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Research Article

Thymoquinone Nanoparticle Induces Apoptosis and Cell Migration Retardation through Modulating of SUMOylation Process Genes in Breast Cancer Cell Line

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Background: Due to the heterogeneity of breast cancer, most advanced-stage patients are resistant to therapy. Disruption of SUMOylation, a post-translational modification, is linked to breast cancer.

Objective: This study aimed to assess the impact of thymoquinone nanoparticles (Liposomal-TQ), an anti-cancer drug, combined with doxorubicin (DXR), the most effective chemotherapeutic drug used to treat breast cancer, on the expression of SENP2 and SENP6, two major components involved in the SUMOylation process, in normal and cancerous breast cell lines.

Materials and Methods: The MCF7 cell line, a breast cancer cell line, and MCF10, a non-tumor epithelial cell line, were separately treated with Liposomal-TQ and DXR. Cell viability and cell migration were assessed using MTT and scratch tests. Apoptosis analysis was performed using annexin-V/PI staining. Gene expression analysis of SENP2 and SENP6 was conducted using quantitative real-time PCR (RT-qPCR). Additionally, the scratch test evaluated the anti-cell migratory effect of Liposomal-TQ.

Results: The findings obtained from RT-qPCR analysis indicated a significant increase in the expression of SENP2 and SENP6 genes in the TQ and DXR treatment groups compared to the control group in MCF7 but not in MCF10 cell lines (p-value < 0.05). Also, after 24 hours of treatment of MCF7 and MCF10 cells with liposomal-TQ, late apoptotic cells were significantly increased compared to the control and liposome groups (p-value < 0.0001) and compared to the control group, both DXR and Liposomal-TQ dramatically reduced the migratory ability of breast cancer cells (p-value = 0.001 and p-value = 0.001, respectively).

Conclusion: Our study indicated that Liposomal-TQ promotes apoptosis in breast cancer cells and inhibits cell migration ability. These findings enhance our understanding of the role of Liposomal-TQ in the carcinogenic activities of SENP2 and SENP6 in the SUMOylation pathway of breast cancer.

Keywords: Breast cancer, Doxorubicin, SENP2, SENP6, Thymoquinone

1. Background

Annually, more than a million new cases of breast cancer are diagnosed in women, accounting for approximately half of all cancer-related deaths among the female population (1). While therapeutic strategies have improved the prognosis of breast cancer in recent years, many patients in advanced stages experience resistance to treatment due to the heterogeneity of the disease. This heterogeneity is driven by sequential genomic and proteomic changes, including post-translational modifications (PTMs)(2). Disruption of SUMOylation, a PTM resulting from molecular alterations in SUMO proteins, is associated with breast cancer (3). Hence, in the pursuit of improved clinical results for individuals with breast cancer, it is essential not only to concentrate on comprehending the molecular intricacies associated with SUMOylation in this disease but also to unveil novel, potent, and efficacious therapeutic agents.

SUMOylation, which falls under the category of reversible PTMs, holds significant importance in various biological processes such as cellular growth, replication, programmed cell death, DNA mending, and cellular viability (4, 5). The proteins involved in the SUMOylation process are called the Small ubiquitin-related modifiers (SUMOs) (5). The regulation of the SUMO pathway appears to alter the balance between SUMOylation and deSUMOylation among various oncoproteins and tumor suppressors, which may contribute to oncogenic transformation (6). One of the most critical components involved in the SUMOylation process is SENPs (Sentrin/SUMO-specific protease) (7).

SENP is one of the most critical components of the SUMOylation process, responsible for converting immature SUMOs into their mature form. Another function of SENPs is the separation of SUMO proteins from substrates through deSUMOylation (8). For instance, SENP2, a member of the SENP family, participates in the maturation and deSUMOylation of SUMO proteins through its second N-terminus, influencing various biological processes. Previous studies have shown that increased SENP2 expression contributes to the invasion of breast cancer cells by enhancing deSUMOylation and affecting NF-KB transcription (9, 10). Similarly, altered SENP2 expression is associated with the incidence and invasion of bladder and hepatocellular carcinoma (11). Another member of the SENP family, SENP6, has been demonstrated to disrupt the balance of SUMOylation, contributing to cancer (12). There is limited evidence suggesting that SENP6 is downregulated in breast cancer despite reports of its upregulation in various other cancers (12, 13).

Thymoquinone (TQ) is a biologically active compound extracted from the black seeds of Nigella sativa, which can modulate the epigenetic traits of cancer cells by affecting processes such as SUMOylation, deSUMOylation, histone acetylation, and anti-DNA mutation. TQ exhibits significant bioactivity and minimal systemic toxicity, making it an effective alternative to conventional medication for managing breast cancer. The administration of TQ supplemented with piperine has been observed to reduce the growth of breast cancer cells by activating apoptosis, suppressing neovascularization, and enhancing the immune response (14). Furthermore, recent studies have shown that TQ can increase PTEN expression and induce apoptosis in human breast cancer cells resistant to doxorubicin (15).

2. Objective

TQ has the potential to function as an anti-breast cancer drug by regulating the gene expression of SENP2 and SENP6 in breast cancer cells, thereby modulating the SUMOylation and deSUMOylation of SUMO target substrates. This research aimed to investigate whether TQ could regulate the gene expressions of SENP2 and SENP6 in the MCF7 and MCF10 cell lines to inhibit cancer growth.

3. Materials and Methods

3.1. Cell Culture and Drug Treatment

MCF7, a widely-used breast cancer cell line, and MCF10, a non-cancerous epithelial cell line, were obtained from the Iranian Biological Resource Center in Tehran, Iran. Subsequently, the samples were incubated in Dulbecco's Modified Eagle Medium (DMEM) enriched with 10% fetal bovine serum (FBS), 100 units.mL^{-1} penicillin, and 100 µg.mL^{-1} streptomycin. Incubation was carried out at 37 °C under an atmosphere with a relative humidity of 95% and supplemented with 5% CO_2.

3.2. Preparation of Thymoquinone-Nanoparticles (Liposomal-TQ)

The stock solution of TQ (Sigma-Aldrich, USA, Cas No: 490-91-5) was prepared in dimethyl sulfoxide (DMSO; Merck, Germany), and an appropriate

working concentration (400 µM) was prepared from the stock using the complete medium. Liposomal-TQ was synthesized using the thin-film hydration method. In this approach, liposomes were created through the conventional film technique, combining DSPEmPEG 2000 (1,2-Distearoyl-phosphatidyl ethanol amine-methyl-polyethylene glycol conjugate-2000 (Na+ salt)) obtained from Nanocs, USA, cholesterol, and Tween-80. The process began by solubilizing a chloroform solution containing TQ, phospholipid, and cholesterol. Next, the solution was dried using a rotary evaporator (ChongYe RE 3000, Shanghai, China) at 50 °C. The resulting film was dissolved and hydrated in phosphate buffer saline (PBS) with a pH of 6.5 for 30 minutes at 60 °C, aided by the presence of Tween-80. Subsequently, all liposome dispersions were combined using a sonicator (Noise Isolating Tamber, Ninbo, JY92-IIN, China) for 3 minutes at 80 watts. Small liposome particles were obtained through the synthesis of liposomal-TQ, utilizing soybean phospholipids (SPC), egg yolk phospholipids (EPC), and hydrogenated soybean phospholipids (HSPC). Cholesterol was included to enhance the fluidity of the lipid membrane, while Tween-80 was added to improve the encapsulation efficiency of the liposomes.

3.3. Thymoquinone-Nanoparticle Size and Zeta Potential Determination (Liposomal-TQ)

The size and zeta potential of the nanoparticles were determined using the Dynamic Light Scattering (DLS) technique and a Zetasizer (Nano ZS3600, Malvern, UK). MCF7 and MCF10 cells were treated with standard working solutions containing various concentrations of Liposomal-TQ to achieve doses of 12.5, 25, 50, and 100 µM.

3.4. Measurement of Metabolic Activity through MTT Assay

The MTT assay was conducted to assess the impact of varying concentrations of Liposomal-TQ on the metabolic activity of MCF7 and MCF10 cells. In brief, MCF7 and MCF10 cells were seeded in a 96-well plate (10×10^3 cells/well) and incubated with 12.5, 25, 50, and 100 µM concentrations of the drug for 24 hours. Following incubation, the medium was aspirated, and MTT solution (5 $mg.mL^{-1}$, Sigma-Aldrich, USA) was added, followed by incubation at 37 °C for 4 hours. Formed formazan crystals were solubilized by adding 100 µL of DMSO to each well, and their absorbance was measured using an ELISA reader at 570 nm. The metabolic activity of treated cells was compared to that of untreated (control) cells.

3.5. Assay of Apoptosis

To investigate the impact of TQ on apoptosis, MCF7 and MCF10 cells were analyzed using flow cytometry. These cells were seeded in 12-well culture plates at a density of 2×10^5 cells per well and were harvested 24 hours after drug treatment. Before staining with the FITC Annexin V Apoptosis Detection Kit II, the cells were washed in PBS following the manufacturer's instructions (BD Biosciences, USA). The ratio of apoptotic cells was determined using a FACS instrument (Becton-Dickinson). Cells showing PI-negative and annexin-V-positive staining were considered to be in the early stages of apoptosis. In contrast, those displaying double-positive staining for PI and annexin-V were considered in the later stages of apoptosis or necrosis.

3.6. RNA Isolation and cDNA Preparation

Total RNA from MCF7 and MCF10 cells was isolated 24 hours after treatment with 50 µM TQ using QIAzol Isolation Reagent following the manufacturer's instructions (QIAGEN GmbH, Germany). The purity of the extracted RNA was assessed by measuring the optical density (A260/A280 ratio) using a NanoDrop 1000 Spectrophotometer (Wilmington, DE, USA), and the quality of the isolated RNA was evaluated through agarose gel electrophoresis. Reverse transcription (RT) was performed using the RevertAid First Strand complementary DNA (cDNA) Synthesis kit purchased from Fermentas (Thermo Fisher Scientific Inc, USA). The reaction volume (20 µL) included 12 µL of nuclease-free water, 1 µL of Random Hexamer Primer, 4 µL of 5x Reaction Buffer, 2 µL of dNTP Mix (10 mM), 1 µL of RiboLock RNase Inhibitor (20 $U.\mu L^{-1}$), and 1 µL of RevertAid M-MuLV Reverse Transcriptase (200 $U.\mu L^{-1}$) as the master mix. Subsequently, 2 µL of total RNA (1 µg for each reaction) was added before initiating the reaction.

3.7. Quantitative Real-Time PCR

Changes in the mRNA expression levels of SENP2 and SENP6 were examined using real-time PCR. The reaction mixture included 5 µL of RealQ Plus 2x Master Mix Green (Ampliqon, DK), 2 µL of cDNA product, 1 µL of forward and reverse primers (10 pmol

each), and 2 µL of nuclease-free water, resulting in a final volume of 10 µL. The reaction mixtures underwent initial activation at 95 °C for 15 minutes, followed by 40 cycles of denaturation at 95 °C for 15 seconds and annealing/elongation at 60 °C for 60 seconds. The reaction was carried out using the Rotor-Gene Q Real-time PCR System (ABI Stepone, USA), and a melting curve analysis was performed to confirm the specificity of the produced results. The fold change in gene expression was calculated relative to the control, with adjustment for the reference gene GAPDH, using the comparative Ct ($2^{-\Delta\Delta CT}$) method. The primer sequences for quantitative real-time PCR (RT-qPCR) are provided in **Table 1**.

3.8. Scratch Test

Initially, approximately 50,000 MCF7 and MCF10 cells were cultured in 24-well plates. After 24 hours of incubation, when the cells had adhered to the bottom of the plate and reached confluency, a scratch was made in the culture medium using a 100 µL sampler tip. Subsequently, the wells were washed once with PBS, and DMEM with 10% FBS was added to the plates. The wells were imaged using reverse microscopy (Italy, 3-XDS, Optika) at the beginning of the experiment. Seventy-two hours later, the cells had migrated from the edges of the scratch to the central area, and the samples were photographed.

Table 1. Primer sequences of quantitative real-time PCR (RT-qPCR)

Gene	Primer
SENP2	F: 5'-CCTGTCCCTGCCAGTGTGTT-3' R: 5'-ATGCCCCAAATCTGTTTCCCTCC-3'
SENP6	F: 5'-ACAAAACTCTTCTGCCAAGCC-3' R: 5'-ACCGTCACTTTCTTCCTGCC-3'
GAPDH	F: 5'-GCAGGGATGATGTTCTGG-3' R: 5'-CTTTGGTATCGTGGAAGGAC-3'

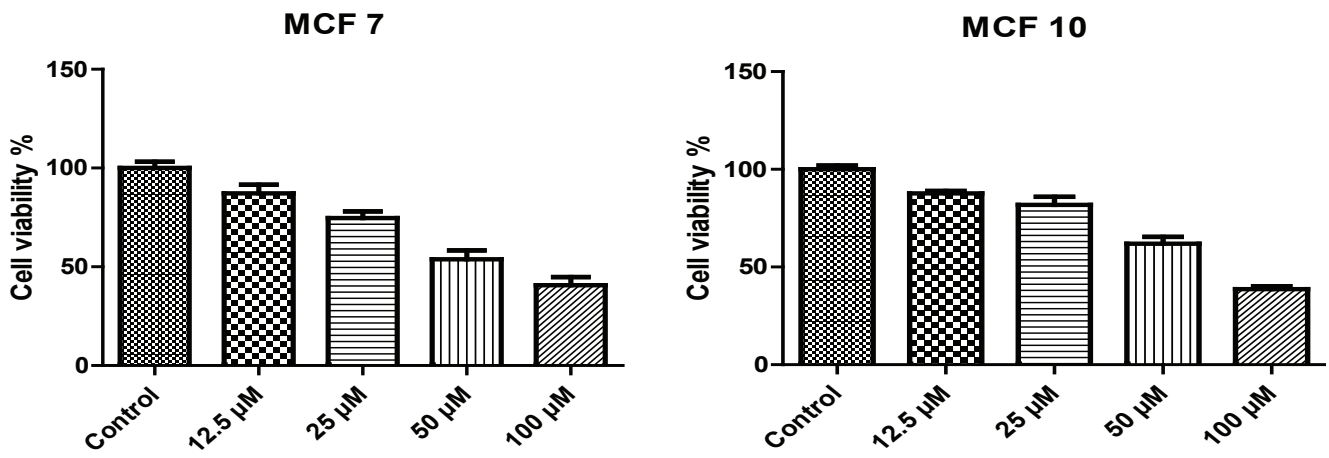

Figure 1. Cell viability of MCF7 and MCF10 cell Lines after Exposure to the Different Doses of Liposomal-TQ for 24 Hours. Liposomal-TQ administration resulted in a dose-dependent reduction in metabolic activity in both MCF7 and MCF10 cells lines which the maximum cytotoxicity was observed following a 24-hour treatment of cells with 50 µM TQ compared to control cells.

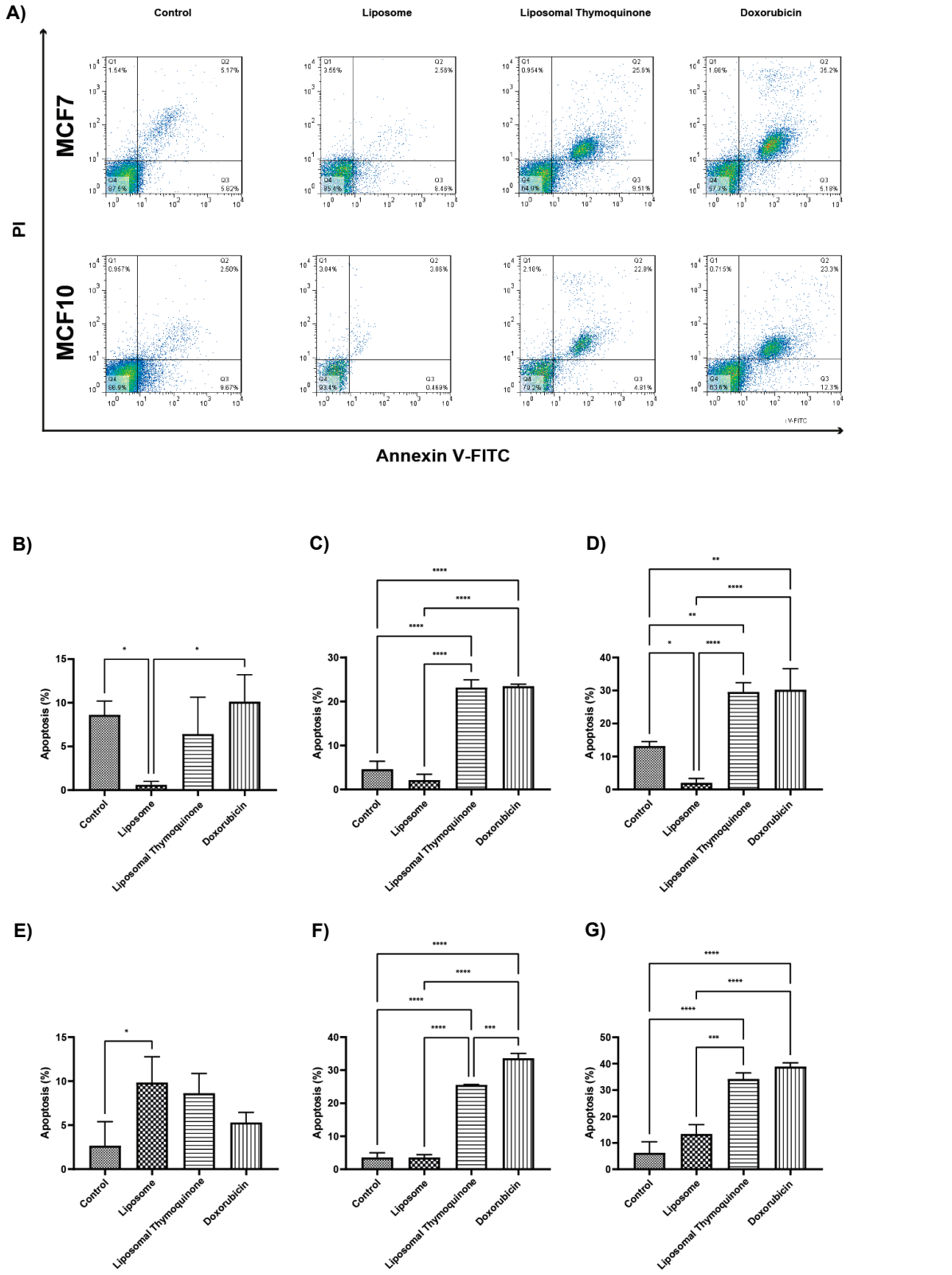

Figure 2. Effects of Liposomal-TQ on Apoptosis Using Flowcytometry. Liposomal-TQ significantly increased late and total apoptosis compared to the liposome and control group. **A)** 24 hours after treatment with DXR and liposomal-TQ, cell apoptosis increased; **B)** early apoptosis in MCF10 cell line; **C)** late apoptosis in MCF10 cell line; **D)** total apoptosis in MCF10 cell line; **E)** apoptosis in MCF7 cell line; **F)** late apoptosis in MCF7 cell line; **G)** total apoptosis in MCF7 cell line (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).

The rate of cell migration was determined by measuring the distance between the two scratches and comparing the control and treatment groups. After data collection, the analysis was performed using GraphPad Prism (GraphPad 9 Software).

3.9. Statistical Analysis

GraphPad Prism software assessed the significance of differences between experimental variables. The MTT and Real-time PCR data were analyzed using Tukey tests and one-way ANOVA, respectively. A statistically significant result was defined as having a p-value less than 0.05.

4. Results

4.1. Particle Size and Z Potential of Polymer-Encapsulated Thymoquinone Nanoparticles (Liposomal-TQ)

Dynamic light scattering (DLS), or photon correlation spectroscopy, is a commonly employed technique for measuring particle size and surface charge. It utilizes a laser beam of a specific wavelength to scatter particles or macromolecules in a liquid medium based on their Brownian motion. The zeta potential of Liposomal-TQ was found to be -66.3 mV, with a mean electrophoretic mobility of -0.000514 cm/Vs.

4.2. Thymoquinone Treatment Exerts a Dose-Dependent Cytotoxic Effect Against MCF7 and MCF10 Cells

The metabolic activity of MCF7 and MCF10 cells treated with various doses of TQ for 24 hours was determined using the MTT colorimetric assay. As shown in **(Fig. 1)**, Liposomal-TQ administration resulted in a dose-dependent reduction in metabolic activity in both MCF7 and MCF10 cells. Notably, the maximum cytotoxicity was observed following a 24-hour treatment of cells with 50 µM TQ compared to untreated cells (approximately 50%, p-value < 0.001). The 50 µM dose of Liposomal-TQ, which exhibited more effective apoptosis effects, was chosen to analyze

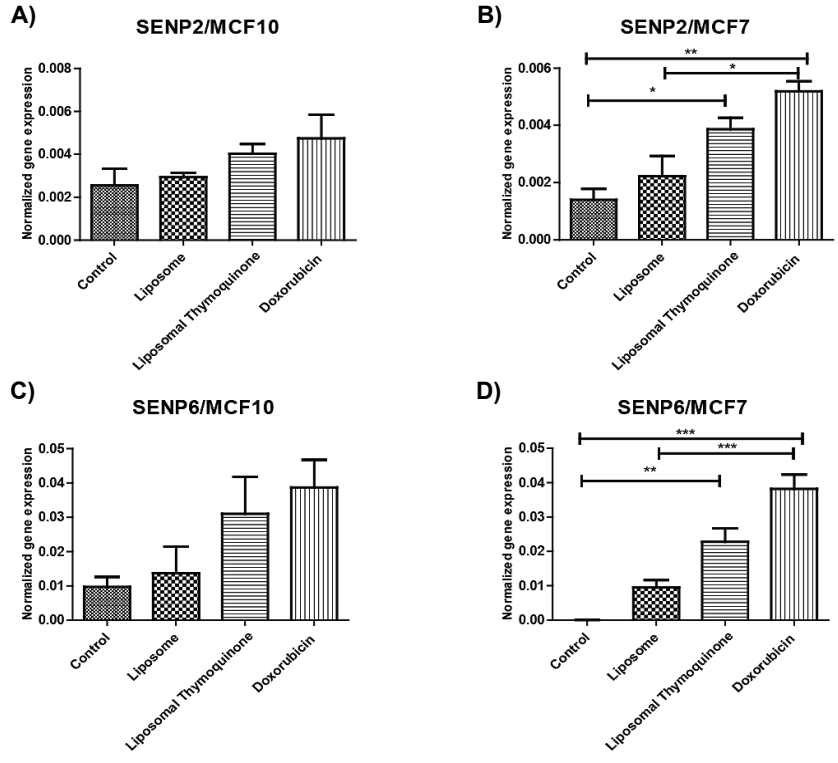

Figure 3. The SENP2 and SENP6 expression in MCF7 and MCF10 cells following treatment with Liposomal-TQ and DXR by RT-qPCR analysis. Both DXR and liposomal-TQ upregulate SENP2 (**A and B**) and SENP6 (**C and D**) genes and downregulates. MCF7 and MCF10 cell lines were treated with DXR and liposomal-TQ. Then, RNA was isolated, and cDNA synthesis was done. All values were normalized to GAPDH (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).

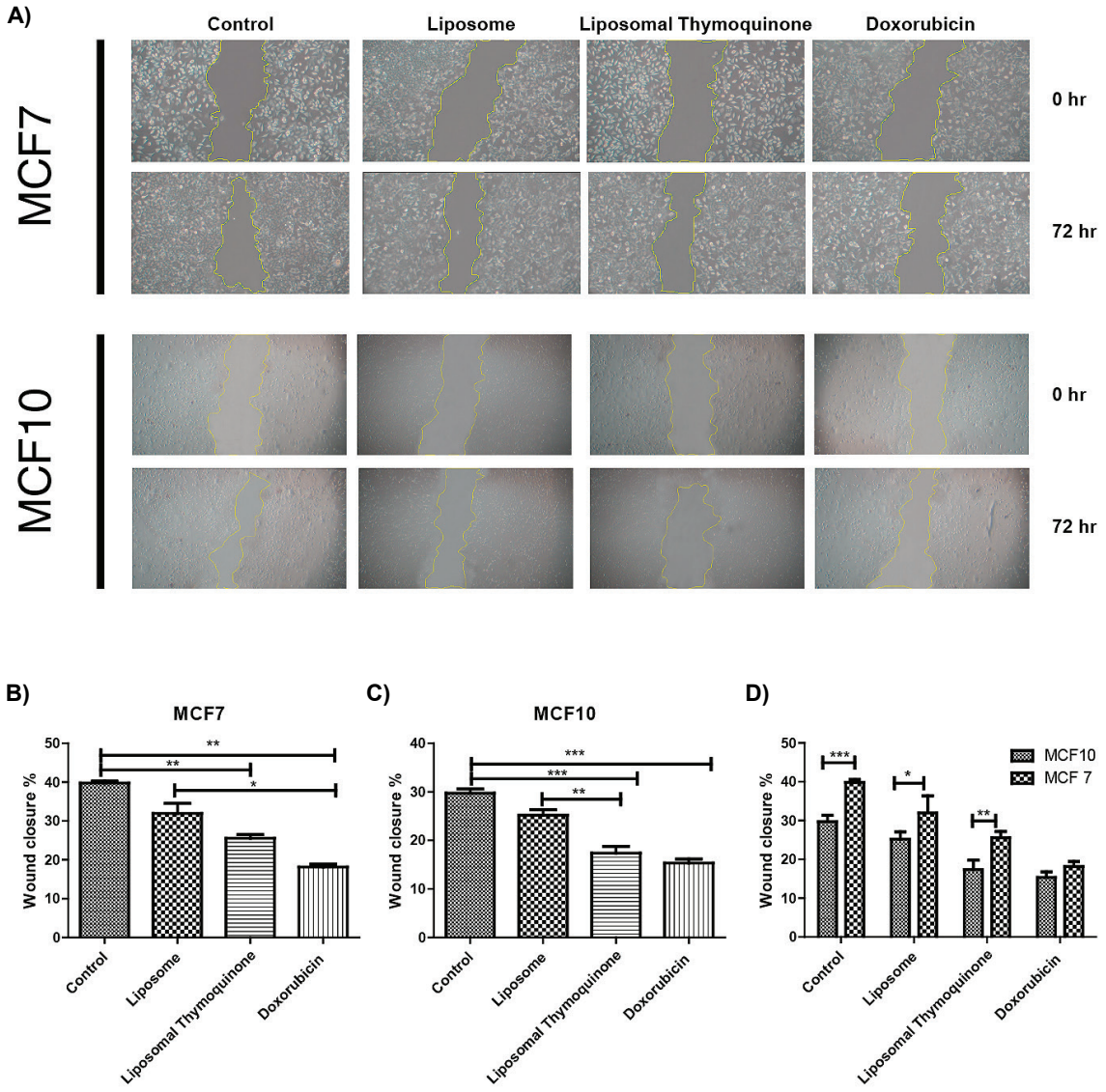

Figure 4. **The scratch test to assess the anti-cell migratory effect of Liposomal-TQ and DXR on both MCF7 and MCF10 cells. A)** Both liposomal-TQ and DXR significantly decreased the percentage of wound closure after 72 hours of treatment; **B**) Wound closure in MCF7 cells in different groups; **C**) Wound closure in MCF10 cells in different groups; and **D**) Comparison of wound closure between MCF7 and MCF10 cells in different groups (*P < 0.05, **P < 0.01, ***P < 0.001).

SENP2 and SENP6 expression.

4.3. Liposomal-TQ-Induced Apoptosis of MCF7 and MCF10 Cells

The initial series of analyses investigated the effects of Liposomal-TQ on apoptosis in MCF7 and MCF10 cells using Annexin-V binding and Annexin-V in combination with PI, employing the FITC Annexin V Apoptosis Detection Kit II. As illustrated in **(Fig. 2A)**, a 24-hour treatment of MCF7 and MCF10 cells with TQ-loaded nanoliposomes significantly increased the proportion of late apoptotic cells (Annexin V-PI double-positive cells) when compared to both the control and liposome-only groups (p-value < 0.0001). The results demonstrate a statistically significant difference in the apoptosis level between the combined treatment with 50 µM Liposomal-TQ and nanoliposome treatment alone for MCF7 and MCF10 cells (35% and 27%, respectively, compared to 11% and 3%, respectively)

(p-value < 0.05) **(Fig. 2B, 2C, 2D, 2E, 2F, 2G).**

4.4. Real-Time PCR Results on Expression of SENP2 and SENP6 in MCF7 and MCF10 Cells with TQ Treatment

RT-qPCR analysis was conducted to assess the expression of SENP2 and SENP6 in MCF7 and MCF10 cells following treatment with Liposomal-TQ and DXR. MCF7 and MCF10 cells were treated with 50 µM of Liposomal-TQ, harvested, and then evaluated for the expression of the SENP2 and SENP6 genes using RT-qPCR. The increased expression of the SENP2 and SENP6 genes was observed in MCF7 cells treated with Liposomal-TQ compared to untreated cells (p-value < 0.05 and p-value < 0.01, respectively) **(Fig. 3A, 3B)**. A one-way ANOVA revealed a significant positive correlation between SENP2 expression in MCF7 cells treated with Liposomal-TQ and untreated cells (p-value = 0.015). However, Liposomal-TQ treatment did not result in a significant increase in the expression of SENP2 and SENP6 in MCF10 cells compared to untreated cells **(Fig. 3C, 3D)**.

4.5. Liposomal-TQ Attenuates Migration Ability of MCF7 and MCF10 Cells

We conducted a scratch test to assess the anti-cell migratory effect of Liposomal-TQ and DXR on both MCF7 and MCF10 cells. In both cell lines, Liposomal-TQ significantly reduced the cell migration ability compared to the cells treated with liposomes alone and untreated cells (MCF7: p-value < 0.01, MCF10: p-value < 0.001) **(Fig. 4)**.

5. Discussion

Despite significant advances in breast cancer diagnostic and therapeutic approaches, many patients eventually relapse due to treatment resistance. Doxorubicin (DXR) is considered the most effective chemotherapeutic agent for breast cancer treatment (16, 17). Nevertheless, research has shown that DXR can induce treatment resistance, promote tumor growth, and lead to unfavorable prognoses and reduced survival rates among patients (18, 19). This resistance is attributed to various mechanisms, including genomic and proteomic changes such as post-translational modifications. Hence, there is a compelling need to discover new, effective drugs for breast cancer treatment. This study assesses the impact of Liposomal-TQ and DXR on the breast cancer cell line MCF7 and the normal epithelial cell line MCF10. Thymoquinone (TQ) has been recognized as an effective anti-cancer drug in various research contexts, and our study, we found that Liposomal-TQ considered a safe drug, significantly increased the expression of SENP2 and SENP6 in breast cancer cells.

Thymoquinone (TQ) has demonstrated various pharmacological benefits, including its anti-oxidative, anti-inflammatory, immunomodulatory, and anti-neoplastic effects (20-24). TQ's anti-cancer effects are attributed to multiple mechanisms, including its ability to inhibit cancer cell growth, induce apoptosis, arrest the cell cycle, generate reactive oxygen species (ROS), and inhibit metastasis and angiogenesis (25). In this study, TQ exhibited time-dependent cytotoxicity on breast cancer cell lines and healthy breast cells, aligning with earlier research findings.

Many malignancies exhibit high upregulation of SUMOylation, with breast cancer cells promoting tumor development through increased cellular substrate conjugation via enhanced SUMOylation (12). The critical role of SENPs is to maintain the balance of SUMO proteins by deconjugating the modified proteins. In this study, we investigated whether Liposomal-TQ can modulate the expression of SENP2 and SENP6 in both normal and cancerous breast cells. Following treatment with Liposomal-TQ and DXR, we observed a significant increase in SENP2 and SENP6 expression in MCF7 cells. However, these genes did not show significant changes in MCF10 cells. Mirecka *et al*. reported that breast cancer patients with single nucleotide polymorphisms (SNPs) in SENP2 are more susceptible to breast cancer (11).

Additionally, breast cancer tissues have been found to exhibit significantly lower levels of SENP6 mRNA expression compared to normal tissues (13). Notably, prior research has indicated that the loss of SENP6 in HeLa cells is associated with decreased sensitivity to radiation and chemotherapy (26). In our study, breast cancer cells treated with Liposomal-TQ and DXR displayed higher expression of SENP6, suggesting that these cells might be more susceptible to therapy.

In this study, both Liposomal-TQ and DXR significantly inhibited the cell migration of MCF7 and MCF10 cells. The anti-migratory properties of TQ have been demonstrated in various cancer types, including colorectal cancer, glioblastoma, melanoma, and lung cancer (27). Previous studies have revealed that SENP2, by deSUMOylating TBL1/TBLR1, can inhibit the MMP13 promoter, suppressing invasion and cell migration in

bladder cancer cells (28). Similarly, increased SENP2 expression inhibits growth and colony-forming ability in hepatocellular cancer cells (29).

In conclusion, our results suggest that thymoquinone nanoparticles (Liposomal-TQ) are effective and safe tumor suppressors. This study demonstrated that Liposomal-TQ induces apoptosis in breast cancer cells and exhibits anti-migratory properties. These findings expand our understanding of the oncogenic functions of SENP2 and SENP6 in the breast cancer SUMOylation process. Liposomal-TQ holds promise as a potential alternative for next-generation chemotherapy, offering the potential for reduced adverse effects in the management of breast cancer patients in the future. Further research is needed to deepen our understanding of the roles of these genes and the SUMOylation process related to breast cancer.

Data Availability

The present study did not involve generating or analyzing any datasets; therefore, data sharing is irrelevant to this article.

Conflicts OF Interest

The authors declare no conflict of interest.

Ethical Approve

The present article lacks any research involving human subjects conducted by any authors.

References

1. Bashmail HA, Alamoudi AA, Noorwali A, Hegazy GA, G AJ, Choudhry H, *et al*. Thymoquinone synergizes gemcitabine anti-breast cancer activity via modulating its apoptotic and autophagic activities. *Sci Rep*. 2018;**8**(1):11674. doi:10.1038/s41598-018-30046-z.
2. Heo K-S. Regulation of post-translational modification in breast cancer treatment. *BMB Reports*. 2019;**52**(2):113. doi:10.5483/BMBRep.2019.52.2.017.
3. Rabellino A, Khanna KK. The implication of the SUMOylation pathway in breast cancer pathogenesis and treatment. *Crit Rev Biochem Mol Biol*. 2020;**55**(1):54-70. doi:10.1080/10409238.2020.1738332
4. Baek SH. A novel link between SUMO modification and cancer metastasis. *Cell Cycle*. 2006;**5**(14):1492-1495. doi:10.4161/cc.5.14.3008.
5. Eifler K, Vertegaal ACO. SUMOylation-Mediated Regulation of Cell Cycle Progression and Cancer. *Trends Biochem Sci*. 2015;**40**(12):779-793. doi:10.1016/j.tibs.2015.09.006.
6. Veltman IM, Vreede LA, Cheng J, Looijenga LH, Janssen B, Schoenmakers EF, *et al*. Fusion of the SUMO/Sentrin-specific protease 1 gene SENP1 and the embryonic polarity-related mesoderm development gene MESDC2 in a patient with an infantile teratoma and a constitutional t(12;15)(q13;q25). *Hum Mol Genet*. 2005;**14**(14):1955-1963. doi:10.1093/hmg/ddi200.
7. Abdel-Hafiz HA, Horwitz KB. Control of progesterone receptor transcriptional synergy by SUMOylation and deSUMOylation. *BMC Mol Biol*. 2012;**13**(1):10. doi:10.1186/1471-2199-13-10.
8. Nayak A, Muller S. SUMO-specific proteases/isopeptidases: SENPs and beyond. *Genome Biol*. 2014;**15**(7):422. doi:10.1186/s13059-014-0422-2.
9. Gao X, Wu Y, Qiao L, Feng X. SENP2 suppresses NF-kappaB activation and sensitizes breast cancer cells to doxorubicin. *Eur J Pharmacol*. 2019;**854**:179-186. doi:10.1016/j.ejphar.2019.03.051.
10. Chang CC, Huang YS, Lin YM, Lin CJ, Jeng JC, Liu SM, *et al*. The role of sentrin-specific protease 2 substrate recognition in TGF-beta-induced tumorigenesis. *Sci Rep*. 2018;**8**(1):9786. doi:10.1038/s41598-018-28103-8.
11. Mirecka A, Morawiec Z, Wozniak K. Genetic Polymorphism of SUMO-Specific Cysteine Proteases - SENP1 and SENP2 in Breast Cancer. *Pathol Oncol Res*. 2016;**22**(4):817-823. doi:10.1007/s12253-016-0064-7.
12. Bawa-Khalfe T, Yeh ET. SUMO Losing Balance: SUMO Proteases Disrupt SUMO Homeostasis to Facilitate Cancer Development and Progression. *Genes Cancer*. 2010;**1**(7):748-752. doi:10.1177/1947601910382555.
13. Mooney SM, Grande JP, Salisbury JL, Janknecht R. Sumoylation of p68 and p72 RNA helicases affects protein stability and transactivation potential. *Biochemistry*. 2010;**49**(1):1-10. doi:10.1021/bi901263m.
14. Talib WH. Regressions of Breast Carcinoma Syngraft Following Treatment with Piperine in Combination with Thymoquinone. *Sci Pharm*. 2017;**85**(3):27. doi:10.3390/scipharm85030027.
15. El-Mahdy M, Zhu Q, Barakat B, Arafa E-S, Wang Q-E, Zhao Q, *et al*. Thymoquinone upregulates PTEN expression and induces apoptosis in doxorubicin-resistant human breast cancer cells. *AACR*; 2008.
16. Barrett-Lee P, Dixon J, Farrell C, Jones A, Leonard R, Murray N, *et al*. Expert opinion on the use of anthracyclines in patients with advanced breast cancer at cardiac risk. *Annals of oncology*. 2009;**20**(5):816-827.
17. Shi Y, Bieerkehazhi S, Ma H. Next-generation proteasome inhibitor oprozomib enhances sensitivity to doxorubicin in triple-negative breast cancer cells. *Int J Clin Exp Pathol*. 2018;**11**(5):2347-2355.
18. Shukla A, Hillegass JM, MacPherson MB, Beuschel SL, Vacek PM, Pass HI, *et al.* Blocking of ERK1 and ERK2 sensitizes human mesothelioma cells to doxorubicin. *Mol Cancer*. 2010;**9**(1):314. doi:10.1186/1476-4598-9-314.
19. Li X, Lu Y, Liang K, Liu B, Fan Z. Differential responses to doxorubicin-induced phosphorylation and activation of Akt in human breast cancer cells. *Breast Cancer Res*. 2005;**7**(5):R589-597. doi:10.1186/bcr1259.
20. Mansour MA, Nagi MN, El-Khatib AS, Al-Bekairi AM. Effects of thymoquinone on antioxidant enzyme activities, lipid peroxidation and DT-diaphorase in different tissues of mice: a possible mechanism of action. *Cell Biochem Funct.* 2002;**20**(2):143-151. doi:10.1002/cbf.968.
21. Umar S, Zargan J, Umar K, Ahmad S, Katiyar CK, Khan HA. Modulation of the oxidative stress and inflammatory cytokine response by thymoquinone in the collagen induced arthritis in Wistar rats. *Chem Biol Interact*. 2012;**197**(1):40-46. doi:10.1016/j.cbi.2012.03.003.
22. Pari L, Sankaranarayanan C. Beneficial effects of thymoquinone on hepatic key enzymes in streptozotocin-nicotinamide induced diabetic rats. *Life Sci*. 2009;**85**(23-26):830-834. doi:10.1016/j.lfs.2009.10.021.
23. Gali-Muhtasib H, Roessner A, Schneider-Stock R. Thymoquinone: a promising anti-cancer drug from natural sources. *Int J Biochem Cell Biol*. 2006;**38**(8):1249-1253. doi: 10.1016/j.

biocel.2005.10.009.
24. Woo CC, Kumar AP, Sethi G, Tan KH. Thymoquinone: potential cure for inflammatory disorders and cancer. *Biochem Pharmacol*. 2012;**83**(4):443-451. doi:10.1016/j.bcp. 2011.09. 029.
25. Banerjee S, Padhye S, Azmi A, Wang Z, Philip PA, Kucuk O, *et al*. Review on molecular and therapeutic potential of thymoquinone in cancer. *Nutr Cancer*. 2010;**62**(7):938-946. doi:10.1080/01635581.2010.509832.
26. Dou H, Huang C, Singh M, Carpenter PB, Yeh ET. Regulation of DNA repair through deSUMOylation and SUMOylation of replication protein A complex. *Molecular Cell*. 2010;**39**(3): 333-345. doi: 10.1016/j.molcel.2010.07.021.
27. Bhattacharya S, Ahir M, Patra P, Mukherjee S, Ghosh S, Mazumdar M, *et al*. PEGylated-thymoquinone-nanoparticle mediated retardation of breast cancer cell migration by deregulation of cytoskeletal actin polymerization through miR-34a. *Biomaterials*. 2015;**51**:91-107. doi:10.1016/j.biomaterials.2015.01.007.
28. Tan M, Gong H, Wang J, Tao L, Xu D, Bao E, *et al*. SENP2 regulates MMP13 expression in a bladder cancer cell line through SUMOylation of TBL1/TBLR1. *Scientific Reports*. 2015;**5**(1):1-8.
29. Shen HJ, Zhu HY, Yang C, Ji F. SENP2 regulates hepatocellular carcinoma cell growth by modulating the stability of beta-catenin. *Asian Pac J Cancer Prev*. 2012;**13**(8):3583-3587. doi:10.7314/apjcp.2012.13.8.3583.