RESEARCH ARTICLE

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Regulation of *NF-κB* Expression by Thymoquinone; A Role in Regulating Pro-Inflammatory Cytokines and Programmed Cell Death in Hepatic Cancer Cells

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Abstract

Background: The miracle herb Nigella sativa (N. sativa) is a member of the Ranunculaceae family that possesses many properties, such as antioxidant, anticancer, analgesic, antibacterial, and anti-inflammatory. Thymoquinone (TQ) is the primary ingredient that makes up N. sativa, which is responsible for its many properties. So, our research focused on the biological role of TQ and its anticancer activities. **Methods:** A wide range of TQ concentrations (50µg/µl, 25µg/µl, and 12.5µg µl) was prepared and evaluated for their potential regulatory role in cell lines of hepatocellular carcinoma (HepG2 cell line) compared with normal hepatocytes cells, untreated and DMSO-treated cells. **Results:** The more significant level of LDH obtained after TQ treatment compared to untreated cells provides evidence of the cytotoxic effects of TQ on HepG2 cells. Notably, the normal hepatocyte cells subjected to the same concentrations of TQ showed neglected influence in cell viability rate, indicating the selective regulatory role of TQ in cancer cell proliferation. Interestingly, as a critical mediator of malignancy transformation, the nuclear factor-kappa B expression level (*NF-κB*) significantly decreased in a time and dose-dependent manner of TQ treatment. Furthermore, we investigated whether TQ regulates the expression of deleted liver cancer 1 (*DLC1*) and Caspase 3 (*Casp3*). Notably, the treatment with TQ showed increased expression levels of *DLC1* and *Casp3* upon treatment. TQ extract sufficiently mediated the secretion of the released pro-inflammatory cytokines from treated cells. This regulation of released cytokines by TQ may affect the activation of *NF-κB* in treated cells. **Conclusion:** These results indicate that TQ mediates the activation of *Casp3*, *DLC1*, and *NF-κB*, providing a new function of TQ in treating hepatocellular carcinoma (HCC).

Keywords: Thymoquinone- Nuclear factor-kappa B- Cell death- HCC- Normal hepatocytes

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Introduction

Cancer is categorized as a fatal disease that supports aberrant cell expansion with the possibility of metastasis; the different characteristics of cancer cells are resistance to growth-inhibiting signals, evasion of apoptosis, reproductive capacity unaffected by telomeres, sustained angiogenesis, metastasis, tissue invasion, and improper metabolic processes, it is forming a significant challenge to overcome it (Fekry et al., 2022). HCC is an aggressive malignancy with early vascular invasion and metastases (Wang et al., 2019; Elimam et al., 2020). The critical cellular change responsible for the cancer phenotype is dysregulated cell proliferation, which is initiated and maintained by a small number of pathways, according to advances in cancer biology (Roberts and Gores, 2005; Mohamed et al., 2022). HCC is distinguished by a mutation in tumor-suppressor gene p53, the most frequently mutated gene in HCC (Roberts and Gores, 2005). Agents targeting the receptor tyrosine kinase pathways, the Wnt/b-catenin signaling pathway, the ubiquitin/proteasome degradation pathway, the epigenetic DNA methylation, and histone deacetylation pathways, the PI3 kinase/AKT/mTOR pathway, the angiogenic pathways, and telomerase are some of the new treatments in development that target several of these critical pathways (Elawdan et al., 2022). Several of these strategies have great potential to improve the long-term prognosis for individuals with advanced HCC (Roberts and Gores, 2005). In clinical trials, several drugs have been examined for the treatment of HCC; these include common chemotherapeutic drugs like doxorubicin, cisplatin, and 5-fluorouracil; hormones like tamoxifen, megestrol, somatostatin, and analogs of statins; and immunological regulators like interferon and a-thymosin. These substances have both been tested singly and in combination. None of these drugs or formulations have been shown to be significantly more effective in extensive randomized studies despite a wealth of evidence of success in a small series of patients. Underlying liver conditions such acute hepatitis, impaired liver synthesis,

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splenomegaly, and cirrhosis can occasionally make it difficult to treat advanced HCC. The ability of patients to undergo surgery, tolerate chemotherapy regimens, and participate in clinical trials of novel chemotherapy drugs is typically constrained by the severity of the underlying liver disease (Roberts and Gores, 2005; Chen et al., 2020).

Ranunculaceae family member Nigella sativa is a miracle herb that possesses a lot of properties as an antioxidant, anticancer, spasmolytic, immunomodulator, analgesic, antibacterial, anti-inflammatory, hepato-protective and gastro-protective (Guan et al., 2006; Majdalawieh et al., 2017). The primary ingredient (up to 50%) in the essential oil of the globally popular medicinal herb Nigella sativa is TQ. A bioactive substance known as TQ (2-methyl-5-isopropyl-1,4-benzoquinone, or TQ) is obtained from black seeds in Eastern Africa, the Middle East, and West Asia (Qin et al., 2020). TQ, which makes up a large portion of the black seeds of N. sativa, has also been shown to have anti-inflammatory and cancer-fighting properties (Almajali et al., 2021; Noorbakhsh et al., 2018). TQ has many actions against many kinds of cancers. For example, in Acute lymphoblastic leukemia, it activates ROS and HSP70, downregulates Bcl-2, increases Bax, and activates caspases 3 and 8, all of which are necessary to cause apoptosis, also in bladder cancer; it decreases PI3K/Akt signaling and mTOR activity, in breast cancer, PI3K/Akt signaling is obstructed, which encourages G(1) arrest; also p53 is upregulated, NF-B is inhibited, the production of ROS downregulates p38 MAPK, TWIST1 expression is inhibited, and cancer cell metastasis is controlled by controlling EMT (Khan et al., 2017). There is a lot of research that confirms the TQ anticancer effect against all types of cancer by different mechanisms (Majdalawieh et al., 2017; Almajali et al., 2021; Yi et al., 2008; Khan et al., 2017). Studies focus on the anticancer activity of TQ against liver cancer, where it induces the expression of the anti-apoptotic gene Bcl-2, the pro-apoptotic gene Bcl-xS, and the TRAIL death receptors, suppresses the expression of NF-B and *IL-8* and increases apoptosis in HepG2 cell line. It also decreases the expression of antioxidant enzymes like glutathione peroxide, glutathione s-transferase, and catalase, regulates the cell cycle transition in animals from the G1/S phase (Noorbakhsh et al., 2018; Khan et al., 2017). The anti-apoptotic gene Bcl-2 was suppressed by TQ treatment, but pro-apoptotic Bcl-xS and TRAIL death receptors were increased. In conclusion, TQ increased the amount of TRAIL that caused HepG2 cells to die, partly by upregulating TRAIL death receptors, suppressing *NF-κB* and *IL-8*, and promoting apoptosis. The variety of molecular processes by which TQ-dependent HCC cell growth is suppressed highlights the promise of this substance as an anti-HCC medication (Khader and Eckl, 2014). Deleted in Liver Cancer1 is a mammalian gene that codes for a protein also recognized as *DLC1* and StAR-related lipid transfer protein 12 (STARD12) (Khader et al., 2009; Porter and Jänicke, 1999). *DLC1*, which has a CpG island and is located on chromosome 8 short arm (8p21.3-22), is essential for the growth of human HCC (Noorbakhsh et al., 2018; Samarghandia et al., 2019). Expression of *DLC1* suppresses cell proliferation, tumorigenicity, and HCC cell invasiveness. For the therapy of HCC, direct or indirect targeting of *DLC1* is possible (Khalil et al., 2019). Immune responses are controlled by *NF-κB* transcriptional regulators., apoptosis, and cell viability and act as central mediators of cancer induction and progression (Maher et al., 2020). In HCC, TQ suppresses tumor development and metastasis by regulating the *NF-κB* signaling pathway (El-fadl et al., 2021). Caspases are crucial mediators of apoptosis, a form of planned cell death. One of these, caspase-3, is a lethal protease frequently active and catalyzes the cleavage of several crucial cellular proteins (Porter and Jänicke, 1999). In lung cancer, The activation of Caspases-3 and 9 by TQ also triggered caspase-dependent apoptosis (Samarghandian et al., 2019). The lysosomal enzyme caspase 3 is involved in the apoptotic pathway, which can be activated by TQ (Abd El Maksoud et al., 2020). TQ has recently been found to have various biological properties, including hepatoprotective, anti-inflammatory, antioxidant, and anti-carcinogenetic properties. Therefore, employing cell lines for HCC, we want to emphasize and validate the significance of TQ as potential anti-proliferation agents in the current research (HCC).

Materials and Methods

Preparing of Thymoquinone (TQ) agent

In order to prepare different concentrations of TQ (Sigma-Aldrich, USA) 10 mg was dissolved in 1 ml of Dimethylsulfoxide (DMSO) to get a final concentration of 100 µg/µl. Different dilutions was prepared from the above concentration using DMSO to get a concentration of 50µg/µl, 25µg/ µl, and 12.5µg µl.

Cell lines

HepG2 cell lines and normal hepatocytes were obtained from VACSERA, Giza, Egypt. Both cell lines were cultured in RPMI medium supplemented with 3 mM L-glutamine, 3 mM sodium pyruvate, and 10% heat-treated fetal calf serum (FCS). Under 5% CO2 conditions, the cell line was incubated at 37°C (Khalil et al., 2017; Abd El Maksoud et al., 2019). All the experiments were held in Khalil's laboratory with biosafety level two instructions. Potential contamination with Mycoplasma was regularly checked during the subculture of cell lines. Zeiss A-Plan 10X inverted microscope was used to analyze the imaging of cultivated cells.

Cell viability and cytotoxic effects

On HepG2 cells, the TQ was examined for cytotoxicity, and the potential CC50 was determined. Accordingly, the cells were incubated at 37°C in a CO2 incubator and cultivated in 96-well plates at a density of 10X103 cells/ well. The cells were treated with TQ at various doses, including 1000g/ml, 500g/ml, 250g/ml, or 125g/ml, and then incubated for an overnight period. By counting the number of survived cells and analysing the inverted microscope images of the cells, cell viability was assessed. By removing the old media, PBS-washing the cells, trypsinizing the attached cells, and then counting the number of cells with a hemocytometer, the number

of treated cells that survived was manually recorded. Additionally, the 50% cytotoxic concentration (CC50) was found in connection to the amount of formazan dye that has been evaluated by the levels of absorbency of treated cells and untreated cells at 570nm using the MTT cell growth test kit (Sigma-Aldrich, Germany) (Khalil et al., 2017). HepG2 cells and normal hepatocytes were plated in 96-well plates at a density of $5X10^4$ cells per well. TQ was then applied to the cells at the appropriate concentration and incubated for an overnight period. The old media was removed after incubation, and the cells were washed with PBS. Then, 10 µl of MTT substrate was added to each well, followed by an hour of incubation at 37 °C. Then 100 µl of DMSO were added to each well. then an incubation period of 4 hours at 37 °C. Finally, the colour changes were assessed at 570nm using ELISA reader (Qadri et al., 2009; Abd El-Hady et al., 2023).

Lactate dehydrogenate (LDH) detection assay

The formation of LDH was observed in the fluid medium obtained from HepG2 cells treated with various doses of TQ using the LDH assay kit (Abc-65393). As a positive control, Triton 100-X was utilized, which had the greatest harmful effect on the treated cells. 100 µl of lysed cells and fluid media were treated with 100 µl of LDH reaction mix per the instructions of manufacturer for an hour at standard temperature. By measuring LDH activity at OD 450 nm, a plate reader was used. The fold change in LDH production was used to calculate the relative LDH production by dividing the mean values of the treated cells by the mock mean values (Khalil et al., 2019; Guirgis et al., 2023).

Enzyme-linked immunosorbent assay (ELISA)

Interleukin-6 (IL-6) and IL-10, as well as TNF-α, are utilized in an ELISA assay to quantify and analyze the anti-inflammatory cytokines that have been released (Abcam ab46042, Abcam ab100549, and Abcam ab18121, respectively). 96-well plates containing HepG2 cells were incubated overnight. The cells were then given treatment with the suggested dose of TQ, and an incubation period of (0, 6, 12, 24, 48, and 72 hrs). The cells were lysed using 1X cell lysis buffer (Invitrogen, USA) at each time point, and then 100 µl of the lysed cells were put onto the ELISA plate reader and incubated for 2 hours at room temperature with 100 µl of control solution and 50 µl of 1X biotinylated antibody. Each well was then filled with 100 µl of 1X streptavidin-HRP solution, and each was left to incubate for 30 minutes in the dark. Each well sample received 100 µl of the chromogen TMB substrate before being incubated at room temperature (RT) without the light for 15 minutes. The reaction was finally stopped by adding 100 µl of stop reagent to each well of the samples. 450 nm was used to quantify the absorbance of each well (Maher et al., 2020; Abd El Maksoud et al., 2020; El-fadl et al., 2021).

Quantitative real time PCR (qRT-PCR)

The cellular RNA was extracted using TriZol (Invitrogen, USA) and purified using an RNA purification kit to quantitatively analyze gene expression using qRT-PCR (Invitrogen, USA). Using M-MLV reverse transcriptase, complementary DNA (cDNA) was created from 1 µg of total RNA (Promega, USA). Using the QuantiTect-SYBR-Green PCR Kit from Qiagen (USA) and the specific primers mentioned in Table 1, the quantification analysis of the mRNA levels of NFκB, *DLC1*, and *Casp3* was accomplished. Each specified gene's cycle threshold (Ct) values were normalized using the housekeeping glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression level. Each sample was treated with 10 µl of SYBR green, 0.25 µl of RNase inhibitor (25 U/l), 0.2 µM of each primer, 2 µl of synthesized cDNA, and nuclease-free water to a final volume of 25 µl for the PCR process. PCR was carried out under the following conditions: 94°C for 5 min, 35 cycles (94°C for 30 sec, 60°C for 15 sec, and 72°C for 30 sec); (Khalil et al., 2017; Khalil et al., 2019; Alalem et al., 2023).

Data analysis

Microsoft Excel was used to evaluate all charts and histograms. Based on the following equations, the quantification analysis of the mRNA obtained from the qRT-PCR test used delta-delta Ct analysis: (1) (delta-delta Ct) = (delta Ct value for experimental -delta Ct for control), (2) (delta-delta Ct) = (delta-delta Ct) = (delta-delta Ct) for quantification of fold change (Rao et al., 2013; Khalil et al., 2017). The two-tailed t-test used by the student was employed for statistical analysis. P-values lower than 0.05 were regarded as statistically significant.

Results

Cell viability and cytotoxic effect of TQ on HepG2

Using a manually counted number of live cells, an MTT and LDH production test kit, and the appropriate TQ extract, we determined the indicated cytotoxic concentration of 50% (CC50) within HepG2 cells. Accordingly, at a density of 10,000 cells per well, the cells were seeded in 96-well plates and left overnight. The cells were subsequently given treatments all night long with varying concentrations of TQ (125–1000 ug/mL). Interestingly, the TQ treatment at a low dosage (125 ug/mL) stopped the viability of HepG2 cells and demonstrated 50% inhibition at a higher dose. At the same TQ treatment concentrations, the ordinary cell viability

Table 1. Sequences of the Oligonucleotides Used to Measure the mRNA Levels of the Identified Genes.

Description	Primer sequences 5'-3'
NFkB1-sense	GAAATTCCTGATCCAGACAAAAAC
NF-kB1 antisense	ATCACTTCAATGGCCTCTGTGTAG
DLC1-sense	GGACACCATGATCCTAACAC
DLC1-antisense	CTCATCCTCGTCTGAATCGT
Casp3-sense	GGACAGCAGTTACAAAATGGATTA
Casp3-antisense	CGGCAGGCCTGAATGATGAAG
GAPDH-sense	TGGCATTGTGGAAGGGCTCA
GAPDH-antisense	TGGATGCAGGGATGATGTTCT

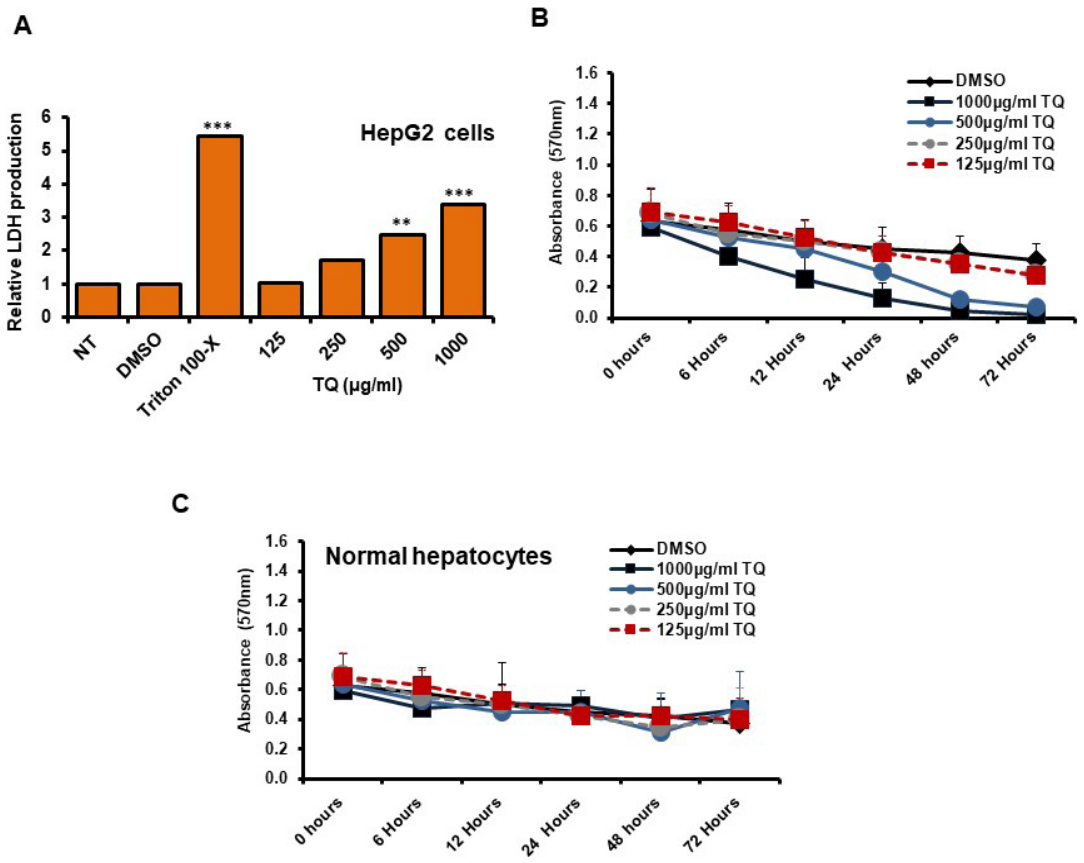

Figure 1. (A) Comparative lactate dehydrogenase (LDH) production between Triton 100X-treated and untreated cells (NT). (B) MTT assay for the cell survival rate of TQ of HepG2 cells pretreated with various concentrations (125 -1000 ug/ml) at the relevant time points. (C) Cell viability rate of normal hepatocyte cells subjected to the indicated concentrations of TQ and incubated for different time points.

rate of normal hepatocytes displayed an undetectable harmful effect (Table 2) (Figures 1A, B, and C). It was determined whether the treated cells produced LDH, which is regarded as a sign of chemical-mediated cytotoxicity in HepG2 cells. The relative LDH production increased significantly in HepG2 cells treated with 500 ug/ml, more than tripling and growing progressively dose-dependent (Table 3) (Figure 2A). The production amount of LDH after treatment compared to untreated cells further supports the cytotoxicity of TQ on HepG2 cells. The effect of TQ on HepG2 cell viability was assessed using the MTT test. As TQ was applied to the cells,

Table 2: Number of cells that survived after TQ treatment.

			TQ concentration (µg/ml)			
	NT	DMSO	125	250	500	1000
Mean	325000	280000	190000	170000	150000	35000
STD	21213.2	7071.07	7071.07	14142.14	14142.14	28284.27
P values		0.21	0.017*	0.013*	0.006**	0.002**

Cells that have not been treated are designated as NT, and the standard deviation of three separate tests is designated as STD; *, significant P values ≤0.05 are indicated; **, high significant P values ≤0.01 are indicated.

Table 3. LDH Generation in TQ-Treated Cells

				TQ concentration (µg/ml)			
	100-X	DMSO	Triton 100-X	125	250	500	1000
Mean absorbance	0.04	0.04	0.42	0.05	0.05	0.13	0.18
STD	0.01	0.01	0.26	0.02	0.01	0.03	0.01
Relative LDH production	1	1.25	12.07	1.53	1.46	3.7	5.1
P values		0.19	0.02*	0.13	0.18	0.001**	0.001**

Cells that have not been treated are designated as NT, and the standard deviation of three separate tests is designated as STD; *, significant P values ≤0.05 are indicated; **, high significant P values ≤0.01 are indicated.

Table 4. Rate of Cell Viability in TQ-Treated Cells

	DMSO	TQ concentration (µg/ml)			
		125	250	500	1000
Mean absorbance	0.52	0.41	0.33	0.18	0.06
STD	0.11	0.1	0.11	0.05	0.02
P values		0.17	0.04*	0.001**	0.001**

Cells that have not been treated are designated as NT, and the standard deviation of three separate tests is designated as STD; *, significant P values ≤0.05 are indicated; **, high significant P values ≤0.01 are indicated.

cell proliferation at the specified time intervals showed substantial inhibition. However, 500 ug/ml and 1000 ug/ml showed highly significant inhibition in TQ-treated cells in a time-dependent manner as compared to untreated cells (controls), as shown in (Table 4) (Figure 2B). Interestingly, normal hepatocyte cells showed negligible differentiation in cell viability rate upon treatment with the same concentrations of TQ and incubated for the same time as the cancer cell viability rate (Figure 1C). These findings indicate the selective regulation of HepG2 cell proliferation by TQ treatment without the detectable toxic effect of the normal hepatocytes.

Cytotoxic consequences of TQ on HepG2 cells

The TQ was then investigated for its potential activity against HepG2 cell growth. The inverted microscopy images of treated cells showed dramatic changes in cell morphology treated with TQ (250µg/ml and 1000µg/ml) (Figure 2A). Likewise, the number of survived cells upon TQ treatment showed a significant decreasing number with a higher concentration of TQ (Figure 2B). This result firstly confirms the cytotoxic effect of the higher concentration of TQ agent on HepG2 cells indicated by cell morphology, number of survived upon treatment in comparison with the control cells.

Inhibition of NFkB expression and regulation of DLC1 & Casp3 activation by TQ

To emphasize the pharmacological effects of TQ fraction on cell signaling, we measured the *NFkB* mRNA levels, *DLC1* and *Casp3* in treated HepG2 cells using qRT-PCR. Gene expression patterns for *NFkB*, *DLC1*, and *Casp3* within HCC (HepG2) cell lines found that expression of gene inhibition for *NFkB* was considerably reduced in TQ-treated cells. In contrast, *DLC1* and *Casp3* expression profile was significantly increased upon treatment with varying amounts of TQ (Figure 3A, B, and C). These results indicate the

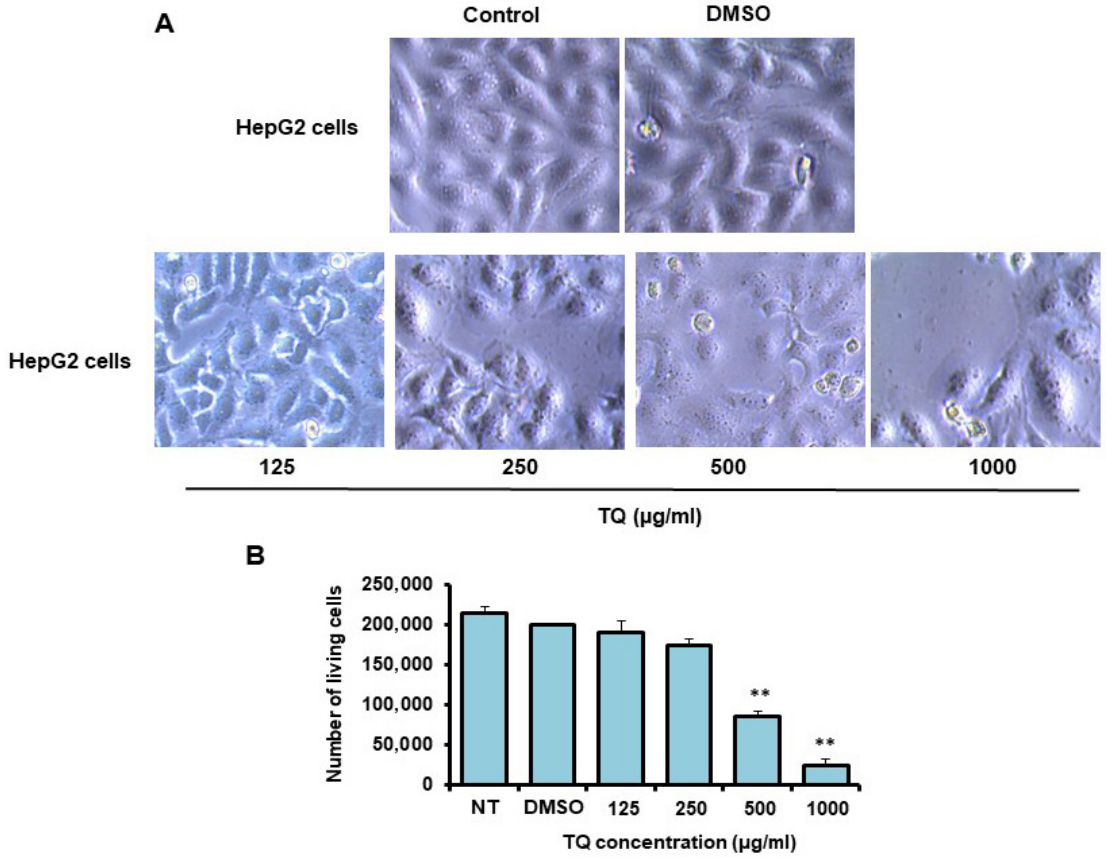

Figure 2. (A) Comparing the cell viability of the HepG2 cell line pre-treated with TQ extract to cells that had not been treated (control) and cells that had been treated with DMSO. (B) Using a hemocytometer, count the number of HepG2 cells that survived after being pre-treated with TQ extract at various concentrations (125–1000 ug/ml).

Table 5. *NFkB, DLC1*, and *Casp3* Quantification Analyses in HepG2-Treated Cells

Genes	Condition	Expression fold changes	Standard deviation	Student two-tails t-test	P-values
NFkB	NT	1	0		
	DMSO	1.15	0.14	0.26	> 0.05
	250 µg/ml TQ	0.7	0.15	0.089	> 0.05
	500 µg/ml TQ	0.22	0.02	0.046	< 0.05*
	1000 µg/ml TQ	0.12	0.09	0.006	< 0.01**
DLC1	NT	1	0		
	DMSO	0.98	0.15	0.86	> 0.05
	250 µg/ml TQ	1.43	0.58	0.4	> 0.05
	500 µg/ml TQ	6.72*	1.17	0.02	< 0.05
	1000 µg/ml TQ	7.84*	1.6	0.02	< 0.05
Casp-3	NT	1	0		
	DMSO	0.97	0.14	0.83	> 0.05
	250 µg/ml TQ	2.66	1.48	0.25	> 0.05
	500 µg/ml TQ	3.27**	0.21	0.004	< 0.01
	1000 µg/ml TQ	5.1**	0.043	0.001	< 0.01

Cells that have not been treated are designated as NT, and the standard deviation of three separate tests is designated as STD; *, significant P values ≤0.05 are indicated; **, high significant P values ≤0.01 are indicated.

potential role of TQ in regulating oncogene expression and stimulation-programmed cell death in HepG2 cells. Before treatment with DMSO and different concentrations of TQ (250–500 g/ml), the expression levels of the *NFkB*, *DLC1*, and *Casp3* genes were assessed in cell lines, along with a comparison to the control. The result showed the downregulation of the *NFkB* gene and the upregulation of *DLC1* & *Casp3* genes upon increasing concentration

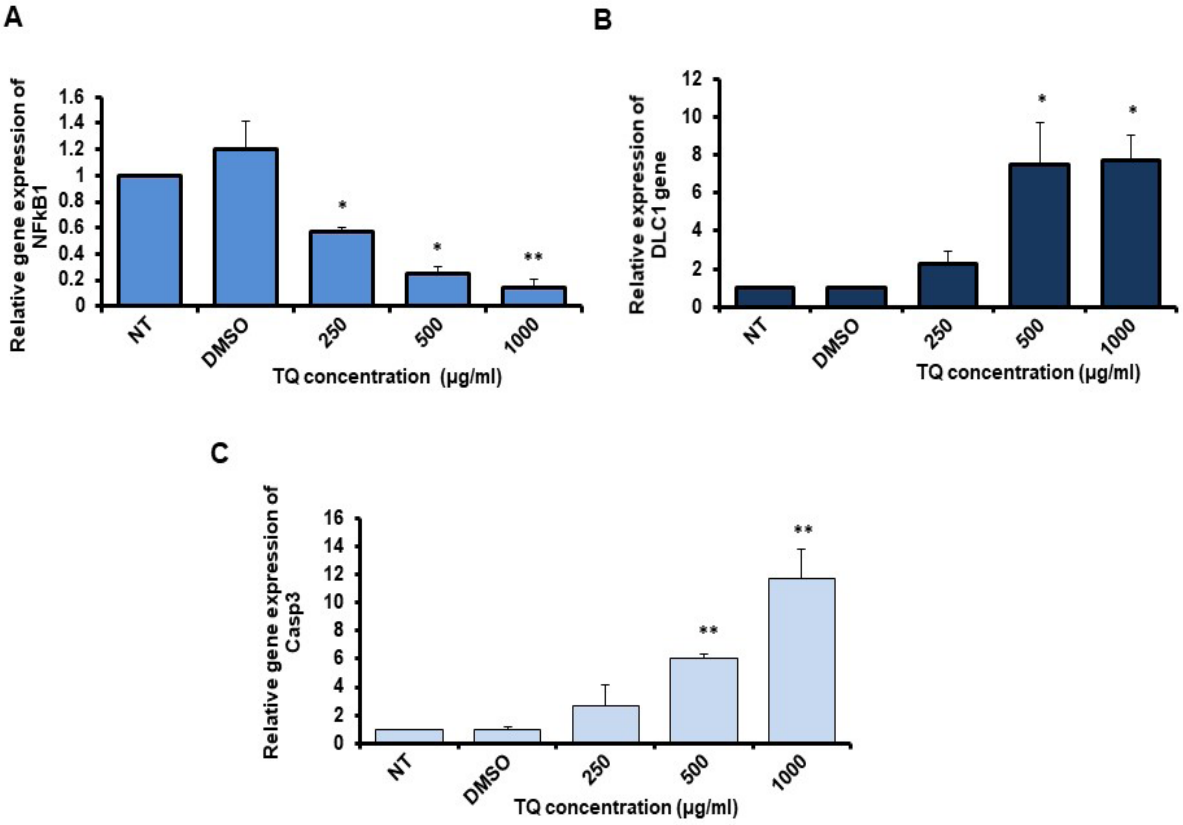

Figure 3. (A) Quantification of gene expression of NFkB1 before the treatment with different concentrations of TQ, along with a comparison to the control using PCR. (B) Quantification of gene expression of DLC1 before the treatment with different concentrations of TQ, along with a comparison to the control using PCR. (C) Casp3 gene expression was quantified using PCR before being treated with various TQ concentrations and compared to the control. The stranded deviation (SD) of two separate trials is shown in the error bars. Gene expression quantification results underwent a significance analysis using a student two-tailed t-test. **P < 0.01 was regarded as extremely significant and *P < 0.05 as statistically significant.

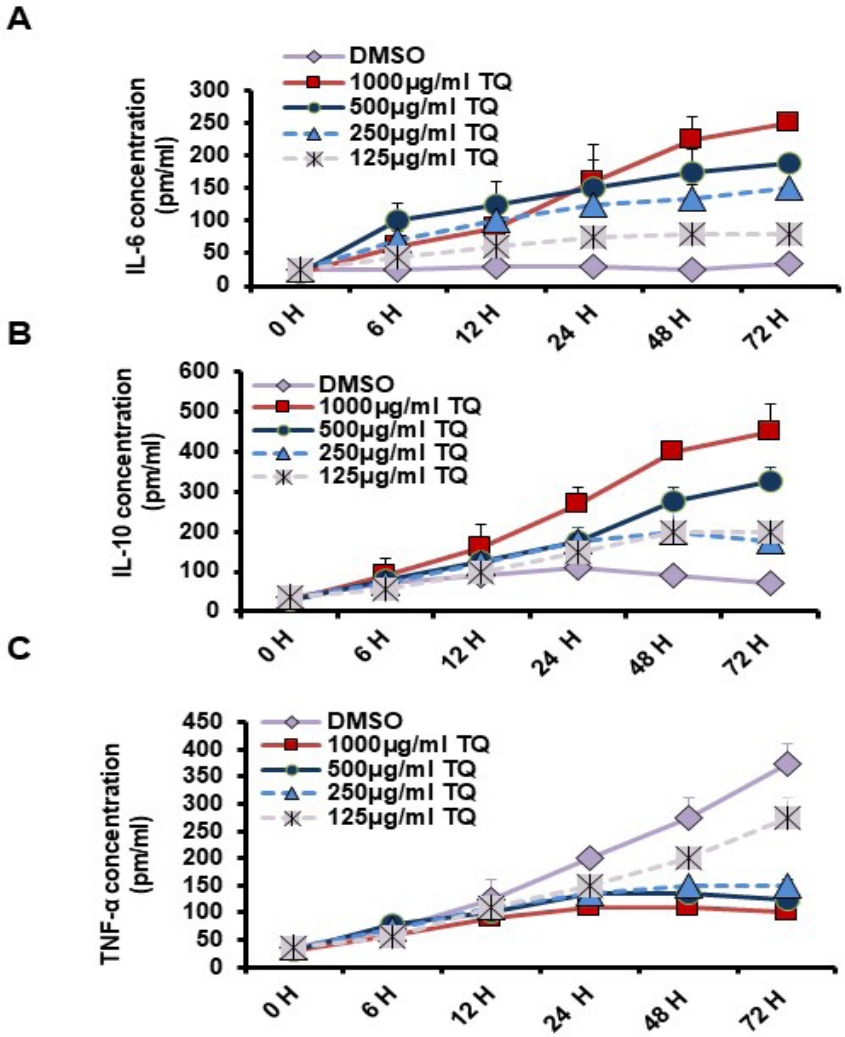

Figure 4. Pro-Inflammatory Cytokine Secretion Indicated by ELISA in a Dose and Time-Dependent Manner: (A) IL-6, (B) IL-10, and (C) TNF-α.

of TQ (Table 5).

TQ-induced pro-Inflammatory cytokine secretion from treated cells

We used the ELISA test to analyze the fluid medium of the treated HepG2 cells at the indicated time points to quantify the pro-inflammatory cytokines secreted (Figure 4A). This analysis showed that the mean concentration of IL-6 increased to 250 pm/ml time-dependent in the TQ-treated cells. When cells were treated with a concentration of 1000 ug/ml TQ extract, the high level of IL-10 produced in TQ-treated cells was significantly created (Figure 4B). Compared to untreated or DMSO-treated cells, the mean concentration of TNF-α was considerably lower in cells treated with TQ (Figure 4C).

Discussion

Due to its capacity to influence several molecular pathways, TQ has the potential to be an efficient therapeutic small molecule in the prevention and treatment of cancer (Majdalawieh et al., 2017). To better understand the chemical basis of TQ activity, laboratory research should continue. This will make it possible to create potent analogs with fewer side effects and more useful drug delivery technologies, ultimately improving the cancer management system. Our goal in conducting this study was to demonstrate TQ's positive and secure benefits in cancer treatment. The biological activity of TQ was compared to that of cells treated with DMSO and untreated cells (control) in HepG2 cells. According to our findings, the potential cytotoxic concentration of TQ on the treated cells was more significant than 500 µg/ml. Additionally, the MTT viability test on HepG2 cells showed that, after 24 hours of treatment, the IC50 for TQ was 500 g/ml. HepG2 cell viability was reduced over time and dose-dependently, and TQ treatment reduced cell proliferation as TQ concentration rose. The increased level of LDH formation in response to treatment compared to untreated cells supports our findings that TQ is cytotoxic to HepG2 cells (Nagi and Mansour, 2000; El-Abhar et al., 2003). Moreover, published research showed that pre-treatment with TQ enhanced cell survival, decreased LDH release, and maintained cell bodies in cerebellar granule neurons (Sarkar et al., 2021).

All cell types contain the transcription factor *NFkB*. (Cartwright et al., 2016). Carcinogenesis is linked to NFκB signaling pathway abnormal activation (Concetti and

Wilson, 2018). The activation of *NF-κB* is well recognized as the cause of apoptosis resistance (Concetti and Wilson, 2018). Our findings demonstrated that the *NF-κB* gene was downregulated as TQ concentration was raised. These findings suggest that TQ, by inducing programmed cell death and reducing the ensuing inflammation, may be able to suppress the division of cancer cells. The overactive stimulation of the *NF-κB* signaling pathway has been observed in a variety of tumor tissues, and persistent inhibition of *NF-κB* results in adverse immune cell development or disrupted cell growth, so downregulation of NFκB by TQ help in preventing tumor cell from continuous growth. Additionally, it was discovered that TQ suppresses NFκB and the molecules that it regulates, which prevents tumor angiogenesis and tumor growth. It was concluded that TQ significantly reduces tumor growth and angiogenesis. Therefore, TQ's anticancer and anti-angiogenic activities may have an underlying mechanism involving the suppression of NFκB and downstream effector molecules (Paul and Paul, 2005). The direct inhibitory effect of TQ treatment on *NF-κB* activation, which has also been seen on several occasions, may be responsible for the downregulation of pro-inflammatory mediators after TQ administration (El Gazzar et al. 2007; Sethi et al., 2008; Zhang et al., 2016). Despite the fact that *NF-κB*-mediated gene transcription is necessary for normal physiological functions, most malignancies are linked to aberrant *NF-κB* signaling, which results in the production of various inflammatory cytokines and the activation of a wide variety of tumor-promoting signaling pathways (Karin, 2009; He and Karin, 2011). Also, the hepato-protective activity of this flavonoid is another way that TQ affects NFκB; several TQ derivative patents for protective effects have recently been created (Noorbakhsh et al., 2018). *DLC1* has been shown to be downregulated in a variety of cancer types, including HCC. (Xie et al., 2015). In addition, the tumor suppressor gene deleted in liver cancer 1 (*DLC1*) encodes a protein that regulates and prevents cell growth, invasion, and angiogenesis (Xue et al., 2008). Our findings demonstrated that *DLC1* and *Casp3* gene expression increased when TQ concentration increased. These findings indicated that TQ could restore the expression of the tumor suppressor genes DLC 1 and *Casp3*, which slow the evolution of HCC. According to other research, loss seems to contribute to the development and oncogenic autophagy of HCC (Xue et al., 2008; Song et al., 2016; Wu et al., 2018). Furthermore, Increased expression of the *Casp3* gene, an effector caspase in apoptosis, is recognized for directing the destruction of cellular structure. The process of programmed cell death, known as apoptosis, is essential for eliminating infected cells and disease pathogenesis. It is reliant on the induction of a proteolytic caspase cascade. (Porter and Jänicke, 1999; Khalil et al., 2020). In this approach, caspase-3 has been acknowledged as a critical modulator of apoptosis (Asadi et al., 2021). According to other research, TQ upregulates the *Casp3* gene, which can promote apoptosis and prevent the formation of HCC in the HepG2 cell line, which agrees with these findings. Curiously, *Casp3* is one of the executioner caspases believed to be in charge of the actual cell damage (Porter and Jänicke, 1999). Finally, TQ not only inhibits cell growth and survival but also causes the death of cancer cells. The deregulation of apoptosis-associated gene expression typically recurs after TQ-induced apoptosis (Gomathinayagam et al., 2020).

Immune system defense against potentially damaging stimuli, such as tissue damage or allergens, includes inflammation. On the contrary hand, an uncontrolled inflammatory response is the root of various disorders, such as autoimmune diseases, cancer, metabolic disease, allergies, and cardiovascular dysfunction. Individuals are financially burdened by these conditions, which have consequences for society (Sarkar et al., 2021). IL (interleukin)-6 is a multifunctional cytokine that regulates inflammation, the acute phase response, hemopoiesis, and immune response. It was first identified as a B-cell distinguishing factor (Pereira et al., 2013; Romano et al., 1997). IL-10 improves natural killer cell performance, which results in greater antigen availability through pathogen destruction (Malefyt et al., 1991; Pereira et al., 2013). Our research showed that the pro-inflammatory mediators IL-6 and IL-10 are produced due to TQ therapy. In agreement with our findings, other research have revealed that high rates of the pro-inflammatory mediators IL-6 and IL-10 in treated cells indicate that TQ may have regulatory effects on cancer cells. These effects boost innate immunity, delay cancer progression, and enhance cell function (Pereira et al., 2013). TNF, a cytokine with immunomodulatory solid properties, is also essential for host defence, inflammation, and immunological homeostasis. Depending on the biological milieu, it can have several consequences, such as apoptosis, necrosis, angiogenesis, immune cell activation, differentiation, and cell migration. (Wajant, 2009) . Tumor necrosis factor (TNF), despite its name, history of discovery, and approval as an anticancer drug, has been linked to both the onset and progression of cancer in some preclinical models (Wajant, 2009). By promoting the creation of genotoxic chemicals, such nitric oxide or ROS, in cancer cells, TNF may damage DNA and prevent DNA repair (Asadi et al., 2021). In agreement with our results, TQ has demonstrated that it can reduce TNF- via a dose-dependent anti-inflammatory impact (Almajali et al., 2021). TQ immunomodulatory activity is one of the fundamental mechanisms behind its anticancer properties. There is evidence that TQ prevents tumour necrosis factor from activating NFκB. (Majdalawieh and Fayyad, 2015). The cumulative actions of TQ significantly prevent the proliferation and development of cancer cells (Gomathinayagam et al., 2020). As previously mentioned, TQ has been shown to block the cellular mechanisms and molecular targets underlying practically all cancer features (Gomathinayagam et al., 2020). Because of its ability to disrupt many signaling cascades implicated in the generation and progression of cancer, TQ prospective as a top candidate for adjuvant therapy is thus well supported. (Gomathinayagam et al., 2020). It is exciting to see that TQ can reduce the intrinsic cytotoxicity of several chemotherapy drugs while increasing their efficacies.

In conclusion, our results demonstrate the therapeutic potential of TQ against HCC by its cytotoxic effect by raising LDH levels, activating the *DLC1* and *Casp3* genes,

and suppressing NF-Kβ, also Proinflammatory cytokines are strongly influenced by TQ by increasing of IL6 and IL10 levels and decreasing of TNF-α which indicate the critical role of TQ in decreasing progression of HCC. As a result of the use of this molecule as a therapeutic agent in a variety of diseases and disorders, we expect that this will herald a promising future for researchers to discover TQ-based alternative medicine.

Author Contribution Statement

Ahmed Salah and Rasha Sleem, performed the experiments. Amal Abd-Elaziz helped in supervision and conceptualizing experiments. Hany Khalil designed the research plan, supervised overall research, provided and interpreted the results. Ahmed Salah and Rasha Sleem organized and wrote the manuscript.

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Ethical statement

The current work has been approved by the Ethical Committee of Genetic Engineering and Biotechnology Research Institute, University of Sadat City.

Availability of data and materials

The data support these findings are available from the corresponding author upon reasonable request.

Conflicts of interest

All authors declare that there are no conflicts of interest.

References

Abd El-Hady A, El-Chennawi FA, El-Fikiy B, et al (2023). Identification of the Anticancer Potential of Vitamin B17 via Targeting Raf-1 and Autophagy-related Gene Expression. *Middle East J Cancer*, **14**, 349-62.

Abd El Maksoud, Ahmed I, Taher RF, et al (2019). Selective Regulation of B-Raf Dependent K-Ras/Mitogen-Activated Protein by Natural Occurring Multi-Kinase Inhibitors in Cancer Cells. *Front Oncol*, **9**, 1220.

Abd El Maksoud, Ahmed I, Elebeedy D, et al (2020). Methylomic Changes of Autophagy-Related Genes by Legionella Effector Lpg2936 in Infected Macrophages. *Front Cell Dev Biol*, **7**, 390.

Alalem M, Dabous E, Awad AM, et al (2023). Influenza a virus regulates interferon signaling and its associated genes; MxA and STAT3 by cellular miR-141 to ensure viral replication. *Virol J*, **20**, 183.

Almajali B, Al-Jamal HAN, Taib WRW, et al (2021). Pharmaceuticals Thymoquinone, as a Novel Therapeutic Candidate of Cancers. *Pharmaceuticals*, **14**, 369.

Asadi M, Taghizadeh S, Kaviani E, et al (2021). Caspase-3: Structure, Function, and Biotechnological Aspects. *Biotechnol Appl Biochem*, **69**,1633-45.

Ates MB, Ortatatli M, Oguz H, et al (2021). The Ameliorative Effects of Nigella Sativa, Thymoquinone, and Bentonite against Aflatoxicosis in Broilers via AFAR and Nrf2 Signalling Pathways, and down-Regulation of Caspase-3. *Br Poult Sci*, **63**, 332–9.

Cartwright T, Perkins ND, Wilson CL (2016). *NFKB*1: A Suppressor of Inflammation , Ageing and Cancer. *Febs J*, **283**, 1812–22.

Chen SL, Liu LL, Wang CH, et al (2020). Loss of RDM1 Enhances Hepatocellular Carcinoma Progression via P53 and Ras/Raf/ERK Pathways. *Mol Oncol*, **14**, 373-86.

Concetti J, Wilson CL (2018). *NFKB*1 and Cancer: Friend or Foe?. *Cells*, **7** , 1–16.

El-Abhar HS, Abdallah DM, Saleh S (2003). Gastroprotective Activity of Nigella Sativa Oil and Its Constituent, Thymoquinone, against Gastric Mucosal Injury Induced by Ischaemia/Reperfusion in Rats. *J Ethnobiol*, **84**, 251–8.

Elawdan KA, Farouk S, Aref S, et al (2022). Association of vitamin B12/ferritin deficiency in cancer patients with methylomic changes at promotors of TET methylcytosine dioxygenases. *Biomark Med*, **16**, 959–70.

El-fadl HMA, Hagag NM, El-shafei RA , et al (2021). Effective Targeting of Raf-1 and Its Associated Autophagy by Novel Extracted Peptide for Treating Breast Cancer Cells. *Front Oncol*, **11**, 682596.

Elimam H, El-Say KM, Cybulsky AV, et al (2020). Regulation of Autophagy Progress via Lysosomal Depletion by Fluvastatin Nanoparticle Treatment in Breast Cancer Cells. *ACS Omega*, **5**, 15476-86

Fekry T, Salem MF, Abd-Elaziz A, et al (2022). Anticancer Properties of Selenium-Enriched Oyster Culinary-Medicinal Mushroom, Pleurotus ostreatus (Agaricomycetes), in Colon Cancer In Vitro. *Int J Med Mushrooms*, **24**, 1–20.

Gazzar Mohamed AEl, El-Mezayen R, Nicolls MR, Dreskin SC (2007). Thymoquinone Attenuates Proinflammatory Responses in Lipopolysaccharide-Activated Mast Cells by Modulating NF-KappaB Nuclear Transactivation. *Bba-Gen Subjects*, **1770**, 556–64.

Gomathinayagam R, Ha JH, Jayaraman M, et al (2020). Chemopreventive and Anticancer Effects of Thymoquinone: Cellular and Molecular Targets. *J Cancer Prev*, **25**, 136–51.

Guan M, Zhou X, Soulitzis N, et al (2006). Aberrant Methylation and Deacetylation of Deleted in Liver Cancer-1 Gene in Prostate Cancer: Potential Clinical Applications. *Clin Cancer Res*, **12**, 1412–9.

Guirgis SA, El-Halfawy KA, Alalem M, et al (2023). Legionellapneumophila induces methylomic changes in ten-eleven translocation to ensure bacterial reproduction in human lung epithelial cells. *J Med Microbiol*, **72**.

He G, Karin M (2011). *NF-KB* and STAT3- Key Players in Liver Inflammation and Cancer. *Cell Res*, **21**, 159–68.

Karin M (2009). NF-KappaB as a Critical Link between Inflammation and Cancer. *Csh Perspect Biol*, **1**, 1–15.

Khader Bresgen MN, Eckl PM (2009). In Vitro Toxicological Properties of Thymoquinone. *Food Chem Toxicol*, **47**, 129–33.

Khader M, Eckl PM (2014). Thymoquinone: An Emerging Natural Drug with a Wide Range of Medical Applications. *Iran J Basic Med Sci*, **17**, 950.

Khalil H, El-Malah T, El Maksoud AI, et al (2017). Identification of Novel and Efficacious Chemical Compounds That Disturb Influenza A Virus Entry in Vitro. *Front Cell Infect Microbiol*, **7**, 304.

Khalil H, Arfa M, El-Masrey S, EL-Sherbini S, Abd-Elaziz A (2017). Single Nucleotide Polymorphisms of Interleukins Associated with Hepatitis C Virus Infection in Egypt. *J Infect Dev Countr*, **11**, 261–8.

Khalil H, Abd El-Maksoud AI, Roshdey T, El-Masry S (2019). Guava Flavonoid Glycosides Prevent Influenza A Virus

Infection via Rescue of P53 Activity. *J Med Virol*, **91** ,45–55.
Khalil H, Abd-Elhady A, Elawdan KA, et al (2020). The Mechanical Autophagy as a Part of Cellular Immunity; Facts and Features in Treating the Medical Disorders. *Immuno Invest*, **00**, 1–24.
Khan Asaduzzaman MD, Tania M, Shangyi Fu, Junjiang Fu (2017). Thymoquinone, as an Anticancer Molecule: From Basic Research to Clinical Investigation. *Oncotarget*, **8**, 51907.
Maher E, Gedawy G, Fathy W, et al (2020). Hsa-MiR-21-Mediated Cell Death and Tumor Metastases: A Potential Dual Response during Colorectal Cancer Development. *Middle East J*, **11**, 483-2.
Majdalawieh AF, Fayyad MW (2015). Immunomodulatory and Anti-Inflammatory Action of Nigella Sativa and Thymoquinone: A Comprehensive Review. *Int Immunopharmacol*, 28, 295–4.
Majdalawieh AF, Fayyad MW, Nasrallah GK (2017). Anti-Cancer Properties and Mechanisms of Action of Thymoquinone, the Major Active Ingredient of Nigella Sativa. *Crit Rev Food Sci Nut*, **5**, 3911–28.
Mohamed ESA, Bassiouny K, Alshambky AA, et al (2022). Anticancer Properties of N,N-dibenzylasparagine as an Asparagine (Asp) analog, Using Colon Cancer Caco-2 Cell Line. *Asian Pac J Cancer Prev*, **23**, 2531–40.
Nagi MN, Mansour MA (2000). Protective effect of Thymoquinone against doxorubicin-induced cardiotoxicity in rats: a possible mechanism of protection. *Pharmacol Res*, 41, 283–9.
Noorbakhsh MF, Hayati F, Samarghandian S, Shaterzadeh-Yazdi H, Farkhondeh T (2018). An Overview of Hepatoprotective Effects of Thymoquinone. *Recent Pat Food Nutr Agric*, **9**, 14-2.
Paul George A, Paul AG (2005). NF-KB: A Novel Therapeutic Target for Cancer NF-KB: A Novel Therapeutic Target for Cancer. *Eukaryon*, **1**.
Pereira DS, Mateo EC, de Queiroz BZ, et al (2013). TNF-α, IL6, and IL10 Polymorphisms and the Effect of Physical Exercise on Inflammatory Parameters and Physical Performance in Elderly Women. *AGE*, **35**, 2455–3.
Porter AG, Jänicke RU(1999). Emerging Roles of Caspase-3 in Apoptosis. *Cell Death Differ*, **6**, 99–104.
Qadri SM, Mahmud H, Föller M, Lang F(2009). Thymoquinone-Induced Suicidal Erythrocyte Death. *Food Chem Toxicol*, **47**, 1545–9.
Qin G, Xinyi T, Li H, et al (2020). Long Noncoding RNA P53-Stabilizing and Activating RNA Promotes P53 Signaling by Inhibiting Heterogeneous Nuclear Ribonucleoprotein K DeSUMOylation and Suppresses Hepatocellular Carcinoma. *Hepatology*, **71**, 112-29.
Rao X, Huang X, Zhou Z, Lin X (2013). An Improvement of The2–Delta Delta CT) Method for Quantitative Real-Time Polymerase Chain Reaction Data Analysis. *Biostat Bioinforma Biomath*, **3**, 71–85.
Roberts LR, Gores JG (2005). Hepatocellular Carcinoma: Molecular Pathways and New Therapeutic Targets. *Semin Liver Dis*, **25**, 212–25.
Romano M, Sironi M, Toniatti C, et al (1997). Role of IL-6 and Its Soluble Receptor in Induction of Chemokines and Leukocyte Recruitment. *Immunity*, **6**, 315–5.
Samarghandian S, Azimi-Nezhad M, Farkhondeh T (2019). Thymoquinone-Induced Antitumor and Apoptosis in Human Lung Adenocarcinoma Cells. *J Cell Physiol*, **234**, 10421–1.
Sarkar C, Jamaddar S, Islam T, et al (2021). Therapeutic Perspectives of the Black Cumin Component Thymoquinone: A Review. *Food Funct*, **12**, 6167–3.
Sethi Gautam, Kwang SA, Aggarwal B (2008). Targeting Nuclear Factor-KB Activation Pathway by Thymoquinone: Role in Suppression of Antiapoptotic Gene Products and Enhancement of Apoptosis. *Mol Cancer Res*, **6**, 1059–70.
Song LJ, Liu Q, Meng XR, et al (2016). DLC-1 Is an Independent Prognostic Marker and Potential Therapeutic Target in Hepatocellular Cancer. *Diagn Pathol*, **11**, 2.
Malefyt W, René De, Haanen J, et al (1991). Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. *J Exp Med*, **174**, 915–4.
Wajant, Harald (2009). The Role of TNF in Cancer. *Results Probl Cell Differ*, **49**, 1–15.
Wang H, Liao P, Zeng SX, Lu H (2019). It Takes a Team: A Gain-of-Function Story of P53-R249S. *J Mol Cell Biol*, **11**, 277-3.
Wu HT, Xie CR, Lv J, et al (2018). The Tumor Suppressor *DLC1* Inhibits Cancer Progression and Oncogenic Autophagy in Hepatocellular Carcinoma. *Lab Invest*, **98**, 1014–4.
Xie, Rong C, Sun HG, et al (2015). Significance of Genetic Variants in *DLC1* and Their Association with Hepatocellular Carcinoma. *Mol Med Rep*, **12**, 4203–9.
Xue Wen, Krasnitz A, Lucito R, et al (2008). *DLC1* Is a Chromosome 8p Tumor Suppressor Whose Loss Promotes Hepatocellular Carcinoma. *Gene Dev*, **22**, 1439–4.
Yi Tingfang, Cho SG, Yi Z, et al (2008). Thymoquinone Inhibits Tumor Angiogenesis and Tumor Growth through Suppressing AKT and Extracellular Signal-Regulated Kinase Signaling Pathways. *Mol Cancer Ther*, **7**, 1789–96.
Zhang L, Bai Y, Yang Y (2016). Thymoquinone Chemosensitizes Colon Cancer Cells through Inhibition of *NF-KB*. *Oncol Lett*, **12**, 2840–5.
Zoheir Khairy MA, Abdelhafez MA, Darwish AM, Mahrous KF (2022). New Approach about the Signaling Crosstalk between IQGAPs/*NF-KB*/*IL-8* and PDCD5/P53/TRAIL Pathways That Modulate Malignant Transformation in Hepatocellular Carcinoma. *Asian Pac J Cancer Prev*, **23**, 271–9.