Cellular responses with thymoquinone treatment in human breast cancer cell line MCF-7

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ABSTRACT

Background: *Nigella sativa* or black seed extract has been reported to show various medicinal benefits. Thymoquinone which is an active compound of its seed has been reported to contain anti-cancer properties. **Objective:** The study addressed the anti-cancer efficiency of long-term *in vitro* treatment with thymoquinone towards human breast cancer cell lines MCF-7. **Materials and Methods:** Cell proliferation was determined with CellTiter 96 Aqueous. Non-Radioactive Cell Proliferation Assay Kit. It was followed with trypan blue exclusion test to determine the percentage of viable cells. The study incorporated cell cycle assay to distinguish cell distribution at various cell cycle phases using Cycletest Plus DNA Reagent Kit. The apoptosis detection kit was used to determine the percentage of apoptotic and necrotic cells using flow cytometry. **Results:** The 50% inhibitory concentration (IC_{50}) value determined using the proliferation assay was 25 μM thymoquinone. Late apoptotic cell percentage increased rapidly when treatment duration was increased to 24 h with 25 and 100 μM thymoquinone. Further analysis using cell cycle assay showed thymoquinone inhibition of breast cancer cell proliferation at minimal dose 25 μM and led to S phase arrest significantly at 72 h treatment ($p = 0.009$). It was also noted elevation sub-G_1 peak following treatment with 25 μM thymoquinone for 12 h. Increase in thymoquinone to 50 μM caused G_2 phase arrest at each time-point studied. **Conclusion:** In general thymoquinone showed sustained inhibition of breast cancer cell proliferation with long-term treatment. Specificity of phase arrest was determined by thymoquinone dose.

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INTRODUCTION

Almost 25% of drugs used during the last 20 years are directly derived from plants, while the other 25% are chemically altered natural products.[1] Thymoquinone is the most active compound found in *Nigella sativa* seed. There has been increasing number of literature on its medical values within the past decade. *N. sativa* is one of the most extensively studied plants both for phytochemical and pharmacological properties.[2] It is perceived to contain medical benefits mainly in Asia and Europe. Numerous animal and cell-based studies have been done and some of which include anti-inflammatory, anti-oxidant, anti-cancer, anti-microbial, anti-parasitic, and anti-hyperglycemic. However, specific effect of long-term exposure of thymoquinone on breast cancer cells has not been reported previously.

Nigella sativa oil or thymoquinone administration was reported to lower cyclophosphamide-induced toxicity by up-regulation of antioxidant mechanisms. This indicated potential clinical applications for use of *Nigella sativa* to minimize toxic effects of treatment with anticancer drugs.[3] Khan and Sultana suggested that *Nigella sativa*, was a potent chemopreventive agent and suppresses ferric nitrilotriacetate (Fe-NTA)-induced oxidative stress, hyperproliferative response, and renal carcinogenesis in Wistar rats.[4] An *in vivo* study using DBA2/P815 mouse model, clearly showed that the injection of the essential oil into the tumor site significantly inhibited solid tumor development.[5]

Thymoquinone significantly and dose-dependent reduced the intrinsic activity of the MCP-1 promoter in pancreatic

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ductal adenocarcinoma cells.[6] Earlier study using human chronic myeloid leukemia cells KBM-5 demonstrated that thymoquinone suppressed tumor-necrosis factor-induced NF-κB activation in a dose and time-dependent manner and also inhibited activation of NF-κB that were induced by various carcinogens and inflammatory stimuli.[7] Results from previous findings may indicate that the anti-cancer and anti-inflammatory activities were assigned to thymoquinone was mediated in part through the suppression of NF-κB activation pathway. These findings demonstrated the possible role of *Nigella sativa* and thymoquinone in cancer treatment.

A major concern based on recent reports was development of drug-resistance cancer cells. This condition develops over time due to prolonged exposure to treatment. Focus of this study was to provide evidence on thymoquinone and its long-term exposure to treat human breast cancer using culture model. Cell proliferation and viability level were determined to select optimal dose for subsequent stage in the study. Percentage of apoptotic and necrotic cells were determined after prolonged exposure to thymoquinone to support the earlier findings. This was followed by cell cycle analysis to determine the cellular response due to long-term thymoquinone exposure.

MATERIALS AND METHODS

Cell line and growth media

Human mammary breast cancer epithelial cells line, MCF-7, (HTB-22) was purchased from American Type Culture Collection (ATCC) in this study. Cell were grown in Roswell Park Memorial Institute (RPMI) 1640 (Invitrogen, Gibco, US) containing L-glutamine was supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Gibco, US) and 1 unit penicillin/streptomycin (Hyclon) was used as medium for MCF-7 cell line. Cells were seeded at a density of $2–4 \times 10^4$ cells/well in fresh complete -medium and incubated overnight at 37°C in a humidified 5% CO_2 atmosphere for 24 hour before treatment. The cells were sub-cultured by washing twice with PBS and tripsinization of adherent cells was done using 1.0 ml 25% Trypsin with 0.53 mM EDTA solution for 25 cm^2 flasks until reaching 80–90% confluence. Thymoquinone was purchased from Sigma-Aldrich and dissolved in distilled water to 1 mM concentration. The thymoquinone concentrations prepared included 25, 50, and 100 μM thymoquinone solutions.

Cytotoxicity assay

Cells were seeded at 5×10^3 per well in 96-well plate with 200 μl of fresh complete medium for 24 h before treatment. Fresh complete medium (200 μl) containing thymoquinone (25, 50 and 100 μM) was added into each well to treat cells for 0, 24, 48, and 72 hours. The Cell Titer 96 AQ_{ueous}. Non-Radioactive Cell Proliferation Assay Kit (Promega, USA) was used according to manufacturer's protocol to determine the number of viable cells after the treatment with thymoquinone. Absorbance was read at 490 nm using Eliza microplate reader (Platos, R496). Percentage of cell proliferation was determined using the following formula:

$$\frac{\text{Absorbance test well}}{\text{Absorbance control well}} \times 100$$

Viability assay

Interaction of trypan blue dye with the membrane damaged cells was considered as the principle of this assay. At least, 1×10^5 cells/well were plated in 6-well plates and incubated at 37 °C until cells adhered to flask surface within overnight. The MCF-7 cells were treated with thymoquinone from 25, 50, and 100 μM, incubated at different time -points. The treated cells were trypsinized and centrifuged at $300 \times g$ for 10 min and re-suspended in 200 μl of phosphate buffered saline (PBS). The total of suspension cells was mixed with 200 μl of trypan blue staining solution for 5 min at room temperature. The cells were loaded into hemocytometer and cells that exhibited dye uptake were counted under an inverted light microscope.

Apoptosis assay

Annexin V-FITC Apoptosis Detection Kit (BD Bioscience, USA) was used in order to determine the percentage of treated and untreated MCF-7 breast cancer cells that were actively undergoing apoptosis after treatment with various concentrations of thymoquinone (25, 50, and 100 μM). The procedure was done according to the manufacturer's protocol. The analysis was done within an hour. The percentage of apoptotic cells was determined using flow cytometry (BD FACSCanto II) and plotted using FACS Diva program. Each experiment was repeated at least three times.

Cell cycle assay

The Cycletest Plus DNA Reagent Kit (BD Bioscience, USA) was used to distinguish distribution of cells between cell-cycle phases before treatment and after treatment with thymoquinone. The protocol recommended by BD Bioscience was followed and flow cytometry (FACSCanto II, BD Bioscience) was used to run samples. The results obtained were analyzed using the ModFit software.

Statistical analysis

Each experiment was done in triplicates. Analysis of results was done using *t*-test. All values were expressed as means ± standard deviation (SD). The results with $p < 0.05$ were considered as significant.

RESULTS

The growth of MCF-7 cells was studied with treatment of thymoquinone at various concentrations ranging from 25,

50, 100, 200 to 300 μM. The results showed that there was significant difference between untreated cells with treated cells at various concentrations using MTS assay [Figure 1a]. Cytotoxicity effect of thymoquinone was seen even at lower thymoquinone concentration. Further time -point effect on cell proliferation was carried out to compare the differences at 24, 48, and 72 h. The 50% inhibitory concentrations (IC_{50}) were 37, 23, and 27 μM, respectively. It was seen that prolonged exposure to thymoquinone sensitized the cells toward lower population. The overall IC_{50} value of thymoquinone was approximately 25 μM for MCF-7 cell line [Figure 1b].

The presence of viable cells was determined by trypan blue exclusion assay. The percentage of viable cells was 90% either after 12 or 24 h treatment with 25 μM. Similarly, treatment with 50 μM thymoquinone for 24 h was found to have 80% viable cells. Prolonged exposure to thymoquinone until 72 h caused significant reduction in viable cell percentage in all the concentrations studied [Figure 2].

The apoptotic effect was revealed that the numbers of viable cells after 24 h treatment with thymoquinone significantly decrease and percentage of apoptotic and necrotic cells were found to increase due to the raising in concentration of treatment. The data were presented in Figure 3.

The flow cytometry analysis on apoptotic cells distribution showed the percentage of viable cells in control did not differ between 12 h and 24 h treatment. We found that after 12 h treatment, the number of viable cells declined to 41% with 100 μM compared to 60-70% viable cells with the other two concentrations. At 24 h the viable cells further dropped to 7% with 100 μM but remain within 60-70% for the other concentrations. Early apoptotic cell percentage at 12 h was higher with 25 μM and 50 μM compared to control and 100 μM. Further exposure to 24 h caused percentage of early apoptotic cells to increase in those treated with 50 μM and 100 μM compared to control. The percentage of late apoptotic cells was highest when treated with 100 μM either for 12 h or 24 h. It was found to be within 24–26% with 50 μM in both treatment durations. Whereas, the percentage of late apoptotic cells -increased from 16% to 24% at 12 h and 24 h, respectively. The 100

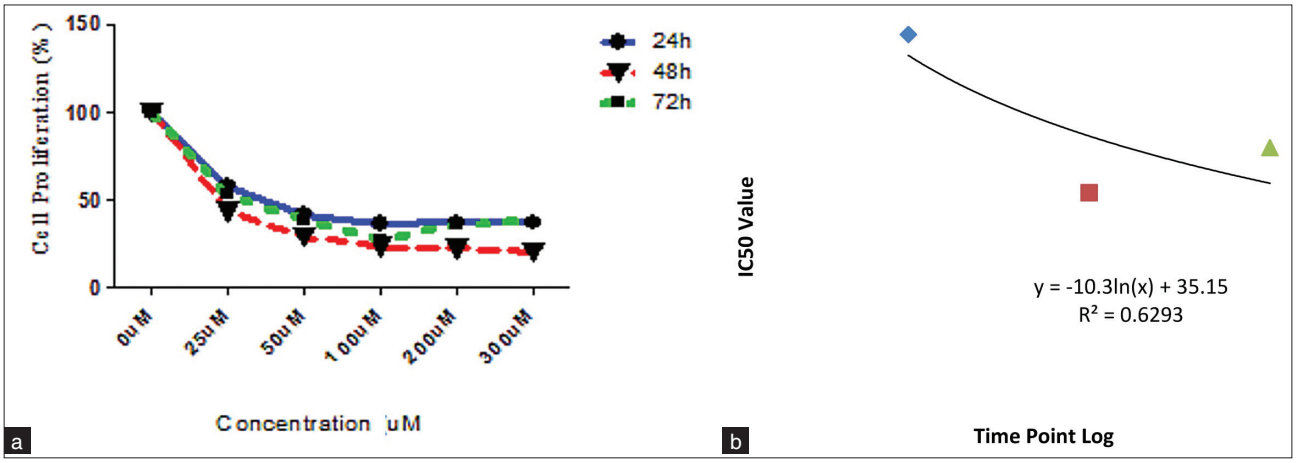

Figure 1: Percentage of cell proliferation doing MTS assay for thymoquinone: Treated MCF-7 cells for 24 h, 48 h, and 72 h was obtained by one-way analysis of variance (ANOVA) with PASW 18, and p-value ≤ 0.05. (b) The constant IC50 value of thymoquinone was 25μM for MCF-7 breast cancer cell line. The experiments were done in quadruplicate for different time manner

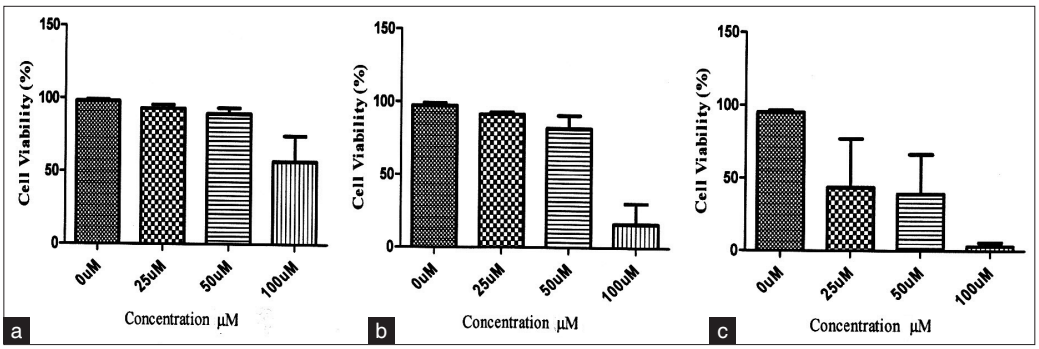

Figure 2: Percentage of cell viability assay with trypan blue extraction test at different concentration (0, 25, 50, and 100μM) after (a) 12 h (b) 24 h (c) 72 h. Mean ± SEM of three independent experiments using GraphPad Prism 5

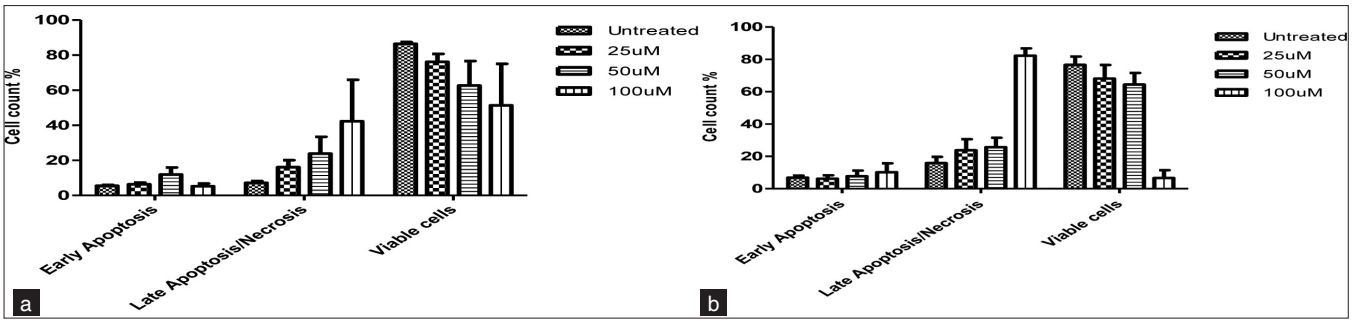

Figure 3: Values are mean ± SEM of three independent experiments, p-value ≤ 0.05 of cell counts at different stages of apoptosis assay obtained by flow cytometry (FACSCanto II) after (a) 12 h and (b) 24 h exposure of MCF-7 breast cancer cells with various thymoquinone concentrations (0, 25, 50 and 100 μM)

μM concentration caused 82.3% cells to be in late apoptotic stage. Necrotic cell percentage was almost similar for 25 μM and 50 μM at both durations studied. However, it was more than control after 24 h treatment.

Comparison was done to determine the difference between tamoxifen and thymoquinone towards apoptosis. This study showed that thymoquinone induced more cells to move into early apoptotic, late apoptotic and necrotic stages.

We selected three different concentrations less than 100 μM thymoquinone from proliferation assay finding for subsequent experiment. Cell cycle assay was performed to particularly identify phases that were regulated by thymoquinone. The results showed that with 25 μM thymoquinone, the S phase was arrested at every time -point studied significantly (p=0.009). In addition, sub-G_1 was noted to be slightly elevated after 12 h. Gradual increase in thymoquinone to 50 μM caused G_2 phase arrest at each time-point was studied, although non-significant. At highest concentration, 100 μM there was G_0/G_1 phase arrest beginning within 12-24 h treatments. Prolonging treatment duration to more than 24 h showed Sub-G_1 arrest [Figure 4]. This showed specificity in thymoquinone action towards cell cycle arrest which depended on its concentration.

The phase arrested by tamoxifen was also determined in this study. The findings showed S phase arrest following 12 h treatment. However, following extended duration to 24 h, the arrest occurred in G_0/G_1 phase. Comparison between tamoxifen and thymoquinone showed that both caused S phase arrest at low dose and G_0/G_1 phase arrest at higher concentration.

DISCUSSION

Cancer drug resistance has been reported to develop following treatment. Therefore, there is need to explore into new anti-cancer drugs. There has been increasing literature on medicinal properties of thymoquinone over the years. This study aims to provide further information to anti-cancer features of thymoquinone. It comprised investigation into extended treatment duration and observation on molecular processes. The initial stage involved study on breast cancer cell proliferation and exposure to wide range of thymoquinone concentration. The long-term treatment with thymoquinone was studied for 72 h in breast cancer cell line MCF-7. Cells were found to be more sensitized to 27 μM thymoquinone following 72 h. The IC_{50} previously reported was 32 μM with 48 h treatment.[8] The proliferation of MCF-7 cell line was regulated by dose of thymoquinone and in overall 50% cell death occured with 25 μM thymoquinone. The prolonged treatment at various time points with specific concentration showed substantial cell death particularly at 72 h. Previous findings on effect of thymoquinone in other cell lines showed similar proliferation inhibition with 10, 25, and 50 μM with increase in sub-G_1 phase within 24 h. However, study was short-term exposure to 24 hours on primary effusion lymphoma cell line (PEL), rat mammary carcinoma cell line (BC1), and lymphoma cell line (BC3).[9]

Our findings showed that the percentage of early apoptotic cells was similar to those reported by Shoieb *et al.*[9] However, we report here the percentage of late apoptotic cell which was higher than early apoptotic cells. This indicated the specificity of thymoquinone to breast cancer cells of human origin rather than canine cells studied by Shoieb *et al.*[9]

Further analysis on cell cycle was carried out for prolonged treatment of 72 h. Thymoquinone was found to inhibit molecules which are involved in the S phase with 25 μM thymoquinone concentration. Furthermore, sub-G_1 phase arrest occurred with either 25 μM when treated for 12 h. It was previously reported more than 60 μM thymoquinone led to sub-G_1 arrest within 12–48 h. This indicated cell growth inhibition could be sustained with minimal dose 25 μM thymoquinone in an extended duration of treatment. It has been reported that effect of thymoquinone on

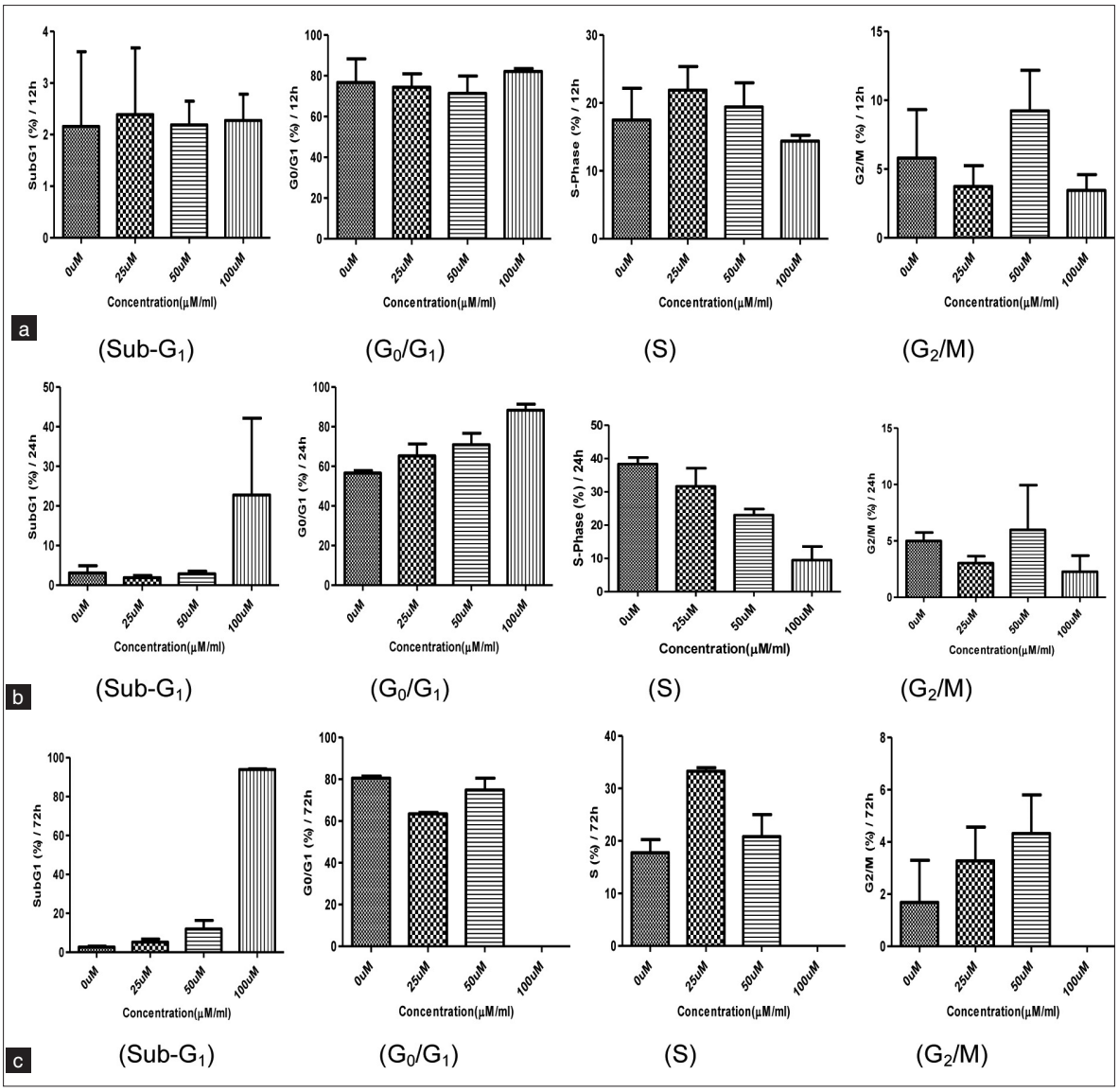

Figure 4: Values are mean ± SEM of three independent experiments, *p*-value ≤ 0.05 of cell counts at different phases of cell cycle (a) 12 h (b) 24 h and (c) 72 h

proliferation of small cell lung cancer cells was found to wane with time with less activity observed at 48 and 72 h, suggesting more frequent dosing may be required to demonstrate a sustained effect.[10]

Shoieb *et al.*,[9] reported that canine osteosarcoma cell line COS31 treated with 100 μM thymoquinone showed G1 phase arrest after 24 and 48 h but not within 12 h of treatment. The same concentration was found to arrest G_0/G_1 phase in this study. However, the accumulation of G_1 phase begun earlier at 12 h in human breast cancer cell line. Thus, indicating thymoquinone interacts with molecules related to G_0/G_1 phase such as cyclin D and cyclin kinases.[11] The activities of these molecules have been inhibited as a result of 50 μM thymoquinone treatment for 72 h. Cyclin D1 is a protein which encoded by the CCND1 gene in humans which was responsible for the transformation of cells from G_1 to S phase of the cell cycle.[12] Over expression of cyclin D1 has been shown in a variety of tumors.[13] It has been stated that activation of cyclin-dependent kinase 4 (CDK4) causes over expression of cyclin D1 gene.[14] Accumulation of CDK4 occurs following increasing in its activity due to protein expression of cyclin D1 and they form a complex cyclin D1-CDK4.[15] Cyclin D1 is one of the most oncogenes which are over expressed in breast cancer.[16] It is overexpressed in almost 50% of human breast cancers especially in 30–60% of primary ductal adenocarcinomas and is expressed early in the disease process.[17] Eventually inhibition of cyclin D1 expression or function inhibits entry into S phase.[18] Sub-G_1 phase arrest appeared within 24 h treatment with 100 μM and continued to increase after 72 h treatment.

Comparison of our findings to study with doxorubicin-resistant MCF-7 cell line showed similar results. Whereby, G_2 phase arrest was seen following 50 μM thymoquinone treatment at each time-point studied. The cell cycle phases regulated by tamoxifen were also studied to compare its mechanism of action with thymoquinone in MCF-7 cell lines. It showed inhibition of cell cycle at S phase. These findings suggest that tamoxifen and thymoquinone have similarity in leading to cell cycle arrest particularly with use of minimal dose, that is, 25 μM thymoquinone. These findings suggested that thymoquinone had specific targeting phases during cell cycle which rely on its concentration.

Thymoquinone induces apoptosis through modulation of multiple targets and hence has been introduced as a potential phytochemical that could be useful for apoptosis to happen among various cancer cells. It has been stated that thymoquinone causes apoptosis in cells by p53-dependent,[19] and p53-independent pathway[20] and drug-induced apoptosis is associated with the activation of caspases,[20,21] increases in p53 expression,[22] up-regulation of anti-apoptotic Bcl-2[11,23] and decrease in cyclins B1 and D1.[11] Recent studies have shown that NF-κB is a convincing target of thymoquinone which was associated with cell growth inhibition and induction of apoptosis in cancer cells.[24] The anti-tumor activity or cell growth inhibition could in part be due to the effect of thymoquinone on cell cycle.[24] Further investigations need to be done to know particularly its exact targeting genes which are related to apoptosis and cell cycle phases.

CONCLUSION

Minimal dose of thymoquinone showed considerable inhibition of breast cancer cell with long-term treatment. The presence of Sub-G_1 peak indicated thymoquinone could halt cancer cell proliferation with 25 μM. Further investigations need to be conducted to relate thymoquinone to its anti-cancer properties and synergistic effect when combined with current anti-cancer drugs.

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