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RESEARCH ARTICLE

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Thymoquinone reversed doxorubicin resistance in U87 glioblastoma cells via targeting PI3K/Akt/mTOR signaling

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Abstract

Natural compounds such as thymoquinone (TQ) have recently gained increasing attention in treating glioblastoma (GBM). However, the effects of TQ in reversing drug resistance are not completely understood. Therefore, we aimed to examine TQ impacts on GBM cells with doxorubicin (DOX) resistance and the involvement of the PI3K/Akt/mTOR pathway. GBM cancer U87 and U87/DOX (resistant cells) cells were exposed to DOX and TQ, and cell proliferation was assessed by the MTT assay. ELISA was applied to evaluate cell apoptosis. The expression of apoptotic mediators such as Caspase-3, Bax, Bcl-2 and PI3K, Akt, mTOR, P-gp, and PTEN was assessed via qRT-PCR and western blot. We found that a combination of TQ and DOX suppressed dose-dependent cell growth capacity in cells and increased the cytotoxic effects of DOX in resistant cells. In addition, TQ treatment increased DOX-mediated apoptosis in U87/DOX cell lines via modulating the pro- and anti-apoptotic markers. A combination of TQ and DOX upregulated PTEN and downregulated PI3K, Akt, and mTOR, suppressing this signal transduction in resistant cells. In conclusion, we showed TQ potentiated doxorubicin-mediated antiproliferative and pro apoptotic function DOX-resistant glioblastoma cells, which is mediated by targeting and suppressing PI3K/Akt/mTOR signal transduction.

KEYWORDS

apoptosis, doxorubicin resistance, glioblastoma, PI3K/Akt/mTOR pathway, thymoquinone

1 | INTRODUCTION

Glioblastoma (GBM) or grade IV astrocytoma, is one of the brain's most common malignancies and the most aggressive type of glioma (Delgado-Martín & Medina, 2020; Gusyatiner & Hegi, 2018). This type of brain malignancy is characterized by significantly low survival time, fast growth, aggressive behavior, and development of drug resistance against conventional chemotherapeutics used to treat GBM (Zeng et al., 2017). Thus, discovering an impressive therapeutic modality is essential for this disease (Ge et al., 2023).

The high developmental potential and diminished apoptosis potential of the GBM cells depend more on the hereditary transformations and alteration in the expression pattern of apoptotic and oncogenic mediators (Wu et al., 2023). Therefore, identifying and designing novel therapeutic agents for the effective and complete removal of GBM cells requires a full understanding of possible genetic or pharmacological alterations in the chief signal transductions involved in the GBM cell growth and survival (Stylli, 2020; van Solinge et al., 2022). In this regard, recent studies have extensively examined the potential

of natural polyphenolic compounds in treating various human malignancies, including GBM (Stylli, 2021).

Thymoquinone (TQ), a flavonoid extracted from *Nigella sativa*, is an effective chemopreventive compound in various cancer types (Mostofa et al., 2017). It possesses various anticancer activities, including antioxidative, angiogenesis inhibitory, antiproliferative, apoptosis-inducing, and anti-metastatic properties (Khan et al., 2019; Khyavi et al., 2022). Inhibition of cancer cell growth, survival and metastasis, downregulation of major proliferative signaling pathways, and induction of cell cycle arrest are among the primary mechanisms by which TQ exerts anticancer and chemopreventive effects in GBM (Chowdhury et al., 2018). Additionally, recent studies have demonstrated that combining TQ with conventional chemotherapeutic agents used for treating GBM is an effective strategy for increasing the anticancer potential of drugs and reversing drug resistance in GBM (Pazhouhi et al., 2016, 2018).

Therefore, in this study, the role of TQ in increasing the amount of cell apoptosis induced alone or together with doxorubicin was investigated on the GBM cancer cell line.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Human GBM cells, U87 and U87/DOX (resistant cells), were provided by the Institut Pasteur Cell Bank (Tehran, Iran). After culturing both cell lines in RPMI-1640 medium containing 100 U/mL penicillin/streptomycin and 10% fetal bovine serum, cells were incubated at 37°C with 5% CO_2 to reach their maximum culture capacity for other steps.

2.2 | Cell proliferation assay

The cell viability after TQ exposure in both cell lines was assessed via the MTT assay. In this method, the cells were affected by increasing concentrations of DOX (0–25 μM) alone and in combination with 45 μM TQ. After 24 h, the fresh medium containing 10% MTT solution was added and cells were kept in the dark in an incubator for 4 h. After the formed formazan crystals were dissolved using DMSO, the microplate reader read the absorption rate and the amount of cell viability was calculated.

2.3 | qRT-PCR

Gene expression for MDR-1, PI3K, Akt, mTOR, PTEN, Bax, Bcl-2, and Caspase-3 was analyzed with qRT-PCR in cells. At first, total cellular RNAs were extracted from cells using the RNeasy Plus mini kit. Then, a reverse transcriptional reaction was performed to obtain cDNA using Prime Script RT Master Mix. Subsequently, the Power SYBR Green PCR Master Mix kits were used to detect the relative mRNA expression by RT-PCR in the ABI 7300 fast Real-Time PCR Systems. Raw data were analyzed, and gene expression changes between the untreated and treated MCF-7 cells were calculated using the $2^{-(\Delta\Delta C_T)}$ method.

2.4 | Western blotting analysis

At the protein level, the expression of P-gp, PI3K, Akt, mTOR, PTEN, Bax, Bcl-2, and Caspase-3 was assessed by western blotting. Centrifugation separates the culture medium from the treated cells to prepare cell lysate containing protein. The cells were washed twice with phosphate buffer; then, the cell suspension was lysed using 500 μL of cell lysate buffer. Then, 50 μg of each protein sample was added to 5 μL of protein sample buffer and boiled for 5 min. Then, the samples were placed in a 10% SDS-PAGE gel on which a 4% polyacrylamide stacking gel was placed, and electrophoresis with a voltage of 120–80 V in Western running buffer was carried out until migration of the colored buffer loaded to the end of the gel. Then, the spread proteins were transferred to PVDF-activated membranes. After blocking, overnight incubation at 4°C with primary antibodies against P-gp, PI3K, Akt, mTOR, PTEN, Bax, Bcl-2, Caspase-3, and β-actin was performed. The membranes were incubated for 1 h with secondary antibodies (anti-rabbit or anti-mouse) conjugated with HRP and diluted in PBS. Then, the membranes were rewashed, and the bands appeared using an ECL kit and x-ray film. Bands were analyzed using ImageJ software.

2.5 | Evaluating cell apoptosis

A photometric enzyme immunoassay was used to assess the effects of TQ on the apoptosis in cells via a Cell Death Detection ELISA kit. After treating cells with various amounts of TQ, the number of apoptotic cells was measured in accordance with the protocol.

2.6 | Statistical analysis

Data were analyzed by GraphPad Prism 6.01 software and presented as mean ± SD. Analysis of variance (ANOVA) was used to evaluate statistically significant differences between groups. $p < .05$ was considered significant.

3 | RESULTS

3.1 | The effects of DOX on the cell viability in U87 and U87/DOX cells

An MTT assay was applied to evaluate the suppressive impacts of DOX on cell viability. In this process, cells were exposed to ascending concentrations of DOX (0–25 μM) for 24, 48, 72, and 96 h. DOX suppressed the cell viability of both cell lines dose-dependently after 48 h (Figure 1). However, the cytotoxic effects of doxorubicin were more prominent in U87 cells than in U87/DOX cells. In other words, DOX exerts cytotoxic effects on U87 cells at lower concentrations, indicating a higher sensitivity of U87 cells to DOX than U87/DOX cells. In addition, IC_{50} values for doxorubicin were 5.12 μM in U87 and 12.38 μM in U87/DOX cells.

3.2 | TQ increased the antiproliferative effects of DOX in U87/DOX cells

Results from the MTT assay reported that both DOX and TQ treatment resulted in the dose-dependent suppression of the proliferation of U87/DOX cells after 48 h (Figure 2). In addition, the combination of 45 μM TQ with DOX increased the antiproliferative effects of DOX in U87/DOX cells such that the IC_{50} value for DOX decreased from 12.38 to 6 μM in U87/DOX cells (Figure 2).

3.3 | TQ downregulated P-gp in cells

P-gp, a pump belonging to the ATP binding cassette superfamily, is involved in developing drug resistance by decreasing the intracellular concentrations of cytotoxic agents. Measuring P-gp expression in U87/DOX cells showed that the expression levels of this pump were significantly higher in resistant cells in comparison with U87 cells ($p < .05$). Resistant cell exposure to the combination of DOX and TQ significantly downregulated MDR-1 ($p < .05$; Figure 3) and P-gp expression at the protein level. In other words, by decreasing the P-gp expression in resistant cells, TQ may increase the intracellular levels of DOX, hence increasing its cytotoxic effects on cells.

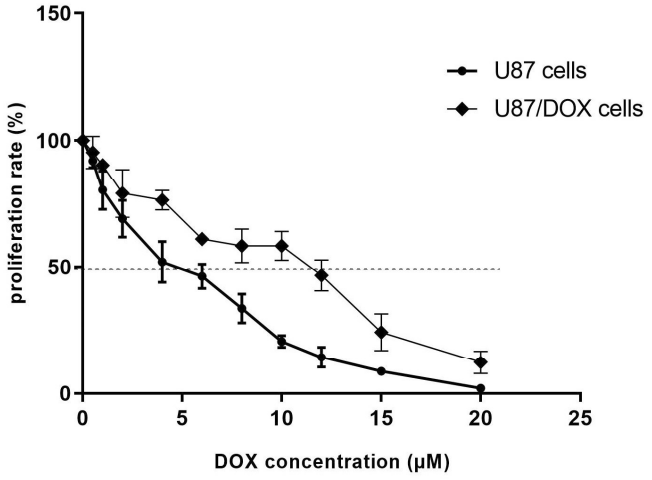

FIGURE 1 Effects of DOX on cell viability in U87 and U87/DOX cells. Results are shown as mean ± SD after three experiments. TQ, thymoquinone; U87/DOX U87 resistant cells.

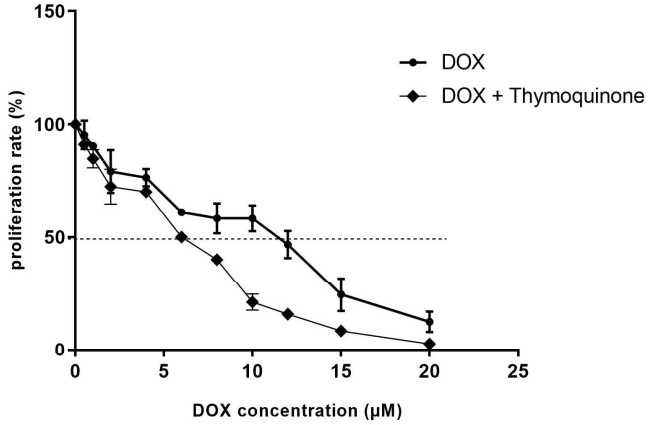

FIGURE 2 Effects of TQ on the antiproliferative effects of DOX in U87/DOX cells. Results are shown as mean ± SD after three experiments. TQ, thymoquinone; U87/DOX U87 resistant cells; DOX, doxorubicin.

3.4 | TQ downregulated the PI3K/Akt/mTOR axis in cells

PI3K/Akt/mTOR signal transduction is one of the key proliferative pathways that commonly showed aberrant expression profiles. We tested whether GBM cancer cell treatment with TQ reversed DOX resistance by exerting an impact on the expression levels of key components of this signaling, including PI3K, Akt, and mTOR. For this purpose, qRT-PCR and western blotting were applied in resistant and sensitive cells exposed to the combination of DOX and TQ. The first important component of this signaling pathway is PI3K. In U87/DOX cells, the PI3K expression was significantly higher than in U87 cells. Resistant cells exposed to a combination of TQ and DOX showed considerably lower levels of PI3K expression ($p < .05$; Figure 4a).

Additionally, in U87/DOX cells, Akt was upregulated compared to sensitive cells. Combining TQ and DOX significantly suppressed Akt expression levels ($p < .05$; Figure 4b). Another main component of PI3K/Akt/mTOR signaling is mTOR. Resistant cells showed significantly higher expression levels of mTOR. Upon treatment with the combination of TQ and DOX, the expression levels of mTOR were significantly decreased ($p < .05$; Figure 4c). Therefore, TQ

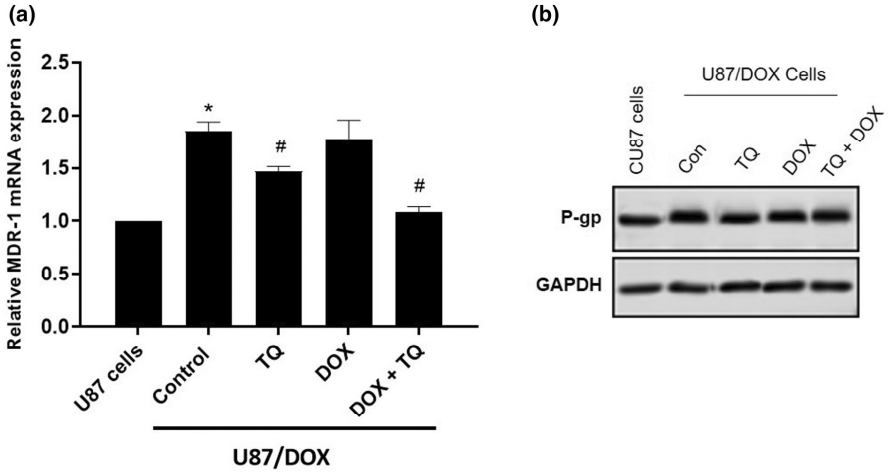

FIGURE 3 Effects of TQ on the expression levels of (a) mRNA (MDR-1) and (b) protein (P-gp) in glioblastoma cells. Results are shown as mean ± SD after three experiments. MDR-1, multidrug resistance mutation 1; P-gp, permeability glycoprotein; TQ, thymoquinone. *$p < .01$ versus U87 sensitive cells, #$p < .05$ versus U87/DOX cells (Control).

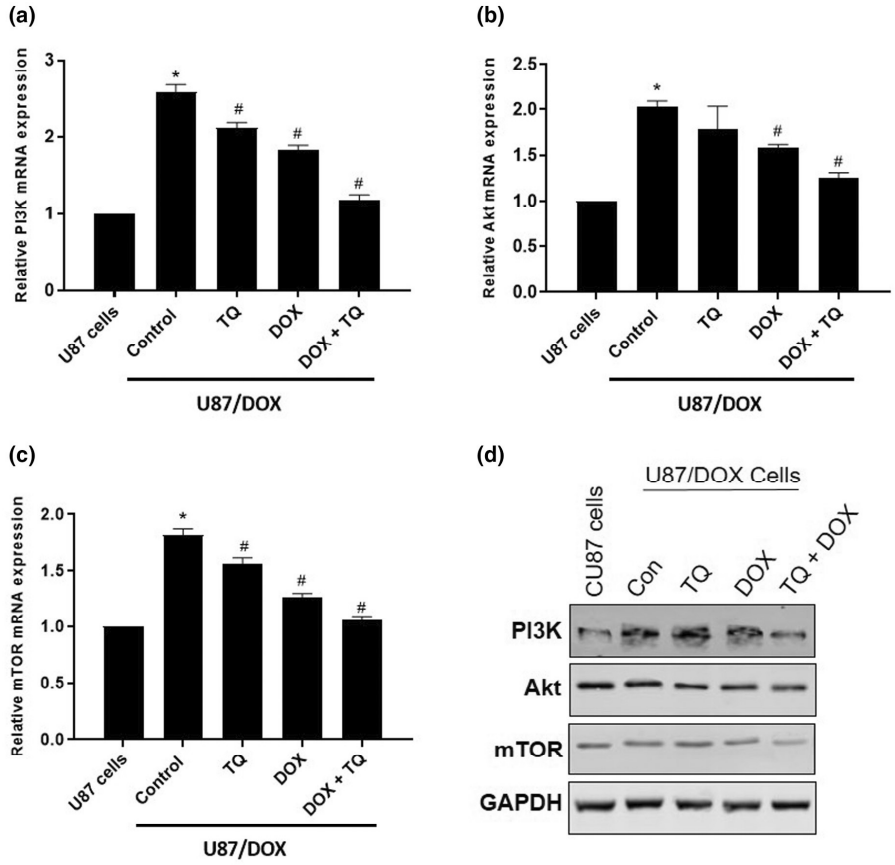

FIGURE 4 Effects of TQ on the PI3k/Akt/mTOR axis in glioblastoma cells. (a–c) mRNA expression levels of PI3K/Akt/mTOR pathway, (b) protein expression levels of PI3K/Akt/mTOR axis in glioblastoma cells. Results are shown as mean ± SD after three experiments. Akt, protein kinase B; mTOR, mechanistic target of rapamycin; PI3k, phosphoinositide 3-kinases; TQ, thymoquinone. *$p < .01$ versus U87 sensitive cells, #$p < .05$ versus U87/DOX cells (Control).

may increase the sensitivity of DOX-resistant cells by inhibiting one of the potent proliferative signaling pathways, PI3K/Akt/mTOR signaling.

3.5 | TQ upregulated the PTEN tumor suppressor in cells

Another main component involved in the PI3K/Akt/mTOR signaling is PTEN, which has tumor suppressor activity by inhibiting this signaling. Measuring PTEN expression in cells showed that resistant GBM cells have lower PTEN levels than sensitive cells ($p < .05$; Figure 5). U87/DOX cell exposure to the combination of TQ and DOX significantly upregulated PTEN expression ($p < .05$; Figure 5). In other words, TQ inhibited PI3K/Akt/mTOR signaling via increasing PTEN expression in cells, hence reversed DOX resistance in GBM cells.

3.6 | TQ potentiated DOX-induced apoptosis in cells

To confirm the results of the MTT assay, the percentage of apoptotic cells was assessed in the U87/DOX cell line

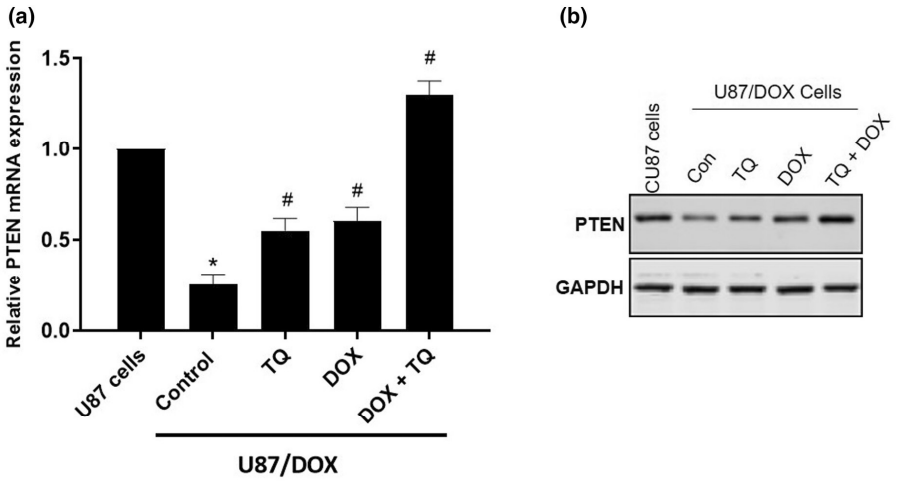

FIGURE 5 Effects of TQ on PTEN expressions are (a) at the mRNA level and (b) at the protein level in glioblastoma cells. Results are shown as mean ± SD after three experiments. PTEN, phosphatase; TQ, thymoquinone; and tensin homolog. *$p < .01$ versus U87 sensitive cells, #$p < .05$ versus U87/DOX cells (Control).

following treatment with a combination of TQ and DOX via ELISA. We demonstrated that apoptotic cell percentages were significantly higher in cells treated with TQ or DOX alone ($p < .05$; Figure 6). Combining TQ and DOX led to a significant elevation in apoptotic cells. As shown in Figure 7a–c, the expression levels of key apoptotic markers were also evaluated in this study. The combination of TQ and DOX upregulated Bax and caspase-3 expression and downregulated Bcl-2 expression ($p < .05$).

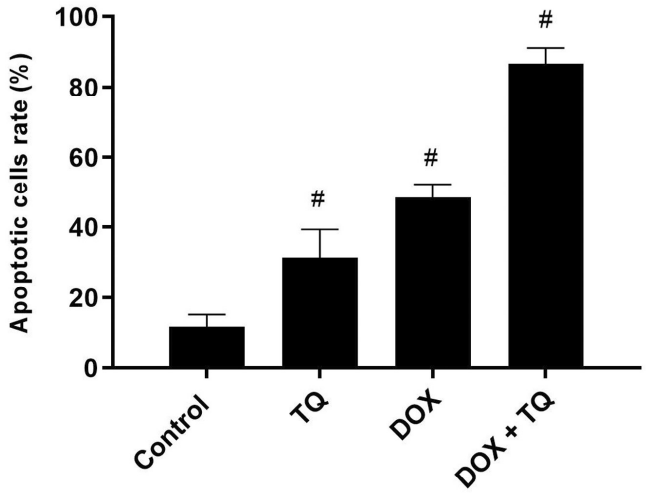

FIGURE 6 Effects of TQ on apoptosis in glioblastoma cells. Results are shown as mean ± SD after three experiments. TQ, thymoquinone. #$p < .05$ versus U87/DOX cells (Control).

4 | DISCUSSION

GBM is the most common and destructive primary brain tumor. It is very resistant to conventional radiation and chemotherapy, and surgical tumor removal is ineffective (Zhang et al., 2019). Surgery and following chemotherapy/radiotherapy are gold standard therapeutic modalities for treating GBM (Di Nunno et al., 2021). However, in most cases, developing drug resistance against chemotherapeutic agents leads to the failure of GBM therapy and increases the need to find innovative and effective therapeutic options (Geng et al., 2023; Hottinger et al., 2016). In this regard, various mechanisms, including evading apoptosis, suppressing oxidative stress, inhibiting autophagy, and modulating cell cycle and GBM stem cells, are important mechanisms underlying the development of drug resistance in GBM cells (Ou et al., 2020; Stavrovskaya et al., 2016). High toxicity for normal cells, presence of severe side effects, lack of sufficient efficacy for patients with high-grade cancers, failure to increase the survival rates of patients, as well as the development of resistance, hence tumor recurrence, are among the important disadvantages and limitations of using conventional chemotherapeutic agents in treating GBM (Haar et al., 2012; Mao et al., 2023; Oliver et al., 2020). Combination therapy, especially with natural compounds such as TQ that have no toxicity to normal cells and side effects, successfully solves these problems (Ghosh et al., 2018).

Accumulating evidence has demonstrated that TQ, alone or in combination with other mainline chemotherapy agents, is highly effective in inhibiting cancer progression and synergistic reduction of tumor initiation in a broad range of human malignancies by targeting and modifying tumorigenic pathways (Banerjee et al., 2010; Schneider-Stock et al., 2014). In particular, various studies have investigated the potential of TQ in reversing drug resistance against the most commonly used chemotherapeutic agents, including temozolomide (TMZ) and DOX in GBM cells (Nonnenmacher et al., 2015). For example, Pazhouhi et al. (2016) indicated that TQ synergistically enhanced the anticancer activity of TMZ in GBM cell line U87MG through inhibition of autophagy. Khazaei and Pazhouhi (2017)

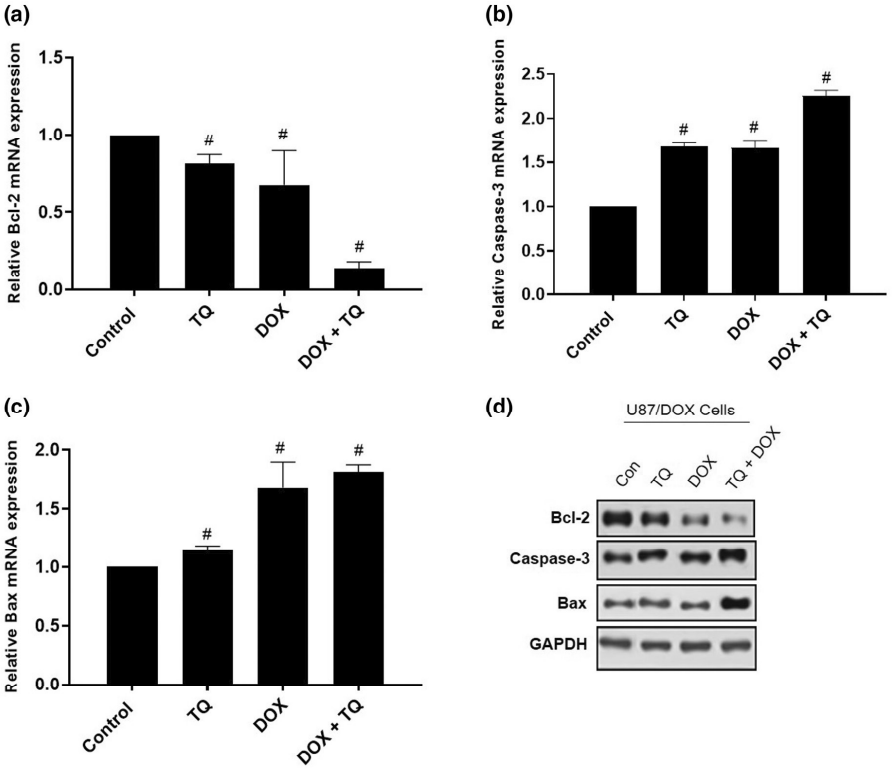

FIGURE 7 Effects of TQ on apoptotic mediators in glioblastoma cells. Results are shown as mean ± SD after three experiments. TQ, thymoquinone. $^{\#}p < .05$ versus U87/DOX cells (Control).

reported that combined exposure of GBM cells with TQ and TMZ led to synergistic induction of apoptosis and inhibition of cancer cell proliferation and growth. Gurung et al. demonstrate similar findings: TQ inhibited proliferation and induced DNA damage, cell cycle arrest, and apoptosis in GBM cells. TQ can promote telomere shortening in GBM cells by inhibiting telomerase activity, which is more potent in GBM cells with DNA-PKc overexpression. TQ was also reported to inhibit tumor growth and enhance the therapeutic function of 5-fluorouracil (5-FU) in a primary rat colorectal tumor model (Gurung et al., 2010). In this model, treatment with 5-FU/TQ combination resulted in a more significant reduction in AOM-induced colorectal tumors and large aberrant crypt foci compared to either agent alone (Gurung et al., 2010). A recent study by Fröhlich et al. (2017) showed increased ROS production and concomitant DNA damage induction in human colon cancer cells treated with a novel hybrid of TQ and artemisinin. In another study by Lei et al. (2012), TQ sensitized 5-FU in treating gastric cancer by enhancing apoptosis induction and growth inhibition. In general, the results obtained from our study are in line with previous studies and consistent with them. Our study showed that the combination of DOX and TQ decreases the IC_{50} values of doxorubicin or, in other words, increases the cytotoxicity of DOX. On the other hand, DOX and TQ increase PTEN gene expression and decrease P-gp expression. However, combining these two has a more substantial effect in promoting apoptosis in the U87 GBM cell line. In other words, TQ increases the sensitivity of U87 cells to apoptosis caused by doxorubicin.

As discussed above, the results of preclinical studies encourage the use of TQ in clinical settings. A significant amount of information about TQ regarding its molecular anticancer activity, drug toxicity, bioavailability and pharmacokinetics, and new drug delivery approaches is now available to researchers.

5 | CONCLUSION

In conclusion, our results emphasize the strong potential of targeting apoptosis as a therapeutic approach to improve doxorubicin response in GBM. However, the choice of drug to combine with doxorubicin is very important, as we have shown that TQ is an up-and-coming therapeutic agent in preclinical cancer models. Since TQ is a potential candidate for future clinical trials in cancer patients, these findings showed its positive effects in increasing the sensitivity of GBM cancer cells to conventional chemotherapy drugs.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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