Research Article

Med Biomedical Journal

Assessment of the Synergistic Effect of Thymoquinone and Cisplatin on MCF-7 Breast Cancer Cells

Fatemehsadat Mousavinasab[1], Nasrin Hashemipour[2*], Shahrad Soghala[3*], Mehrdad Talebi [4*], Maryam aghazadeh [5*],Elaheh Baghalzadeh Salmasi[6], Mohammad Yahya Vahidi Mehrjardi[7*,] Poopak Farnia[8*]

1. Department of Medical Immunology, School of Medicine, Shahid Beheshti University, Tehran, Iran
2. Department of Biology, School of Molecular Genetic, Yazd University, Yazd, Iran6
3. Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran
4. Department of Medical Genetics, Faculity of Medicine, Shahid Sadoughi University of Medical Science, Yazd, Iran
5. Faculty of new technologies engineering, Department of Life Sciences and Biotechnology, university of Shahid Beheshti, Tehran, Iran
6. Department of cellular and molecular biology, faculty of microbiology, Zanjan azad university, Iran
7. Diabetes Research Center, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
8. Mycobacteriology Research Centre, National Research Institute of Tuberculosis and Lung Disease, Shahid Beheshti University of Medical Sciences, Tehran, Iran
9. Department of cellular and molecular biology, faculty of microbiology, Zanjan azad university, Iran
10. Department of Medical Genetics, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
11. Mycobacteriology Research Centre, National Research Institute of Tuberculosis and Lung Disease, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Corresponding Authors: Shahrad Soghala (Shahrad_soghala@ yahoo.com), Mohammad Yahya Vahidi Mehrjardi (mmvahidi@gmail.com), Poopak Farnia (p.farnia@sbmu.ac.ir), and Nasrin Hashemipour (Ns652629@gmail.com)

Citation:

Soghal Sh., Mehrjardi M., Farnia P. and Hashemipour N.(2024). Assessment of the Synergistic Effect of Thymoquinone and Cisplatin on MCF-7 Breast Cancer Cells. *Med Biomedical J*,1(1).6-18.

Abstract

Background: Breast carcinoma is still one of the most common cancers globally. Neutraceuticals have become a focus in anticancer treatment due to increasing evidence. Thymoquinone is one of Nigella Sativa's most active compounds and has various therapeutic properties. In the current work, the combined effects of thymoquinone and cisplatin on treating the breast malignancy cells MCF-7 were examined.

Material and methodology: Using the MTT technique, the amount of cytotoxicity of the drugs was assessed independently and in combination. Furthermore, the interaction between cisplatin and

thymoquinone was investigated using the intermediate effect method, and the western blot and Real-Time Quantitative Reverse Transcription PCR methods had been employed to assess the apoptotic gene expression levels in the cells after 24 hours of drug exposure.

Result: The MTT results demonstrated that Cisplatin and thymoquinone each reduce cell survivability in a manner that varies with time and dose. Given that none of the therapies' combined indices (CIs) exceeded 1, it can be said that anticancer medications work synergistically to slow the proliferation of cancer cells in breast tissue.

Conclusion: The study showed that a combined treatment of Cisplatin and TQ induces an interplay among PPARγ (ID: 5468), P53 (ID: 7157), BAX (ID: 581), BCL-2 (ID: 596), Caspase-3 (ID: 836), and Caspase-9 (ID: 842) in favor of apoptosis induction in breast cancer cells. According to these findings, this approach might improve breast cancer treatment effectiveness and decrease some side effects in the future.

Keywords: Cisplatin, Thymoquinone, Breast Cancer, Synergism, Apoptosis

Introduction

One of the most prevalent oncological diseases, breast cancer has an increasing demand for study [1-6]. The breast is impacted by breast cancer, which has the potential to metastasize to the bones, lungs, and brain. Cisplatin has proven to be a compelling agent, accounting for about half of all platinum (II)-complex chemotherapeutic treatments [8 ,7]. Furthermore, Cisplatin has a response rate of more than 40% in breast cancer metastasis if used as a first-line medication for metastatic disease. Despite its interaction with nucleophilic chemicals in cancer cells, it could swiftly eliminate pharmacokinetic tolerance due to its binding with plasma proteins. Therefore, new medicinal compounds are needed to increase existing medicines' known effectiveness while lowering undesired side effects [10 ,9].

Black seed belongs to the Ranunculaceae family, and themoquinone (TQ) is the primary active ingredient in its oil (2-isopropyl-5-methyl-1, 4-benzoquinone, TQ). In traditional medicine, TQ is used to treat dysentery, asthma, digestive problems, high blood pressure, and obesity. Among the activities of thymoquinone, it is possible to mention the cleaning of free and superoxide radicals [11]. Moreover, It keeps GSTs, catalases, and GPx —all antioxidant enzymes—active. The antineoplastic properties of thymoquinone are mediated by a number of different pathways, including antiproliferative, ROS production, cell cycle arrest, apoptosis induction, and antimetastasis/antiangiogenesis. In this context, Dastjerdi *et al.* studied the impact of TQ on the P53 gene expression as a tumor inhibitor and apoptosis inducer in the MCF-7 breast cancer cell line. Their results demonstrated that TQ causes MCF-7 cells to undergo apoptosis by causing a time-dependent increase in P53 expression [12].

Whether using a single drug or a mix of numerous different drugs, a treatment that, in addition to being safe and effective, has a high ability to treat BC and also deal with the side effects of chemotherapy is needed. Combining conventional chemotherapy drugs with natural compounds has attracted many scientists' attention because of the significant increase in anticancer properties without harming healthy tissue. We investigate the effect of TQ and Cisplatin as anticancer agents, both individually and in combination, on the BC cell line (MCF-7)

Method and material

Chemicals and reagents

Thymoquinone (TQ) was bought from Sigma-Aldrich (Vienna, Austria), diluted in 80 mM stock solution in DMSO (dimethylsulfoxide) and kept at -20°C. The following substances have been supplied by Sigma (St. Louis, Missouri, United States): dimethyl sulfoxide, also known as (DMSO), secondary conjugated antibody to Horse-radish peroxidase (HRP), Cisplatin, and 5-diphenyltetrazolium bromide (MTT), 3-[4, 5-dimethylthiazol-2-yl]-2. Santa Cruz Biotechnology (Santa Cruz, CA, USA) provided the

initial anti-Bcl-2, anti-p53, and anti-Cas9 antibodies. Penicillin and streptomycin antibiotics, as well as Fetal Bovine Serum (FBS) and Dulbecco Modified Eagle Medium (DMEM), were purchased from Invitrogen (Grand Island, NY, the USA).

Cell culture

From 2019 to 2020, Shahid Sadoughi University of Medical Sciences conducted an in vitro investigation using the MCF-7 cell line of human breast cancer. The Roswell Park Memorial Institute (RPMI) medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°Cin 5% CO2 has been used in order to culture MCF-7 cells, which were procured from the Pasteur Institute (Tehran, Iran).

Determining cell survivability

The MTT method has been employed to examine how Cisplatin, TQ, and their combination affected the viability of MCF-7 cells. In short, 104 cells per well were used to seed MCF-7 cells onto 96-well plates, and confluence was maintained at 37°. Application of the MTT solution (5 mg/ml in PBS) and cultivation at 37 °C for four hours were done ensuing a treatment of Cisplatin (1-100 l), TQ (1-50 l), and their combination to trigger cytotoxicity. The formazan crystals were dispersed into one hundred l of DMSO, and then added to each well after the MTT solution had been removed. Utilizing a microplate reader, the optical density (OD) of the walls at 540 nm has been determined (BioTek® ELx800, USA).

Calculation of combination index values

To determine any potential synergistic effects of TQ and Cisplatin, cancer cells were subjected to progressively higher concentrations of the two drugs. The Chou-Talalay equation was used to create dose-response curves and calculate interactions using the computer programme ComboSyn (ComboSyn Inc., Paramus, NJ, USA). Indicators of antagonistic, additive, and synergistic effects were given the values CI>1, CI=1, and CI1.

Quantitative real-time polymerase chain reaction (qRT-PCR)

From the MCF-7 cells, total RNA was obtained after they had been treated for 24, 48, and 72 hours with TQ, Cisplatin, and their combination. To differentiate between the mRNA expression of apoptotic and anti-apoptotic markers, the producer followed the manufacturer's instructions and used Trizol reagent (Invitrogen). Using a superscript II reverse transcriptase kit (Takara, Japan) in accordance with the recommended producer's protocol, total RNA (1 g) was employed for cDNA synthesis. The internal amplifying agent in each experiment was GAPDH cDNA. First, the mixture was denatured for 10 minutes at 95 °C. The following PCR conditions were used: 95°C for 1 min, 60°C for 2 min, 72°C for 2 min for 30 cycles, then 72°C for 10 min after that.

Western Blot analysis

Following the treatment with CDDP, TQ, and their mix, we utilized western immunoblotting to examine at the cellular expressions of important apoptotic-promoting proteins (p53 and caspase-9) and proteins that suppress apoptosis (Bcl-2) in the cells. To create whole-cell lysates, The IC50 concentrations of CDDP, TQ, and their combined use were applied to treated MCF-7 cell lines for a period of twenty-four hours. To lyse the cells, RIPA buffer (Sigma, USA) was combined with a cocktail of protease and phosphatase inhibitors (Cell Signaling Technologies, Beverly, MA). Bicinchoninic acid (BCA) assay has been used to detect protein concentrations (Merck, Germany). Wet protein transfer was the method employed to sort proteins (40 mg) on 6-12% sodium dodecyl sulphate (SDS)-polyacrylamide gels before transferring them to membranes made of PVDF. 5% skimmed milk was used to block the blots, then afterward probed with the necessary antibodies at the vendor-recommended dilutions.

Statistic evaluation

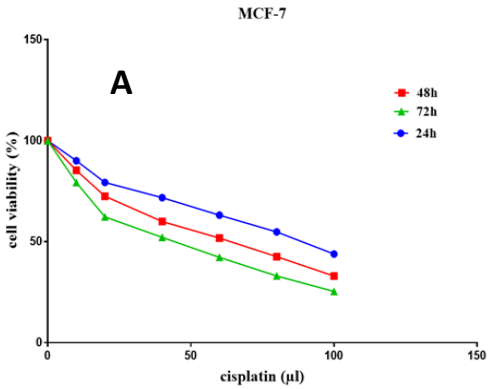
ANOVA one-way testing was applied for statistical evaluation analysis in all studies followed by Tukey or Dunnett analysis using Graph Pad Prism 7. Results 5.

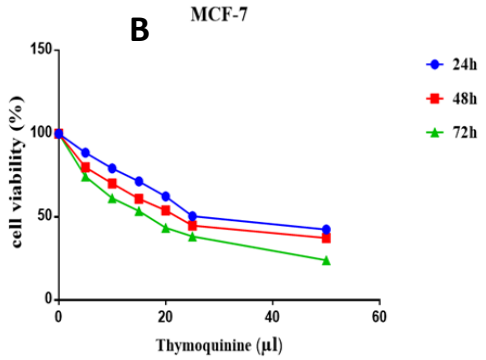
Result

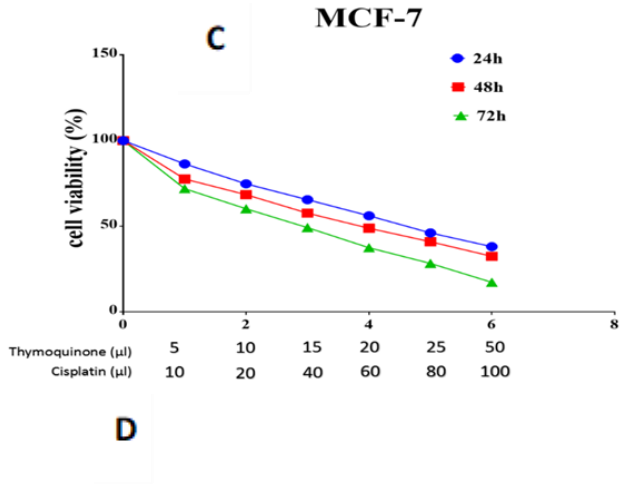
Impact of TQ and cisplatin on Cell Viability

Compared to each of them alone, combining these agents reduces cell viability much more strongly. It was also found that this effect is at its maximum activity level in 72 hours. The IC50 value was estimated to be about 80.84 µl in MCF-7 cells after 24 h incubation with Cisplatin. Moreover, the IC50 value was reduced to 76.93 and 65.06 µl after treatment with Cisplatin for 48 and 72h, respectively. The IC50 value of Thymoquinone was 30.78, 28.92, and 22.55 that are statistically different are represented by a P-value of less than 0.0

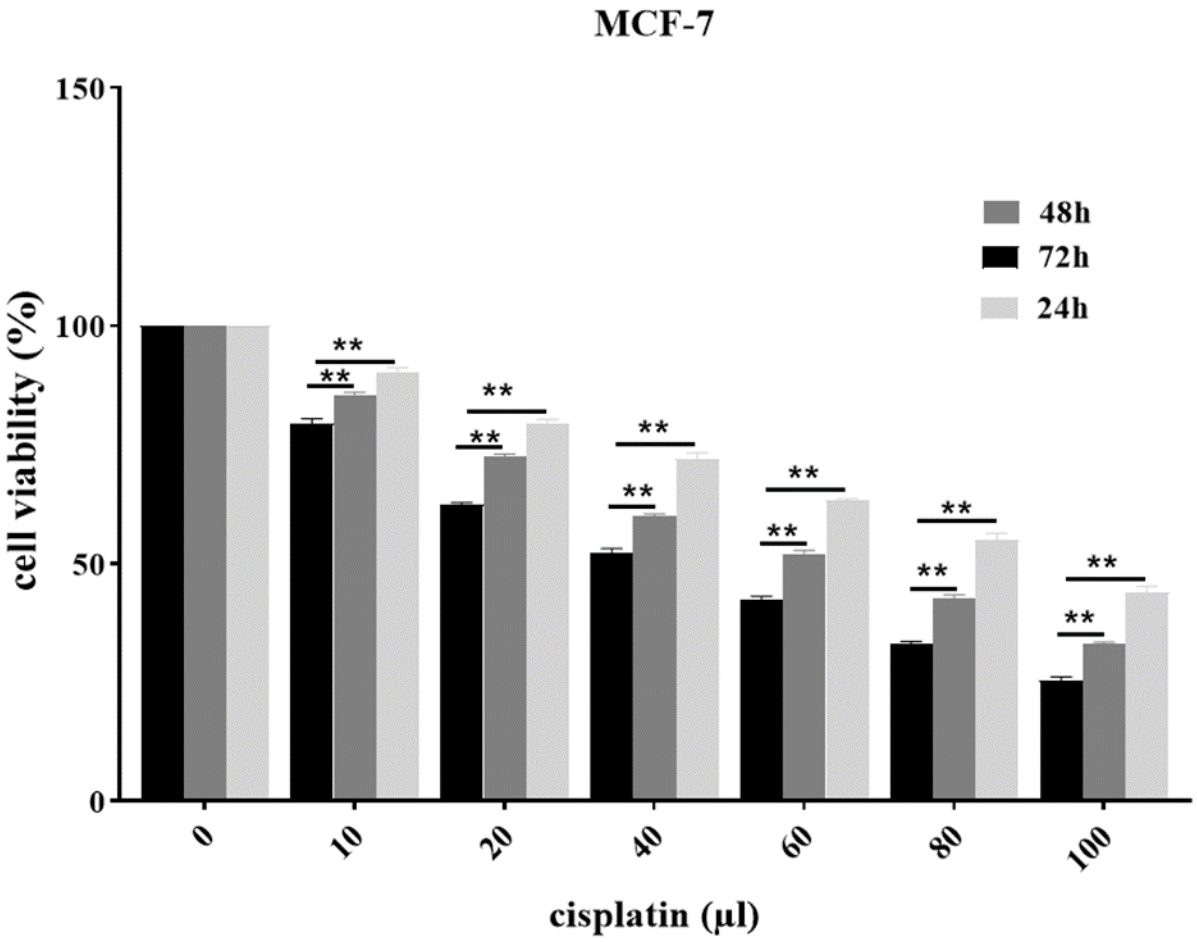
µl for 24, 48, and 72 hours, respectively. CDDP had a stronger cytotoxic impact on MCF-7 cell lines improved by combination with Thymoquinone and, after exposure for 24, 48, and 72 hours, reduced its IC50s to 40.3, 38.39, and 34.74 µl respectively (Fig.1).

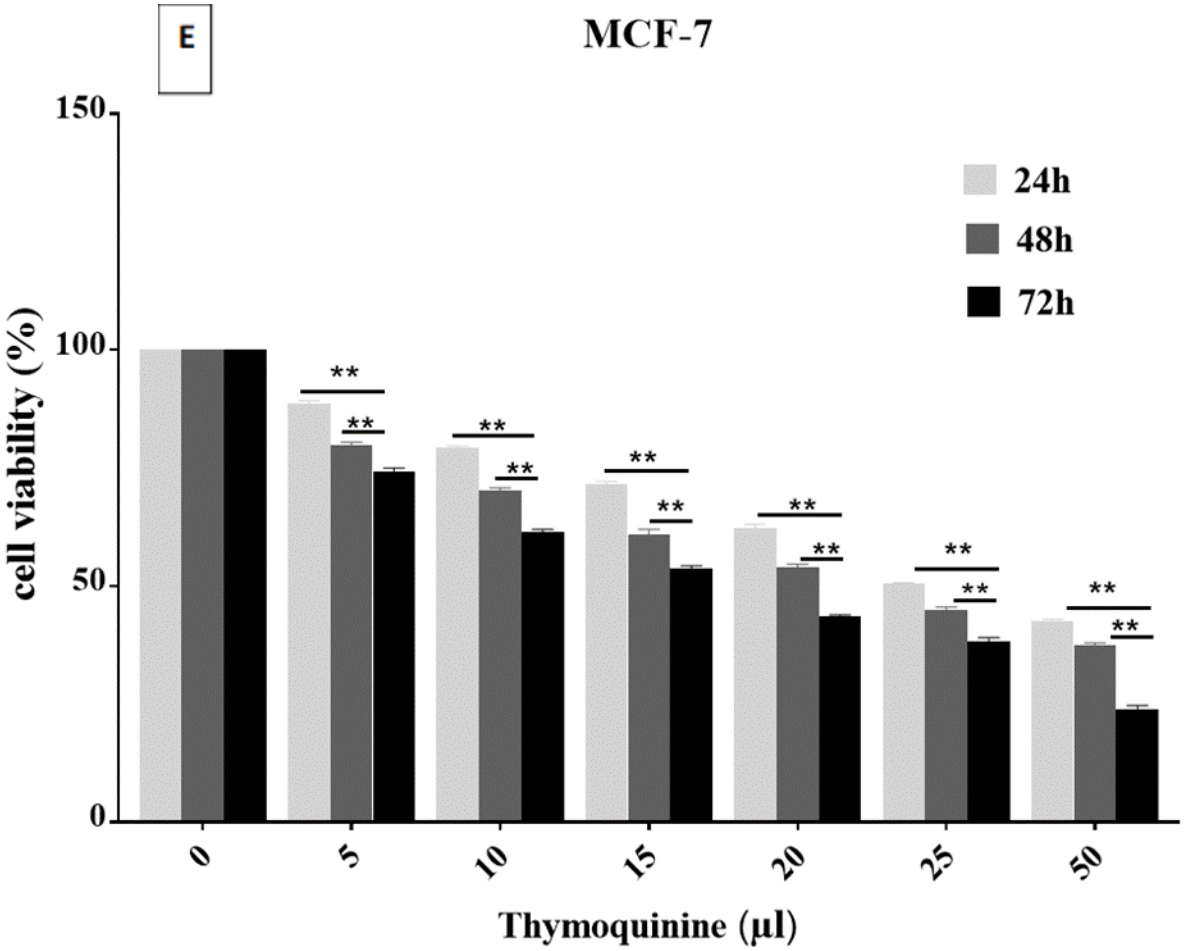






D


MCF-7
150
100
50
0
cell viability (%)
48h
72h
24h
**
0
10
20
40
60
80
100
cisplatin (µl)


E
MCF-7
150
100
50
0
cell viability (%)
24h
48h
72h
**
0
5
10
15
20
25
50
Thymoquinine (µl)

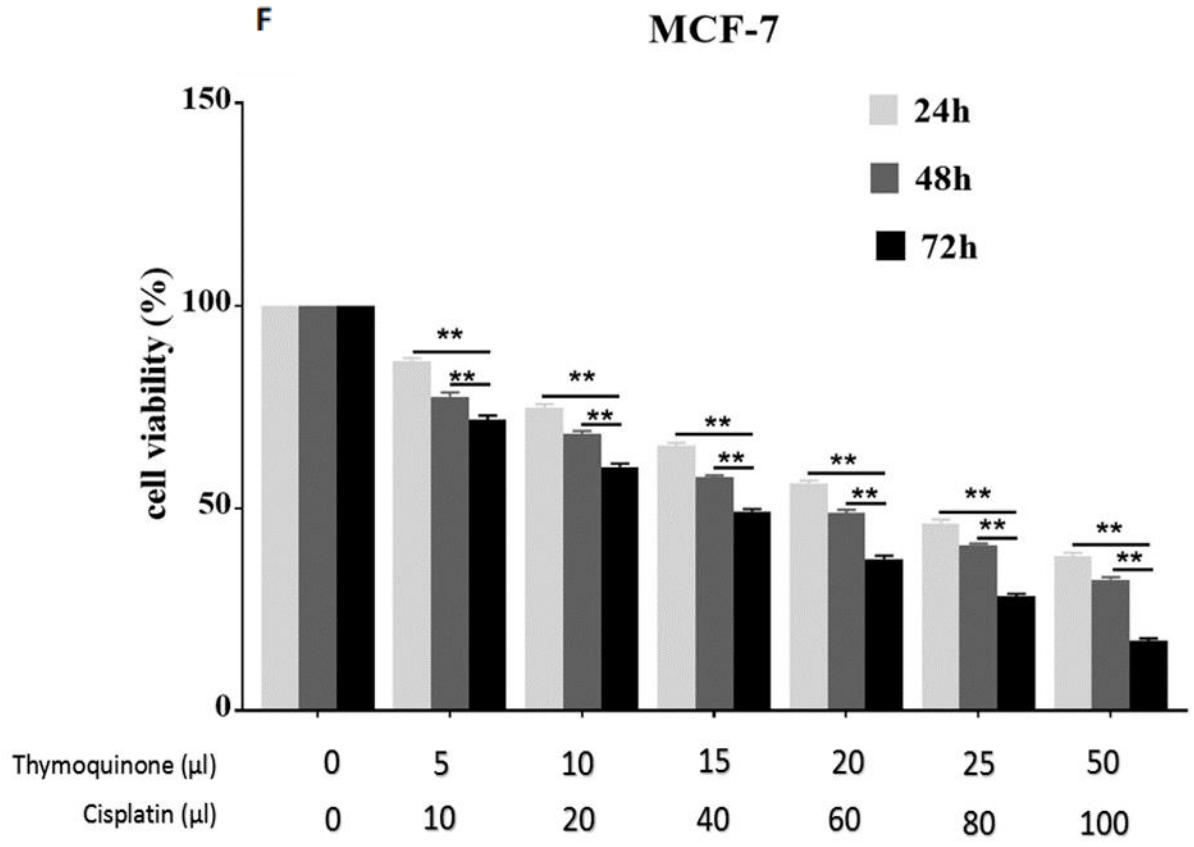


Figure 1 demonstrates the combined impact of TQ and cisplatin on the survivability of MCF-7 cells. Cisplatin's impact on MCF-7 at 24, 48, and 72 hours (A, D). Impact of TQ on MCF-7 at 24, 48, and 72 h (B, E) (B, E). Impact of TQ+cisplatin on MCF-7 at 24, 48, and 72 h (C, F). *, P 0.05; values are shown as the mean SD of at least three separate studies.

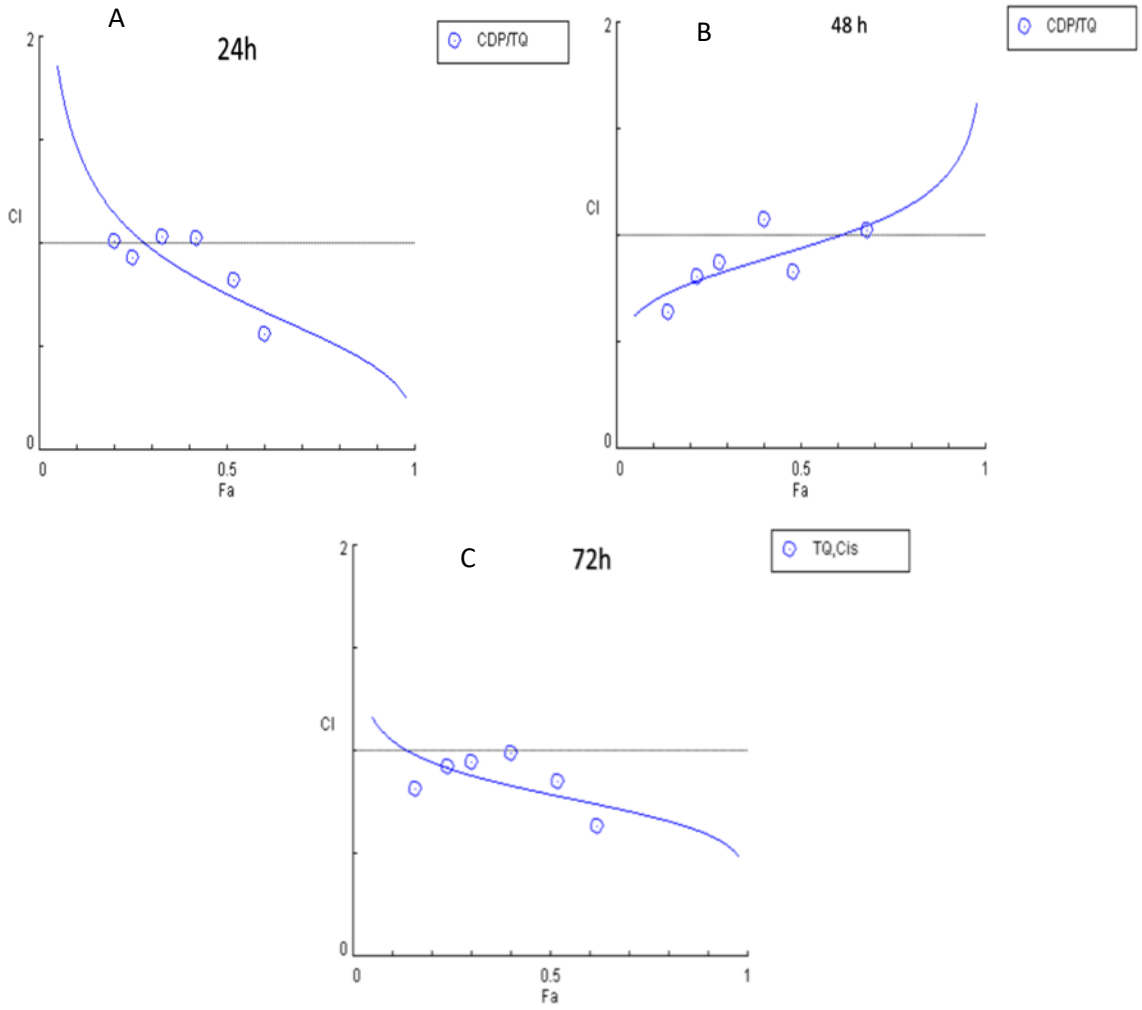


Figure 2: Combination Index Plot. A, B and C represents combination index plots of MCF-7 upon treatment with different concentrations of cisplatin and Thymoquinone at 24, 48,and 72 h.

Impact of TQ and cisplatin combination on p53, Bax, and Bcl-2 levels in MCF-7 cell lines

Following contact with single- and combined-effect therapies, the anti-apoptotic protein (Bcl-2) and apoptotic inducer proteins (p53 and BAX) expression was examined by Western immunoblotting with the hope of further understanding the cytotoxic effect mechanism of TQ's alone and coupled with CDDP against MCF-7 cells. Our data revealed that after 24 h, under combination treatment with TQ and Cisplatin, p53 protein expression was considerably enhanced when compared to separate groups. Moreover, in comparison to the control group, TQ (30.78 l) and Cisplatin (80.84 l) boosted Bax expression levels. However, when combined, TQ and Cisplatin induced a greater rise in Bax protein expression than each drug did separately. The expression of the Bcl-2 was reduced in the cisplatin- and -TQ- treated cells as opposed to these levels in the cells resolved with cisplatin (80.84) or TQ (30.78 M) alone (Fig.3).

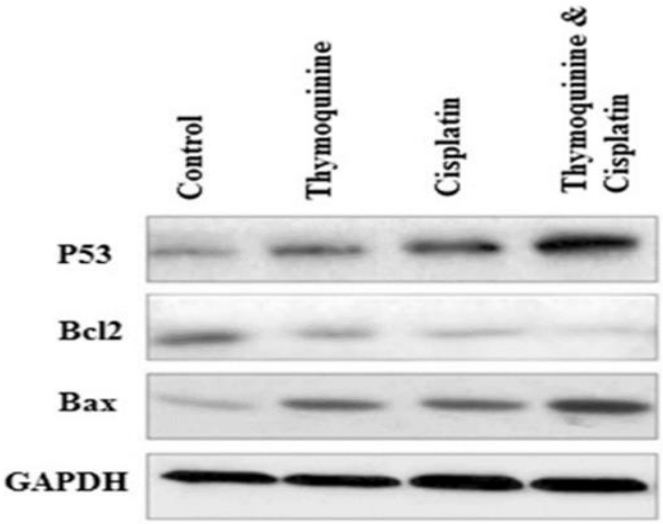
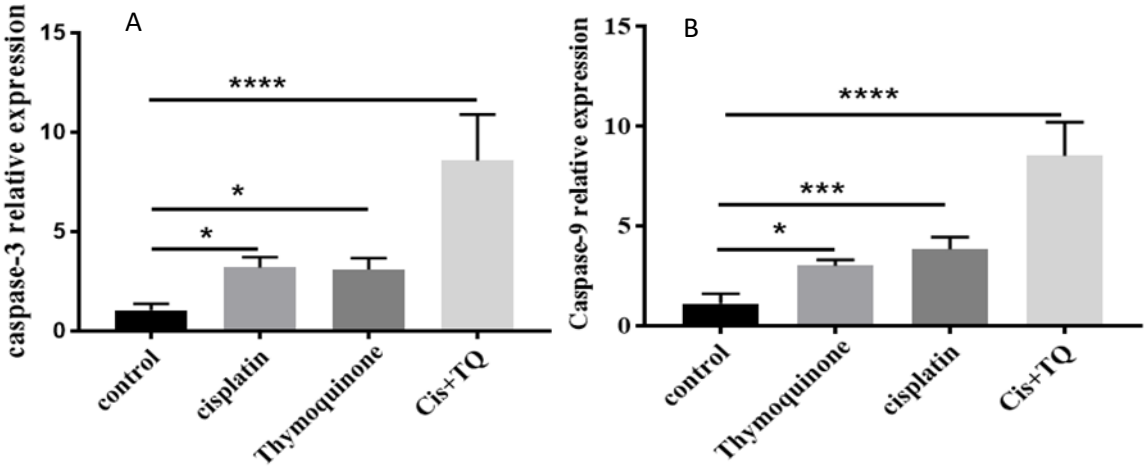


Figure 3: Western blot examination of the anti-apoptotic protein Bcl-2 and the pro-apoptotic proteins (p53 and BAX) expression levels after 24 hours of exposure to solo and combined therapies.

The effect of cisplatin and TQ on the caspase-9, caspase-3, and PPAR-γ expression

Using qRT-PCR, the expression levels of caspase-9, caspase-3, and PPAR- in MCF-7 cells were examined. Cisplatin and TQ increased caspase-9, caspase-3, and PPAR- levels in comparison to the control group (Fig. 4A and Fig. 4B). Caspase-9, Caspase-3, and PPAR- expression was higher when Cisplatin and TQ were combined than when these two drugs were used separately. The synergistic effect previously verified at the cellular level of the MCF-7 cell line was confirmed by these results at the molecular level (Fig.4).



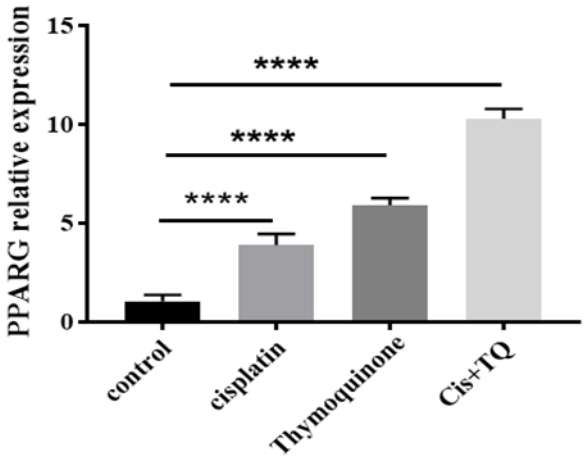


Figure 4: Impact of therapy with cisplatin, Thymoquinone and cisplatin + Thymoquinone on the mRNA expression levels for caspase-3, caspase-9, and PPARγ in the MCF-7 human breast cancer. Values are calculated as means ±SEM from three independent experiments. * P < 0.05, *** P < 0.0005, **** P < 0.00001 compared to the control.

Discussion

The most common cancer among women diagnosed globally is breast cancer, which is also linked to increased rates of mortality as well as morbidity. Surgery, hormone therapy, radiation, and chemotherapy are all alternatives for treatment. Cisplatin is a platinum medication that causes DNA damage, G1/S arrest, and apoptosis by alkylating DNA by creating platinum-DNA adducts [13]. Furthermore, Cisplatin, by blocking early EMT, prevents breast cancer metastasis, and it is a promising therapeutic for eliminating breast cancer and preventing tumor spread, according to a recent comprehensive study [14]. Despite Cisplatin's success in treating breast cancer, its usage in chemotherapy is limited due to the resilience of cancer cells to it and its harmful effects on normal cells, like kidney damage [15]. Various natural compounds mixed with synthetic anticancer medications, such as Cisplatin, are effective candidates for cancer therapy because they improve chemotherapeutic drug efficacy while reducing side effects [16]. Consequently, in the current investigation, we examined how Cisplatin and TQ worked together to affect the apoptotic processes in breast cancer cells. The results indicate that Cisplatin and TQ combined therapy interplay between PPAR, P53, BAX, Caspase-3, and Caspase-9 in the breast cancer cell, favoring apoptotic induction.

Strong TQ anticancer effects on different tumor cells and malignancies have been demonstrated in several in vivo and in vitro works. TQ was discovered to have anti-metastatic effects predominantly through the down-regulation of NF-B-regulated CXCR4 expression, suggesting that it could be used to treat breast cancer [17]. Also, using an MTT assay, Alobaedi et al. assess the anti-proliferative effectiveness of TQ, RES, and their combo on three breast cancer cell lines and a normal cell. They discovered that combining TQ and RES to treat breast cancer in mice could synergiz. Similarly, our data revealed that TQ's cytotoxic effects were primarily dose-dependent [18]. We may conclude that existing research findings support TQ's efficacy in treating breast cancer. However, this was the first time in BC that a study on TQ's capacity to produce synergistic cytotoxicity when paired with anticancer medications like Cisplatin was conducted (Fig.2).

Although the molecular mechanisms behind TQ's anticancer effectiveness are unclear, study results indicate that TQ has an antitumor impact because it promotes apoptosis [19]. A common target for cancer treatment is apoptosis, or the programmed death of cells. For cancer survivors, the p53 gene is crucial because it causes apoptosis in the aftermath of damage to the DNA. Bcl-2 family members, including Bax, have crucial roles as either promoters or suppressors of apoptosis (e.g., Bcl-2). We looked into the possible apoptotic impact of TQ on MCF-7 cells using Western Blot analysis. A considerable overexpression of apoptosis executive proteins, such asp53, and BAX, was discovered. On the other side, there was a drop in the anti-apoptotic Bcl-2 protein expression.

Furthermore, the qRT-PCR analysis revealed that the combination therapy group had higher mRNA levels of caspase-3 and caspase-9 than the individual treatment group. These findings may shed light on apoptotic activation's mechanism in response to TQ therapy. Furthermore, the significant apoptosis in combination therapy is due to TQ and Cisplatin's integrative apoptosis induction mechanisms. The ligand-activated transcription factor PPAR is part of the PPARs family, including PPAR and PPAR/. PPAR appears to be a possible therapeutic target for type 2 diabetes, inflammation, and cancer, according to growing research [20]. Both natural and artificial PPAR ligands are known to prevent the spread of cancer cells by modulating the protein expression of several cell cycle regulators. Tamoxifen and the PPAR ligand troglitazone have been shown to inhibit cell growth, arrest the cell cycle, and trigger apoptosis in ER-positive MCF-7 breast cancer cells [21]. Moreover, the tumor suppressor p53's promoter gene is transactivated by PPAR agonists, which increases the p53 protein expression and its target gene p21. This accelerates caspase-9 division and cytochrome c discharge into the cytoplasm.

It's interesting to note that PPAR agonists can trigger intrinsic apoptosis by upregulating the pro-apoptotic Bid, and they can also increase apoptosis by establishing a p53-Bid complex at the mitochondria. Also, it has been demonstrated in numerous studies that natural products can activate PPAR to cause apoptosis in breast cancer cells and decrease inflammation in leukaemia cells [22]. In this circumstance, CC Woo et al. discovered that TQ coupled with doxorubicin and 5-fluorouracil enhanced cytotoxicity and had a potent anti-proliferative impact on breast cancer cells [23].

They interestingly found that TQ was able to up-regulate PPAR- activity and reduce Bcl-2, Bcl-xL, and survivin gene expression in breast cancer cells for the first time. The combination team in this particular study exhibited the highest level of PPAR among all the groups. These findings are consistent with earlier research, implying that TQ increases PPAR-transactivation somehow.

In conclusion, our findings imply that combining TQ and Cisplatin in the treatment of BC can have considerable synergistic effects, which may give a solution to Cisplatin's insufficient efficacy and drug resistance in some cases. One plausible explanation for the synergistic effects found in this study could be synergistic apoptotic activation, with PPAR-transactivation playing a pivotal role.

Contributors

Contributed equally to this work with: Fatemehsadat Mousavinasab, and Mehrdad Talebi

Acknowledgements

This study supported by Shahid Sadoughi University of Medical Sciences.

Conflicts of Interest

The authors declare no conflict of interest, financial or otherwise.

Funding

This study was funded by Shahid Sadoughi University of Medical Sciences.

References

1. Fattahi, M., et al., *microRNA-382 as a tumor suppressor? Roles in tumorigenesis and clinical significance.* International journal of biological macromolecules, 2023: p. 125863.
2. Pordel, S., et al., *The role of microRNA-185 in the pathogenesis of human diseases: a focus on cancer.* Pathology-Research and Practice, 2023: p. 154729.
3. Khasraghi, L.B., et al., *MicroRNA-206 in human cancer: Mechanistic and clinical perspectives.* Cellular Signalling, 2023. **101**: p. 110525.
4. Huang, C., et al., *Non-coding RNAs/DNMT3B axis in human cancers: from pathogenesis to clinical significance.* Journal of Translational Medicine, 2023. **21**(1): p. 621.
5. Faramin Lashkarian, M., et al., *MicroRNA-122 in human cancers: from mechanistic to clinical perspectives.* Cancer Cell International, 2023. **23**(1): p. 1-25.
6. Shirvani, H., et al., *MiR-211 plays a dual role in cancer development: from tumor suppressor to tumor enhancer.* Cellular Signalling, 2023. **101**: p. 110504.
7. Pasha, N. and N.C. Turner, *Understanding and overcoming tumor heterogeneity in metastatic breast cancer treatment.* Nature Cancer, 2021. **2**(7): p. 680-692.
8. Sheikhpour, E., et al., *A survey on the role of interleukin-10 in breast cancer: A narrative.* Reports of biochemistry & molecular biology, 2018. **7**(1): p. 30.
9. Fang, C.-y., et al., *Natural products: Potential treatments for cisplatin-induced nephrotoxicity.* Acta Pharmacologica Sinica, 2021. **42**(12): p. 1951-1969.
10. Zahedian, S., et al., *The impacts of prepared plasma-activated medium (pam) combined with doxorubicin on the viability of mcf-7 breast cancer cells: a new cancer treatment strategy.* Reports of Biochemistry & Molecular Biology, 2022. **10**(4): p. 640.

many characters of a critical transcription factor. International Journal of Molecular Sciences, 2018. **19**(11): p. 3339.

21. Lewoniewska, S., et al., *Troglitazone-Induced PRODH/POX-Dependent Apoptosis Occurs in the Absence of Estradiol or ERβ in ER-Negative Breast Cancer Cells.* Journal of clinical medicine, 2021. **10**(20): p. 4641.
11. Malik, S., et al., *Thymoquinone: A small molecule from nature with high therapeutic potential.* Drug Discovery Today, 2021. **26**(11): p. 2716-2725.
12. Dastjerdi, M.N., et al., *Effect of thymoquinone on P53 gene expression and consequence apoptosis in breast cancer cell line.* International journal of preventive medicine, 2016. **7**.
13. Moo, T.-A., et al., *Overview of breast cancer therapy.* PET clinics, 2018. **13**(3): p. 339-354.
14. Varghese, E., et al., *The "yin and yang" of natural compounds in anticancer therapy of triple-negative breast cancers.* Cancers, 2018. **10**(10): p. 346.
15. Ghafari, M., et al., *Development of a novel liposomal nanoparticle formulation of cisplatin to breast cancer therapy.* Journal of Cellular Biochemistry, 2020. **121**(7): p. 3584-3592.
16. Safi, A., et al., *miRNAs modulate the dichotomy of cisplatin resistance or sensitivity in breast cancer: an update of therapeutic implications.* Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents), 2021. **21**(9): p. 1069-1081.
17. Shanmugam, M.K., et al., *Thymoquinone inhibits bone metastasis of breast cancer cells through abrogation of the CXCR4 signaling axis.* Frontiers in Pharmacology, 2018. **9**: p. 1294.
18. Alobaedi, O.H., W.H. Talib, and I.A. Basheti, *Antitumor effect of thymoquinone combined with resveratrol on mice transplanted with breast cancer.* Asian Pacific journal of tropical medicine, 2017. **10**(4): p. 400-408.
19. Zhang, M., et al., *Thymoquinone induces apoptosis in bladder cancer cell via endoplasmic reticulum stress-dependent mitochondrial pathway.* Chemico-Biological Interactions, 2018. **292**: p. 65-75.
20. Liu, Y., et al., *The role of PPAR-δ in metabolism, inflammation, and cancer:*
22. Weng, J.-R., et al., *A sterol from soft coral induces apoptosis and autophagy in MCF-7 breast cancer cells.* Marine drugs, 2018. **16**(7): p. 238.
23. Woo, C.C., et al., *Anticancer activity of thymoquinone in breast cancer cells: possible involvement of PPAR-γ pathway.* Biochemical pharmacology, 2011. **82**(5): p. 464-475.