Toxicon 250 (2024) 108103

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Copper (II) increases anti-Proliferative activity of thymoquinone in colon cancer cells by increasing genotoxic, apoptotic, and reactive oxygen species generating effects

Vildan Betul Yenigun [a,b], Abdurrahim Kocyigit [a,*], Ebru Kanimdan [a,b], Ezgi Balkan [a], Ayse Zehra Gul [a]

[a] Bezmialem Vakıf University, Faculty of Medicine, Department of Medical Biochemistry, Istanbul, Turkey
[b] Bezmialem Vakif University, Vocational School of Health Services, Istanbul, Turkey

ARTICLE INFO

Handling editor: Ray Norton

Previous Presentation at Conferences: This manuscript was presented as a poster presentation at the American Society of Human Genetics Annual Meeting on October 15–19, 2019, in Houston, USA.

Keywords:
Colon cancer
Thymoquinone
Copper
ROS
Cell death

ABSTRACT

Thymoquinone is the main active compound derived from the essential oil of the *Nigella sativa* plant seed. While thymoquinone is an antioxidant, it has been reported in several studies that thymoquinone has dose-dependent pro-oxidant activity with the Fenton reaction in the presence of transition elements such as iron and copper. This study aimed to investigate cytotoxic, apoptotic, genotoxic, and reactive oxygen species (ROS) generating effects of thymoquinone treated with copper in colon cancer cells. HT-29 cells were treated with pro-oxidant-acting doses of thymoquinone alone and together with the non-toxic dose of Copper (II) Sulfate for 24 h. Cytotoxic, apoptotic, genotoxic, and ROS production activities were analyzed by MTT viability test, Acridine Orange/Ethidium Bromide (AO/EB) staining, alkaline single cell gel electrophoresis and H2DCF-DA assay, respectively. Viability results showed that thymoquinone and copper synergistically affect cancer cells, and DNA damage was increased with the synergic effect. The intracellular ROS was increased when thymoquinone and copper were applied together. Applying redox-active copper (II) with thymoquinone increases DNA damage, apoptosis, and cell death by increasing the amount of intracellular ROS through pro-oxidant activity. Treatments targeting copper-related pathways may open new therapeutic avenues for cancer treatment.

1. Introduction

Colon cancer is the third most common cancer type in both men and women and the second leading cause of cancer-related deaths worldwide (Siegel et al., 2023). While advanced colon cancer typically presents with symptoms, early-stage colon cancer and premalignant adenomatous polyps frequently remain asymptomatic, making their detection challenging (Cappell, 2008). Treatment plans for colon cancer may include a combination of chemotherapy, targeted therapy, immunotherapy, surgery, and radiation therapy. However, the efficacy of these treatments remains a challenge (Fabregas et al., 2022), so efforts to discover new anticancer compounds with high sensitivity to cancer cells are extending. Some medicinal plants contain a diverse range of molecules that target different aspects of cancer cell growth and survival against several types of cancers, including colorectal cancer (Benarba et al., 2018). These bioactive molecules belong to a wide array of phytochemical families, each with its unique properties, and they activate diverse signaling pathways, so more research is going on these molecules for anti-cancer activities.

Thymoquinone (TQ; 2-isopropyl-5-methylbenzo-1, 4-quinone) is a bioactive compound found in the seeds of *Nigella sativa*, a plant commonly known as black cumin or black seed. This plant has been used for centuries in traditional medicine, particularly in Middle Eastern and Southeast Asian cultures. TQ is considered one of the major active components responsible for the therapeutic properties associated with black seed oil, so it is the most widely studied bioactive compound of *N. sativa* seeds (Malik et al., 2021). TQ has been studied for its various biological and pharmacological activities and is known for its antioxidant, anti-inflammatory, antimicrobial, and anticancer properties (Gomathinayagam et al., 2020; Zari et al., 2020; Darakhshan et al., 2015), besides its cardioprotective, hepatoprotective, neuroprotective, and several other diverse activities (Farkhondeh et al., 2017; Noorbakhsh et al., 2018; Isaev et al., 2020). TQ, a p-quinone, exerts protective effects by stimulating the activity of cytoprotective enzymes,

* Corresponding author. Department of Medical Biochemistry, Faculty of Medicine, Bezmialem Vakif University, 34093, Fatih, Istanbul, Turkey.
E-mail addresses: abdurrahimkocyigit@yahoo.com, akocyigit@bezmialem.edu.tr (A. Kocyigit).

https://doi.org/10.1016/j.toxicon.2024.108103
Received 26 August 2024; Received in revised form 12 September 2024; Accepted 12 September 2024
Available online 13 September 2024

resulting in protection against cellular damage caused by oxidative stress. However, in addition to its antioxidant activity, studies demonstrated that thymoquinone acts as a pro-oxidant to induce oxidative damage and apoptosis in cancer cells (Chae et al., 2020; El-et al., 2010).

In the presence of transition metals like copper and iron, natural antioxidants derived from plants can induce oxidative damage to DNA (Perron et al., 2009). The pro-oxidant activity of polyphenol compounds is thought to arise from their ability to reduce Fe^{3+} or $Cu2^{+}$ ions. Iron and copper generate hydroxyl radicals (•OH) through a Fenton-like reaction with H_2O_2, and these generated •OH species can cause damage to DNA (Gunther et al., 1995). Therefore, experiments investigating the pro-oxidant properties of polyphenol compounds are commonly conducted in the presence or absence of these metals or their chelators (Puppo, 1992; Laughton et al., 1989). Additionally, these experiments often utilize oxidized metal ions like Fe^{3+} or Cu^{2+} to demonstrate the pro-oxidant effect of phenolic compounds (Hayakawa et al., 2004).

In accordance with this information, the present study aimed to study the pro-oxidant effects of TQ by investigating the genotoxic, apoptotic, cytotoxic, and reactive oxygen species (ROS) generating effects given together with copper in colon cancer cells.

2. Materials and methods

2.1. Cell culture

Colon cancer cell line HT-29 (ATCC® HTB-38) was used in this study. The cells were cultured in McCoy's 5A medium (Biochrom, Germany) supplemented with 10% fetal bovine serum (FBS) (Biochrom, Germany) and 1% penicillin/streptomycin (100 U/ml penicillin and 100 μg/ml streptomycin) (Biochrom, Germany). Cell cultures were maintained at 5% CO_2 and 37 °C, providing optimal conditions for cell growth.

2.2. MTT cell viability test

HT-29 cells were seeded at a density of 5×10^3 cells per well in 96-well culture plates in 100 μl complete medium. After overnight incubation, 10–60 μM TQ (Cat # 274666, Sigma-Aldrich, United States) doses were administered to the cells alone and together with 150 μM Copper (II) Sulfate ($CuSO_4$, Cat # 102792, Merck Millipore, Germany). After 24 h incubation, 10 μl of 5 mg/ml Thiazolyl Blue Tetrazolium Bromide (TBTB, Sigma-Aldrich, United States) solution was added to each well and incubated further for 4 h at 37 °C. The supernatant was aspirated, and the MTT formazan, formed by metabolically viable cells, was dissolved in 100 μL Dimethyl Sulfoxide (DMSO, Applichem, Germany) by mixing for 20 min on a shaker. The absorbance was then measured at 570 nm by using the microplate reader (Varioskan Flash Microplate Reader, Thermo Scientific, USA). The absorbance of control cells without treatment was considered 100% viable. Each treatment had at least four replicate wells, and the mean values were plotted.

2.3. Acridine Orange/Ethidium Bromide double staining

The apoptotic impact of Cu over TQ in colon cancer cells was investigated using the Acridine Orange/Ethidium Bromide (AO/EB) double staining technique. This technique involves a straightforward and cost-effective approach to differentiate apoptotic cells from viable ones. AO and EB are fluorescent dyes used to stain nucleic acids. AO stains all nucleated cells green, while ethidium bromide only enters cells with damaged membranes, staining them orange-red. To analyze apoptotic ratios followed by administration of TQ w/wo $CuSO_4$ for 24 h, the cells were removed from the culture plates, subjected to staining using the AO and EB dyes (Sigma Aldrich, USA), and then observed using a fluorescent microscope (Leica DM-1000 Germany). Potential ratios of apoptotic cells were determined by evaluating a minimum of 100 cells for each treatment sample.

2.4. Genotoxicity assay

The genotoxicity of TQ treated colon cancer cells w/or w/o $CuSO_4$ was assessed using the alkaline single-cell gel electrophoresis assay according to Singh et al. with slight modifications (Singh et al., 1988). Initially, HT-29 cells were seeded in 6-well cell culture plates, with each well containing around 2×10^5 cells. These plates were then incubated at 37 °C in an environment with 5% CO2 for 24 h. Subsequently, the cells were treated with TQ alone and together with $CuSO_4$, and the cells were then subjected to another 24-h incubation period. The cells were then detached from the wells using trypsin/EDTA, followed by a PBS wash. The cell density was adjusted to 2×10^5 cells/ml using cold PBS, and 10 μL cell suspension was transferred to an Eppendorf tube and mixed with 90 μL of 0.6% low melting point agarose (Sigma Aldrich, USA). The mixture was placed on 1% normal melting point agarose (Sigma Aldrich, USA) pre-coated slides and incubated at 4 °C for about 15 min. Then, slides were put in cold lysis buffer pH = 10 (1% Triton X-100, 2.5 M NaCl, 10 mM Tris, 0.1 M EDTA, Sigma-Aldrich, USA) for 1 h at 4 °C in the dark. After that, slides were incubated in alkaline solution (0.3 M NaOH, 1 mM EDTA, Sigma-Aldrich, USA) for 40 min in the dark at 4 °C to unwind the DNA. Then, electrophoresis was performed at 0.72 V/cm (26 V, 300 mA) for 25 min at 4 °C. Slides were then neutralized with 0.4 M tris buffer (pH = 7.5) for 5 min, washed with 1XPBS, stained with EB (2 μg/mL), and then examined utilizing a fluorescence microscope (Leica, Germany). The acquired data from the cells' DNA were analyzed using a software package called Comet IV, developed by Perceptive Instruments in Suffolk, UK.

2.5. Intracellular reactive oxygen species (iROS) measurement

The iROS levels were measured using a fluorometric technique employing a probe, 2′,7′-dichlorofluorescein diacetate (H2DCF-DA) obtained from Sigma, USA. HT-29 cells were seeded at a density of 5×10^3 cells per well in a black 96-well plate and incubated for 24 h. Then, the cells were treated with various concentrations (10–60 μM) of TQ w/or w/o $CuSO_4$ for another 24 h. After this treatment period, the cells were washed once with 1XPBS and subsequently exposed to 5 μM H2DCF-DA for 30 min at a temperature of 37 °C in a dark environment. Then, the fluorescence that is indicative of the iROS present within the cells was detected by using a fluorimeter (Varioskan Flash Multimode Reader, Thermo Scientific) at excitation and emission wavelengths 485 nm and 535 nm, respectively. The results were normalized to the viable cells.

2.6. Statistical analysis

The results were presented as mean ± standard deviation (mean ± SD). IC_{50} values were calculated by nonlinear regression analysis. To assess the statistical significance of all experimental data, variance analysis using One-way ANOVA was conducted. A p-value of less than 0.05 was considered statistically significant. The IBM SPSS package program for Windows (Version 20, Armonk, NY IBM Corp) was used for all statistical analyses.

3. Results

3.1. Cytotoxicity of TQ, Cu, and Cu + TQ on colon cancer cells

The HT-29 cells were treated with different concentrations of only TQ, Cu, and Cu addition to TQ for 24 h, and the impact on cell viability was assessed through the MTT cell viability test. Cu's non-toxic dose was chosen as 150 μM, the highest concentration where cell viability difference was insignificant (depicted in supplements). The data, as shown in Fig. 1, demonstrated a notable decline ($p < 0.05$) at 20 μM when the cells were treated only with TQ. However, when the cells were treated with Cu + TQ, a significant reduction in cell viability was observed even at 10 μM. Additionally, Cu treatment of cancer cells with TQ lowered the

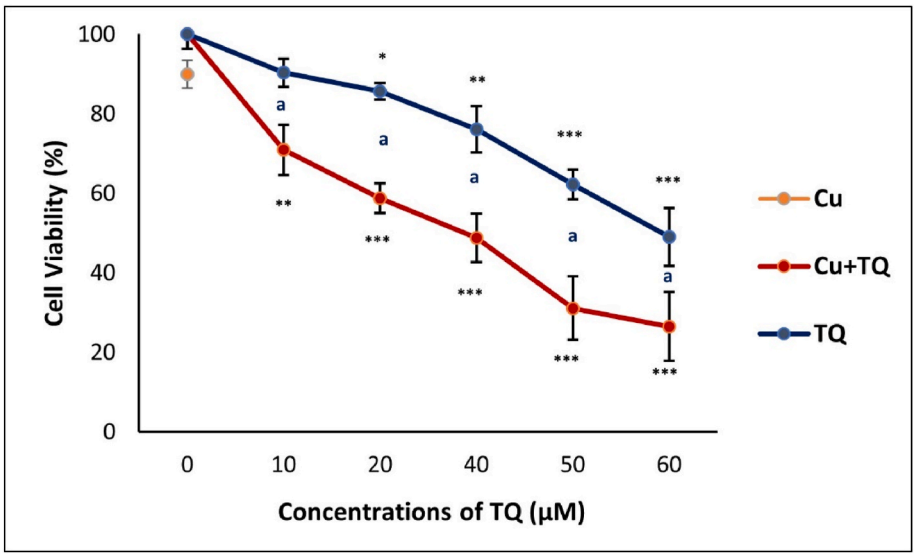
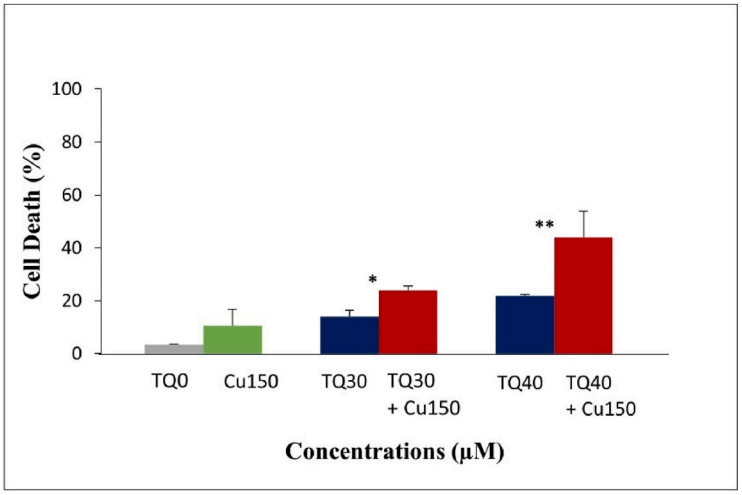


Fig. 1. Effect of Cu (150 μM) alone and addition to different doses of TQ treatment on HT-29 colon cancer cells. Cell viability was determined by the MTT cell viability test and calculated as a percentage relative to the non-treated group. * indicates significance values of TQ and TQ&Cu administration relative to control cells (* = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$). a shows significant difference between the cytotoxicity of cells treated with TQ alone and TQ&Cu co-administration.

IC_{50} value of TQ from 58.14 μM to 34.62 μM. On the other hand, alone Cu treatment (150 μM) did not cause a significant cell death in HT-29 cells ($p = 0.079$).

3.1.1. *The apoptotic effect of TQ with Cu treatment*

The current study evaluated the apoptotic effects of Cu addition to TQ treatment using the AO/EB double staining method. According to AO/EB double staining, the viable cell ratios decreased, and the apoptotic cell ratios increased with the increasing doses of TQ and the addition of Cu (Fig. 2).



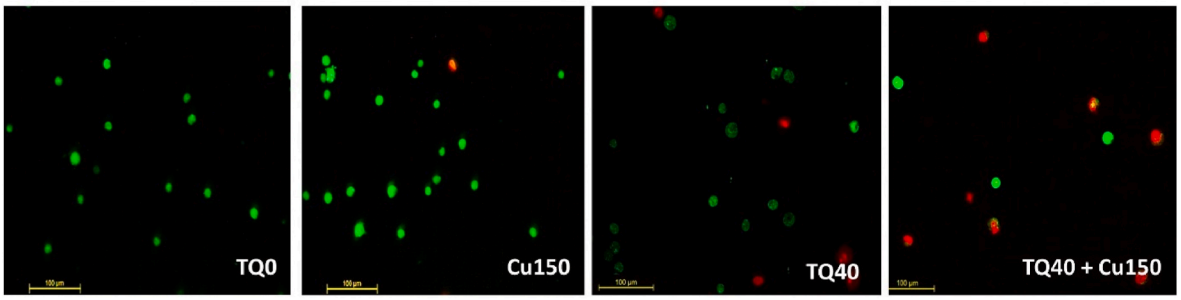


Fig. 2. Apoptotic activity of Cu addition to TQ treatment on HT-29 colon cancer cells. Cells were treated with 30 and 40 μM TQ alone or together with Cu for 24 h, then stained with AO/EB, and analyzed using fluorescence microscopy by evaluating a minimum of 100 cells for each treatment sample. Results are given as percentages. * and ** show statistical differences between only TQ treated and TQ/Cu treated cells. * means that $p < 0.05$ and ** means $p < 0.01$.

3.2. The genotoxic effect of TQ with Cu treatment

DNA damage of HT-29 cells after TQ treatment alone or with Cu was measured through the Comet Assay method. Damaged DNA of treated cells was visualized as a comet, and tail intensity was calculated using a software package called Comet IV. Our findings showed that the percentage of tail density was increased in cancer cells with the treatment of TQ and Cu together (Fig. 3).

3.3. iROS of cells treated with TQ and Cu

The iROS levels generated by TQ and TQ/Cu were analyzed through the fluorometric method using the H_2DCF-DA probe. The results showed that iROS levels significantly increased with the addition of Cu to TQ treatment (Fig. 4). Cu treatment alone (150 μM) did not increase iROS significantly.

4. Discussion

TQ is a bioactive compound found in *Nigella sativa* seeds (black cumin) and has been used in traditional medicine for centuries. As a major active component of black seed oil, TQ has garnered extensive research attention (Malik et al., 2021). TQ has been studied for its anticancer properties, including colon cancer, in addition to its various other beneficial activities (Attoub et al., 2013). Here in the current study, we also investigated the effects of TQ in combination with copper on cytotoxic, apoptotic, genotoxic, and iROS production in colon cancer cells.

Hsu et al. (2017) showed that 20 μM TQ significantly reduced human LoVo colon cancer cell proliferation and suppressed the migration by reducing the levels of p-PI3K, p-Akt, p-GSK3β, and β-catenin and thereby inhibiting the downstream COX-2 expression (Hsu et al., 2017). Another study demonstrated that TQ resulted in 50% cell death at 53.3 ± 1.7 μM in HT-29 colon cancer cells at 24hrs (Rooney et al., 2005). Our results also supported these findings that TQ significantly reduced viability after 20 μM dose and reduced the viability by 50% at 58.14 μM concentration.

TQ's antioxidant properties have been shown in many studies, as in Adinew et al., that TQ exhibits considerable antioxidant activity by upregulation of Nrf2 and downregulation of PD-L1 in Triple-Negative Breast Cancer Cells and decreases the generation of H_2O_2, at the same time increasing catalase (CAT) activity, superoxide dismutase (SOD) enzyme, and glutathione (GSH) (Adinew et al., 2022). However, TQ does not only exert protective effects by enhancing the activity of cytoprotective enzymes to guard against oxidative stress-induced cellular damage but also exhibits pro-oxidant properties (Mahmoud et al., 2019). Studies have highlighted TQ's ability to induce oxidative damage and apoptosis in cancer cells (El-et al., 2010), adding another dimension to its multifaceted therapeutic potential.

Phenolic compounds are believed to exhibit pro-oxidant activity by generating iROS at elevated phenolic concentrations, elevated pH levels, or in the presence of transition metal ions due to their capacity to reduce ions such as Cu^{2+} or Fe^{3+} (Rajashekar, 2023). It is thought that polyphenols' anticancer mechanism involves the mobilization of endogenous copper and the demonstration of pro-oxidant activity (Azmi et al., 2006). Several reports in the literature have also shown that both serum (Huang et al., 1999; Zuo et al., 2006) and tumor (Kuo et al., 2002; Yaman et al., 2007) copper levels in cancer patients are significantly elevated. For these reasons, studies examining the pro-oxidant characteristics of polyphenol compounds are frequently carried out in the presence or absence of transition metals like Fe^{3+} or Cu^{2+} or their chelators (Puppo, 1992; Hayakawa et al., 2004). Procyanidin B2 (epicatechin-(4β-8)-epicatechin) that is present in grape seeds, apples, and cacao beans and has strong antioxidant properties was shown to induce DNA damage in the presence of Cu(II) (Sakano et al., 2005). In another study, it was also shown that copper mediated oxidation of -(−)

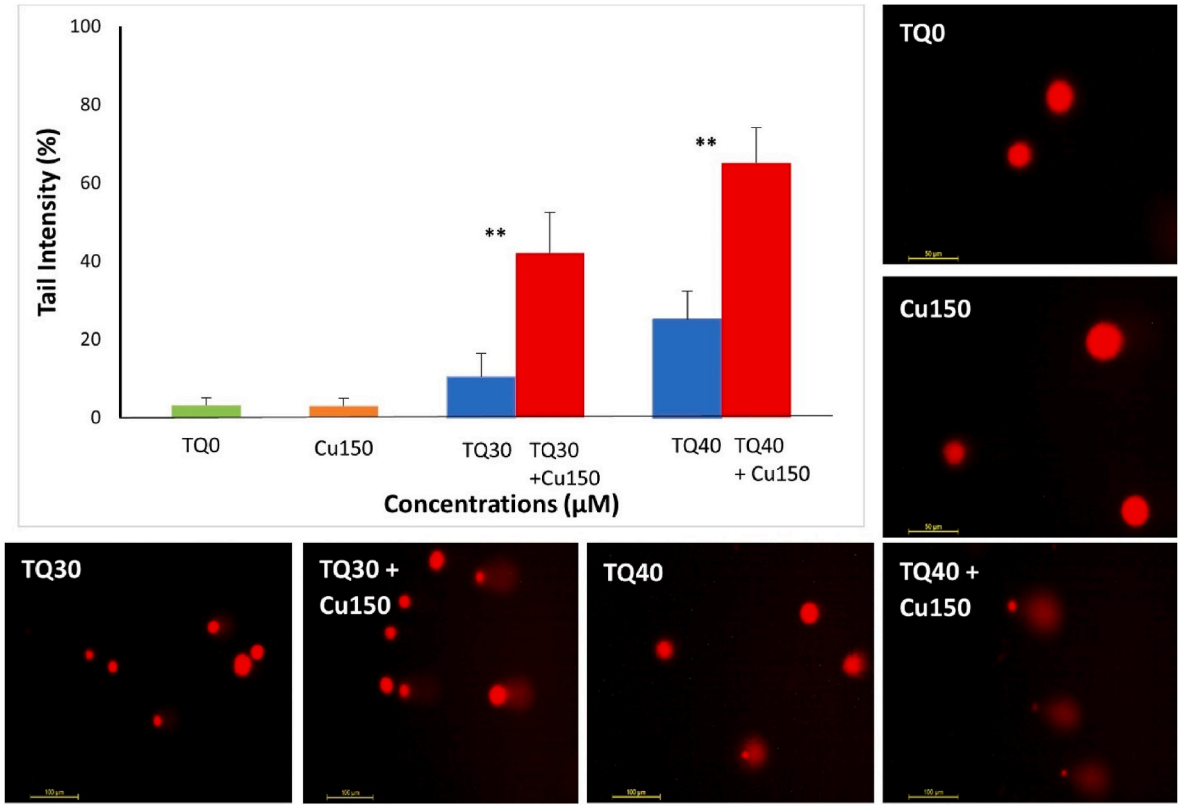


Fig. 3. DNA damage was found to be significantly higher in the group where TQ and Cu were given together compared to the group given only TQ. The data presented were mean ± SD. ** shows the statistical difference between only TQ treated and TQ/Cu treated cells and means $p < 0.01$.

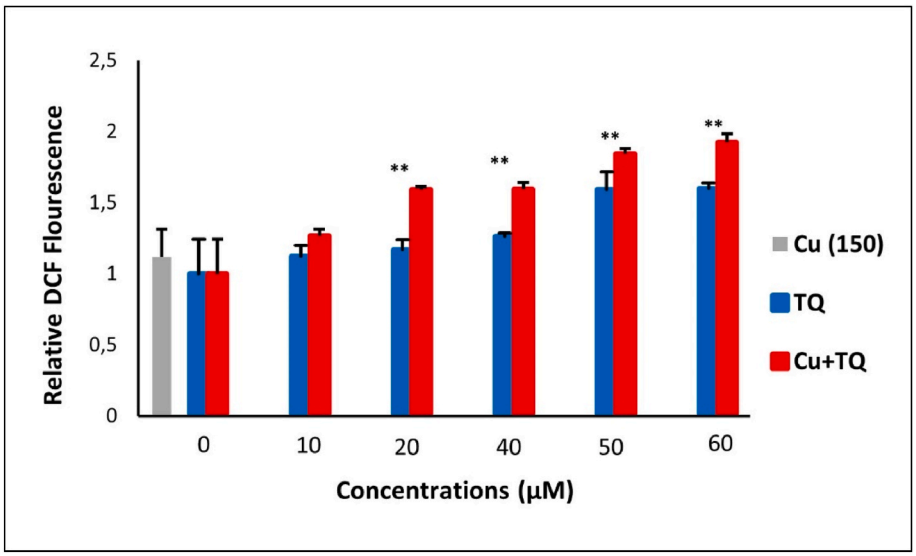


Fig. 4. iROS generating effects of Cu addition (150 µM) to TQ treatment (10–60 µM) in HT-29 cells. The data presented were mean ± SD. ** indicates statistical differences between only TQ treated and TQ/Cu treated cells ($p < 0.01$).

epigallocatechin-3-gallate (EGCG), a green tea polyphenol among the most effective antioxidants, possibly leads to the formation of polymerized polyphenols, and copper oxidized catechins were more efficient pro-oxidants as compared with their unoxidized forms. These results also support the hypothesis that the pro-oxidant action of plant polyphenols may be an important mechanism in their anticancer properties (Azam et al., 2004). Perron et al. tested 12 phenolic compounds for their effects on Cu+/H_2O_2 mediated DNA damage using gel electrophoresis, and based on their experiments, a copper redox-cycling mechanism was proposed for the pro-oxidant activity (Perron et al., 2011). Simunkova et al. aimed in their study (2021) to elucidate the antioxidant vs. pro-oxidant properties of Kaempferol, a flavonoid that occurs in tea and many vegetables and fruits, in the presence of copper (II) ion through the Fenton reaction, and they proposed that the pro-oxidant properties of Cu-kaempferol complexes may provide anticancer activity of these substances (Simunkova et al., 2021).

In light of this knowledge, we tested TQ's pro-oxidant activity in colon cancer cells in the presence of Cu^{2+}. Previous studies demonstrated that copper ions stimulate the proliferation of human umbilical artery and vein endothelial cells. The human umbilical vein endothelial cells were shown to twofold increase in cell number when incubated with 500 µM CuSO4 in a serum-free medium without exogenous growth factors for 48 h results (Hu, 1998). Wang et al. (2016) showed in their study that Copper levels between 30 and 120 µM could enhance the viability and promote proliferation of brain microvascular endothelial cells (Wang et al., 2016). Tchounwou et al. (2008) reported that 220.5 ± 23.8 µg/mL copper sulfate was needed to achieve a 50% reduction in cell viability in HepG2 cells upon 48 h of exposure (Tchounwou et al., 2008). In our study, we used a 150 µM $CuSO_4$ addition to TQ treatment, the highest concentration showing an insignificant reduction in cell death.

Our results demonstrated that the addition of Cu to TQ treatment resulted in increased cell death at the same concentrations of TQ, nearly halving the IC50 of TQ. In addition, it was also shown that the addition of Cu to TQ treatment increased the DNA damage of colon cancer cells analyzed by Comet assay. Zubair et al. (2013) also have shown that TQ can cause oxidative cellular DNA breakage, which can be inhibited by copper-chelating agents using human peripheral lymphocytes and comet assay (Zubair et al., 2013). Further, this group also applied 5 µM TQ with neocuproine (Cu-specific chelator) to prostate cancer cell lines PC3, LNCaP, DU145, and C42B and observed that chelation of copper inhibited the cytotoxic effect of TQ and concluded that TQ targeted cellular copper in prostate cancer cell lines leading to a pro-oxidant cell death (Zubair et al., 2013).

DNA damage can arise from various external and internal factors, such as exposure to chemicals, radiation, and free radicals. Both preclinical and clinical evidence demonstrate that ROS plays a significant role in modulating the genotoxic stress induced by chemotherapy agents and ionizing radiation (Srinivas et al., 2019). Also, it has been shown in many studies that flavonoids result in DNA damage and, eventually, apoptosis by inducing ROS production in cancer cells (Wang et al., 2019; Jomova et al., 2022; Chekuri et al., 2023). It is also stated in the literature that TQ causes DNA damage by inducing intracellular ROS production (Zubair et al., 2013). In our study, we demonstrated that Cu addition to TQ treatment significantly increased the iROS levels in HT-29 cells compared to TQ treatment alone, so we propose that DNA damage, cytotoxicity, and apoptosis caused by the treatment are caused by ROS production induction.

While the results of our study are promising, it is important to acknowledge that our experiments were conducted *in vitro* using a colon cancer cell line, which may not fully replicate the complexity of tumor microenvironments *in vivo*. The pro-oxidant mechanisms of redox-active copper (II) and TQ observed in this controlled setting may behave differently in living organisms due to factors such as immune responses, tissue heterogeneity, and copper homeostasis, which could influence the outcomes. Furthermore, we demonstrated enhanced DNA damage and apoptosis through iROS generation in cancer cells, but toxicity to non-cancerous cells was not fully explored. Further *in vitro* studies involving non-cancer cells, as well as *in vivo* investigations, are essential to thoroughly assess the safety, efficacy, and potential side effects of this combination therapy.

5. Conclusion

In conclusion, our research demonstrated that utilizing redox-active copper (II) in conjunction with TQ amplifies DNA damage and apoptosis by augmenting iROS levels through pro-oxidant mechanisms in colon cancer cells. Investigating therapeutic approaches that focus on the copper-related pathway could present innovative possibilities in cancer treatment.

Declaration of financial/other relationships

The Authors declare that there is no conflict of interest.

Institutional review board (IRB) approvals

Since this study only covers *in vitro* analysis, no IRB approval is required.

CRediT authorship contribution statement

Vildan Betul Yenigun: Writing – original draft, Investigation. **Abdurrahim Kocyigit:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Ebru Kanimdan:** Investigation. **Ezgi Balkan:** Investigation. **Ayse Zehra Gul:** Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.toxicon.2024.108103.

References

Adinew, G.M., et al., 2022. Anticancer effects of thymoquinone through the antioxidant activity, upregulation of Nrf2, and downregulation of PD-L1 in triple-negative breast cancer cells. Nutrients 14 (22).

Attoub, S., et al., 2013. Thymoquinone as an anticancer agent: evidence from inhibition of cancer cells viability and invasion in vitro and tumor growth in vivo. Fundam. Clin. Pharmacol. 27 (5), 557–569.

Azam, S., et al., 2004. Prooxidant property of green tea polyphenols epicatechin and epigallocatechin-3-gallate: implications for anticancer properties. Toxicol. Vitro 18 (5), 555–561.

Azmi, A.S., et al., 2006. Plant polyphenols mobilize endogenous copper in human peripheral lymphocytes leading to oxidative DNA breakage: a putative mechanism for anticancer properties. FEBS Lett. 580 (2), 533–538.

Benarba, B., Pandiella, A., 2018. Colorectal cancer and medicinal plants: principle findings from recent studies. Biomed. Pharmacother. 107, 408–423.

Cappell, M.S., 2008. Pathophysiology, clinical presentation, and management of colon cancer. Gastroenterol Clin North Am 37 (1), 1–24.

Chae, I.G., et al., 2020. Thymoquinone induces apoptosis of human renal carcinoma Caki-1 cells by inhibiting JAK2/STAT3 through pro-oxidant effect. Food Chem. Toxicol. 139, 111253.

Chekuri, S., et al., 2023. Isolation and anticancer activity of quercetin from Acalypha indica L. against breast cancer cell lines MCF-7 and MDA-MB-231. 3 Biotech 13 (8), 289.

Darakhshan, S., et al., 2015. Thymoquinone and its therapeutic potentials. Pharmacol. Res. 95–96, 138–158.

El-Najjar, N., et al., 2010. Reactive oxygen species mediate thymoquinone-induced apoptosis and activate ERK and JNK signaling. Apoptosis 15 (2), 183–195.

Fabregas, J.C., Ramnaraign, B., George, T.J., 2022. Clinical updates for colon cancer care in 2022. Clin. Colorectal Cancer 21 (3), 198–203.

Farkhondeh, T., Samarghandian, S., Borji, A., 2017. An overview on cardioprotective and anti-diabetic effects of thymoquinone. Asian Pac J Trop Med 10 (9), 849–854.

Gomathinayagam, R., et al., 2020. Chemopreventive and anticancer effects of thymoquinone: cellular and molecular targets. Journal of Cancer Prevention 25 (3), 136–151.

Gunther, M.R., et al., 1995. Hydroxyl radical formation from cuprous ion and hydrogen peroxide: a spin-trapping study. Arch. Biochem. Biophys. 316 (1), 515–522.

Hayakawa, F., et al., 2004. Prooxidative activities of tea catechins in the presence of Cu2+. Biosci. Biotechnol. Biochem. 68 (9), 1825–1830.

Hsu, H.H., et al., 2017. Thymoquinone suppresses migration of LoVo human colon cancer cells by reducing prostaglandin E2 induced COX-2 activation. World J. Gastroenterol. 23 (7), 1171–1179.

Hu, G.f., 1998. Copper stimulates proliferation of human endothelial cells under culture. J. Cell. Biochem. 69 (3), 326–335.

Huang, Y.-L., Sheu, J.-Y., Lin, T.-H., 1999. Association between oxidative stress and changes of trace elements in patients with breast cancer. Clin. Biochem. 32 (2), 131–136.

Isaev, N.K., et al., 2020. Thymoquinone as a potential neuroprotector in acute and chronic forms of cerebral pathology. Biochemistry (Mosc.) 85 (2), 167–176.

Jomova, K., et al., 2022. The effect of Luteolin on DNA damage mediated by a copper catalyzed Fenton reaction. J. Inorg. Biochem. 226, 111635.

Kuo, H.W., et al., 2002. Serum and tissue trace elements in patients with breast cancer in Taiwan. Biol. Trace Elem. Res. 89, 1–11.

Laughton, M.J., et al., 1989. Antioxidant and pro-oxidant actions of the plant phenolics quercetin, gossypol and myricetin. Effects on lipid peroxidation, hydroxyl radical generation and bleomycin-dependent damage to DNA. Biochem. Pharmacol. 38 (17), 2859–2865.

Mahmoud, Y.K., Abdelrazek, H.M.A., 2019. Cancer: thymoquinone antioxidant/pro-oxidant effect as potential anticancer remedy. Biomed. Pharmacother. 115, 108783.

Malik, S., et al., 2021. Thymoquinone: a small molecule from nature with high therapeutic potential. Drug Discov. Today 26 (11), 2716–2725.

Noorbakhsh, M.F., et al., 2018. An overview of hepatoprotective effects of thymoquinone. Recent Pat. Food, Nutr. Agric. 9 (1), 14–22.

Perron, N.R., Brumaghim, J.L., 2009. A review of the antioxidant mechanisms of polyphenol compounds related to iron binding. Cell Biochem. Biophys. 53 (2), 75–100.

Perron, N.R., et al., 2011. Antioxidant and prooxidant effects of polyphenol compounds on copper-mediated DNA damage. J. Inorg. Biochem. 105 (5), 745–753.

Puppo, A., 1992. Effect of flavonoids on hydroxyl radical formation by Fenton-type reactions; influence of the iron chelator. Phytochemistry 31 (1), 85–88.

Rajashekar, C.B., 2023. Dual role of plant phenolic compounds as antioxidants and prooxidants. Am. J. Plant Sci. 14 (1), 15–28.

Rooney, S., Ryan, M.F., 2005. Effects of alpha-hederin and thymoquinone, constituents of Nigella sativa, on human cancer cell lines. Anticancer Res. 25 (3b), 2199–2204.

Sakano, K., et al., 2005. Procyanidin B2 has anti- and pro-oxidant effects on metal-mediated DNA damage. Free Radic. Biol. Med. 39 (8), 1041–1049.

Siegel, R.L., et al., 2023. Colorectal cancer statistics, 2023. CA Cancer J Clin 73 (3), 233–254.

Simunkova, M., et al., 2021. Antioxidant vs. Prooxidant properties of the flavonoid, kaempferol, in the presence of Cu(II) ions: a ROS-scavenging activity, Fenton reaction and DNA damage study. Int. J. Mol. Sci. 22 (4).

Singh, N.P., et al., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175 (1), 184–191.

Srinivas, U.S., et al., 2019. ROS and the DNA damage response in cancer. Redox Biol. 25, 101084.

Tchounwou, P.B., et al., 2008. Copper-induced cytotoxicity and transcriptional activation of stress genes in human liver carcinoma (HepG(2)) cells. Met Ions Biol Med 10, 285–290.

Wang, J., et al., 2016. The effects of copper on brain microvascular endothelial cells and claudin via apoptosis and oxidative stress. Biol. Trace Elem. Res. 174, 132–141.

Wang, S., et al., 2019. Induction of ROS and DNA damage-dependent senescence by icaritin contributes to its antitumor activity in hepatocellular carcinoma cells. Pharm. Biol. 57 (1), 424–431.

Yaman, M., Kaya, G., Yekeler, H., 2007. Distribution of trace metal concentrations in paired cancerous and non-cancerous human stomach tissues. World J. Gastroenterol.: WJG 13 (4), 612.

Zari, A., Zari, T.A., 2020. Antioxidant, anti-inflammatory and anticancer activities of thymoquinone: a review. Word Appl. Sci. J 38, 250–263.

Zubair, H., et al., 2013. Redox cycling of endogenous copper by thymoquinone leads to ROS-mediated DNA breakage and consequent cell death: putative anticancer mechanism of antioxidants. Cell Death Dis. 4 (6), e660.

Zuo, X., et al., 2006. Levels of selenium, zinc, copper, and antioxidant enzyme activity in patients with leukemia. Biol. Trace Elem. Res. 114, 41–53.