Indian J Hematol Blood Transfus (Oct-Dec 2017) 33(4):516–524
DOI 10.1007/s12288-016-0758-8

ORIGINAL ARTICLE

Antiproliferative and Apoptosis-Inducing Activities of Thymoquinone in Lymphoblastic Leukemia Cell Line

Amin Soltani[1] · Batoul Pourgheysari[2] · Hedayatollah Shirzad[3] · Zahra Sourani[4]

Received: 26 June 2016 / Accepted: 25 November 2016 / Published online: 8 December 2016

Abstract Acute lymphoblastic leukemia is one of the malignant proliferations of lymphoid cells in the early stages of differentiation and accounts for about 80% of all cases of childhood leukemia. Side effects of available treatment are still main concern. Thymoquinone (TQ), a natural compound isolated from *Nigella sativa*, induces growth inhibition and apoptosis in several cancer cell lines. The aim of the present study was to investigate the effect of TQ alone and in combination with doxorubicine on the proliferation inhibition and apoptosis induction of TQ in a lymphoblastic leukemia cell line. Jurkat cell line was cultured in standard condition and with concentrations of TQ (0–30 μm) and doxorubicine for 24, 48 and 72 h. Cell viability was measured by MTS assay. Apoptosis induction by TQ was assessed by annexin V-FITC/PI and flow cytometry analysis. TQ and DOX decreased cell viability with a time and dose dependent manner. The IC50 values were 19.461 ± 1.141, 17.342 ± 1.949 and 14.123 ± 1.874 μM in 24, 48 and 72 h, respectively for TQ. IC50 values for DOX were. 075 ± .0124, .028 ± .007 and.007 ± .001 μM in 24, 48 and 72 h, respectively. The level of cell apoptosis in all used concentrations of TQ (4, 8, 12, 16 and 20 μm) was higher than control group (10.2, 14.1, 36.6, 87.5 and 93.3% respectively after 24 h; 10.7, 13.9, 64.6, 92.2 and 93.1 respectively after 48 h; 2.83, 5.83, 41.4, 71.6 and 86.6% respectively after 72 h) and reached to a significant level at 12, 16 and 20 μm concentration for 24 and 48 h and 16 and 20 μm for 72 h incubation. Combination of doxorubicine and TQ lead to a synergistic cytotoxicity as compared to any of them alone. The study indicated that TQ is effective on proliferation inhibition and is a strong apoptotic inducer in Jurkat lymphoblastic cell line and has synergistic effect in combination with DOX. This combination strategy can be an alternative way for more powerful anticancer effects. Therefore, the study of the mechanism of apoptosis induction of TQ can be a step forward to in target therapy which might be considered in the future studies.

Keywords Thymoquinone · Lymphoblastic leukemia · Apoptosis

Introduction

Leukemias are heterogeneous malignancies [1], comprise 8% of cancers. About 80% of children leukemia cases are acute lymphoblastic leukemia [2]. Acute lymphoblastic leukemia is a malignant expansion of lymphoid precursor cells within the bone marrow, blood and extra medullary location [3], influences both children and adolescence, with a peak between the ages of 2 and 5 years and again after age of 50 [4]. Patients with T cell acute lymphoblastic leukemia (T-ALL) experience a higher recurrence and early relapse [5]. Intensification the treatment to promote survival prompts the patients to more unfavorable effects [6]. This fact derived us to new therapeutic target for the disease [5]. Over the recent years, there has been

✉ Batoul Pourgheysari
bat238@yahoo.com

1 Department of Immunology, Shahrekord University of Medical Sciences, Shahrekord, Iran

2 Medical Plants Research Center, Shahrekord University of Medical Sciences, Shahrekord, Iran

3 Cellular and Molecular Research Center, Shahrekord University of Medical Sciences, Shahrekord, Iran

4 Department of Medical Laboratory Technology, Shahrekord University of Medical Sciences, Shahrekord, Iran

developing interest for naturally phytochemical components. Studies showed a regimen rich in phytochemical mixes find in plant and natural products (such as vegetables, blooms, entire grains, herbs, nuts, and seeds) are linked with cancer prevention and treatment. More than 25% of medications utilized during the last 20 years are straightforwardly gotten from plants, while the other 25% are artificially modified natural products [7]. Thymoquinone (2-isopropyl-5-methyl-1,4-benzoquinone), is a phytochemical compound derived from the plant *Nigella sativa* or black cumin, which is utilized widely by Middle and Far Eastern nations [8, 9]. Different studies have exhibited that thymoquinone (TQ) has several therapeutic effects including: anti-bacterial, anti-parasitic, anti-viral, anti-inflammatory, immunomodulatory and anti-tumor properties [8, 9] and acts as an intense cytotoxic drug over a variety of human cancer cells [10] such as human breast and ovarian adenocarcinoma [11] squamous carcinoma [12], fibrosarcoma [12], laryngeal neoplastic cells [13], prostate and pancreatic cancer (PC) cell lines [14–17]. It has been recommended that TQ, as a DNA damaging factor, is a possible reactive oxygen species (ROS) producer which applies its anti-cancer properties by repressing cell growth, migration, invasion, angiogenesis and induction of apoptosis [18, 19]. The proliferation inhibitory effects of TQ is specific to cancer cells while it is less noxious to and keeps non-tumor normal cells from chemotherapy-induced damage [10, 20].

Apoptosis, also named as endogenous programmed cell death, is a basic pathway for controlling homeostasis and morphogenesis and involves in pathogens of cancers [21]. Induction of apoptosis is the most important mechanism of action in anticancer agents and its defect not only leads to progression of tumors but also increases their resistance to treatment [22, 23]. Thus, promoting apoptosis in tumors is one of the best ways for anticancer treatments [24]. We have recently reported the anti-proliferative and apoptosis effects of Pterostilbene, a phytonutrients, on T-cell lymphoblastic leukemia cell (jurkat) [25], here we examined the anti-proliferative and apoptosis induction of TQ on this cell line.

Materials and Methods

Cell Culture and Treatment

The human T-cell leukemia line (Jurkat, E6.1) was cultured in standard condition (95% humidity, 5% CO_2, 37 °C) in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/mL penicillin, 0.1 mg/mL streptomycin (PS) and 0.3 mg/ml L-glutamine. A 200 mM solution of TQ (Sigma-Aldrich) and DOX (Sigma-Aldrich) was prepared in 100% DMSO (DiMethylSulfOxide, Sigma-Aldrich) and appropriate working concentrations were prepared with the cell culture medium; the final concentration of DMSO was less than 0.1% in both control and treated cells.

Cell Viability Assay

Jurkat cells were seeded in a concentration of 10×10^3 cells per well in 96-well plates for 24 h before treatment and exposed to TQ and DOX at different concentrations (0–30 μM), (0–0.2 μM), respectively, for different periods. Cell viability was then examined by colorimetric assay using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS), according to manufacturer's instruction (Promega, USA). Briefly 20 μL of MTS (5 mg/mL in phosphate buffered saline) was added to each well and incubated for 3 h, in a dark place. The absorbance was measured at 490–620 nm by an Elisa reader (stat fax-2100 awarenes).

Apoptosis Assay

Cells were seeded in 6-well plates at a density of 2×10^6 cells per well, grown for 24 h before treatment with TQ at different concentrations for 24, 48 and 72 h. The apoptosis was examined by flow cytometry (Partec system, Germany) using the Annexin V-FITC/propidium iodide (PI) apoptosis kit (BD Biosciences), according to manufacturer's instruction. Briefly cells were washed twice with cold PBS, then cells resuspended in 1 ml of 1 × binding buffer (provided with kit) at density of 10×10^5 100 μL of cell suspension incubated with 5 μl of Annexin V-FITC and PI for 15 min at dark at room temperature, then examined by flow cytometry. Flowjo 7.6 software was used for the analysis of the data. At least 10,000 events were recorded and represented as dot plots and histograms.

Cytotoxicity Assay for the Combination of Thymoquinone and Doxorubicine

Jurkat cells were seeded in 96 well plate at a density of 10×10^3 for 24 h before treatment. TQ and DOX were added at specific concentrations, and cells were incubated for 24, 48 and 72 h. Cell viability assay was performed as described above. Synergistic effect was determined by combination-index methods, derived from the median effect principle of Chou and Talalay [26] using the following formula: $CI = C_{A,50}/IC_{50,A} + C_{B,50}/IC_{50,B}$. $C_{A,50}$ and $C_{B,50}$: were the concentrations of drug A and drug B used in combination to achieve 50% drug effect; $IC_{50,A}$ and $IC_{50,B}$ were the concentrations of individual agents to achieve the 50% drug effect. The combination-index (CI)

method is a mathematical and quantitative representation of a two-drug pharmacologic interaction. A CI of 1 indicated an additive effect between the two agents, whereas a CI < 1 or CI > 1 indicated, synergism or antagonism effect, respectively.

Statistical Analysis

Data were analyzed by SPSS using Kruskal–Wallis test and are presented as the mean ± standard deviation from at least three independent experiments. IC50 value was calculated using probit analysis.

Results

Reduction of Cell Viability by TQ

To determine the effect of TQ on viability of Jurkat cells, cells were incubated for 24, 48 and 72 h in the absence or presence of TQ at different concentrations (0–30 μM). The ODs obtained from the absorption at 490 nm was converted to a percentage. Figure 1 shows the reduction of cell viability as concentration increased (dose-dependent). A significant reduction of Jurkat cell viability was also observed with a time-dependent manner at a specific concentration. IC50 (inhibition concentration) was 19.461 ± 1.141, 17.342 ± 1.949 and 14.123 ± 1.874 μM in 24, 48 and 72 h, respectively.

Reduction of Cell Viability by DOX

Jurkat cells, cells were incubated for 24, 48 and 72 h in the absence or presence of DOX at different concentrations (0–.2 μM). Figure 2 shows the reduction of cell viability as concentration increased (dose-dependent). The reduction of viability was also time dependent. IC50 values were

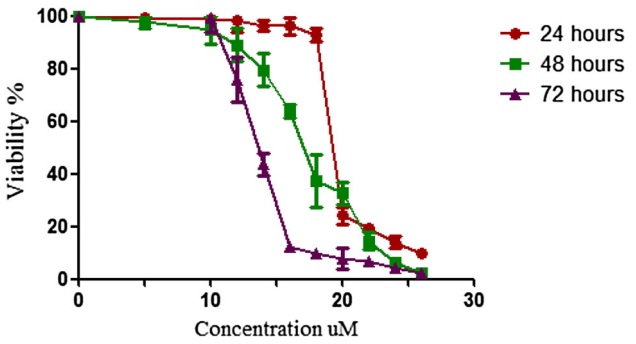

Fig. 1 Effect of TQ on Jurkat cell viability by MTS assay. Cells were incubated with different concentrations of TQ for 24, 48 and 72 h and cell viability was determined compared to untreated cells. Reduction of cell viability was time and concentration dependent

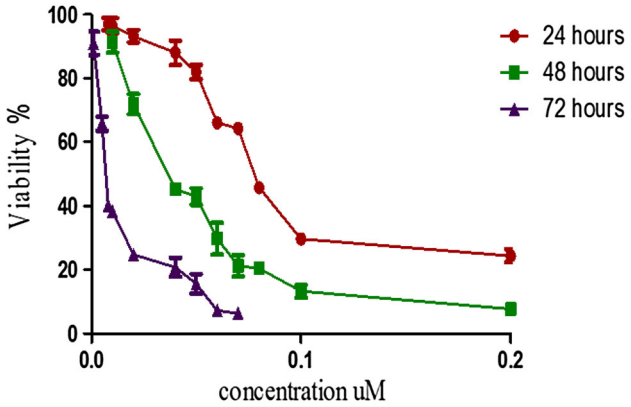

Fig. 2 Effect of DOX on Jurkat cell viability by MTS assay. Cells were cultured in the presence of different concentrations of DOX for 24, 48 and 72 h and viability was determined compared to untreated cells. Reduction of cell viability was time and dose dependent

0.075 ± .0124, .028 ± .007 and .007 ± .001 μM in 24, 48 and 72 h, respectively.

Induction of Apoptosis by TQ

To assess whether TQ could induce apoptosis in Jurkat cells, apoptosis induction was determined by annexin-V/PI apoptosis detection kit. Cells were incubated for 24, 48 and 72 h at various concentrations (4, 8, 12, 16, and 20 μM) of TQ. Cells that were positive just for annexin-V and those were positive both for annexin V and PI considered as early and late apoptotic cells, respectively. As shown in Fig. 3, amount of apoptotic cells are enhanced with increasing the concentration of TQ. After 48 h treatment with above concentrations, about 5.6, 2.15, 6.4, 6.45 and 3.3% of cells were early apoptotic cells and 4.81, 11.5, 57.9, 85.6 and 89.6% of cells were late apoptotic cells, Fig. 4, shows the continuous process of apoptosis. The other two time groups (24 and 72 h) showed this continuous process (data not illustrated). These data showed a significant increase in the number of apoptotic cells post treatment ($P < 0.05$ considered significant) (Fig. 5).

As shown in Fig. 5, TQ lead to increase amount of apoptotic cell and the difference are significant at 3 concentrations (12, 16 and 20 μM) for 24 and 48 h and 2 concentrations (16 and 20 μM) for 72 h, compared to control. Also in each group, different concentrations were compared to each other, and were significant at 4 and 16 ($P < 0.05$), 4 and 20 ($P < 0.01$), 8 and 20 μM ($P < 0.05$) for 24, 48 and 72 h, respectively.

Synergistic Effect of TQ and DOX

Cell viability was evaluated in the presence of different combinations of TQ and DOX. Stronger cytotoxicity was

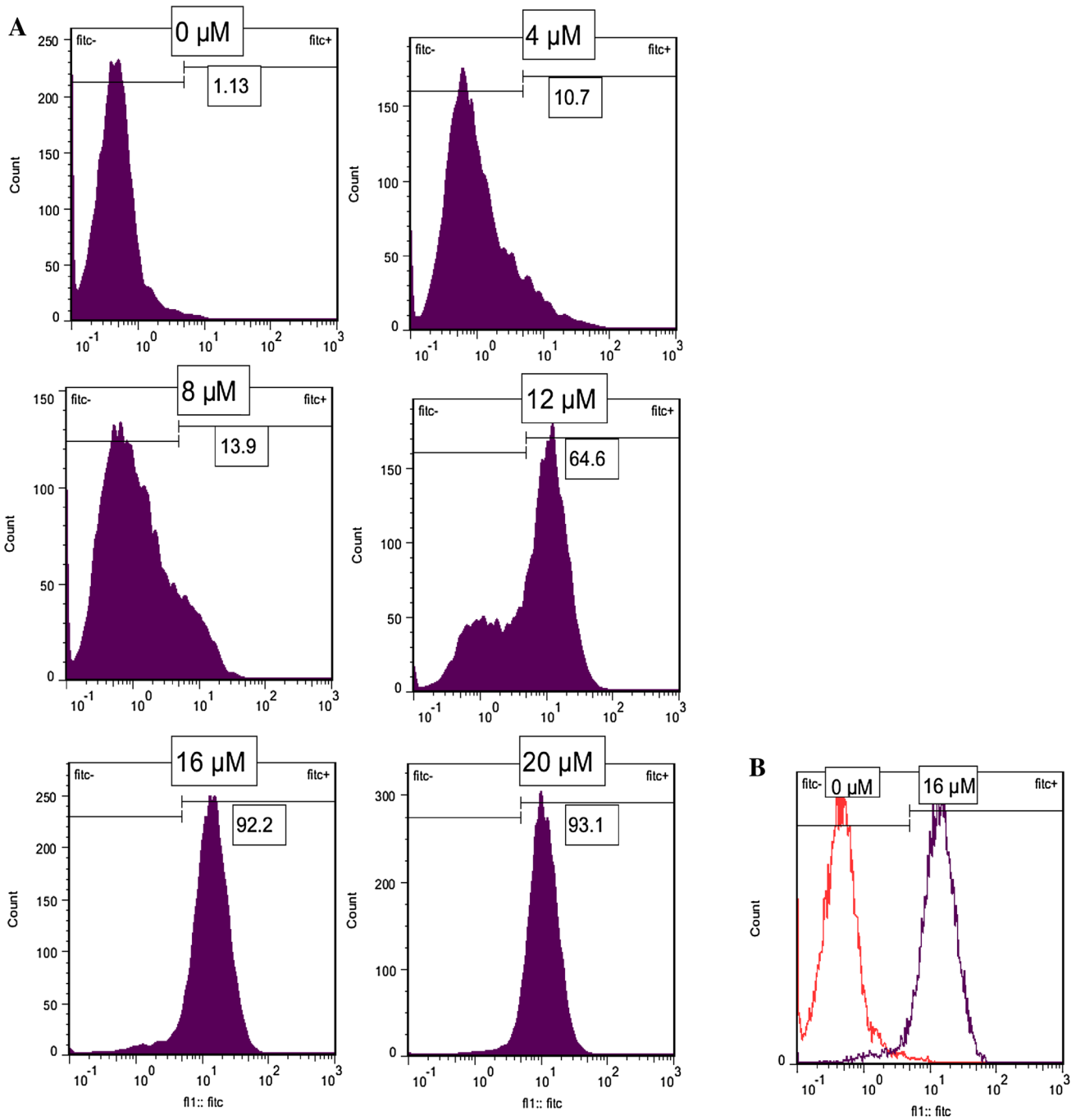

Fig. 3 Concentration-dependent apoptosis induction by TQ in Jurkat cells. Cells were exposed to TQ at the indicated concentrations and incubated for 48 h. Apoptosis induction was assessed by flow cytometry after annexin V-FITC/PI stating. **a** Each histogram the levels of FITC fluorescence in each histogram. **b** Fluorescence curves obtained for untreated and TQ-treated (16 μM) cells in one chart. The *data* are representative of three independent experiments

observed at all used combinations compared to each drug alone (Fig. 6). The results showed synergistic toxicity at 24 (data not illustrated) and 48 h. While 50% viability was observed at 0.28 μM concentration of DOX and 16 μM of TQ, a combination of 0.01 μM of DOX and 9 μM of TQ caused 50% decrease in viability (Fig. 6b). CI (combination value) were represented in Table 1.

Discussion

Induction of apoptosis in cancer cells is an imperative system of activity of some chemo-preventive agents [27]. Our study investigates the anti-proliferative and apoptosis induction of TQ on a lymphoblastic Leukemia cell line. TQ has already been shown to have very lower cytotoxicity on

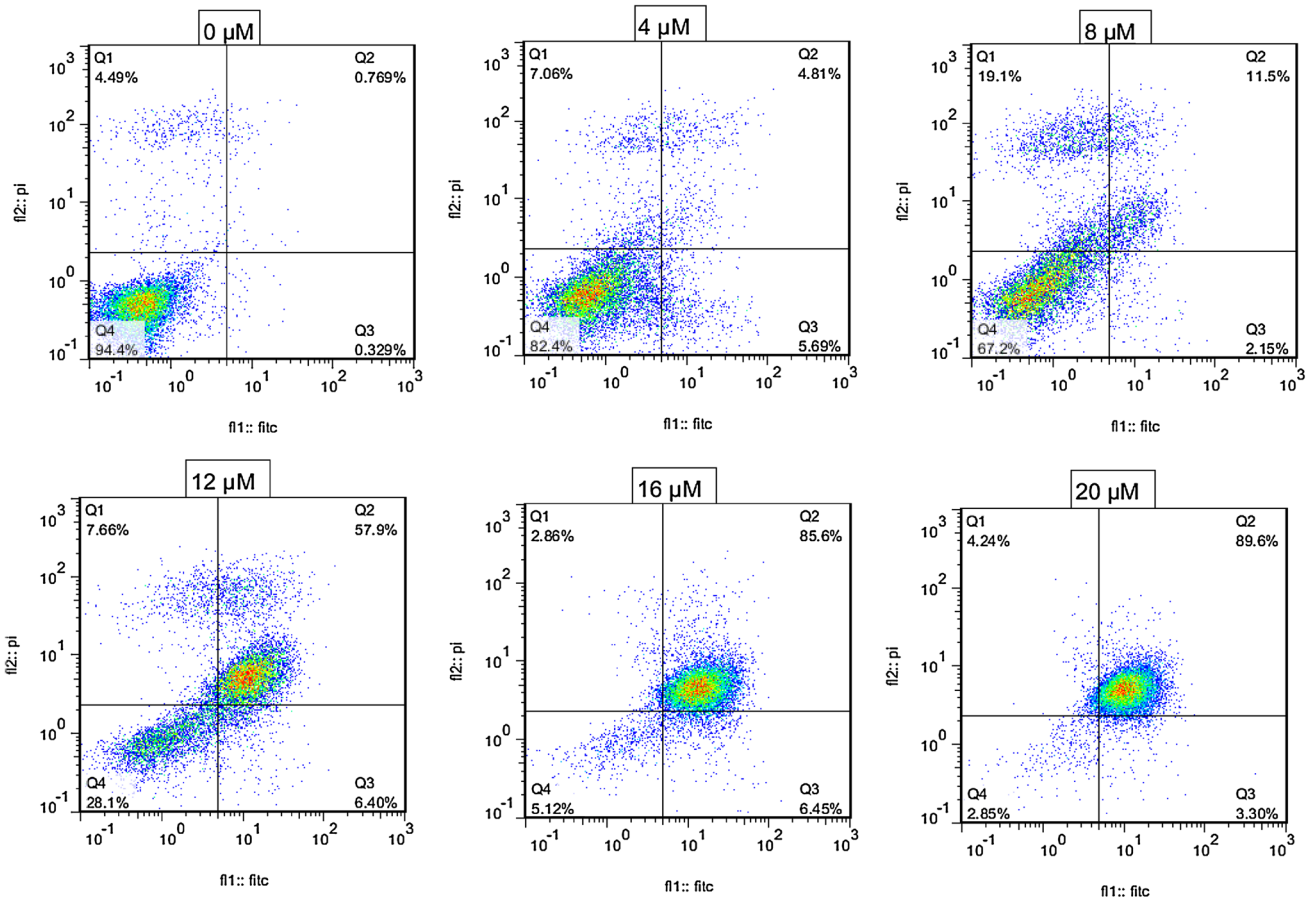

Fig. 4 Concentration-dependent effects of TQ on the number of apoptotic cells, in Jurkat cells. Cells were exposed to different concentration of TQ (4, 8, 12, 16 and 20 μM) for 48 h. *Dot plots* shows the results of a representative apoptosis assay. Cells of the *lower left quadrant* are viable (*Q4*); cells of the *lower right quadrant* are in early apoptosis (*Q3*); cells of the *upper left* are in necrosis (*Q1*); cells of the *upper right* are in late apoptosis (*Q2*). The number of cells in apoptosis, expressed as percentage relative to the total cell number, is indicated

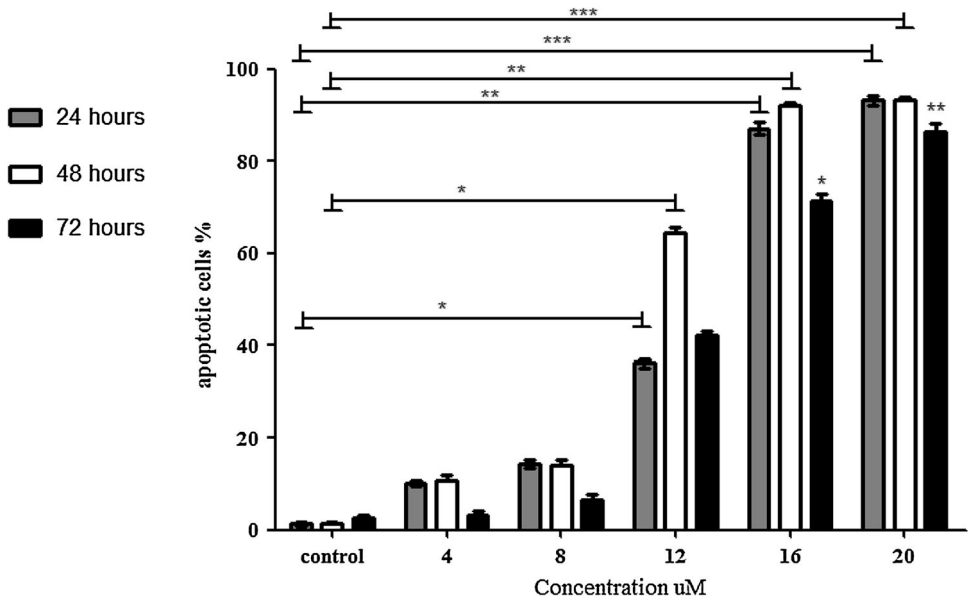

Fig. 5 Effect of TQ on apoptosis at various concentration after 24, 48 and 72 h. Treated cells with TQ at concentration of 12, 16 and 20 μM for 24 and 48 h and at 16 and 20 μM for 72 h incubation, showed significant increase in the amount of apoptotic cells compared to control group (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

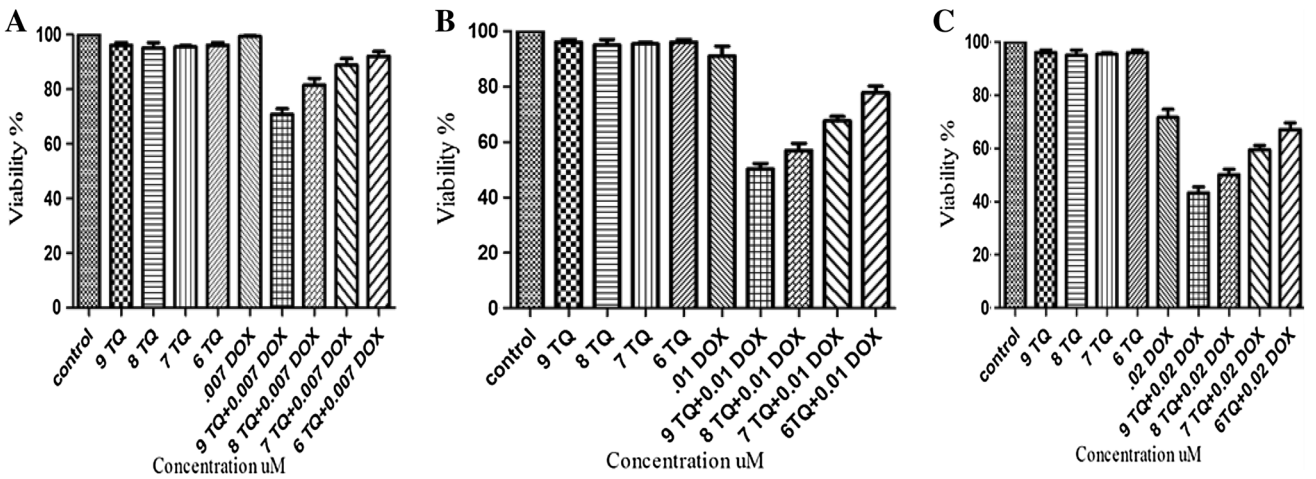

Fig. 6 Jurkat cells were treated with different concentrations of TQ and DOX. A combination of DOX and TQ at 48 h, showed significant reduction in cell viability compared to each drug alone

Table 1 Combination index values of DOX in combination with TQ on the viability of jurkat cells

Concentration of drugs	Time (h)	CI value*
10 μM TQ + 0.03 μM DOX	24	0.9263
9 μM TQ + 0.01 μM DOX	48	0.9196

* CI = 1: additive effect, CI < 1: synergism effect, CI > 1: antagonism effect

normal PBMC than cancer cells [28, 29]. The anticancer effects of TQ have been reported to be mediated through various mechanisms such as anti-proliferation, apoptosis induction, cell cycle arrest, ROS generation and anti-metastasis/anti-angiogenesis [8]. Anti-proliferative effect of TQ has been shown in various cell lines and tumors [30–33] including drug resistant cell lines [30, 34]. Abdelfadil et al. [35] showed that TQ treatment of T28 oral cancer decrease the viability in dose-dependent manner which is consistent with our findings on Jurkat cells as we had decrease of cell viability with increase of concentration. On the other hand TQ had significant cytotoxic consequences for N28 non-tumor cells only at high concentration (100 μM), while it was cytotoxic at dose of 50 μM for T28 cells [35]. Chern Chiuh Woo reported that TQ can suppress the growth of various breast cancer cell lines including MCF-7, MDA-MB-231 and BT-474. This study showed the dose and time dependent anti-proliferative effect of TQ, and IC50 values for above cell lines were 32, 11 and 21 μM for 48 h, respectively [34]. To show the anti-proliferative potency of TQ, some studies compared this effect with chemotherapy drugs, as IC50 values against Siha cells were 14.67 and 18.73 μM for TQ and cisplatin, respectively, as Ng et al. reported [36]. Norfazlina et al. [37] found that TQ can inhibit cell proliferation and induces apoptosis in HL-60 cells (human myeloid leukemia), and the inhibition concentration was about 19 μM, that is consistence with our findings. Our findings showed that TQ can suppress cell proliferation with a time-dependent manner as the IC50 was 19.461, 17.342 and 14.123 for 24, 48 and 72 h respectively. While TQ has potent cytotoxic effects on cancer cells, it had a little effect on normal cells including mouse fibroblasts (L929) [12], prostate epithelial cells (BPH-1) [33], human normal intestinal cells (FHs74Int) [38] and human normal lung fibroblast cells (IMR90) [39], which is favorable for anti-cancer therapy.

Doxorubicine like other DNA synthesis inhibitor agents has very side effects that heart toxicity is the most important of them. This drug is used in treatment of wide range of cancers such as leukemia, lymphoma and solid tumors and often used in combination with other drugs for treatment of cancers in chemotherapy regimen [40]. In this study the anti-proliferative effect of DOX on a lymphoblastic Leukemia cell line was investigated. Different studies have reported the relationship between the concentration of the drug and cytotoxic effect, as a study on breast cancer cells has shown that increasing the concentration of the drug significantly increases the rate of cell death [41]. Several studies demonstrated the effect of drug concentration and treatment duration on various cancer cells including endothelial cells, myocytes, lymphoblastic leukemia cells [42, 43]. A study conducted by sakalar and et al. [44] on T cells showed that doxorubicin lead to a reduction in DNA replication by blocking the cells in G0/G1 and also leads to cancer cell death by activating the receptors Fas and caspase-dependent pathway. The results of our study was in consistent with the results of other studies.

TQ-induced apoptosis can be triggered by various ways and targeting different proteins. Previous studies

showed that induction of apoptosis by TQ can be caspase cascade dependent, as in myeloblastic leukemia HL-60 [45], or independent, as in prostatic cancer cell line [46]. Our findings showed the TQ as a powerful apoptosis inducer in jurkat cells. As shown in Fig. 3, following 24 and 48 h incubation, it derived almost all the cells to apoptosis at 16 and 20 μM concentrations. The present study demonstrated that apoptosis induced by TQ, followed a time and dose dependent manner which is consistent with our data for cell viability. The results of our study are in agreement with the other works, showed the apoptosis induction by TQ in some other cell lines such as neuroblastoma [47], human myeloblastic leukemia [31], human breast carcinoma [34, 48], human colon cancer [38], hepatic cancer [49], and osteosarcoma cell lines [50]. Hussain et al. [51] reported the apoptotic effect of TQ in several primary effusion lymphoma (PEL) in a dose-dependent manner which is again in agreement with our study showed.

In examining the effects of DOX in combination with TQ on viability of the cells, TQ effectively increases the cytotoxic effects of doxorubicin that was very significant at 24 and 48 h. A combination of two drugs at IC50, significantly induce cytotoxic effects in cells, compare with each drug alone at the same concentration. A study by Naus et al. has shown that the combination of PolyFenol has a synergistic effect with chemotherapy (gemcitabine, 5-fluorouracil and mitomycin). Such combinations can be used to improve the efficacy of chemotherapy agents in the treatment of cancer and to enhance the cytotoxic effect given dose and to minimize side effects of the chemotherapy drugs used as single compounds or can be used as a dietary supplement with chemotherapy [52]. Another study demonstrated the effect of TQ along with gemcitabine and oxaliplatine on the pancreas cells. The results showed 2 and 3 times more cytotoxic effect in combination of drugs with TQ than any of them alone [42].

The effect of the combination of TQ and DOX on T cell lymphoblastic leukemia has not been investigated and our data demonstrated the synergistic effect of the combination.

Conclusion

The study demonstrated that TQ can induce apoptosis in this lymphoblastic leukemia cell line which is a favorable effect of anti-cancer therapy It was also shown that TQ enhance the effects of doxorubicin on Jurkat cells. This compound can be considered in combination with common chemotherapy agents in vitro in other cell lines and ALL lymphoblasts and experimental invivo models.

Acknowledgements This research was supported by Medical Plant Research Center of Shahrekord University of medical sciences. We wish to thank Deputy of Research and Technology, Shahrekord University of medical science for financial supports.

Compliance with Ethical Standards

Conflict of interest The authors declared no competing interests.

Ethical Approval This article does not contain any studies with human participants performed by any of the authors.

References

1. Peregud-Pogorzelski J (2014) How have advances in our understanding of the molecular genetics of paediatric leukaemia led to improved targeted therapies for these diseases? Clin Exp Med 23(3):469–474
2. Pyatt D, Hays S (2010) A review of the potential association between childhood leukemia and benzene. Chem Biol Interact 184(1):151–164
3. Narayanan S, Shami PJ (2012) Treatment of acute lymphoblastic leukemia in adults. Crit Rev Oncol Hematol 81(1):94–102
4. Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C et al (2006) Cancer statistics, 2006. CA Cancer J 56(2):106–130
5. Roti G, Stegmaier K (2014) New approaches to target T-ALL. Front Oncol 4:201–211
6. Ebinger M, Witte K-E, Ahlers J, Schäfer I, André M, Kerst G et al (2010) High frequency of immature cells at diagnosis predicts high minimal residual disease level in childhood acute lymphoblastic leukemia. Leuk Res 34(9):1139–1142
7. Vuorela P, Leinonen M, Saikku P, Tammela P, Rauha J-P, Wennberg T et al (2004) Natural products in the process of finding new drug candidates. Curr Med Chem 11(11):1375–1389
8. Woo CC, Kumar AP, Sethi G, Tan KHB (2012) Thymoquinone: potential cure for inflammatory disorders and cancer. Biochem Pharmacol 83(4):443–451
9. Abel-Salam BK (2012) Immunomodulatory effects of black seeds and garlic on alloxan-induced diabetes in albino rat. Allergol Immunopathol 40(6):336–340
10. Gali-Muhtasib H, Roessner A, Schneider-Stock R (2006) Thymoquinone: a promising anti-cancer drug from natural sources. Int J Biochem Cell Biol 38(8):1249–1253
11. Shoieb AM, Elgayyar M, Dudrick PS, Bell JL, Tithof PK (2003) In vitro inhibition of growth and induction of apoptosis in cancer cell lines by thymoquinone. Int J Oncol 22(1):107–113
12. Ivankovic S, Stojkovic R, Jukic M, Milos M, Milos M, Jurin M (2006) The antitumor activity of thymoquinone and thymohydroquinone in vitro and in vivo. Exp Oncol 28(3):220–224
13. Womack K, Anderson M, Tucci M, Hamadain E, Benghuzzi H (2005) Evaluation of bioflavonoids as potential chemotherapeutic agents. Biomed Sci Instrum 42:464–469
14. Banerjee S, Kaseb AO, Wang Z, Kong D, Mohammad M, Padhye S et al (2009) Antitumor activity of gemcitabine and oxaliplatin is augmented by thymoquinone in pancreatic cancer. Cancer Res 69(13):5575–5583
15. Kaseb AO, Chinnakannu K, Chen D, Sivanandam A, Tejwani S, Menon M et al (2007) Androgen receptor-and E2F-1-targeted thymoquinone therapy for hormone-refractory prostate cancer. Cancer Res 67(16):7782–7788
16. Yi T, Cho S-G, Yi Z, Pang X, Rodriguez M, Wang Y et al (2008) Thymoquinone inhibits tumor angiogenesis and tumor growth through suppressing AKT and extracellular signal-regulated kinase signaling pathways. Mol Cancer Ther 7(7):1789–1796

17. Sethi G, Ahn KS, Aggarwal BB (2008) Targeting nuclear factor-κB activation pathway by thymoquinone: role in suppression of antiapoptotic gene products and enhancement of apoptosis. Mol Cancer Biol 6(6):1059–1070
18. Worthen DR, Ghosheh OA, Crooks P (1997) The in vitro anti-tumor activity of some crude and purified components of black-seed, *Nigella sativa* L. Anticancer Res 18(3A):1527–1532
19. Gali-Muhtasib H, Kuester D, Mawrin C, Bajbouj K, Diestel A, Ocker M et al (2008) Thymoquinone triggers inactivation of the stress response pathway sensor CHEK1 and contributes to apoptosis in colorectal cancer cells. Cancer Res 68(14):5609–5618
20. Banerjee S, Padhye S, Azmi A, Wang Z, Philip PA, Kucuk O et al (2010) Review on molecular and therapeutic potential of thymoquinone in cancer. Nutr Cancer 62(7):938–946
21. Goldsworthy TL, Conolly RB, Fransson-Steen R (1996) Apoptosis and cancer risk assessment. Mutat Res Rev Genet 365(1):71–90
22. Ciocca DR, Calderwood SK (2005) Heat shock proteins in cancer: diagnostic, prognostic, predictive, and treatment implications. Cell Stress Chaperones 10(2):86–103
23. Khalil AA, Kabapy NF, Deraz SF, Smith C (2011) Heat shock proteins in oncology: diagnostic biomarkers or therapeutic targets? Biochim Biophys Acta 1816(2):89–104
24. Lee W-J, Hsiao M, Chang J-L, Yang S-F, Tseng T-H, Cheng C-W et al (2015) Quercetin induces mitochondrial-derived apoptosis via reactive oxygen species-mediated ERK activation in HL-60 leukemia cells and xenograft. Arch Toxicol 89(7):1103–1117
25. Rahimnejad T, Beshkar P, Shirzad H, Rafieian- Kopaei M, Safdari V, Asgarian N et al (2014) Effect of pterostilbene in cellular proliferation inhibition and induction of apoptosis in lymphoblastic leukemia cell line. J Babol Univ Med Sci 16(12):32–38
26. Chou T-C, Talalay P (1984) Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 22:27–55
27. Bauer J, Wekerle H, Lassmann H (1995) Apoptosis in brain-specific autoimmune disease. Curr Opin Immunol 7(6):839–843
28. Dergarabetian E, Ghattass K, El-Sitt S, Al-Mismar R, El-Baba C, Itani W et al (2012) Thymoquinone induces apoptosis in malignant T-cells via generation of ROS. Front Biosci 5:706–719
29. Zihlif MA, Mahmoud IS, Ghanim MT, Zreikat MS, Alrabadi N, Imraish A et al (2013) Thymoquinone efficiently inhibits the survival of EBV-infected B cells and alters EBV gene expression. Integr Cancer Ther 12(3):257–263
30. Effenberger-Neidnicht K, Schobert R (2011) Combinatorial effects of thymoquinone on the anti-cancer activity of doxorubicin. Cancer Chemother Pharmacol 67(4):867–874
31. El-Mahdy MA, Zhu Q, Wang QE, Wani G, Wani AA (2005) Thymoquinone induces apoptosis through activation of caspase-8 and mitochondrial events in p53-null myeloblastic leukemia HL-60 cells. Int J Cancer 117(3):409–417
32. Richards L, Jones P, Hughes J, Benghuzzi H, Tucci M (2005) The physiological effect of conventional treatment with epigallocatechin-3-gallate, thymoquinone, and tannic acid on the LNCaP cell line. Biomed Sci Instrum 42:357–362
33. Li Q, Yu D, Liu G, Ke N, McKelvy J, Wong-Staal F (2008) Selective anticancer strategies via intervention of the death pathways relevant to cell transformation. Cell Death Differ 15(8):1197–1210
34. Woo CC, Loo SY, Gee V, Yap CW, Sethi G, Kumar AP et al (2011) Anticancer activity of thymoquinone in breast cancer cells: possible involvement of PPAR-γ pathway. Biochem Pharmacol 82(5):464–475
35. Abdelfadil E, Cheng Y-H, Bau D-T, Ting W-J, Chen L-M, Hsu H-H et al (2013) Thymoquinone induces apoptosis in oral cancer cells through p38β inhibition. Am J Chin Med 41(03):683–696
36. Ng WK, Yazan LS, Ismail M (2011) Thymoquinone from *Nigella sativa* was more potent than cisplatin in eliminating of SiHa cells via apoptosis with down-regulation of Bcl-2 protein. Toxicol In Vitro 25(7):1392–1398
37. Norfazlina MN, Zuraina MYF, Rajab NF, Nazip SM, Rumiza AR, Zaila CFS, Mun LL et al (eds) (2014) Cytotoxicity study of *Nigella sativa* and *Zingiber zerumbet* extracts, thymoquinone and zerumbone isolated on human myeloid leukemia (HL60) cell. In: The Open Conference Proceedings Journal 4(2):99–107
38. El-Najjar N, Chatila M, Moukadem H, Vuorela H, Ocker M, Gandesiri M et al (2010) Reactive oxygen species mediate thymoquinone-induced apoptosis and activate ERK and JNK signaling. Apoptosis 15(2):183–195
39. Gurung RL, Lim SN, Khaw AK, Soon JFF, Shenoy K, Ali SM et al (2010) Thymoquinone induces telomere shortening, DNA damage and apoptosis in human glioblastoma cells. PLoS ONE 5(8):121–124
40. Keizer H, Pinedo H, Schuurhuis G, Joenje H (1990) Doxorubicin (adriamycin): a critical review of free radical-dependent mechanisms of cytotoxicity. Pharmacol Ther 47(2):219–231
41. Czeczuga-Semeniuk E, Wolczynski S, Dabrowska M, Dzieciol J, Anchim T (2004) The effect of doxorubicin and retinoids on proliferation, necrosis and apoptosis in MCF-7 breast cancer cells. Folia Histochem Cytobiol 42(4):221–228
42. Dirican A, Atmaca H, Bozkurt E, Erten C, Karaca B, Uslu R (2015) Novel combination of docetaxel and thymoquinone induces synergistic cytotoxicity and apoptosis in DU-145 human prostate cancer cells by modulating PI3K-AKT pathway. Clin Transl Oncol 17(2):145–151
43. Suwei W, Kotamraju S, Konorev E, Kalivendi S, Joseph J, Kalyanaraman B (2002) Activation of nuclear factor-κB during doxorubicin-induced apoptosis in endothelial cells and myocytes is pro-apoptotic: the role of hydrogen peroxide. Biochem J 367(3):729–740
44. Sakalar C, Yuruk M, Kaya T, Aytekin M, Kuk S, Canatan H (2013) Pronounced transcriptional regulation of apoptotic and TNF-NF-kappa-B signaling genes during the course of thymoquinone mediated apoptosis in HeLa cells. Mol Cell Biochem 383(1–2):243–251
45. El-Mahdy MA, Zhu Q, Wang QE, Wani G, Wani AA (2005) Thymoquinone induces apoptosis through activation of caspase-8 and mitochondrial events in p53-null myeloblastic leukemia HL-60 cells. Int J Cancer 117(3):409–417
46. Koka PS, Mondal D, Schultz M, Abdel-Mageed AB, Agrawal KC (2010) Studies on molecular mechanisms of growth inhibitory effects of thymoquinone against prostate cancer cells: role of reactive oxygen species. Exp Biol Med 235(6):751–760
47. Paramasivam A, Sambantham S, Shabnam J, Raghunandhakumar S, Anandan B, Rajiv R et al (2012) Anti-cancer effects of thymoquinone in mouse neuroblastoma (Neuro-2a) cells through caspase-3 activation with down-regulation of XIAP. Toxicol Lett 213(2):151–159
48. Arafa E-SA, Zhu Q, Shah ZI, Wani G, Barakat BM, Racoma I et al (2011) Thymoquinone up-regulates PTEN expression and induces apoptosis in doxorubicin-resistant human breast cancer cells. Mutat Res Fundam Mol Mech Mutagen 706(1):28–35
49. Rooney S, Ryan M (2005) Modes of action of alpha-hederin and thymoquinone, active constituents of *Nigella sativa*, against HEp-2 cancer cells. Anticancer Res 25(6B):4255–4259
50. Roepke M, Diestel A, Bajbouj K, Walluscheck D, Schonfeld P, Roessner A et al (2007) Lack of p53 augments thymoquinone-

induced apoptosis and caspase activation in human osteosarcoma cells. Cancer Biol Ther 6(2):160–169
51. Hussain AR, Ahmed M, Ahmed S, Manogaran P, Platanias LC, Alvi SN et al (2011) Thymoquinone suppresses growth and induces apoptosis via generation of reactive oxygen species in primary effusion lymphoma. Free Radic Biol Med 50(8):978–987
52. Naus PJ, Henson R, Bleeker G, Wehbe H, Meng F, Patel T (2007) Tannic acid synergizes the cytotoxicity of chemotherapeutic drugs in human cholangiocarcinoma by modulating drug efflux pathways. J Hepatol 46(2):222–229