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Original Research

Thymoquinone induces apoptosis and increase ROS in ovarian cancer cell line

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Abstract: *Nigella sativa* is also known for its properties as a traditional herbal healing for many ailments. In this study, the anticancer properties of thyomquinone (TQ), the active ingredient of *N. sativa*, were studied using ovarian cancer cell line (Caov-3 cells). The anti-proliferative activity of TQ was determined using MTT and the apoptosis was investigated using Flowcytometry and Annexin-V Assays. Multiparameteric cytotoxicity bioassays were used to quantify the changes in cell permeability and mitochondrial membrane potential. Reactive oxygen species (ROS) and apoptosis-involved cell markers were examined to verify cell death mechanism. The MTT-assay showed that TQ induces anti-proliferative activity on Caov-3 with an IC_{50} of 6.0±0.03 µg/mL, without any cytotoxic activity towards WRL-68 normal hepatocytes. A significant induction of early phase of apoptosis was shown by annexin-V analysis. Treatment of Caov-3 cells with TQ induces decreases in plasma membrane permeability and mitochondrial membrane potential. Visible decrease in the nuclear area was also observed. A significant decrease is observed in Bcl-2 while Bax is down-regulated. TQ-triggered ROS-mediated has found to be associated with Hsp70 dysregulation, an indicator of oxidative injury. We found that TQ induced anti-cancer effect involves intrinsic pathway of apoptosis and cellular oxidative stress. Our results considered collectively indicated that thyomquinone may be a potential agent for ovarian cancer drug development.

Key words: Thymoquinone, cell death, ovarian cancer, oxidative stress.

Introduction

In the ancient time of medicine, black cumin [*Nigella sativa* L. (Ranunculaceae)] is considered as an important remedy for a variety of ailments. The seeds have been used by traditional healers in the Middle East and Southeast Asian countries for a variety of diseases (1, 2). The attractiveness of this plant was highly improved by the ideologys' believes in the herb as a cure for multiple diseases. *N. sativa* (black seed) oil contains an abundance of thymoquinone, nigellone (dithymoquinone), conjugated linoleic acid, damascenine, melanthin, nigilline, and tannins (3, 4). Thymoquinone (Figure 1A), extracted from the seed oil of *N. sativa*, has been demonstrated to possess *in vivo* and *in vitro* anti-neoplastic effects (5, 6).This natural compound has been shown to protect through antioxidant mechanism, exert anti-inflammatory effects, and trigger apoptosis of the tumor cells (7).

Ovarian cancer is the second most widespread gynecologic female cancer and the deadliest in terms of absolute number (8). Symptoms of this malignant disease include pelvic pain, bloating, lack of appetite and frequent urination (9, 10). It has many complications such as spread of the cancer to other organs, progressive function loss of various organs, accumulation of abdominal fluids and intestinal obstructions (11). Ovarian cancer usually has a comparatively poor prognosis. The five-year survival rate for all stages of ovarian cancer is 47% (12). Treatment regularly involves chemotherapy and surgery, and sometimes radiotherapy (13). An alternative treatment with larger safety margins are needed to avoid the side effects of chemotherapy. Medicinal plants with rich ethno-knowledge are considered on the biggest source of natural anticancer agents (14). In this study we have chosen *Nigella sativa* as source of natural anticancer agent(s). The reputation of black seed was highly promoted by religious believes in the herb as a cure for multiple diseases. Reports from the Prophet Muhammad (PBUH) of Islam emphasize the significance of black cumin (15, 16). There are few studies focused specifically and in general regarding the anti-cancer mechanism of TQ in ovarian cancer in vitro (17-20). Therefore, the current study was designed to investigate the anticancer mechanisms of thymoquinone against ovarian cancer in vitro focused on ROS and apoptosis mechanism.

Materials and Methods

Chemicals and Reagents

Thymoquinone (>99% purity) was purchased from Sigma (St. Louis, MO, USA). Caov-3 ovarian cancer cell line (ATCC®HTB-75™;Organism:Homo sapiens/ Tissue:ovary/Disease:adenocarcinoma) and WRL-68 (normal hepatocytes) used in this study were obtained from ATTC, USA (ATCC®HTB-75™;Organism: Homo sapiens /Tissue:ovary/ Disease: adenocarcinoma). RPMI

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1460, fetal bovine serum (FBS) and streptomycin-penicillin were procured from Bioscience Ltd. (Wokingham, UK). Phosphate buffered saline (PBS), ethanol (95%), 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell Culture and Viability Assay

Caov-3 and WRL-68 cell lines were cultured in a humidified chamber with 5% CO_2 at 37°C. MTT assay was used to study the proliferation and survival of cancer cells with or without TQ-treatment. In this assay, cells were seeded on a 96-well microplate at $2x10^5$ cells/mL in 100 µL culturing medium. They were plated in triplicate. Concentrations of TQ (0-50µg/mL) were prepared by serial dilution and were transferred to the cells in the 96-well plate and incubated for 24 h. After incubation, the viability of the cells was assessed using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, 5 mg/mL); 20 µL were added to the cells in a dark place, and the cells were then covered with aluminium foil and incubated for 4 h. After incubation, all of the media were removed and 100 µL of DMSO were added to the cells in order to solubilise the formazan crystals. Subsequently, the absorbance was read at a wavelength of 570 nm using a micro-plate reader. The potency of cell growth inhibition for the test agent was expressed as an IC_{50} value.

Multiple Cytotoxicity Assay

Cellomics Multiparameter Cytotoxicity-3 Kit was carried out as clarified earlier in details (21). This kit allows instant and direct quantification in the same cell of different autonomous parameters that scrutinize cell health, including nuclear size, morphological changes, mitochondrial membrane potential changes, alterations in cell permeability and cell loss. Multiparameter Cytotoxicity-3 Plates were examined using the CellReporter™ cytofluorimeter system (Gentix/Molecular devices, United Kingdom).

Image Acquisition and Cytometric Analysis

CellReporter™ cytofluorimeter system is a computer-based and automated fluorescence imaging microscope that automatically recognizesflouro-stained cells and reports the strength and allocation of fluorescence in individual cells. The Array-Scan HCS system scans numerous microfields in individual microwells to obtain and evaluate images of single cells according to distinct algorithms. In each microwell, 1,000 cells were analyzed. Automatic repeated focusing was carried out in the nuclear channel to guarantee focusing in spite of staining intensities in the other channels. Images were obtained for each fluorescence channel, using Array-Scan HCS appropriate filters. Images and data regarding strength and consistency of the fluorescence within each cell, as well as the average fluorescence of the cell population within the well were kept in a Microsoft SQL database for easy recovery. Data were captured, extracted, and analyzed with Data Acquisition and Data Viewer software (Gentix/Molecular devices).

Determination of Reactive Oxygen Species (ROS)

The assembly of intracellular reactive oxygen species was detected using 2',7'-dichlorofluorescin diacetate (DCFH-DA). Briefly, 10 mM flourstain (DCFH-DA) stock solution (in methanol) was diluted 500-fold in Hank's Balanced Salt Solution (HBSS) without serum or other additives to give a 20 µM working solution. After 24 h of exposure to TQ-treatment, the cells in the black microplate were flushed twice with HBSS and then kept in 100 µL working solution of DCFH-DA at 37°C for 30 min. Fluorescence was then determined at 485-nm excitation and 520-nm emission using a fluorescence microplate reader (Tecan Infinite M 200 PRO, Männedorf, Switzerland).

Annexin V Assay

Cells ($2x10^5$ cells/mL) were treated with the IC_{50}concentration for 24, 48 and 72 h. This assay was done using the Annexin V-FITC Apoptosis Kit (BD Pharmingen, BD, USA). Briefly, TQ-exposed and unexposed cells (control) were washed and centrifuged to eliminate the culturing media. Afterward, the cells were rinsed with binding buffer (1X). The rinsed cells were re-suspended in binding buffer and annexin V, and propidium iodide (10 µL) were added and the cells were then incubated at room temperature in the dark for 15 min. Flow cytometric analysis was carried out using a BD FACSCanto™ II instrument. The binding buffer supplied by the manufacturer was used to bring the reaction volume to at least 500 µL for the flow cytometry analysis. DMSO-treated (0.1%, v/v) cells were used as control.

Protein Detection by Western Blotting

Cells at a concentration of 2×10^5 cells/mL were cultured in RPMI 1640 (PAA, Coelbe, Germany) medium containing 10% FBS, seeded into a 75-mm culture flask (TPP Brand) and then treated with IC_{50} concentration for 3, 6, 12 and 24 h. After incubation, the cells were spun down at 1,000 rpm for 10 min. The supernatant was discarded and the pellet was washed twice with phosphate buffered saline (PBS) to remove any remaining media. Estimation of the packed cell pellet volume was done and 20 volumes of mammalian cell lysis reagent (Proteo JET, Fermentas Life Sciences, Burlington, ON, Canada) were added to 1 volume of packed cells. The cells were then incubated for 10 min at room temperature on a shaker (900–1,200 rpm) and centrifugation was done at 16,000–20,000 × g for 15 min to clarify the lysate. The resultant lysate was then transferred to a new tube and stored at −70 °C until analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Statistical Analysis

Results were reported as means ± SEM of at least three analyses for each sample. Normality and Homogeneity of variance assumptions was checked. Statistical analysis was performed according to the SPSS-16.0 package.

Results and Discussion

TQ induced cell viability inhibition in Caov-3 cells

The cytotoxic effects of TQ on the viability of ovarian cancer cell (Caov-3) were determined using the MTT test. Cellular viability following 24h of contact to TQ showed considerable inhibition in TQ-exposed cells

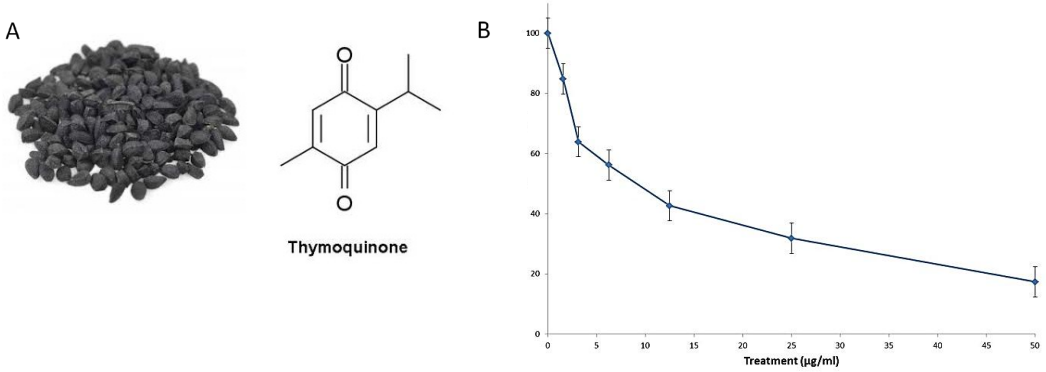

Figure 1. (A) *Nigella sativa* seeds (black seeds) and its active compound (thymoquinone). (B) MTT assay.

compared to control cells (untreated). The IC_{50}value of TQ was6.0± 0.03µg/mL (Figure 1B). TQ has been shown previously to exert *in vitro* anti-neoplastic effects (22). TQ did not show any cytotoxic activity on WRL-68 normal hepatocytes.

TQ-induced apoptosis in Caov-3 cells

To confirm the presence of apoptosis, we examined the cell with the help of the annexin V. This assay revealed the early stages of apoptosis induced by TQ. In the assay, the healthy cells showed 90.66 % viable cells, 4% early apoptosis and 4.65% late apoptosis, whereas after 24 hours treatment TQ in Caov-3 cells showed 90.00 % viable cells, 6.23% early apoptosis and 4.33% late apoptosis. As the treatment time increased to 48 h and 72 h, the percentage of both early and late apoptotic cells continued to increase substantially (Fig 2). The date showed that the TQ has the ability to induce apoptosis time dependently.

Concurrent assessment of cell behavior with nuclear stain showed that the nuclear intensity, which is directly corresponding to apoptotic chromatin changes has increased from 69.45 to 138.07 at 48 hr treatment (fig 3 A, B). Meanwhile, concurrent significant ($p<0.05$) increase in the cell permeability was also observed (Fig. 3C).

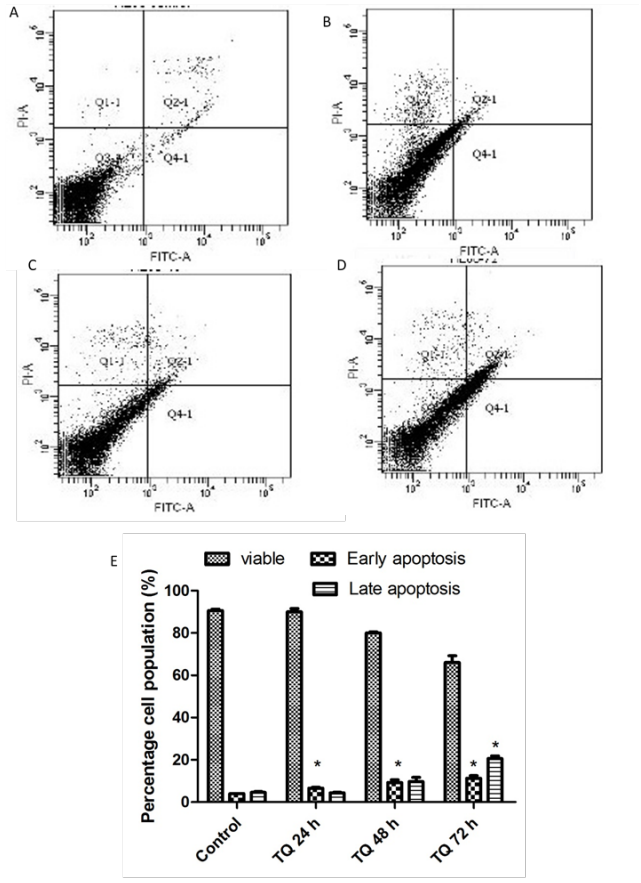

Figure 2. The effect of thymoquinone on early apoptosis of CAOV3 ovarian cancer cell line for (A) Untreated (B) 24 h, (C) 48 h, (D) 72 h and (E) histogram. CaoV3 cells were staining with FITC-conjugated Annexin V and PI, cells were analyzed by flow cytometry. The early apoptotic events (Annexin+/PI-) are shown in lower right quadrant (Q4-1) of each panel. Quadrant (Q2-1) represents Annexin+/PI+ late stage of apoptosis/dead cells.

TQ-induced MMP disruption in Caov-3

The changes in MMP in Caov-3 cells have been

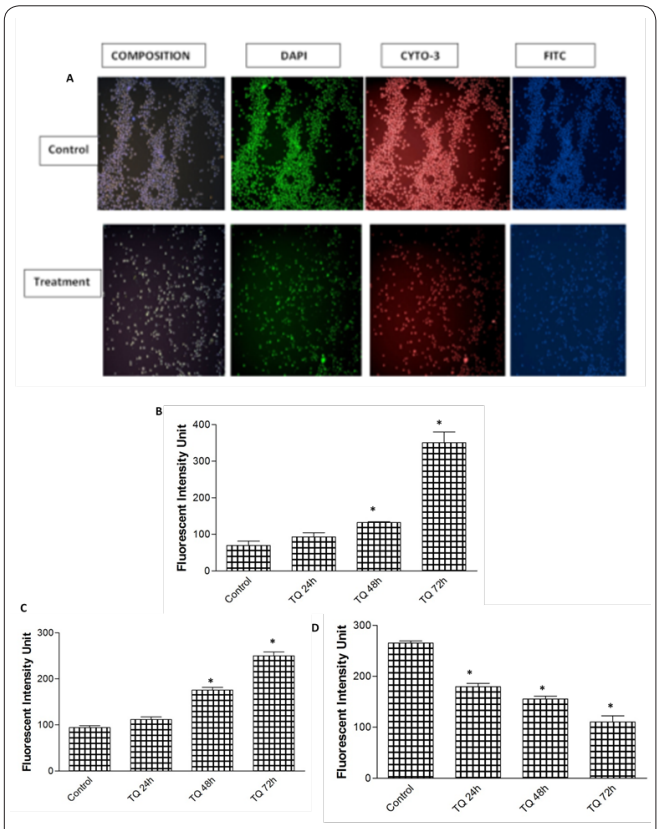

Figure 3. Treated cells shown thymoquinone induced increase in nuclear area intensity and increase in plasma membrane permeability and reduction in mitochondrial membrane potential. (A-C). (D) time-dependent quantitative analysis of cells treated with TQ apoptosis parameters. Multiple Cytotoxicity Assay was conducted using Cellomics Multiparameter Cytotoxicity-3 Kit. Plates were analyzed using the CellReporter™ cytofluorimeter system.

observed after treatment with TQ. Figure 3A showed significant ($p<0.05$) reduction of MMP occurred upon treatment. Changes of mitochondrial membrane potential in Caov-3cells treated with TQ 6µg/ml for 24 h and 48 hr had showed a significant reduction of fluorescence intensity, 179 for 24 hr and 155 for 48 hr, while control cells was 265 (Fig. 3D).

TQ induced cell death includes increased ROS formation

Reactive oxygen species (ROS) are a diversity of free radicals and molecules resulting from molecular oxygen which are continually produced and eradicated in the cytoplasm and have significant responsibilities in cellular signaling and homeostasis (23). Disproportionate quantities of these ROS can lead to oxidative injury to proteins, lipids and nucleic acid (DNA), leading to tumorigenesis or cellular death (24, 25). As depicted in Figure 4, TQ exerted a catalyst role in producing more ROS in a dose-dependent trend, which augmented the ROS level on CAOV3 cell lines.

Western Blots

The Bcl-2 and Bax family proteins participate majorly in the induction of apoptosis and its regulation. These cellular signaling proteins are known to be membrane-bound and their tendency to experience both heterodimerization and homodimerization has been proved to control programmed cell death (26). Apart from the major machinery, which is composed of a cascade of caspases that execute the apoptosis program, for the apoptosis, Bax and Bcl-2 are two master regulators to regulate the on and off of apoptotic cascade, respectively (27, 28). To examine the possible mechanism relatedto the TQ-induced cell death (apoptosis), we examined protein expression of Bax, Bcl-2 and Hsp70 in ovarian cells prior to TQ-induction. Quantification of these proteins (Bax, Bcl-2 and Hsp70) was normalized to β-actin. Bcl-2 and Hsp70 reduced significantly while Bax protein level increased significantly in a concentration-dependent manner (Figure 6). The ratio of Bax and Bcl-2 also dramatically augmented in a time-dependent manner, whereas Bax intensity has been increased from 3 to 18 after 72 hour. Meanwhile Bcl-2 has been decreased from 16 to 3.5 intensity unit (29).

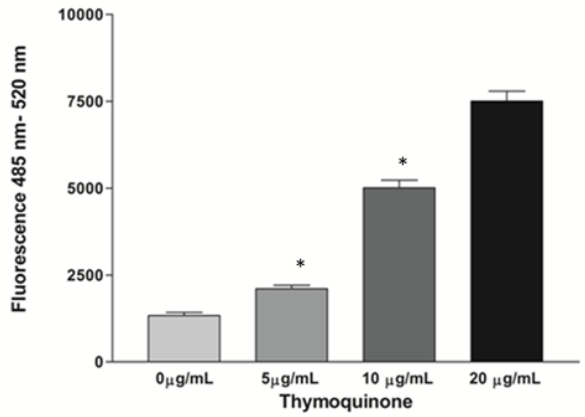

Figure 4. Effect of thymoquinone treatment on ROS. ROS was measured on DCFH-DA-stained CaoV-3 cells with fluorescence micro plate reader.

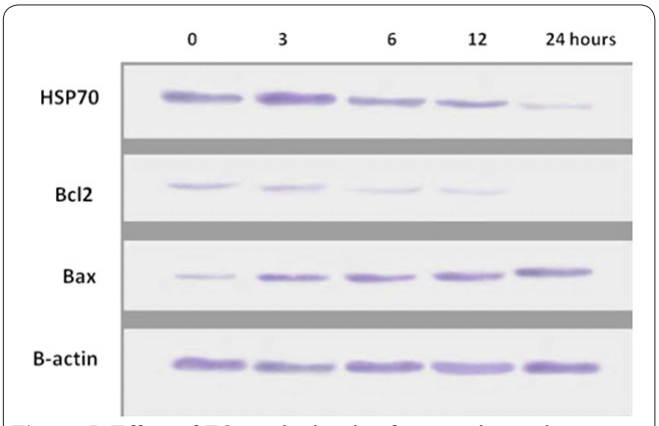

Figure 5. Effect of TQ on the levels of apoptosis regulatory proteins and HSP70 at 3, 6, 12 and 24 h with β- actin as a loading control. Protein levels were quantified using image processing software based on western blot band intensity as shown in Figure 6.

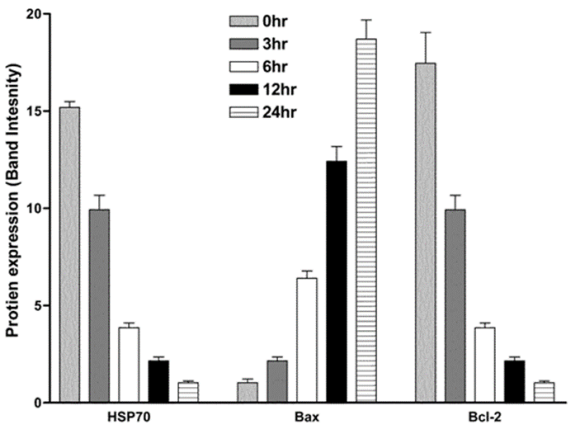

Figure 5. Effect of TQ on the levels of apoptosis regulatory proteins and HSP70 at 3, 6, 12 and 24 h with β- actin as a loading control. Protein levels were quantified using image processing software based on western blot band intensity.

The current findings offered a novel imminent into the pharmacological mechanism of therapeutic potentials of *Nigella sativa*. Moreover, our result indicates that TQ induces intrinsic pathway of apoptosis that is potentially mediated by up-regulating Bax and down-regulating Bcl-2. These results underlined that thymoquinone could be a nominee for an anticancer agent for the treatment of ovarian tumors.

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