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## Cytotoxicity of Thymoquinone (TQ) from *Nigella sativa* Towards Human Cervical Carcinoma Cells (HeLa)

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### ABSTRACT

Thymoquinone (TQ), the bioactive constituent of the volatile oil of *Nigella sativa*, has been shown to exert anti-inflammatory, anti-oxidant and anti-neoplastic effects both *in vitro* and *in vivo*. In this study, the cytotoxicity of TQ was evaluated on human cervical carcinoma cells (HeLa). Results showed that TQ exhibited cytotoxic and anti-proliferative activities towards the cells with IC<sub>50</sub> value of 2.80±0.10mg/ml and 5.37±0.12mg/ml after 72 hours incubation time as being detected by trypan blue dye exclusion test and MTT assay, respectively. Significant decrease in the percentage of cell viability was observed after the treatment with 1.0, 3.0, 10 and 30mg/ml (p<0.05) indicating that TQ induced cytotoxicity in a dose-dependent manner. IC<sub>50</sub> values determined by the trypan blue dye exclusion test were significantly decreased from 5.93±0.81mg/ml (24 hours) to 2.80±0.10mg/ml (72 hours) suggesting that TQ induced cytotoxicity in a time-dependent manner. HeLa cells treated with TQ for 72 hours showed a significant decrease in cell population at G<sub>0</sub>/G<sub>1</sub> phase and significant increase of cell population at sub-G<sub>1</sub> phase at 6.0, 10 and 30mg/ml (p<0.05), suggesting that TQ inhibited cell proliferation by induction of apoptosis in the cells. Expression of p53 detected by using the Human p53 ELISA showed that HeLa cells incubated with 10mg/ml of TQ for 72 hours resulted in significant up-regulation of the expression of the protein (p<0.05) compared to the control untreated sample. It is concluded that TQ was cytotoxic towards HeLa cells in a dose- and time-dependent manner and induced apoptosis via p53-dependent pathway.

**Key words:** Thymoquinone (TQ); Cervical cancer; Cell cycle arrest; Apoptosis; p53

### 1. INTRODUCTION

Cancer is defined as an abnormal mass of tissue which its growth is uncoordinated, persists in excessive manner after the cessation of the stimuli which evoked the change [1]. In 2007, cancer was the second leading cause of death in economically developed countries (following heart diseases) and the third leading cause of death in developing countries (following heart diseases and diarrhoeal diseases) [2]. Cervical cancer is malignant tumour of cervix, second most common and aggressive malignancy in female [3]. Effective prevention and screening programs in developed countries have resulted in a 75% decrease in the incidence and mortality of cervical cancer over the past 50 years [4]. However, in less developed countries where prevention and screening programs are not available, cervical cancer continues to be one of the most common causes of cancer-related morbidity and mortality among women [5].

*Nigella sativa* L., commonly known as black cumin seed, belongs to the botanical family of *Ranunculaceae*. It has diverse biological effects that include anti-microbial, anti-viral, anti-helminthic, anti-inflammatory and immunomodulatory activities. In addition, the volatile oil of *Nigella sativa* has efficacy as a chemotherapeutic agent.

The chemotherapeutic and chemoprotective effects of *N. sativa* extract may be due to quinones that include thymoquinone (TQ) and dithymoquinone (DIM) that are present in the oil of this seed [6]. Thymoquinone (TQ), the bioactive constituent of the volatile oil of black cumin seed, has been shown to exert anti-inflammatory, anti-oxidant and anti-neoplastic effects both *in vitro* and *in vivo* [7].

Although has been documented as having the anti-tumour and chemoprotective effects in several cell lines, the cytotoxicity of thymoquinone (TQ) from *Nigella sativa* towards human cervical carcinoma cells (HeLa) and its effects on the regulation and expression of p53 have not been investigated yet.

In this study, the cytotoxic effect of thymoquinone (TQ) from *Nigella sativa* was determined. The mode of cell death involved and its effects on the regulation of p53 activity were also investigated.

### 2 Materials and methods

#### 2.1. Chemicals

The thymoquinone, TQ (99.9%) was kindly provided by



Professor Dr. Maznah Ismail from Laboratory of Molecular Biomedicine in Institute of Bioscience, Universiti Putra Malaysia.

## 2.2. Cell culture

Human cervical carcinoma cell line (HeLa) was purchased from the American Type Culture Collection (ATCC), USA. The cells were maintained in monolayer cultures in RPMI 1640 culture medium (PAA Laboratories, Austria) with 10% foetal bovine serum (FBS) (PAA Laboratories, Austria) and 1% antibiotics (100IU/ml penicillin and 100mg/ml streptomycin) (PAA Laboratories, Austria). The cells were grown at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

## 2.3. Trypan Blue Exclusion Test

The trypan blue exclusion assay is based on the interaction of trypan blue dye with the cell if the cell membrane is damaged [8]. HeLa cells were treated with TQ ranging from 0.3 to 30mg/ml and incubated for 24, 48 and 72 hours. Control (without TQ) was also included. Cell suspensions 0.4% trypan blue (Sigma Chemicals, USA) were mixed gently at the ratio of 1:1 and the number of viable and dead cell were determined with a haemocytometer under an inverted light microscope. The percentage of viability was calculated using the formula (unstained cell in each well/total viable cells in control well) × 100%. The concentration that gave 50% inhibition of cell viability (IC<sub>50</sub>) was determined from the dose-response graph.

## 2.4. MTT Assay

In the MTT assay, cell respiration (an indicator of cell viability) was assessed by the mitochondrial-dependent reduction of MTT to formazan [9]. HeLa cells were treated with TQ ranging from 0.3 to 30mg/ml and incubated at for 24, 48 and 72 hours. Control (without TQ) was also included. MTT solution (0.5 mg/ml) (Sigma Chemicals, USA) was added and incubated for 4 hours. The dark-blue formazan crystals formed in intact cells were dissolved in DMSO, and the absorbance at 570 nm was measured with a microplate reader. The percent viability of the treated cells was calculated using the formula (A<sub>570</sub> of treated cells/A<sub>570</sub> of untreated cells) × 100%. The concentration that gave 50% inhibition of cell viability (IC<sub>50</sub>) was determined from the dose-response graph.

## 2.5. Cell Morphological Studies

The TQ treated (3.0, 10, and 30mg/ml) and untreated HeLa cells were grown on 6-well plate for 72 hours. The changes in cell morphology were observed under an inverted light microscope (Olympus, Japan).

## 2.6. Cell cycle analysis

The cells were treated with TQ (6.0, 10 and 30mg/ml) for 72 hours. Control (without TQ) was also included. Cells were harvested by centrifugation at 1200 rpm for 10 min at 4°C. The supernatant was discarded. The pellet was washed twice with ice-cold PBS and fixed with ice-cold 70% ethanol and incubated at -20°C for 2 hours. Cells were again centrifuged at 1200 rpm, for 10 min at 4°C, and supernatant

(ethanol) was discarded. The cells were washed with ice-cold PBS and centrifuged at 1200 rpm for 10 min at 4°C. The supernatants were withdrawn. The pellet was resuspended in a solution containing with 425ml PBS, 25ml PI (1mg/ml) (Sigma Chemicals, USA) and 50ml RNase A (1mg/ml) (Sigma Chemicals, USA) and incubated at 4°C for 20 minutes. DNA content was analyzed by FACScan flowcytometer (CyAn ADP, USA) and the population of cells in each cell-cycle phase was determined by using the Submit v3.4 software (CyAn ADP, USA).

## 2.7. Expression of p53

The level of p53 was determined by using the Human p53 ELISA kit (Bender MedSystem, Austria). The activated p53 from the cell lysate specifically bound to an oligonucleotide containing p53 consensus binding site. The oligonucleotide was immobilized on the 96-well plate and detected by primary antibody. Horseradish peroxidase (HRP) conjugated secondary antibody provided sensitive colorimetric. The absorbance or optical density (OD) of each well was read at 450nm by using ELISA microplate reader. The level of p53 was determined from the standard graph.

## 2.8. Statistical Analysis

All the data were expressed in mean ± standard error of mean (SEM). The data were analysed with 1 way analysis of variance (ANOVA) using Statistical Package for Social Science (SPSS) version 14.0. In all statistical analysis, probability of p<0.05 was considered significant.

## 3. Results

### 3.1. Trypan Blue Exclusion Test and MTT Assay

In the dose-response curves obtained from the trypan blue dye exclusion test and MTT assay, a significant decrease (p<0.05) in the percentage of cell viability was observed after the HeLa cells were treated with TQ at 1.0, 3.0, 10 and 30mg/ml for 24, 48 and 72 hours incubation time. Both assays indicated that the cytotoxicity of TQ was concentration- and time-dependent. TQ (30mg/ml) showed the strongest cytotoxicity towards the cells. The IC<sub>50</sub> values determined by the trypan blue exclusion test and MTT assay were shown in Table 1.

**Table 1: IC<sub>50</sub> values of TQ towards HeLa cells at various incubation times as determined by using the trypan blue dye exclusion method and MTT assay**

Incubation Time (hours)	IC <sub>50</sub> (mg/ml)	
	Trypan Blue	MTT Assay
24	5.93 ± 0.81 <sup>a</sup>	9.07 ± 0.62 <sup>1</sup>
48	3.83 ± 0.35 <sup>b</sup>	6.30 ± 0.10 <sup>2</sup>
72	2.80 ± 0.10 <sup>c</sup>	5.37 ± 0.12 <sup>2</sup>

Values were the means of three replicate samples (n=3) and the data were presented as mean ± SEM. a, b, c, 1 and 2 were significantly different (p<0.05)

### 3.1. Cell Morphological Studies

The morphological changes by TQ-treated cells for 72 hours were observed under an inverted light microscope as illustrated in

Fig. 1. Detached cells at high concentration of TQ (10 and 30mg/ml) indicated that TQ induced cell death.

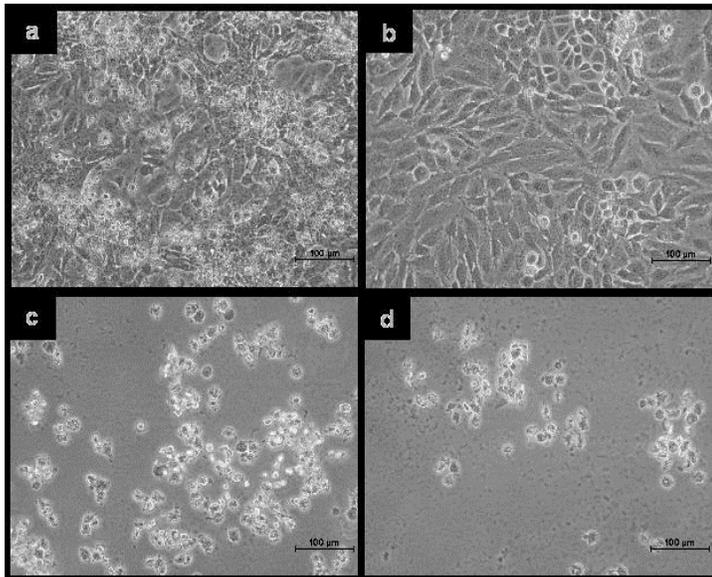


Fig. 1. Morphological changes of HeLa cells treated with TQ for 72 hours. (a) Control, (b) 3.0mg/ml, (c) 10mg/ml, and (d) 30mg/ml. (magnification 200x)

### 3.1. Cell Cycle Analysis

The induction of apoptosis in TQ-treated HeLa cells were determined by flow cytometric analysis of DNA content. The sub-G<sub>1</sub> population indicated apoptotic, and loss of DNA is a typical feature of apoptotic cells [10]. The population of HeLa cells in sub-G<sub>1</sub> phase significantly increased ( $p < 0.05$ ) after the cells treated with TQ (6.0, 10, 30µg/ml) for 72 hours as compared to the control (Fig. 2). On the other hand, the population of cells in G<sub>0</sub>/G<sub>1</sub> phase decrease significantly ( $p < 0.05$ ) after 72 hours after treatment with TQ (6.0, 10, 30µg/ml) (Table 2). These results suggested TQ inhibited cell proliferation by induction of apoptosis in the cells in a dose-dependent manner.

### 3.1. Expression of p53

The effect of TQ on the expression p53 was determined by using the human p53 ELISA technique. Table 3 shows the increase in p53 expression in HeLa cells with the increase of the concentration of TQ after 72 hours treatment. High concentration of TQ (10mg/ml) induced the p53 expression (Table 3).

## 4. Discussion

Over the recent years, there has been growing interest in naturally occurring phytochemical compounds with anti-cancer potential, because they are relatively non-toxic, inexpensive and available in an ingestive form. More than 25% of drugs used during the last 20 years are directly derived from plants, while the other 25% are chemically altered natural products [11]. Many investigations are being carried out worldwide to discover naturally occurring compound which can suppress or prevent the process of carcinogenesis [12].

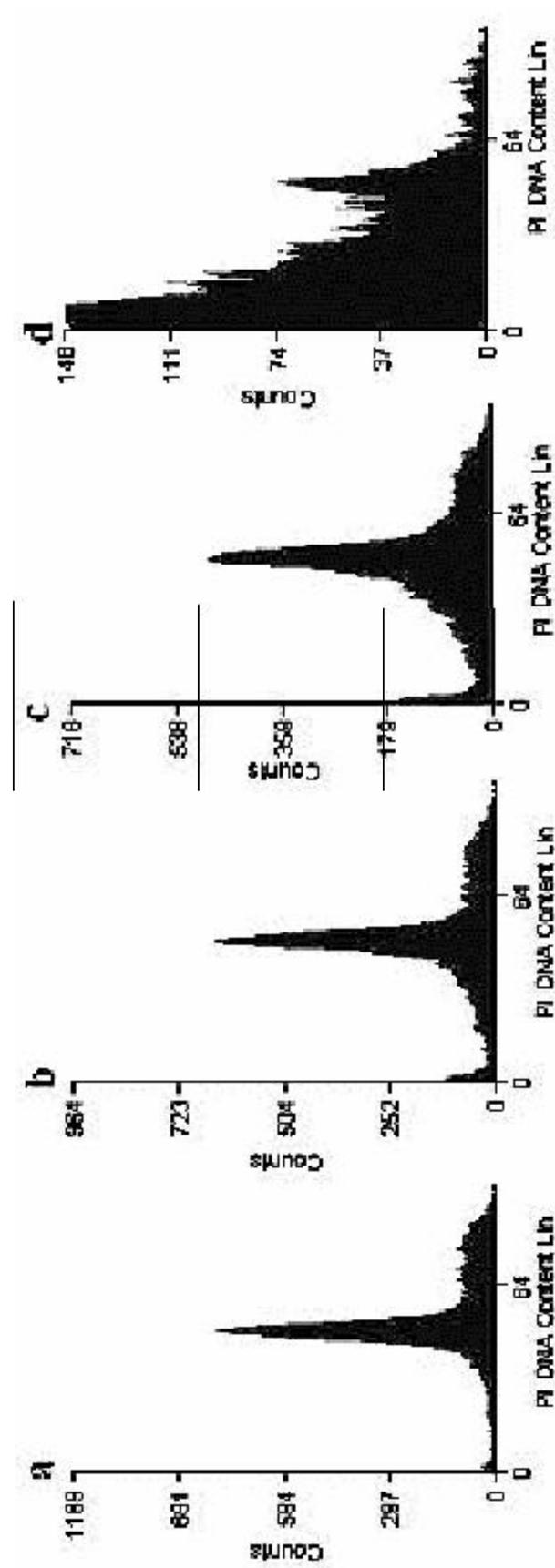


Fig. 2. Cell cycle analysis of HeLa cells treated with TQ for 72 hours. (a) Control, (b) 6.0µg/ml (c) 10µg/ml (d) 30µg/ml. TQ induced apoptosis (increasing sub-G<sub>1</sub> population) in HeLa cells.

**Table 2: Dose-dependent effects of TQ on the cell cycle of HeLa cells after 72 hours treatment.**

Concentration (µg/ml)	Apoptotic Cell (%)		Non-apoptotic Cells (%)	
	sub-G <sub>1</sub>	G <sub>0</sub> /G <sub>1</sub>	S	G <sub>2</sub> /M
Control	11.75 ± 0.32	65.01 ± 1.39	9.63 ± 1.49	15.57 ± 0.02
6.0	25.39 ± 2.63*	56.56 ± 0.29*	9.45 ± 0.20	11.86 ± 2.47
10	36.40 ± 0.59*	49.94 ± 1.11*	7.52 ± 0.53	9.07 ± 1.38*
30	91.92 ± 1.05*	5.65 ± 1.17*	1.75 ± 0.05*	1.22 ± 0.17*

Values were the means of duplicate samples (n=2). Data were presented as mean ± SEM. \* were significantly different from control (p<0.05).

**Table 3: Effects of TQ on the expression of p53 in HeLa cell after 72 hours treatment.**

Concentration(µg/ml)	Expression of p53
Control	16.73 ± 0.50 <sup>a</sup>
1.0	18.62 ± 1.26 <sup>a</sup>
3.0	19.70 ± 1.11 <sup>a</sup>
6.0	19.72 ± 1.83 <sup>a</sup>
10	26.63 ± 3.01 <sup>b</sup>

Values were the means of three replicate samples (n=3). Data were presented as mean ± SEM. a and b were significantly different (p<0.05).

Researches on plants help in the identification with anti-cancer activity that has been subsequently developed into clinical useful drugs [13].

The cytotoxicity of thymoquinone (TQ) was tested and determined by using the trypan blue dye exclusion method and MTT assay. Both the tests indicated that human cervical carcinoma cells (HeLa) were sensitive towards TQ. The trypan blue exclusion test was used to indicate cytotoxicity, where dead cells take up the blue stain of trypan blue. It was a standardized and accepted test to monitor possible cytotoxic effects although it does not give any detailed information other than membrane stability of the cell [14]. Figure 1 shows the percentage of viability of HeLa cells decrease with the increasing concentration of TQ and incubation time while table 1 shows that the IC<sub>50</sub> values determined by both trypan blue dye exclusion test and MTT assay decrease with the increasing incubation time. In short, cytotoxicity of TQ towards HeLa cells was dose- and time-dependent.

The IC<sub>50</sub> values determined by the trypan blue dye exclusion test were different from the MTT assay. The main reason was both assays have different principle. The trypan blue dye exclusion test was based on the principle that in cells with compromised plasma membranes, the trypan blue dye penetrates into the cell and strongly stains the nucleus blue, which was indicated the cell viability directly [15]. On the other hand, MTT assay measured the metabolism of 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide to form formazan precipitate by mitochondrial dehydrogenase which only present in viable cells. Formazan accumulation directly reflected

mitochondrial activity, which was an indirect measure of cell viability [9].

TQ inhibited the proliferation of HeLa cells through a mechanism that involves apoptosis. As compared to control, population of HeLa cells in the sub-G<sub>1</sub> group was significantly increased after HeLa cells were treated with TQ for 72 hours. Figure 3 and table 2 reveals that TQ efficiently induced apoptosis of HeLa cells in a dose-dependent manner.

Induction of cell apoptosis is a useful approach in cancer therapies [16]. Apoptosis is a cell suicide program that has been conserved through the evolution. It leads to a cell death through a tightly regulated process resulting in the removal of damaged or unwanted tissue [17]. Impairment of the apoptotic mechanism ultimately generates a pathological condition that includes developmental defects like, autoimmune diseases, neurodegeneration or cancerous neoplasia [18]. The morphological changes of apoptosis include membrane blebbing, cell shrinkage, chromatin condensation, DNA fragmentation and formation of apoptotic bodies [19].

TQ-induced apoptosis in HeLa cells was accompanied by up-regulating the expression of p53. Table 3 shows that TQ up-regulated the expression of p53 in a dose-dependent manner when treated with 1.0, 3.0, 6.0 and 10mg/ml of TQ. The finding suggested that apoptosis of HeLa cells induced by TQ was p53-mediated apoptotic pathway. The finding was supported with other result indicating that TQ up-regulated the expression of p53 which mediated the apoptosis in human colon cancer cells [7].

The p53 protein acts as a powerful transcription factor and it binds to as many as 300 different promoter elements in the human genome [20], involving in the control of cell cycle, programmed cell death (apoptosis), senescence, differentiation, DNA replication, DNA repair and maintenance of genomic stability [21]. Once damage occurs, p53 is expressed and undergoes phosphorylation, dephosphorylation and acetylation into its active isoforms. Active p53 acts as a transcription factor and as a structural component of protein complexes. Under the regulatory influence of p53, cells are halted at the various p53-regulated checkpoints to allow the removal or repair of DNA damage. When DNA damage is not repaired, the cells are then targeted for death [22].

The present study shows that thymoquinone (TQ) was cytotoxic towards the HeLa cells in a dose-, and time-dependent manner. In addition, TQ induced apoptosis in HeLa cells by the



upregulation of the expression of p53. Therefore, the study suggested TQ induced apoptosis via p53 dependent pathway in HeLa cells.

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