Involvement of NF-κB and HSP70 signaling pathways in the apoptosis of MDA-MB-231 cells induced by thymoquinone: An in vitro study

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Abstract: In this study, we found that thymoquinone potently inhibited the viability of the human invasive breast cancer cell lines, MDA-MB-231 (IC□□6.13µg/mL (24h), 3.28µg/mL (48h) and 1.76µg/mL (72h). Thymoquinone had a cytotoxic effect and induced apoptosis in MDA-MB-231 breast cancer cells as indicated by acridine orange/propidium iodide-stained cells at different time points after thymoquinone treatment. In addition, we demonstrated that thymoquinone induced cell cycle arrest at G0 phase. Treatment of MDA-MB-231 cells with thymoquinone significantly elevated intra-cellular ROS formation, which plays a pivotal role in cell death. Thymoquinone induced activation of caspase-9, caspase-8 and caspase-3/7. We also found that thymoquinone, at high concentration, could inhibit nuclear factor kappa B activation, a key molecule in tumor progression and metastasis. In addition, Western blotting analysis showed that treatment with thymoquinone resulted in down regulation of Bcl-2 and heat shock protein 70 and up regulation of Bax in the MDA-MB-231 cell line. Our results suggest that thymoquinone is a potentially useful agent for the treatment of breast cancer.
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Keywords: Thymoquinone, breast cancer, caspase cascade, reactive oxygen species, Bcl-2/Bax, heat shock protein 70, nuclear factor kappa B

1. Introduction

Cancer is the major health problem of global concern that afflicts a substantial portion of the people all around the world (Atawodi et al., 2011). Breast cancer is a complex and heterogeneous disease, which has become the leading diagnosed cancer among women worldwide and also of the most frequent causes of cancer death among women, especially in western populations and industrialized countries (Jemal et al., 2007). So far, over 1.3 million cases of invasive breast cancer have been reported worldwide. Statistical reviews revealed a total death rate of up to 450,000 cases annually, in women (Garcia et al., 2007). Despite marked improvement of breast cancer treatment options during the past decades, there are still considerable death rates of women in US (15% of all cancer deaths) and other parts of the world (Jemal et al., 2010; Paydar et al., 2013). Among the reasons for this situation are the development of drug resistance and severe side effects of chemotherapy, which still are unresolved problems in clinical oncology. Therefore, the search for novel anti-cancer compounds with improved features is mandatory. Different breast cancer cell lines have been frequently applied by investigators for drug discovery purposes and among these cells estrogen non-dependant MDA-MB-231 is one of the most extensively used models.

In the recent decades, plant-derived drugs have been widely introduced to the modern medicine and the importance of phytomedicines has had a drastic upward trend. This is mostly due to the public awareness of the harmful side-effects of chemically synthetic drugs which has generated a desire to find natural alternatives to replace these chemical compounds.

Thymoquinone (Figure 1) is a phytochemical compound found in the plant Nigella sativa, which has shown promising pharmacological and therapeutic effects against in vitro and in vivo cancer models. It has been studied for its potential effects on colon cancer cells. It reduces mouse colon tumor cell invasion and inhibits tumor growth in animal models of colon cancer. Besides, there is an increasing research interest in thymoquinone to evaluate its anticancer activity against breast cancer. Recent studies demonstrated interesting inhibitory

mechanisms imposed by thymoquinone in breast cancer cell lines MCF-7, MDA-MB-231 and BT-474. Hence, this study was performed to entirely study the possible mechanisms involved in the anti-carcinogenic effects of thymoquinone.

2. Materials and Methods

2.1 Materials

Thymoquinone (99%), Insulin, HEPES, and epidermal growth factor were purchased from Sigma-Aldrich (St Louis, MO, USA). Cell culture medium, fetal bovine serum, penicillin, and streptomycin were obtained from Gibco (Invitrogen, Life Technologies, Inc., Rockville, MD, USA).

2.2 Cell culture

Human invasive **breast cancer** cell line (MDA-MB-231) and non-invasive MCF10A human breast cell line were purchased from American Type Culture Collection (ATCC, Manassas, VA). MDA-MB-231 cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Inc, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO), 1% penicillin and streptomycin. MCF10A cells were grown in Ham's F12: DMEM (50:50), 2.5 mM l-glutamine, 20 ng/ml epidermal growth factor (EGF) (Sigma), 0.1 µg/ml cholera toxin (CT) (Sigma), 10 µg/ml insulin (Sigma), 500 ng/ml hydrocortisone (Sigma) and 5% horse serum (Atlanta Biologicals). Cells were cultured in **tissue culture** flasks (Corning, USA) and were kept in incubator at 37°C in a humidified atmosphere with 5% CO2. For experimental purposes, cells in exponential growth phase (approximately 70-80% confluency) were used.

2.3 MTT cell viability assay

The cytotoxic effect of thymoquinone was assessed by MTT cell viability assay(Gummadi et al., 2013). The assay was performed for different treatment time points. Briefly, 8.0 × 103 cells were seeded in a 96-well plate and incubated overnight at 37 °C in 5% CO2. On the next day, the cells were treated with a two-fold dilution series of six concentrations of thymoquinone, and then they were incubated at 37 °C in 5% CO2 for 24, 48 and 72 hours. MTT solution (4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazoliumbromide) was added at 2 mg/mL and after 2 hours of incubation at 37 °C in 5% CO2, DMSO was added to dissolve the formazan christals. The plates were then read in Chameleon multi-technology micro-plate reader (Hidex, Turku, Finland) at 570 nm absorbance. The cell viability percentage after exposure to the extract for 48 hours was calculated by previously described method (Looi et al., 2011). The ratio of the absorbance of thymoquinone-treated cells to the absorbance of DMSO-treated control cells was determined as percentage of cell viability. IC50 value was defined as the concentration of thymoquinone required to reduce the absorbance of treated cells to 50% of the DMSO-treated control cells. The experiment was carried out in triplicates.

2.4 Acridine orange/propidium iodide double-staining

Cells seeded in 25 cm2culture flasks (1.0×106 cells/flask) were treated with dimethylsulfoxide or boldine at the IC50 dose and incubated at 37°C in 5% CO2for different time periods (24, 48, and 72 hours). The staining solution was prepared by adding 100 µL of 1 mg/mL propidium iodide (Sigma-Aldrich Company Ltd, Gillingham, UK) and 100 µL of 1 mg/mL Acridine orange (Sigma-Aldrich Company Ltd) to 10 mL of phosphate-buffered saline. The cell suspensions were mixed1:1 with the staining solution in micro-titer wells, incubated for 20 minutes, and observed under a BX51 ultraviolet fluorescent microscope (Olympus, Tokyo, Japan).

2.5 Cell cycle analysis

1×104 MDA-MB-231 cells per well were seeded in a 96-well plate and incubated overnight at 37 °C in 5% CO2. Cells were treated with different concentrations of thymoquinone or DMSO (negative control) for 24 hours. Brd U and Phospho-Histone H3 dyes were added into live cells for 30 minutes. Cells were fixed and stained as described by the manufacturer's instruction. Stained cells were visualized and acquired using Cellomics Array Scan HCS reader (Thermo Scientific). Target activation bio-application module was used to quantify the fluorescence intensities of dyes (Mohan et al., 2012).

2.6 Reactive Oxygen Species (ROS) assay

ROS assays was carried out to determine the influence of thymoquinone on the production of ROS level of the treated MDA-MB-231 cells. 1×104 cells per well were seeded onto 96-well plate and incubated overnight at 37 ºC 5% CO2. The cells were then treated with different concentrations of the compound for 24 hours and then dihydroethidium (DHE) dye was added into live culture for 30 minutes. Cells were fixed and washed with wash buffer as described by the manufacturer's instruction. The DHE dye probe is oxidized to ethidium in the presence of superoxides. The fluorescence intensity was measured using a fluorescent plate reader at an extention wavelength of 520 nm and an emission wavelength of 620 nm. The values are represented as means ± SD of three sets of experiments (Arbab et al., 2012).

2.7 Bioluminescent Assays for Caspase-3/7,-8 and -9 Activities

A dose-dependent study of caspase-3/7, -8 and -9 activities was performed in triplicates using assay kits Caspase-Glo® 3/7, 8 and 9 (Promega, Madison, WI) on a white 96-well micro-plate. A total of

1×104 MDA-MB-231 cells was seeded per well and incubated with thymoquinone for different time points. Caspase activities were investigated according to the manufacture protocol. Briefly, 100 µl caspase-Glo reagent was added and incubated at room temperature for 30 minutes. Presences of active caspases from apoptotic cells cleaved the amino luciferin-labeled synthetic tetrapeptide thus release substrate for the luciferase enzyme. The caspase activities were measured using a Tecan Infinite®200 Pro (Tecan, Männedorf, Switzerland) microplate reader (Ahmadipour et al., 2015).

2.8 Quantitative PCR analysis

Analysis of mRNA expression by RT-PCR total RNA from thymoquinone-treated and untreated MDA-MB-231 cells were extracted using RNeasy Mini Kit (Qiagen, Germany). 1 g of total RNA was used to perform their verse transcription with the Quanti Tect Rev. Transcription Kit (Qiagen, Germany). The transcribed cDNA (1 l) was used for polymerase chain reaction (PCR) amplification with specific primers of Bax and Bcl2 genes, and the PCR reaction were carried out. β-actinm RNA served as the loading control. The primers for Bax: sense, 5-TTT GCT TCA GGG TTT CAT CC-3, and antisense, 5-GCC ACT CGG AAA AAG ACC TC-3. The primers for Bcl2: sense, 5-ATG AAC TCT TCC GGG ATG G-3, and antisense, 5-TGG ATC CAA GGC TCT AGG TG-3. The primers for β-actin: sense,5-CGG GAAATC GTG CGT GAC-3, and antisen, 5-GCC TAG AAG CATTTG CGG TG-3. PCR amplification was performed with a thermalcycle, and the reaction was started with the initial denaturation at95 ◦C for 5 min, followed by 30 cycles of denaturation, annealingand extension at 95 ◦C for 30 s, 60 ◦C for 40 s, and 72 ◦C for 1 min, respectively. The reaction was terminated after final extension at72 ◦C for 10 min. The sizes of the amplification products of Bax, Bcl2 and β-actin were 213, 166 and 515 bp, respectively. The PCR products were subjected to 1.5% agarose gel electrophoresis and were stained with ethidium bromide. It was visualized under UVlight using Gel Doc XR System (Bio-Rad, USA) (Ibrahim et al., 2014b).

2.9 Western blotting

Western blotting was used to investigate the expression of apoptosis-related proteins, including Bax, Bcl-2, heat-shock protein HSP70 and NF-κB. MDA-MB-231 cells were treated with boldine in a concentration-dependent manner for 24 hours. Untreated cells served as the negative control. The total protein content in the cells was extracted with cell lysis buffer (50mM Tris-HCl pH 8.0, 120 mM NaCl, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride); 40 µg of protein extract was separated by 10% sodium dodecyl sulfate polyacrylamidegel electrophoresis and then transferred to a polyvinylidenedifluoride membrane (Bio-Rad, Hercules, CA, USA) using a semi-dry transfer unit (TE 70X, Hoefer Inc., Holliston, MA, USA) blocked with 5% nonfat milk in TBS-Tween buffer(0.12 M Tris-base, 1.5 M NaCl, 0.1% Tween 20) for one hour at room temperature, and incubated overnight at 4°C with the appropriate primary antibodies, ie, β-actin (1:5,000), Bcl2(1:1,000), Bax (1:1,000), HSP70 (1:1,000) and NF-κB (1:1,000) purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA), followed by incubation with alkaline phosphatase-conjugatedgoat anti-mouse and goat anti-rabbit secondary antibodies (i-DNA, Promega, Madison, USA) for 30 minutes at room temperature, and washing in Tris-buffered saline with Tween20 for 10 minutes three times on an orbital shaker. The blots were then developed using BCIP®/NBT solution (Santa Cruz Biotechnology Inc.) for a period of 5–30 minutes to detect the target protein band as a precipitated dark-blue color (Ibrahim et al., 2014a).

2.10 Statistical Analysis

Experimental values were presented as the means ± standard deviation (SD) of the number of experiments indicated in the legends. Analysis of variance (ANOVA) was performed using Graph Pad Prism 5 software. Statistical significance was defined when $P<0.05$.

3. Results

3.1 MTT cell viability assay

The cytotoxic effect of thymoquinone was evaluated on MDA-MB-231 human invasive cancer cells using MTT assays. The IC50 values after 24, 48 and 72 hours of treatment with thymoquinone have been shown in Table 1. Significant cytotoxicity and cell inhibitory effect was observed in the MDA-MB-231 cells.

3.2 Quantification of apoptosis using phase-contrast microscopy and AO/PI double-staining

Morphological changes in MDA-MB-231 cells treated with thymoquinone were observed under a fluorescent microscope at 24, 48 and 72 h. The cells were scored under a fluorescent microscope to analyze viable cells, early apoptosis and late apoptosis. Early apoptosis, defined as intervening AO within the fragmented DNA, was observed under bright green fluorescence. At the same time, control cells were visualized with a green intact nuclear structure (Figure 2A). As it is clearly presented in Figure 2B, C, moderate apoptosis in the form of cell membrane blebbing and nuclear chromatin condensation was noticed in thymoquinone-treated cells after 24 and 48 h of treatment. Presence of a reddish-orange color in the thymoquinone-treated cells for 72 h might be due to the binding of PI to

denatured DNA, which indicates the late stage of apoptosis (Figure 2C). The results demonstrated morphological features of apoptosis induced by thymoquinone in a time-dependent manner in MDA-MB-231 human breast adenocarcinoma cells.

3.3 Cell cycle analysis

In order to elucidate the mechanisms underlying the observed anti-proliferative effect of thymoquinone on MDA-MB-231 cells, we examined the cell cycle distribution of MDA-MB-231 cells treated with different concentrations of thymoquinone. Cells were fixed and stained with propidium iodide, and the cell cycle distribution was determined by flow cytometry. Our results showed that treatment with thymoquinone induced cell cycle arrest at G0 phase (Figure 3). The percentage of cell population in G0 phase was 6.26% in control cells. Treatment with thymoquinone induced a gradual increase of the percentages to 29.95%, 83.54%, and 62.35% in response to higher dosages of the compound, suggesting dose-dependent arrest of cells at G0 phase.

3.4 Reactive Oxygen Species (ROS) assay

MDA-MB-231 cells were pretreated with the solvents for 24 hours and stained with DHE dye to determine the influence of the compound exposure on ROS production. The fluorescent intensities of DHE oxidization by ROS were measured using a fluorescence micro-plate reader. As shown in Figure 4, exposure to the compound caused an increase in the ROS level of the thymoquinone-treated MDA-MB-231 cells.

3.5 Bioluminescent Assays for Caspase-3/7,-8 and -9 Activities

The excessive production of ROS from mitochondria and the collapse of MMP may activate downstream caspase molecules and consequently lead to apoptotic cell death. To examine this, we measured the bioluminescent intensities of caspase-3/7, -8, -9 activities of the MDA-MB-231 cells treated with different concentrations of the extract for 24 hours. As shown in Figure5, significant dose-dependent increase in Caspase -8, caspase -9 and -3/7 activity was detected in the thymoquinone-treated MDA-MB-231 cells. Hence, the apoptosis induced by thymoquinone in MDA-MB-231 cells is mediated via both the intrinsic, mitochondrial-caspase-9 pathway which might be p53-mediated (Soengas et al., 1999; Cui et al., 2002; Wu and Ding, 2002) and the extrinsic, death receptor-linked caspase-8 pathway (Figure 5).

3.6 RT-PCR analysis of Bax and Bcl2 mRNA

The expression levels of Bax and Bcl2 mRNA (Figure 7) was evaluated in thymoquinone-treated MDA-MB-231 cells, shown by RT-PCR analysis. Expression of Bax was significantly increased and Bcl2 expression was markedly down regulated in the thymoquinonetreated group as compared to the control untreated cells ($p < 0.05$).

3.7 TQ up-regulated Bax and suppressed the expression of Bcl2 and HSP70 protein

To investigate the mechanism underlying thymoquinone-induced cell death, we performed Western blotting to examine the expression level of Bcl-2, Bax and HSP70 in MDA-MB-231 cells with/without thymoquinone treatment for 24 h. Interestingly, we found that thymoquinone dose-dependently down regulated Bcl-2 and HSP70 expression and up regulated Bax expression in MDA-MB-231 cells (Figure 8).

4. Discussion and conclusion

In the present study, thymoquinone was evaluated for its ability to inhibit the growth of human invasive breast cancer cell line (MDA-MB-231) and human normal breast (MCF-10A) cells using MTT cell viability assay. We observed that MDA-MB-231 cells were more sensitive to the compound compared to MCF-10A cells, indicating the selectivity of thymoquinone between normal and cancer cells.

Cancer is often considered as a disease of cell cycle deregulation. Cell size, extracellular growth signals and DNA integrity are tightly regulated by multiple checkpoints in cell cycle progression. Cancer can originate from perturbation in the expression of positive or negative regulators of cell cycle machinery leading to abnormal proliferation of cancer cells. Thus, induction of cell cycle arrest in cancer cells is considered to be one of the crucial cancer treatment strategies. We found that treating the MDA-MB-231 cells with thymoquinone causesa significant G0 phase increase in a dose-dependent manner demonstrating that thymoquinone arrested cell cycle progression at the G0 phase (Figure 3).

Downstream caspase molecules could be activated by enhanced ROS and declined mitochondrial membrane potential (MMP), which leads to apoptotic cell death through intrinsic, mitochondrial-caspase-9 pathway. Initiator caspase-8 is known to be activated through extrinsic pathway, whereas caspase-9 is activated in the event of mitochondrial cytochrome c leakage. Both initiator caspases can activate caspase-3 or -7, which commit cells to apoptosis. After the binding of cytochrome c to apoptotic activating factor-1, caspase-9 is activated via apoptosome formation which leads to active caspase-3/7, the most effective caspase with many cellular targets (Li et al., 2001). As an example, FAS ligand interacts with FAS receptor, leading to the activation of caspase-8 (El-Ghany et al., 2009). Caspase-8 activation leads to cleavage and activation of the downstream executioner caspases, such as caspase-3/7 (Hyer et al., 2008; Qi et al., 2012). In our

study, thymoquinone exhibited significant elevation in the caspase-9 activities compared to the control. Besides, It showed activation of caspase-8 suggesting that the apoptosis induced by thymoquinone in MDA-MB-231 cells is mediated via both the intrinsic mitochondrial-caspase-9 pathway which might be p53-mediated (Soengas et al., 1999; Cui et al., 2002; Wu and Ding, 2002) and extrinsic, death receptor-linked caspase-8 pathway. Highly elevated caspase-3/7 in thymoquinone-treated cells confirmed that the apoptosis induced by the compound is through caspase-dependent pathway.

Members of the Bcl-2 family include major cell survival and cell death regulators. Bcl-2 acts as apoptosis inhibitors in the cells, while Bax inhibits cell survival as a pro apoptosis factor. Our data showed that thymoquinone treatment caused a significant decline in the expression of pro-survival protein Bcl-2 in the MDA-MB-231 cells treated for 24h. We observed a dose dependent increase in the expression of the pro-apoptotic molecule, Bax. The importance of Bc1-2 for protection of mitochondria during cell death process has been previously studied (Shimizu et al., 1996). Excessive expression of Bax may form Mitochondrial Apoptosis-Induced Channel (MAC) and mediates the release of cytochrome c, that anti-apoptotic factor; Bcl-2 has the ability to block it through inhibition of Bax and/or Bak. Bcl-2 expression decline may lead to loss of MMP which enhances apoptosis-associated release of cytochrome c from the mitochondria (Yang et al., 1992). Hence, upregulation of Bax and down regulation of Bcl-2 molecules caused by thymoquinone treatment may lead to MMP loss and cytochrome c release and induce apoptosis. Another anti-apoptotic protein is HSP70, which is highly expressed in breast cancer cells. HSP70 induces resistance against apoptosis, thus enhances the survival of cancer cells. We showed that thymoquinone suppressed HSP70 in a dose-dependent manner, which further supports its apoptosis-inducing properties.

Tables & Figures:

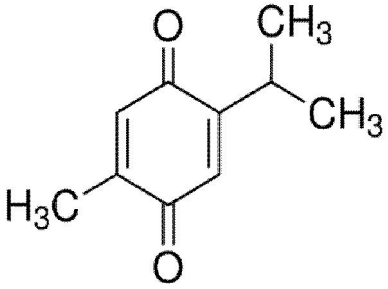


Figure 1. The chemical structure of thymoquinone; 2-isopropyl-5-methyl-1,4-benzoquinone (C10H12O2); Mwt 164.2

Table 1. IC50 of TQ or Tamoxifenin breat cancer MDA-MB-231 cells determined by MTT assays after 24, 48 and 72 h treatment.

Compound	24h	48h	72h
TQ	6.13 ± 0.87	3.28 ± 0.77	1.76 ± 0.25
Tamoxifen	14.45 ± 1.32	10.21 ± 1.21	8.68 ± 1.03

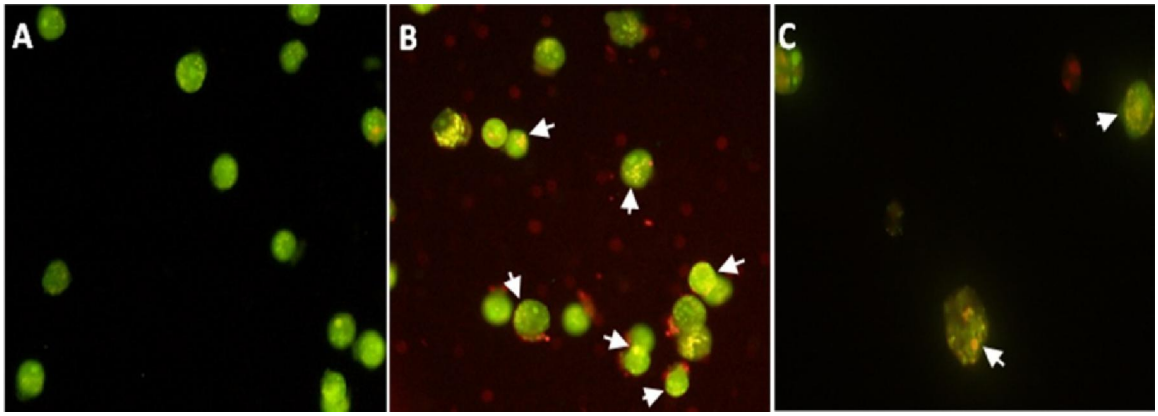
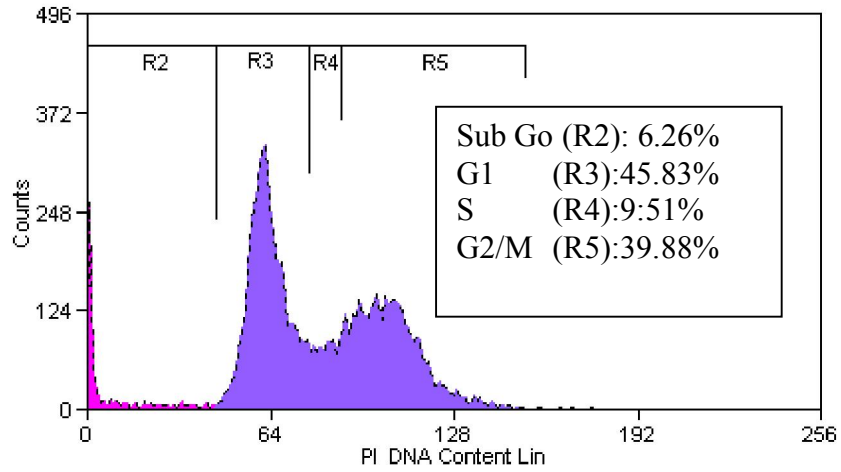
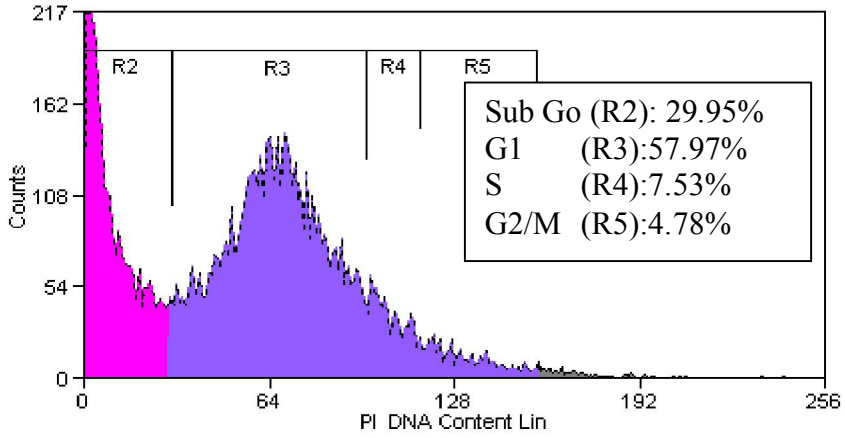


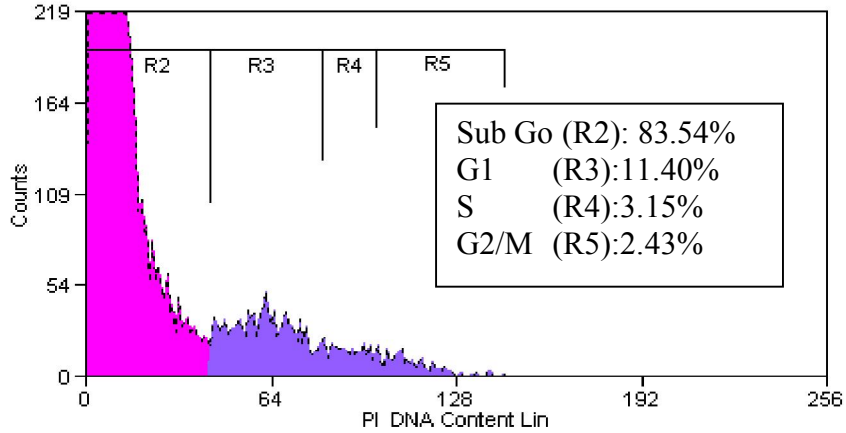
Figure 2. TQ-treated MDA-MB-231 cells after AO/PI staining. Panel (A) shows the green viable MDA-MB-231 cells with diffused chromatin as control (Magnification, 20X). Panel (B) and (C) show the TQ-treated MDA-MB-231 cells with condensed chromatin (white arrow) at 24 hr with magnifications of 20X and 40X, respectively.



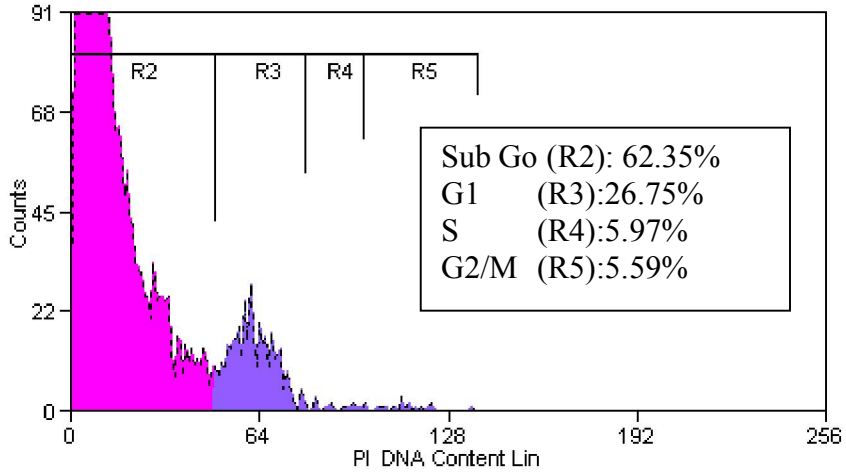
Control 72 hours



24 hours



48 hours (medium dose)



72 hours (high dose)

Figure 3. Effect of thymoquinone on cell cycle progression in MDA-MB-231cells. Thymoquinoneinduces cell cycle arrest at G0 stage.

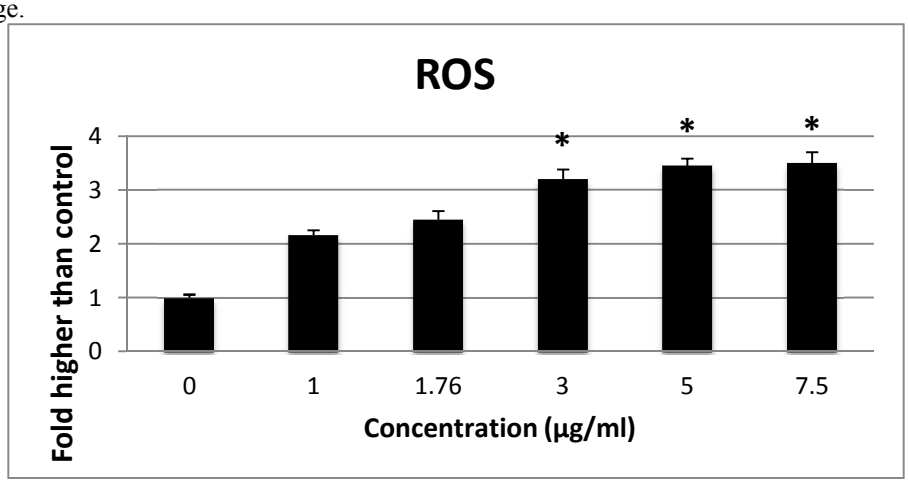


Figure 4. ROS generation in MDA-MB-231cells in the presence of different concentrations of thymoquinone compared to untreated cells. Starting at concentration of 3 µg/mL, thymoquinone caused significant ROS formation in MDA-MB-231cells. All of the data are expressed as the means ± standard error of triplicate measurements. *P <.05 compared with the no-treatment group.

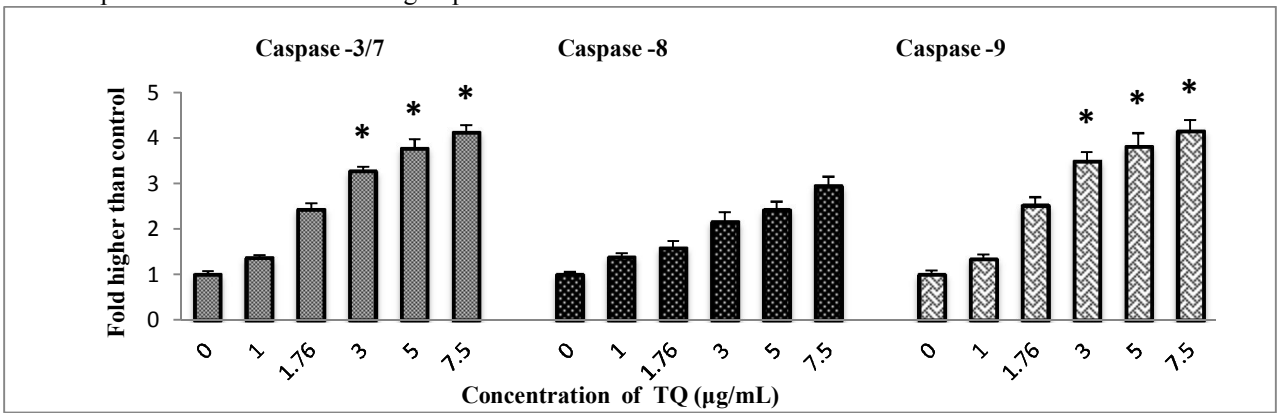


Figure 5. Effect of various concentrations of thymoquinone on caspase 3/7, 8, and 9 activation in MDA-MB-231 cells after 24 h of treatment. The results revealed significant activation of caspases-3/7, -8, and -9. All of the data are expressed as the means ± standard error of triplicate measurements. *P < 0.05 compared with the no-treatment group.

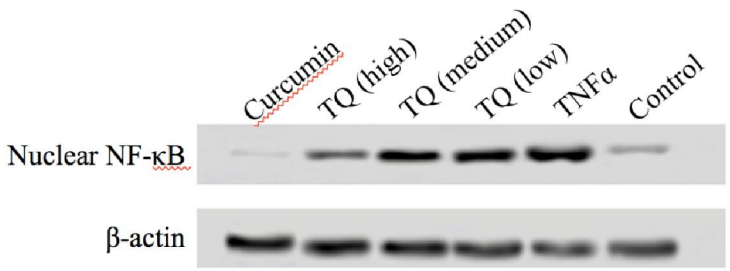


Figure 6. Western blot analysis of thymoquinone-treated MDA-MB-231 cells. Cells were treated with thymoquinone for 24 hours before being lysed and subjected to separation by sodiumdodecyl sulfate polyacrylamide gel electrophoresis. Proteins were then transferred to membrane and probed with antibodies against nuclear NF-κBand β-actin. The membrane was re-probed with anti-β-actin antibody as the loading control. Thymoquinone, at high concentration, decreased nuclear NF-κB in the MDA-MB-231 cells.

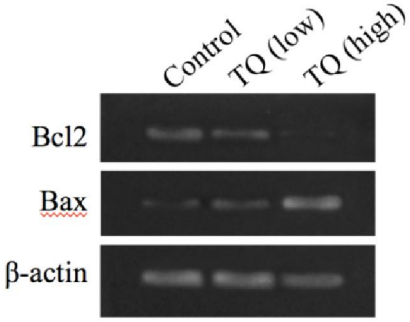


Figure 7. Effect of thymoquinone (TQ)on the Bax and Bcl2 mRNA expression level in MDA-MB-231 cells. TQ caused upregulation of Bax and downregulation of Bcl2 in the treated MDA-MB-231 cells. Triplicates of each treatment group were used in each independent experiment.

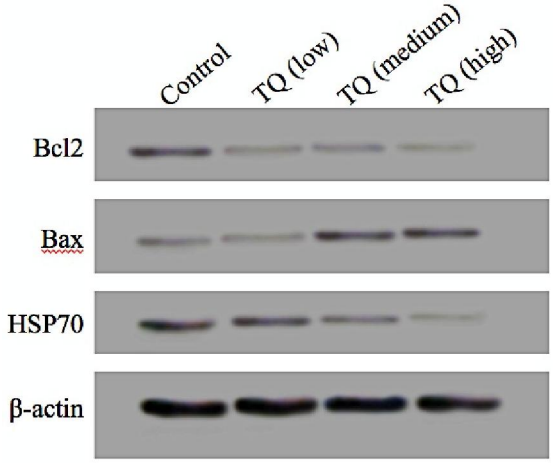


Figure 8. Western blot analysis of thymoquinone-treated MDA-MB-231 cells. Cells were treated with thymoquinone for 24 hours before being lysed and subjected to separation by sodiumdodecyl sulfate polyacrylamide gel electrophoresis. Proteins were then transferred to membrane and probed with antibodies against Bax, Bcl-2, heat shock protein 70 and β-actin. The membrane was re-probed with anti-β-actin antibody as the loading control. Thymoquinone treatment resulted in downregulation of Bcl-2 and heat shock protein 70 and upregulation of Bax in the MDA-MB-231 cells.

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