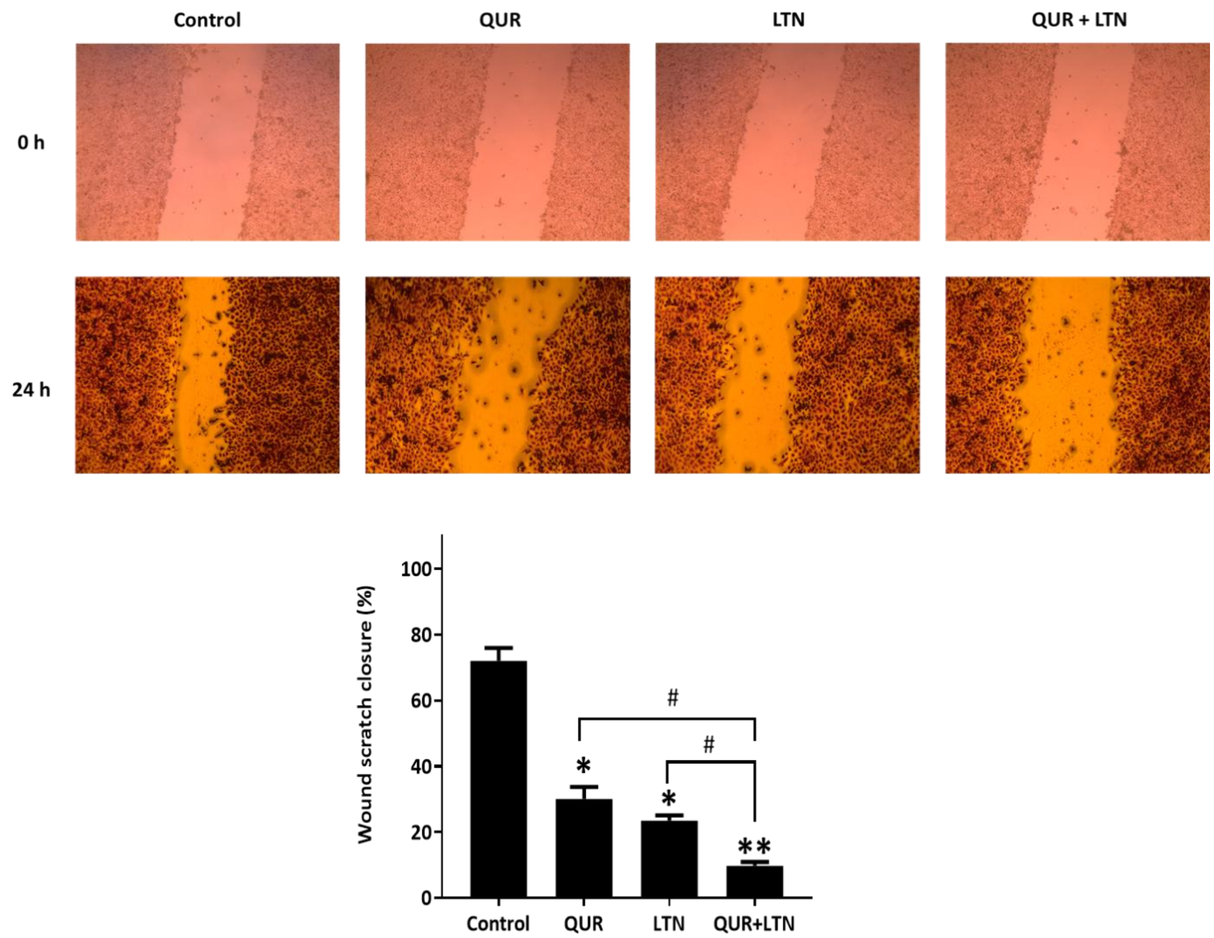


Figure 3. Cell migration analysis findings. The effect of QUR and LTN mono- and combo- treatments on cell migration in MCF-7 cells was determined by wound healing analysis. The rate of wound closure was given as a percentage by taking microscope images at 0 and 24 h of treatments and normalized each group with its own control. QUR; quercetin, LTN; luteolin. Data represent the results of three replicate experiments as mean+SD. *P<0.05, ** P<0.01; compared to control. # P<0.05; compared to QUR or LTN treatments.

Apoptosis is known as programmed cell death, and avoiding apoptosis is a characteristic feature of cancer cells. Therefore, it is suggested that the regulation and activation of molecular mechanisms that will direct cells to apoptosis should be at the center of new treatment approaches to cancer (Hanahan, 2022). Despite the great therapeutic potential of phytochemicals, insight into the molecular effects of their combined use on different cellular signaling pathways is critical to understanding their interactions. Increasing evidence from clinical and preclinical studies reveals the inhibitory role of phytochemicals in cancer development and progression through their positive regulatory effect on apoptotic signaling pathways (Choudhari et al., 2020). The effects of QUR and LTN mono- and combo-therapies on the morphology of cancer cells were determined by AO/EB dual staining, and analyzed under a fluorescence-attached inverter microscope. After incubation with the 40 µM QUR and 40 µM LTN mono- and combo-treatments for 24 h, some morphological changes were observed in the cells, such as condensation of chromatin material and formation of apoptotic bodies, which are cellular signs of apoptosis. While live cells form a green color because they take up AO and do not take up EB, apoptotic cells turn orange-red when EB penetrates the cell (Figure 4.A). As shown in Figure 4.B, the number of apoptotic cells increased significantly in cancer cells that received both monotherapy and combination therapy (P<0.05 and P<0.01, respectively). In addition, combined treatment significantly triggered the formation of apoptotic cell morphology compared to both the monotherapies (P<0.01). Figure 4.C shows the effect of treatments on *Caspase-3* activity, an important apoptotic factor. While QUR and LTN increased the amount of *Caspase-3* by 2.8 and 3.9 fold, respectively, this increase was not found to be statistically significant (P>0.05). However, *Caspase-3* activity increased significantly in cells that received combination therapy (p<0.01), and this increase was also greater compared to monotherapies (P<0.05 and P<0.01). The expression levels of *p53* gene, an important tumor suppressor, increased with monotherapies (P<0.05), but exhibited a sharper increase with combined treatment compared to control (P<0.01), and monotherapies (P<0.01) (Figure 4.D). In addition, anti-apoptotic *Bcl-2* gene expression decreased with QUR and LTN treatments (P<0.05), while a significant decrease was observed with QUR plus LTN combined treatment compared to both control (P<0.01), and QUR treatment (P<0.05) (Figure 4.E).

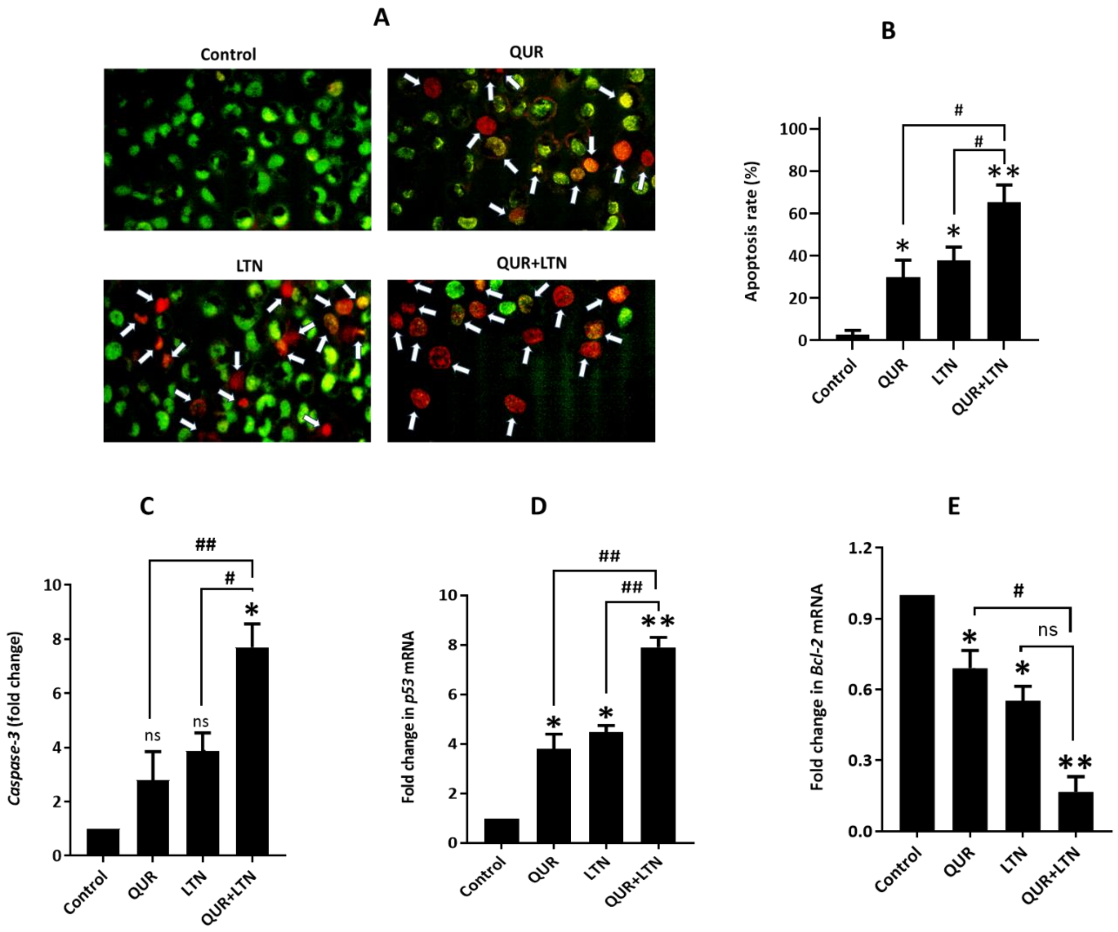


Figure 4. Apoptotic effect on MCF-7 cells of QUR, LTN and QUR+LTN treatments. **A.** Microscope images of cells exposed to AO/EB dual staining after treatments. White arrows mark apoptotic cells. **B.** The ratio of apoptotic cells visualized by AO/EB dual staining analysis was expressed graphically. **C.** *Caspase-3* levels were given as fold change. *p53* (**D**), and *Bcl-2* (**E**) mRNA levels were quantitatively calculated as fold change. *P<0.05, ** P<0.01, ns:not significant; compared to control. # P<0.05, ## P<0.01, ns:not significant; compared to QUR or LTN treatments.

LTN acts by inducing apoptosis and autophagy, and inhibiting cell invasion and migration in cancer cells. Studies have demonstrated the anticancer activity of QUR in cancer cells through inhibition of angiogenesis, arrest of the cell cycle, induction of apoptosis and DNA damage (Talib et al., 2022). It has been reported that the combined use of plant-derived natural compound treatments at low doses exhibits more effective anticancer activity than their monotherapeutic use at high doses (Sauter, 2020). The combination approaches are interesting in cancer treatment as they target different cellular pathways in a distinct and synergistic manner (Mokhtari et al., 2017). Many preclinical studies have shown that combined phytochemical treatments significantly increase anticancer activity (Fantini et al., 2015; Nikanjam et al., 2017). The combined use of phytochemicals, including quercetin, curcumin, and resveratrol, on breast cancer cell lines has been shown to have a synergistic effect by suppressing proliferation and cell migration, arresting the cell cycle, and increasing apoptotic activity (Rizeq et al., 2020). A study revealed that the combination of LTN and QUR with 5-Fluorouracil had synergistic apoptotic and antiproliferative effects on HT-29 colorectal cancer cells, thereby minimizing the side effects of 5-FU (Erdogan et al., 2022). The combination of QUR and LTN was reported to inhibit the invasion and migration of squamous carcinoma (Fan et al., 2019), and led to metastatic inhibition of A431 cervical cancer cells (Lin et al., 2017). In line with all these studies, in this study, QUR plus LTN combination showed cytotoxic and apoptotic activity in MCF-7 cells, while inhibiting colony survival and cell migration. Considering the side effects of current conventional treatments, this study contributes to revealing the great potential of combined use of phytochemicals to minimize these undesirable effects. More studies are needed to reveal effective phytochemical combinations that can support conventional treatments.

CONCLUSION

In conclusion, this study, which was conducted to determine the cytotoxic and apoptotic effects of the combination of QUR plus LTN, revealed that the combined treatment showed critical cytotoxic activity on MCF-7 cells, and that there was a synergistic interaction between these two phytochemicals. It was also observed that the combination therapy significantly inhibited colony survival and cell migration compared to monotherapy. All these effects suggest that the combination therapy, which was determined to have a regulatory role on some pro-apoptotic markers such as *Caspase-3* and *p53*, and anti-apoptotic markers such as *Bcl-2*, triggered apoptosis. Supporting the interesting findings obtained from this study with comprehensive *in vitro* and *in vivo* experiments in the future may pave the way for the evaluation of the combination of QUR and LTN as a chemotherapeutic agent.

Acknowledgements: The author would like to thank Bingöl University Cancer Research Laboratory and its staff.

Conflict of Interest Statement: The author declare that there are no conflicts of interest.

Contribution Rate Statement Summary of Researchers: The author confirms that he has sole responsibility for the design of the study, methodology, conduct of experiments, evaluation and interpretation of results, and preparation of the manuscript.

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