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BIOMEDICAL SCIENCES

Potentiation of the Effect of Lonidamine by Quercetin in MCF-7 human breast cancer cells through downregulation of MMP-2/9 mRNA Expression

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Abstract: Combination therapies are becoming increasingly important to develop an effective treatment in cancer. Lonidamine is frequently used in cancer treatment, but it's often preferred to be used in combination with other drugs because of its side effects. In the present study, the efficacy of the combination of lonidamine with quercetin, a flavonoid of natural origin, on human MCF-7 breast cancer cells was evaluated. The results showed that the combined use of the compounds significantly increased cytotoxicity compared to administration alone ($p<0.0001$). In addition, while lonidamine induced a cell cycle arrest in the G2/M phase, administration of quercetin and its combination with lonidamine arrested the cell division at S point, indicating the synergistic strength of quercetin on cytotoxicity. The combination of quercetin and lonidamine significantly induced apoptosis of MCF-7 cells ($p<0.0001$) and increased caspase levels ($p<0.0001$). In this study, the combination of quercetin and lonidamine has been evaluated for the first time and the combination treatment decreased MMP-2/-9 mRNA expression more potently than the effects of the compounds alone. The results showed that lonidamine was more effective when combined with quercetin, and their combination may be a candidate for a novel strategy of treatment for breast cancer.

Key words: Apoptosis, Lonidamine, MCF-7, MMP-2, MMP-9, Quercetin.

INTRODUCTION

Breast cancer is the most widely encountered malignancy among women worldwide and is the primary cause of cancer-related death in women (Tao et al. 2015). Although there are several active compounds used in the treatment of this disease, their efficiency is restricted due to drug resistance. Therefore, developing novel therapeutic agents remains an important goal in cancer studies.

Lonidamine (LND) or 1-(2,4-dichlorobenzyl)-1H-indazole-3-carboxylic acid was first introduced as an antispermatogenic agent in 1979 but later found to be effective on cancer cells as well (Nath et al. 2016). It has been reported that LND affects a wide range of solid tumors via interfering with energy metabolism by altering the plasma and mitochondrial membranes and affecting DNA repair as well as cellular acidification (Bhutia et al. 2016, Morais-Santos et al. 2013, Nath et al. 2015). While it exhibits limited anticancer activity when administered alone, it shows a remarkable potential in increasing the effect of conventional chemotherapeutic agents. Several studies have revealed that lonidamine may increase the response of tumor cells to various compounds such as doxorubicin and melphalan (Jin et al. 2019, Nath et al. 2013).

Flavonoids are active phytochemical compounds with a broad spectrum of pharmacological effects. One of these compounds known as quercetin (3,3',4',5,7 pentahydroxy flavone) is a major member of this family that can be found in many fruits and vegetables. Several studies have shown that quercetin displays a wide range of pharmacological activities such as anti-oxidant, anti-inflammatory, anti-ulcer, immuno-modulatory and vasodilation (Li et al. 2016, Marunaka et al. 2017, Shoskes & Nickel 2011).

Researchers have also reported that quercetin has a strong growth inhibitory effect on a number of cancer cells such as liver, colon, breast, lung, pancreatic and ovarian cancers (Rauf et al. 2018). Besides having a significant antioxidant activity, quercetin also exhibits proapoptotic effects on tumor cells through blocking the cell cycle at different phases in several types of tumors (Wu et al. 2018).

Quercetin has also become a subject of combination therapies. When administered with a number of chemotherapeutic agents, quercetin has been observed to potentiate their anticancer activities. For instance, it has been reported that quercetin sensitizes breast cancer cells as well as hepatocellular carcinoma cells to doxorubicin (Minaei et al. 2016, Wang et al. 2012). Moreover, quercetin has been reported to decrease the expression of multidrug resistance related protein (MRP1) in prostate cancer cells and enhance the therapeutic effect of docetaxel treatment (Wang et al. 2015). Similarly, administration of quercetin on multidrug resistant K562 leukaemia cells resulted in enhanced apoptotic activity in a dose-dependent manner. Additionally, a combination of quercetin with adriamycin has exhibited a synergistic cytotoxic effect on these cells (Chen et al. 2015). In line with this data, we aimed to evaluate the anticancer effects of a combination treatment of quercetin and lonidamine in MCF-7 breast cancer cells.

MATERIALS AND METHODS

Cell culture and treatment

Human breast carcinoma cell line MCF-7 (HTB-22) was purchased from American Type Culture Collection. Cells were grown in Dulbecco's Modified Eagle's medium (DMEM) (Sigma, Germany) supplemented with 10% fetal bovine serum FBS (Sigma, Germany), 1% L-glutamine (Sigma, Germany) and 1% penicillin/streptomycin (Sigma, Germany), and incubated in a humidified atmosphere containing 5% CO_2 at 37°C. Lonidamine and quercetin (Sigma, Germany) were dissolved in 0.01% dimethylsulfoxide, and cells were treated with 20-100 µM of quercetin, 0.01-10 µM of lonidamine and according to the results of pure compounds, the cells were then treated with quercetin+lonidamine combinations at 80/0.1, 80/1 and 80/5 µM concentrations.

Cell proliferation assay

The effects of quercetin and LND, alone and in combination, on proliferation of MCF-7 cells were assessed by MTT assay. Briefly, cells were treated with 20, 40, 60, 80 and 100 µM of quercetin, 0.01, 0.1, 1, 5 and 10 µM of LND alone for 24 hr. The combination treatment was applied with 80 µM of quercetin and 0.1, 1 and 5 µM of LND while untreated cells were used as control. After 24 h of incubation, cells were treated with MTT solution (5 mg/ml) and incubated at 37°C for 4 hr. The absorbance at 540 nm was measured with a microplate reader (Thermo, Germany). The data represent mean ± standard deviation (SD) of two independent experiments. The effect of the combination treatment has been assessed via calculating the combination index (CI). The individual IC_{50} values of both compounds have

been used in the calculation according to the following equation (Chou & Talalay 1984):

$CI=(D)com_1/(D)_1+(D)com_2/(D)_2$ in which (D) com1 (or (D)com2) is the IC_{50} value for drug1 (or 2) in the combination and (D)1 (or (D)2) is the IC_{50} value of the single treatment. The resulting combination index (CI) was used to determine the effect of the quercetin+lonidamine combination as CI=1, CI<1, and CI>1 as additive, synergism, or antagonism.

Cell Cycle Analysis

The effects of the compounds on cell cycle arrest was evaluated using Muse Cell Cycle Assay Kit (Merck Millipore, Germany). The cells were treated with 80 µM of quercetin, 5 µM of LND and Q+LND combination (80/5 µM). After 24 hr of incubation, cells were fixed and prepared for analysis according to the manufacturer's instructions. The cell population at G0/G1, S and G2/M phases of the cell cycle was analysed by Muse Cell Analyzer (Millipore, Germany).

Annexin-V binding assay

MCF-7 cells were seeded at a density of 2×10^5 cells per well and incubated for 24 hr. Cells were then treated with 80 µM of quercetin, 5 µM of LND and Q+LND combination (80/5 µM) for 24 hr. After the incubation period, cells were harvested and rinsed with 1xPBS, and Annexin V assay kit (Millipore, Germany) was applied according to the manufacturer's instructions. The apoptotic cell population was determined using Muse Cell Analyzer (Millipore, Germany).

Measurement of caspase levels

Total caspase levels were measured by Muse Multicaspase Assay Kit (Millipore) according to user's manual. MCF-7 cells treated with different concentrations of quercetin, LND and their combinations and cell lysates were diluted in multicaspase working solution and incubated for 30 min at 37°C. 150 µl of 7-AAD solution were added to all tubes, mixed thoroughly and run on Muse Cell Analyzer (Millipore, Germany). Four populations of cells have been determined; caspase() and 7-AAD(-) as live cells, caspase(+) and 7-AAD(-) cells exhibiting Pan Caspase activity, caspase(+) and 7-AAD(+) cells exhibiting late stage of caspase activity and caspase(-) and 7- AAD(+) as necrotic cells.

Fluorescence imaging

The apoptotic effects of quercetin, LND and Q+LND combination on MCF-7 cells were visualized using Annexin V-FITC Fluorescence microscopy kit (BD, Germany). After the cells were treated with the compounds, Annexin V-FITC dye was applied on cells for 20 min at room temperature in the dark. Images were acquired using an inverted fluorescence microscope (Olympus, Germany).

RT-PCR experiments

Total RNA was isolated from the cells using RNEasy plus mini kit (Qiagen, Germany) according to the manufacturer's instructions. The cDNA of each sample was synthesized from the total RNA extracts in order to use in PCR amplification. For real-time PCR analysis, reactions were carried out in triplicate using Rotor Gene System (Qiagen). RT^2 qPCR Primer Assay numbers for MMP-2, MMP-9 and GAPDH genes were PPH00151B, PPH00152E and PPH14985F, respectively (Qiagen, Germany). Data analysis has been performed using the comparative ΔΔCt method calculating the difference between the threshold cycle (Ct) values of the target and reference gene of each sample.

Statistical analysis

GraphPad Prism 6.0 version (GraphPad Software Inc.) was used for the statistical analysis. Data were represented as mean ± SD, and differences

between groups were analyzed using oneway ANOVA test with Bonferroni's multiple comparisons.

RESULTS

Cell proliferation experiments in MCF-7 cells

The effects of quercetin, LND and Q+LND combinations on the proliferation of MCF-7 cells were determined by MTT assay and the results were given in Fig. 1. Quercetin was cytotoxic on MCF-7 cells at 80 and 100 µM concentrations ($p<0.05$). The proliferation of the cell population was determined as 91.87±2.06% and 86.99±5.74%, respectively. LND induced significant cytotoxicity at 5 and 10 µM concentrations and the rate of the cell proliferation was detected as 83.57±14.34% ($p<0.05$) and 68.03±8.21% ($p<0.001$), respectively.

On the other hand, the Q+LND combination treatment has shown the most potent cytotoxicity that the cell proliferation was decreased to 66.15±3.79%, 65.56±6.81% and 61.26±5.75% at 80/0.1, 80/1, and 80/5 µM concentrations, respectively ($p<0.0001$). Besides, the combination effect was evaluated by calculation of combination index (CI), and all combinations of quercetin with lonidamine gave a CI value less than 1, indicating a synergistic effect.

Quercetin/LND combination induced a cell cycle arrest at S phase

The effects of Q and LND on cell cycle in MCF-7 cells were investigated. For this purpose, cells were treated with Q (80 µM), LND (5 µM) and Q + LND combination (80 + 5 µM). After 24 h of incubation, cell cycle analyses were performed using the cell cycle assay kit. The cell population percentage in G0/G1, S and G2/M stages was determined using the software of the instrument. According to the results, LND has been found to induce a cell cycle arrest at G2/M phase of MCF-7 cells. However, the effects of quercetin alone

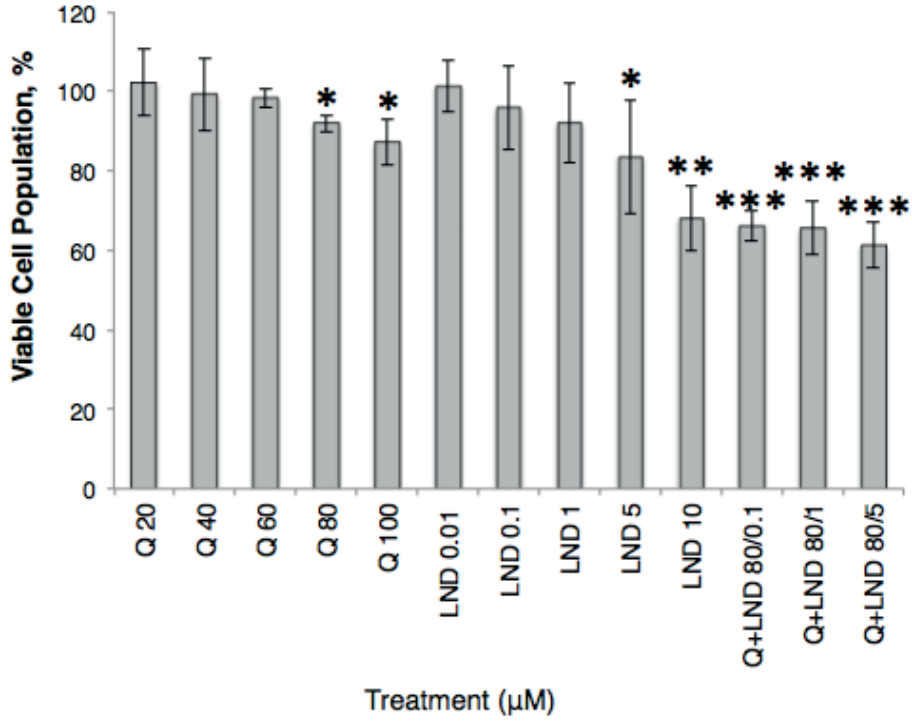

Figure 1. The effects of Quercetin, LND and Q+LND combination on the proliferation of MCF-7 human breast cancer cells. The cells were treated with quercetin (20, 40, 60, 80, 100 µM), LND (0.01, 0.1, 1, 5, 10 µM) and Q+LND combination (80/0.1, 80/1, 80/5 µM) for 24 hr, and the proliferation of the cells was determined by MTT assay. The results are expressed as percentage of the cell proliferation compared with untreated control. The data represent the mean ± SD in four replicates of two independent experiments. The differences are *p < 0.05 and **p < 0.01, ***p<0.0001 compared with control.

and Q+LND combination were similar as they arrest the cell division cycle at the S regulatory point which revealed that the proliferation was stopped at chromosomal DNA synthesis phase (Fig. 2). The results revealed that quercetin has stronger effect on cell cycle arrest in MCF-7 cells.

Induction of apoptosis by quercetin, LND and Q+LND combination in MCF-7 cells

To evaluate the apoptotic nature of the cell death caused by Q+LND, the annexin-V binding assay was performed (Fig. 3). The total apoptotic cell population % was determined as 8.25±1.44% and 10.80±1.80% in quercetin and LND treated groups (p=0.0439, p=0.001, respectively). On the other hand, the measured apoptotic cell population was significantly increased to 33.65±3.05% in combination treatment group including 80 μM of quercetin and 5 μM of LND (p<0.0001), indicating the synergistic effect of the treatment. The increased apoptotic cell population in the Q+LND treated group was also shown by fluorescence imaging studies (Fig. 4).

Following the detection of apoptotic efficiencies of quercetin and lonidamine, the effects of these compounds alone and their combination on caspase levels of MCF-7 cells were also evaluated. For that purpose, multicaspase assay was performed which allows to detect the presence of multiple caspases including caspase-1, 3, 4, 5, 6, 7, 8, and 9. The total caspase levels were significantly higher in all treated groups compared to control. The total caspase levels were recorded as 16.8±1.36% (p=0.0114), 18.48±0.93% (p=0.0009) and 25.08±0.53% (p<0.0001) in quercetin, LND and Q+LND groups, respectively (Fig. 5).

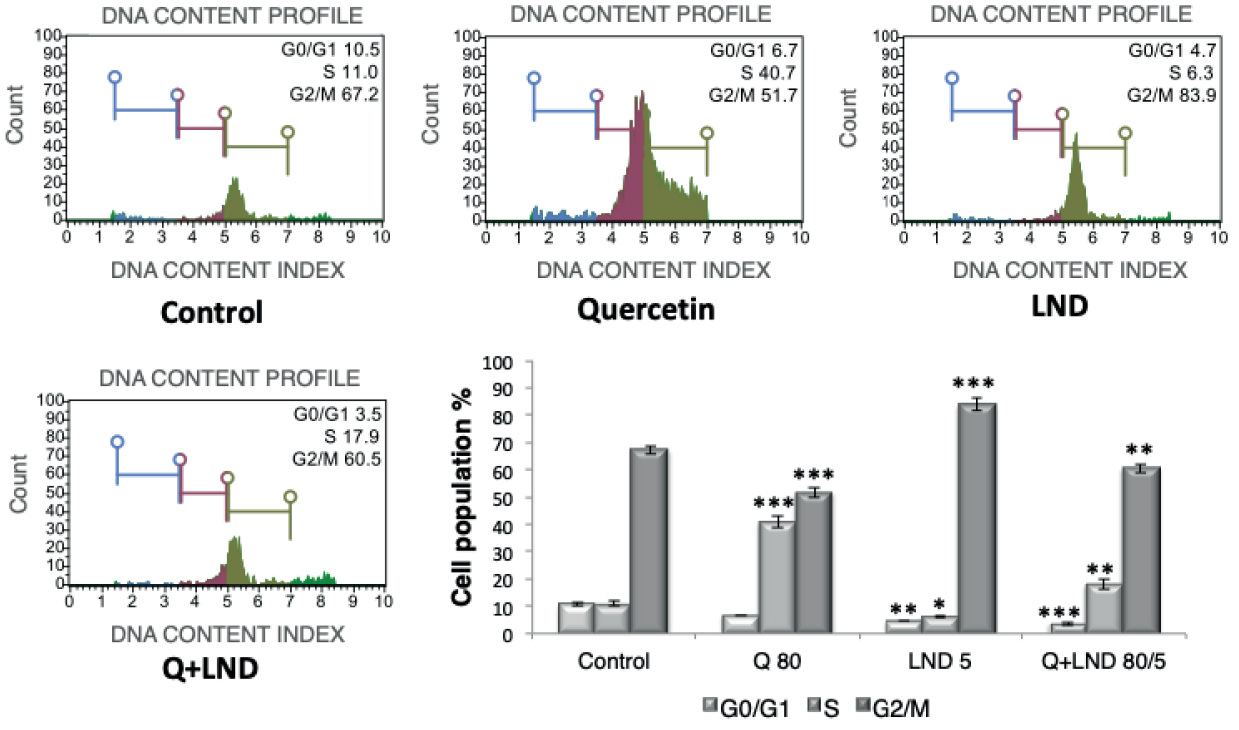

Figure 2. The results of cell cycle analysis. The MCF-7 cells were treated with 80 μM of quercetin, 5 μM of LND and 80/5 μM of Q+LND combination for 24 hr and the cell population percentage at different phases of cell cycle including G0/G1, S, and G2/M were detected by Muse Cell Analyzer (Millipore, Germany). The results were given for three independent experiments.

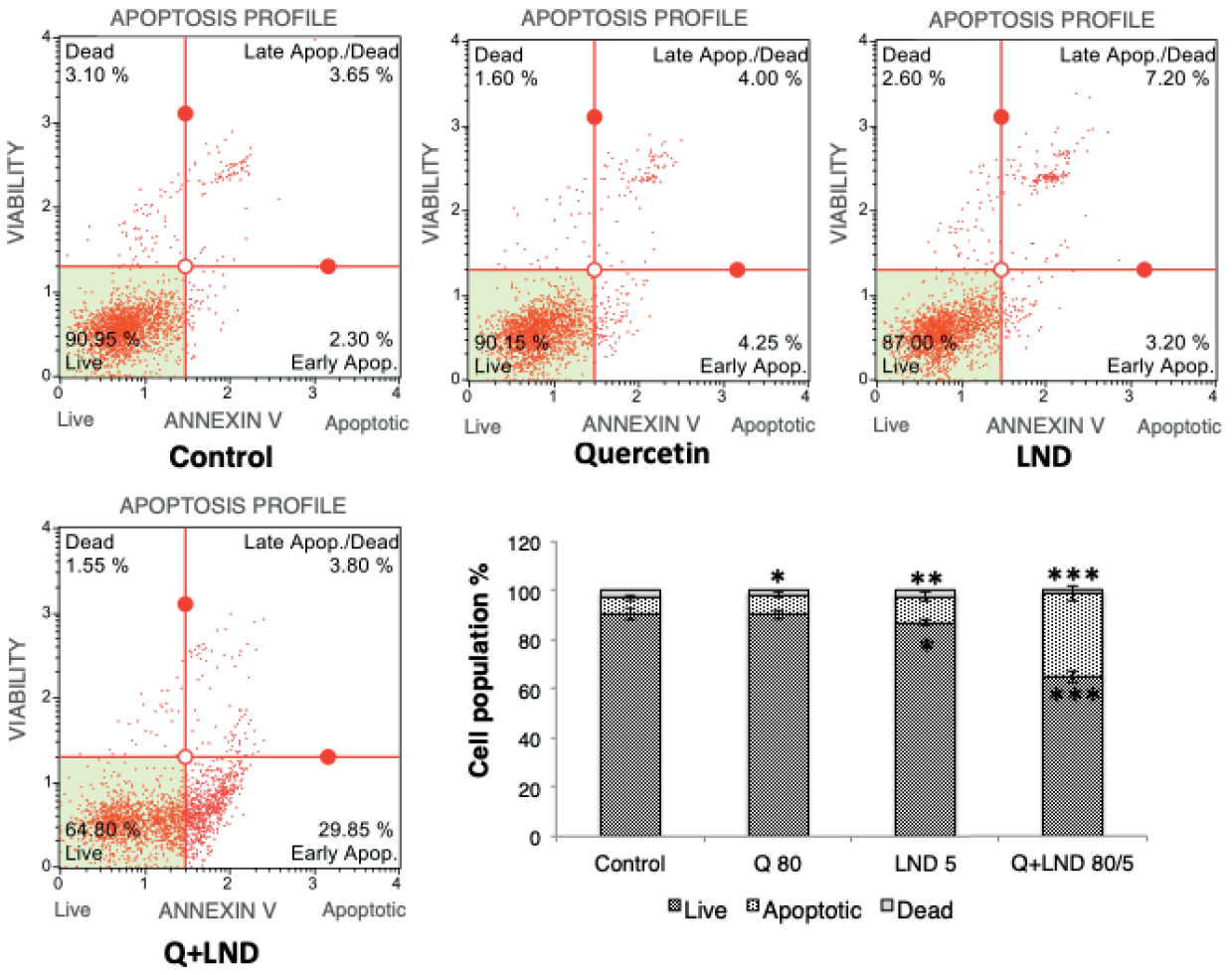

Figure 3. The results of Annexin V binding assay. The cells were treated with 80 μM of quercetin, 5 μM of LND and 80/5 μM of Q+LND combination for 24 hr, and the apoptosis was detected by Muse cell analyzer (Millipore). Nontreated cells were used as control. The apoptotic cells were determined by the Annexin V positivity based on phosphatidylserine exposure and dead cells were determined by the nuclear dye 7aminoactinomycin D (7-AAD) positivity. Four different cell population were enabled to determine by cytofluorometric separation on a Muse cell analyzer: non-apoptotic live (lower left (LL): 7-AAD negative, apoptosis negative), non-apoptotic dead (upper left (UL): 7-AAD positive, apoptosis negative), apoptotic live (lower right (LR): 7-AAD negative, apoptosis positive), and apoptotic dead (upper right (UR):7-AAD positive, apoptosis positive) cells. The figure represents cytofluorimetric dot plots of three independent experiments performed for annexin V detection and mean ± SD of late apoptotic population % (bar graph). The differences are *, ** and * from control: $p < 0.05$, $p<0.01$ and $p < 0.0001$, respectively.**

Relative mRNA expression of MMP-2/-9

In the present study, the antimetastatic efficiency of quercetin, LND and Q+LND combination in MCF7 cells was evaluated by gene expression (Fig. 6) measurements of MMP-2 and MMP-9. The cells were treated with quercetin (80 μM) and LND (5 μM) alone and with Q+LND combination (80/5 μM). The results of PCR analysis showed that all compounds significantly decreased the relative mRNA expression of MMP-2/-9 when compared with control ($p<0.0001$). On the other hand, the relative mRNA expression of MMP-2 in treated groups were significantly lower than the results of MMP-9 gene ($p<0.05$).

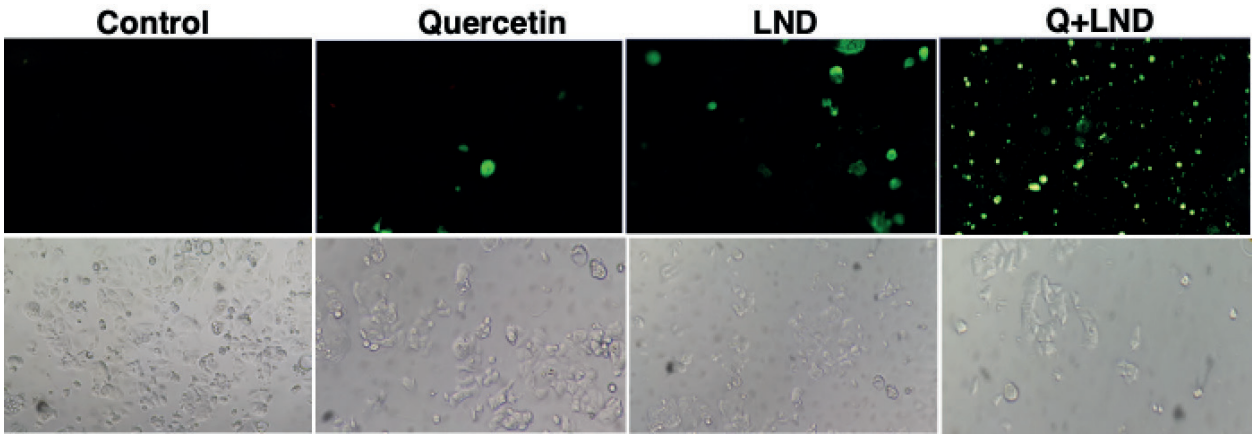

Figure 4. Fluorescence microscopy analysis of Annexin V-FITC staining. MCF-7 breast cancer cells were left untreated (control) and treated with 80 µM of quercetin and 5 µM of LND alone, and 80/5 µM Q+LND combination for 24 hr, and the fluorescence images were obtained by Annexin V staining protocol according to the instructions (BD Biosciences, Germany) through fluorescence microscope (Olympus, Germany).

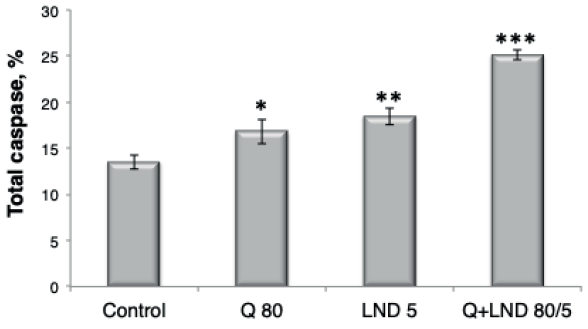

Figure 5. Q+LND combination increased total caspase levels of MCF-7 cells significantly. The cells were treated with 80 µM of quercetin, 5 µM of LND and 80/5 µM of Q+LND combination for 24 hr and total caspase levels were detected with multicaspase assay kit. Values are mean±SD of three samples of medium from wells containing MCF-7 cells. The differences are *, ** and *** from control: $p < 0.05$, $p<0.01$ and $p < 0.0001$, respectively.

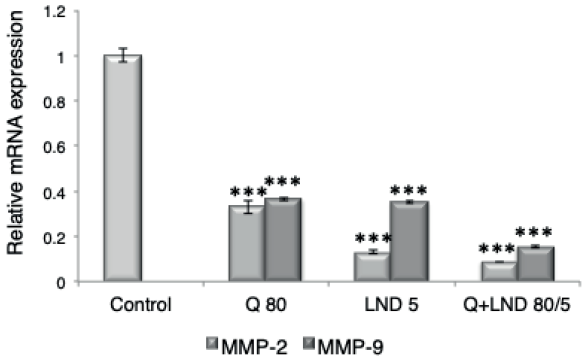

Figure 6. Q+LND combination downregulated MMP2/9 mRNA expression in a dose-dependent manner. ΔΔCt method was used to determine relative mRNA expression of MMP-2/9 gene in MCF-7 cells. Mean ± SD are shown (n = 3). The differences are *** from control, $p < 0.0001$.

DISCUSSION

Breast cancer is the most frequently diagnosed cancer among women with a high incidence rate (Tao et al. 2015). Although combinations of the available drug molecules may yield significant results, high toxicity still stands as a problem in the treatment. Therefore, studies based on the combination of chemotherapeutics with various compounds of natural origin have gained importance over the years. In this regard, we have explored the effect of an anticancer agent lonidamine in combination with a plant-derived compound, quercetin, with the aim of providing an effective treatment using lower concentrations.

Matrix metalloproteinases (MMPs) are a zinc-dependent endopeptidase family which play a significant role in tissue remodelling through degrading extracellular matrix components, thereby providing a convenient environment for cellular growth (Lu et al. 2012). However, multiple members of MMPs are overexpressed in several cancers in order to support invasion and migration of tumour cells. Among these, MMP-2 and -9 have been reported to significantly contribute to angiogenesis, cell proliferation and metastasis (Baspinar et al. 2017, Wang et al. 2014). Therefore, a large number of studies have been conducted to suppress the expression of MMPs in cancer. In this regard, compounds of natural origin have yielded promising results as well. For instance, a natural flavonoid, luteolin-7-O-glucoside, has been reported to inhibit migration and invasion in oral cancer cells through regulating MMP-2 expression (Velmurugan et al. 2020). Another dietary flavonoid, myricetin, has inhibited migration of A549 lung cancer cells via downregulating MMP-2 and -9 expression (Kang et al. 2020). Similarly, a member of cucurbitacin family, cucurbitacin B, inhibited MMP-2 expression in both MCF-7 breast cancer cells as well as SW480 colorectal cancer cells and enhanced the anticancer activity of imatinib mesylate (Bakar 2016).

Quercetin is one of the natural compounds which is reported to have important anticancer activities in the literature. In several studies, the inhibitory role of quercetin on proliferation of various cancer cells have been demonstrated. Its mechanism of action has been linked to multiple cellular processes. For instance, it has been reported that quercetin inhibits tumor growth significantly via interfering in multiple signalling pathways such as PI3K, Akt, mTOR and AMPK (Su et al. 2016, Wang et al. 2011, Xiang et al. 2014). A number of studies have demonstrated that this interference leads to altered expression of MMPs in various cancers, thereby preventing migration and invasion. A study conducted on human glioma cells has revealed that quercetin inhibits the expression of MMP-9 through Akt and Erk signalling pathways (Pan et al. 2015). Similarly, it has been reported that quercetin suppresses Akt phosphorylation which resulted in reduced MMP-2 and -9 expression in hepatocellular carcinoma (Lu et al. 2017). It has also been reported that quercetin prevents migration and invasion of human oral cancer cells through inhibiting NF-kB which leads to downregulated MMP-2 and -9 expressions (Lai et al. 2013). Due to these promising results, quercetin is considered an important candidate in cancer treatment (Zheng et al. 2012). Several studies have been conducted on the efficacy of quercetin in combination with other chemotherapeutics. For instance, the cytotoxic activity of topotecan alone and in combination with quercetin on MCF-7 and MDA-MB-231 breast cancer cell lines were investigated. Results showed that a combination with quercetin reduced the IC_{50} value of topotecan significantly (Akbas et al. 2005). It has also been reported that doxorubicin in combination with quercetin decreases the viability of MDA-MB231 breast cancer cells synergistically as well as reducing the toxicity of doxorubicin (Staedler et al. 2011). Moreover, in vivo studies have demonstrated that doxorubicin-generated cardiotoxicity may be prevented by quercetin through inhibiting oxidative stress induced by Bmi-1 protein (Dong et al. 2014). On the other hand, it has been reported that quercetin potentiates a pro-autophagic effect of temozolomide and exhibits a potent synergistic effect when administered in combination on human glioma cells (Jakubowicz-Gil et al. 2010). Despite these promising results, due to its poor aqueous solubility and low bioavailability, quercetin has recently been a subject of nanomedicine in order to provide an

effective way of drug delivery (Guan et al. 2016, Nam et al. 2016).

Lonidamine is an indazole-3-carboxylic acid derivative that exhibits antiglycolytic activity in several cancers via interfering with ATP production in tumour cells. Studies have demonstrated that it has limited anticancer activity when administered alone; however, it has a great potential to enhance the efficacy of conventional chemotherapeutic agents (Nath et al. 2015). Even though some adverse effects have been observed in clinical trials of lonidamine, these effects were limited and investigations on various cancers continued. Overall data suggest that lonidamine is a promising candidate in cancer treatment with limited toxicity to healthy tissues (Cervantes-Madrid et al. 2015, Nath et al. 2015). Additionally, the effect of lonidamine on endothelial cell functions involved in angiogenesis has been investigated in vitro. Experiments have indicated that lonidamine reduces endothelial cell proliferation, migration, invasion, secretion of metalloproteinases, MMP-2 and MMP-9, and vessel formation in a dose dependent manner (Del Bufalo et al. 2004). These results clearly indicate a possible synergistic effect between lonidamine and quercetin in terms of suppressing MMP-2 and -9 expressions, thus providing a stronger anticancer activity on breast cancer cells.

In the present study we have investigated the effect of quercetin and lonidamine, alone and in combination, on MCF-7 human breast cancer cells. Experiments have revealed that the cell proliferation rate decreases significantly following a treatment with quercetin at 80 and 100 μM and with lonidamine at 5 and 10 μM. However, a combined treatment with quercetin (80 μM) and lonidamine (5 μM) has yielded a stronger antiproliferative effect on the cells compared to individual treatments. Previous studies have reported relatively higher doses or longer durations of exposure for both compounds in order to obtain a significant cytotoxic activity on breast cancer cells (Maurya & Vinayak 2019, Morais-Santos et al. 2013); however, some studies have reported a relatively lower dose for quercetin. For instance, Hanikoglu et al. 2020 reported the IC_{50} value of quercetin as 73.63 μM and Niazvand et al. 2019 reported it as 73.8 μM, both after 24 h of incubation (Hanikoglu et al. 2020, Niazvand et al. 2019). Overall data indicate that the doses we have used in our study are within the spectrum of doses used in the previous and/or recent studies. However, the selectivity of this combined treatment in breast cancer is yet to be elucidated. In this regard, an in vitro study has reported a selective cytotoxic effect of lonidamine on MCF-7 cells at a dose of 300 μM when compared with primary mammary epithelial cells, HMEC (Golding et al. 2013). On the other hand, multiple studies have reported that quercetin is selectively cytotoxic on breast cancer cells without affecting non-tumorigenic epithelial cells when applied in low doses (Jeong et al. 2009, Li et al. 2018) whereas increased doses of quercetin can be toxic on non-cancerous tissues (Wang et al. 2020). However, this problem can be eliminated via developing nanoformulations of the compound (Niazvand et al. 2019). Therefore, the proposed combination therapy can be considered for targeted drug delivery studies in order to improve its bioavailability and reduce the possible adverse effects.

In conclusion, lonidamine is a widely used compound in cancer treatment, but it brings along some difficulties in treatment because of its adverse effects. The present study has focused on the efficacy of lonidamine in combination with quercetin on MCF-7 breast cancer cells. The results have demonstrated that the combination of quercetin and lonidamine inhibited cell proliferation synergistically

in MCF-7 cells and induced apoptosis. Gene expression analyses have shown that this effect is likely due to the downregulating effect on MMP-2/-9 protein, which increases metastasis in cancer. These results suggest that quercetin may be considered a promising candidate in combination therapies as a chemosensitizer that would aid in dose reduction and minimizing drug toxicity in breast cancer treatment.

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