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Research Article

In vitro AND *in silico* EVALUATION OF THYMOQUINONE AS POTENTIAL ANTICANCER AGENT IN HUMAN ACUTE MYELOID LEUKEMIA HL-60 CELLS

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Abstract: This study aims to explore the cytotoxic, apoptotic and autophagic effects of thymoquinone on human acute myeloid leukemia. The cytotoxic effects of thymoquinone were determined with 3-(4, 5-dimethylthiazol-2-yl)-2 and 5-diphenyltetrazolium bromide (MTT) tests. B-cell lymphoma 2 associated X protein (Bax), B-cell lymphoma 2 (Bcl-2), caspase 3, mammalian target of rapamycin (mTOR), phosphatidylinositol-3-kinase (PI3K), and protein kinase B (AKT) gene expression analyzes were studied with quantitative real-time polymerase chain reaction (qRT-PCR). AutoDock Tools 4.2 software was applied to research the potential binding of thymoquinone in the active sites of Bax, Bcl-2, caspase 3, mTOR, PI3K, and AKT proteins. Thymoquinone caused a cytotoxic effect on HL-60 cells (Human leukemia cell line) with a value of 16.35 µM. Bcl-2 expression was decreased in all concentrations applied compared to the control. A decrease in caspase 3 expression level was detected in the cells treated with 10 µM, 15 µM, and 25 µM thymoquinone compared to the control. Thymoquinone induced an important decrease in mTOR and PI3K expressions compared to the control at all doses, while AKT decreased at a dose of 15 µM. The docking outcomes showed that thymoquinone interacts with the active site amino acids of apoptotic and autophagic proteins via hydrophobic interactions and hydrogen bonding. The present findings suggest that thymoquinone can stimulate autophagy by prevention of PI3K/AKT/mTOR pathway in HL-60 cells and may become a new target for the therapy of acute myeloid leukemia.

Özet: Bu çalışmada, insan akut miyeloid lösemisinde timokinonun sitotoksik, apoptotik ve otofajik etkilerinin araştırılması amaçlandı. Timokinonun sitotoksik etkileri 3-(4,5-dimetiltiyazol2-yl)-2,5-difeniltetrazolyum-bromür (MTT) testi ile belirlendi. B hücre lenfoma 2 ile ilişkili X proteini (Bax), B hücre lenfoma 2 (Bcl-2), kaspaz 3, rapamisinin memeli hedefi (mTOR), fosfatidilinositol-3-kinaz (PI3K) ve protein kinaz B (AKT) gen ekspresyon analizleri kantitatif gerçek zamanlı polimeraz zincir reaksiyonu (qRT-PCR) ile yapıldı. Bax, Bcl-2, kaspaz 3, mTOR, AKT ve PI3K proteinlerinin aktif bölgesindeki timokinonun potansiyel bağlanmasını araştırmak için AutoDock Tools 4.2 yazılımı kullanıldı. Timokinon, HL-60 hücrelerinde (İnsan lösemi hücre hattı) 16.35 µM değerinde sitotoksik etkiye neden olmuştur. Uygulanan tüm dozlarda kontrole göre Bcl-2 ekspresyonu azalmıştır. 10 µM, 15 µM ve 25 µM timokinon ile tedavi edilen hücrelerde kontrole göre kaspaz 3 ekspresyon seviyesinde bir azalma tespit edildi. Timokinon, tüm dozlarda kontrole kıyasla mTOR ve PI3K ifadelerinde önemli bir azalmaya neden olurken, AKT 15 µM'lik bir dozda azaldı. Yerleştirme sonuçları, timokinonun apoptotik ve otofajik proteinlerin aktif bölge amino asitleri ile hidrofobik etkileşimler ve hidrojen bağı yoluyla etkileşime girdiğini belirtti. Bulgularımız, timokinonun HL-60 hücrelerinde PI3K/AKT/mTOR yolunu önleyerek otofajiyi uyarabildiğini ve akut miyeloid lösemi tedavisi için yeni bir hedef olabileceğini düşündürmektedir.

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Key words:
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Apoptosis
Autophagy
Molecular docking
PI3K/AKT/mTOR
Thymoquinone

Introduction

Acute myeloid leukemia (AML) is the most common type of leukemia in which myeloblast accumulation takes place due to both differentiation failure and excessive proliferation of stem cells (Maha *et al.* 2008). The incidence of AML increases substantially with age. Most of the aged patients are not suitable to be cured with intense chemotherapy or bone marrow transplantation. Lack of adequate and less damaging treatments leads to a

very low therapy proportion of only 5-15% of these patients (Kahl *et al.* 2019). AML chemotherapy drugs produce side effects such as infection, bleeding, and anemia and can damage healthy cells and tissues (Bashmail *et al.* 2020). Therefore, a new therapeutic agent with fewer side effects needs to be found to treat AML and scientists are exploring natural materials that can be used for this purpose.

Natural compounds and even raw herbs pose promising alternative drug sources in cancer treatment. They are known to suppress or block carcinogenic processes (Bashmail *et al.* 2018). Thymoquinone is one of the main bioactive constituents isolated from *Nigella sativa* L., and it has anti-oxidant, anti-inflammation, anti-hypertensive and anti-tumor properties (Ahmad *et al.* 2013, Bashmail *et al.* 2018). Earlier studies have shown that thymoquinone has antitumor effects on various cancer types such as cervical squamous carcinoma (Ng *et al.* 2011), breast cancer (Woo *et al.* 2011), pancreatic cancer (Banerjee *et al.* 2009), skin cancer (Gali-Muhtasib *et al.* 2004b), osteosarcoma (Peng *et al.* 2013), colorectal carcinoma (Gali-Muhtasib *et al.* 2004a), glioblastoma (Gurung *et al.* 2010) and bladder cancer (Zhang *et al.* 2018a). On the other hand, it remains unclear whether thymoquinone has anti-cancer activity in AML. Although the effect of thymoquinone has been studied on many cancer types, the molecular mechanisms underlying its effect remain to be elucidated.

In some recent studies, thymoquinone has been reported to exhibit anticancer activities including induction of apoptosis (El-Mahdy *et al.* 2005) and autophagy (Zhang *et al.* 2018b) in different cancer cells. Autophagy and apoptosis are important mechanisms that determine the survival or death of cancer cells (Sun *et al.* 2019). Supporting and regulatory genes are included in apoptotic pathway. As the failure of the apoptotic process can cause drug resistance or tumor development, it is important to investigate new drugs that may affect the process (Artun & Karagöz 2021). Apoptosis is arranged by Bcl-2 family proteins and caspases. Autophagy is a survival mechanism stimulated in a problematic environment to preserve cell integrity by metabolic stress and/or food starvation. Autophagy is activated in conditions such as cell stress and damage, aging, pathogen infection (Chang *et al.* 2017) and deprivation of oxygen and growth factors (Meng *et al.* 2020). Studies show that two different cell deaths can be induced by communal signs that influence the development and treatment of cancer such as B-cell lymphoma 2 (Bcl-2), phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway, and p53 (Ge *et al.* 2013, Krumar *et al.* 2015, Liu *et al.* 2018, Saiki *et al.* 2011, Zhou *et al.* 2015). PI3K/AKT/mTOR pathway shows a central function in cell metabolism, cell proliferation, cell cycle progression, angiogenesis, autophagy and apoptotic cell death. It represents one of the main survival pathways that is unregulated in different cancer types and contributes to cancer pathogenesis and treatment resistance. This pathway, which is structurally active in most malignancies, leads to inhibition of programmed cell death and promotion of cell survival (Liu *et al.* 2018). Thus, the prevention of PI3K/AKT/mTOR signaling pathway could have enormous potential to induce cell death related with apoptosis and/or autophagy. On the other hand, the detailed mechanisms of diverse antitumor agent therapies, specifically natural remedies, which may contain diverse programmed cell deaths to some extent, are still not understood.

How thymoquinone induces cell death and whether the genes involved in these pathways play a role in thymoquinone induced cell death are not completely known. The present study explored the cytotoxic effect of thymoquinone and its effect on apoptotic and autophagic mechanisms by using the acute myelogenous leukemia cell line HL-60 (Human leukemia cell line). Additionally, molecular docking was also done to define the mechanism of anticancer activity of thymoquinone. The findings propose that thymoquinone prevents the growth of HL-60 cells inducing autophagy through down-regulation of Bcl-2 and PI3K/AKT/mTOR pathways.

Materials and Methods

Cell Culture

HL-60 cell line which modeled acute myeloid leukemia was used as cancer cells (Al-Otaibi *et al.* 2019). HL-60 cell line was obtained from İzmir Institute of Technology (İzmir, Turkey). HL-60 cells were grown in the presence of RPMI 1640 medium (Life Technologies), 10% warmth inactivated fetal bovine serum (Life Technologies), and 1% penicillin-streptomycin (100 U/mL) (Life Technologies). The cells were cultivated at 37°C in a humidified incubator with 5% CO_2 and 95% air atmosphere. The passage was done every 2 days.

A stock solution of thymoquinone (10 mM) was prepared by dissolving a defined amount of thymoquinone in dimethyl sulfoxide (DMSO) and stored at 4°C. The *last volume* of *DMSO* in the cells was less than 0.1%.

Methyl Thiazolyl Tetrazolium (MTT) Cell Viability Assay

Cell viability was analyzed based on MTT method according to the assay of Mossman (1983). From the cells in the appropriate medium, 7.5 x 10^3 cells per 100 µl per well were distributed into 96-well microplates. After 24 h of incubation, different concentrations of thymoquinone (1 µM, 5 µM, 10 µM, 15 µM, and 20 µM) were added on the cells, and the total volume was completed to 200 µL. Thymoquinone treated cells were incubated at 37°C in the presence of 5% CO_2 and 95% humidity for 24 h. After incubation, 20 µL of MTT (5 mg/mL phosphate buffer saline, pH 7.2) solution was added to each well, and the platelets were kept in a CO_2 oven for 4 h. Then, the medium on the cells was aspirated, and 100 µL of DMSO was added to each well and incubated at 37°C for 10

minutes. The colored solution was quantified at a wavelength of 570 nm via a microplate reader (Bio-Rad model 680 microplate reader). All investigations were done in triplicates for thymoquinone. Cell viability was measured by (Test OD/Control OD)×100. Polynomial regression analysis was performed through Microsoft Excel to determine the half-maximum inhibitory concentration (IC_{50}) values of thymoquinone.

RNA Isolation and cDNA Synthesis

Total RNA for cDNA synthesis was extracted from the cells using the PureLinkTM Mini Kit (Life Technologies, Carlsbad, CA, USA) procedure. Quality and quantity of the whole RNA samples were measured through NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA). The first-strand cDNA was produced through reverse transcriptase using total RNA isolated from the cells using High Capacity cDNA reverse Transcription Kit (Applied Biosystems, Foster City, CA) following the process specified in the user manual. cDNA products were diluted in *nuclease-free water* at 42°C for 1 h and stored at -20°C before further analysis.

Gene Expression Analysis by Quantitative Real-Time PCR

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) for specification of B-cell lymphoma 2 associated X protein (Bax), Bcl-2, caspase 3, PI3K, AKT, and mTOR gene expressions were done via a 7500 Fast Real Time PCR (Applied Biosystems). Bax, Bcl-2, caspase 3, PI3K, AKT, and mTOR gene expression quantities were obtained using TaqMan gene expression kits (Applied Biosystems, Foster City, CA) following manufacturer's protocols (Table 1). PCR mix solution was contained in diluted complementary DNA (cDNA) gene-specific TaqMan gene expression kit and TaqMan master mix (Applied Biosystems) in a final volume. qRT-PCR data were assessed through an ABI PRISM 7500 Sequence Detection system (Applied Biosystems). Every sample was assayed in triplicate.

Table 1. IDs for tagman gene expression assay.

Target gene	Assay ID
Bax	Hs00180269_m1 FAM-MGB
Bcl-2	Hs00608023_m1 FAM-MGB
caspase 3	Hs00234387_m1 FAM-MGB
mTOR	Hs00234522_m1 (FAM-MGB)
PI3KCA	Hs00907957_m1 (FAM-MGB)
AKT1	Hs00178289_m1 (FAM-MGB)

Molecular Docking Studies

Docking analysis was applied within Bax (PDB code:1F16), Bcl-2 (PDB code:2W3L), caspase 3 (PDB code:3KJF), PI3K (PDB code: 5JHB), AKT (PDB code: 3MVH), and mTOR (PDB code: 3JBZ) active sites, which were obtained from the protein data bank server (http://www.rcsb.org). The two-dimensional (2D) structure of thymoquinone was downloaded from Pubchem compound database (http://www.ncbi.nlm.nih.gov/search). The three dimensional (3D) structure for thimoquinone was generated using ACD/ChemSketch (Fig. 1). The structures of thymoquinone were submitted to the protein preparation module of AutoDock Tools 4.2 molecular modeling software (Morris *et al.* 2009). In molecular docking simulations, Glide/XP docking protocols were applied to predict the topology of thymoquinone in the active site of the target structure. Thymoquinone was docked to the active site of targets (Bax, Bcl-2, caspase 3, PI3K, AKT, and mTOR) using AutoDock Tools 4.2 molecular modeling software (Schrödinger Release 2016-2: Schrödinger, LLC, New York, NY, USA). The binding energy of docked molecules was computed via X-Score. The amino acid residues hydrophobic interactions and making hydrogen bonding were calculated making hydrogen bonding via Getneares, which is a device obtainable with DOCKv5.1.1.

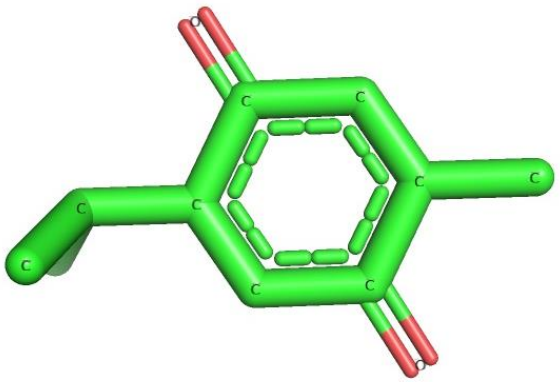

Fig. 1. Structure of thymoquinone.

Statistical Analysis

IC_{50} value was determined using Microsoft Excel program. All statistical analyses were done by Statistical Package for Social Sciences (SPSS) version 18.0 statistic software package (SPSS Inc, Chicago, Illinois). Statistical significances were compared by one-way analysis of variance (ANOVA) with Tukey's post-hoc test. Differences among the treated doses with $p<0.05$ was regarded as statistically significant.

Results

Cell Viability

In order to evaluate the cytotoxicity ability, MTT analysis of thymoquinone at diverse concentrations against HL-60 cells were conducted for the incubation duration of 24 h, and the outcomes are presented in Fig. 2. It was determined that the cell viability decreased by 1.3, 2, and 2.7 times in the groups treated with 10 µM, 15 µM and 20 µM thymoquinone, respectively, compared to the control group ($p=0.006$, $p<0.001$, $p<0.001$). IC_{50} concentrations for the thymoquinone were calculated from the dose response curves. Thymoquinone was exposed to cytotoxicity against HL-60 cell line with an IC_{50} value of 16.35 µM.

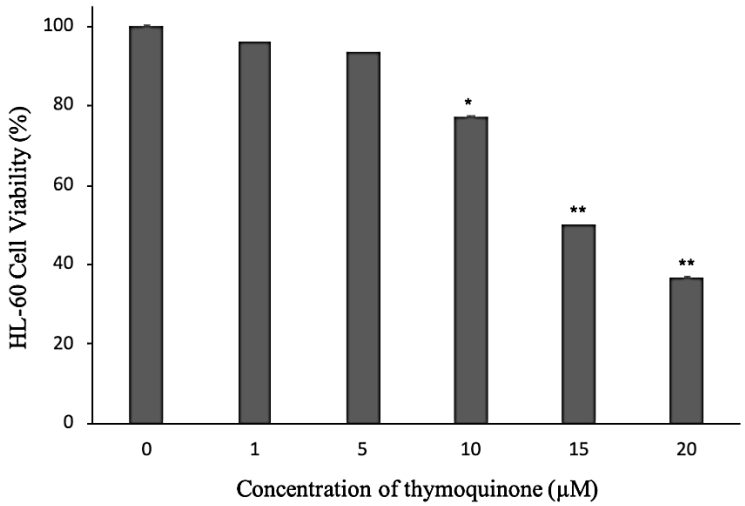

Fig. 2. The effect of thymoquinone on HL-60 cell viability after 24 h exposure. The results are expressed as means ± SD from three independent experiments. * indicates p<0.01 and ** indicates p<0.001 versus control.

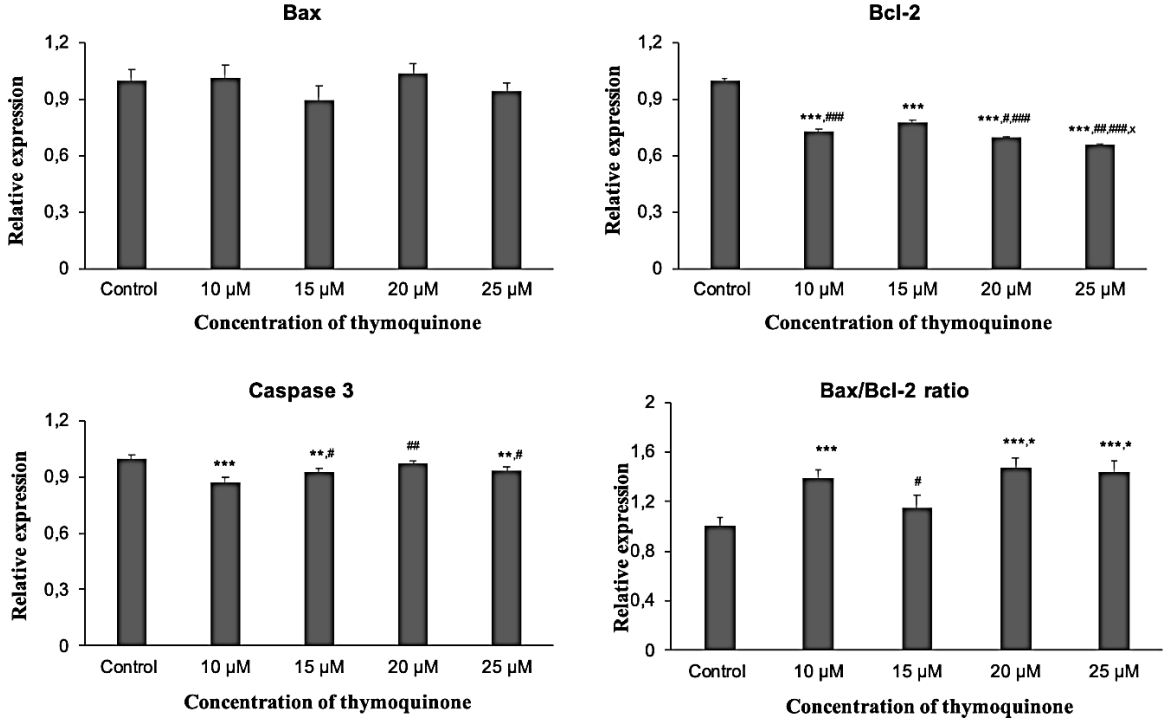

Fig. 3. The effects of thymoquinone on expression levels of Bax, Bcl-2, caspase 3, and the ratio of Bax/Bcl-2 genes in HL-60 cells. The values of each group were given as mean±standard deviation (SD). ***p <0.001 significant difference compared to the control, **p <0.01 significant difference compared to the control, # p <0.05 significant difference compared to 10 μM treatment group, ##p <0.001 significant difference compared to 10 μM treatment group, ### p <0.001 significant difference compared to 15 μM treatment group, *p <0.01 significant difference compared to 15 μM treatment group, ^{x}p <0.001 significant difference compared to 20 μM treatment group.

The Effect of Thymoquinone on Gene Expression Levels of Bax, Bcl-2, caspase 3, and Ratio of Bax/Bcl-2 Genes in HL-60 Cells

Bax and Bcl-2 genes are members of Bcl-2 family and exhibit a significant function in apoptotic cell death (Chen *et al*. 2017). Therefore, the expression of these genes were evaluated. The results are presented in Fig. 3. Bax and Bcl-2 gene expression levels were determined in HL-60 cells treated with control and four different doses of thymoquinone (10 μM, 15 μM, 20 μM, and 25 μM). There was no statistically significant difference in Bax gene expression levels between the groups (p=0.999, p=0.256, p=0.944, p=0.764). No significant difference was also

found in Bax values between the groups treated with thymoquinone. Bcl-2 gene expression levels were found to be lower in the groups treated with thymoquinone compared to the control group (p <0.001, p <0.001, p <0.001, p <0.001). Bcl-2 gene expression level in the cells which were treated with 25 µM thymoquinone was determined to be lower than the other experiment groups (p <0.001, p <0.001, p <0.001). However, it was determined that Bcl-2 gene expression level was lower in the group treated with 20 µM thymoquinone compared to the groups treated with 10 µM and 15 µM thymoquinone (p=0.021 for 10 µM, p <0.001 for 15 µM). Bcl-2 family shows proapoptotic or antiapoptotic effects by affecting caspase 3 activity (Chen *et al.* 2017). Therefore, the gene expression level of caspase 3 was also analyzed in the present study. The results are presented in Fig. 3. Caspase 3 gene expression levels were determined in HL-60 cells treated with the control and four different doses of thymoquinone (10 µM, 15 µM, 20 µM, and 25 µM). When caspase 3 gene expression level was evaluated, there was no statistically significant difference among the control group and the cells treated with 20 µm (Ꝑp=0.430). The treatments with 10 µm, 15 µm, and 25 µm thymoquinone significantly reduced caspase 3 levels compared to the control (p <0.001, p=0.004, p=<0.006). A reduction was stated on caspase 3 gene expression level at 10 µM thymoquinone treatment compared to other thymoquinone treated groups (p=0.032, p <0.001, p=0.020). The findings also showed a significant increase in Bax/Bcl-2 ratio in cure by thymoquinone (10 µM, 20 µM, and 25 µM) (p=0.001, p <0.001 p <0.001). The ratio of Bax/Bcl-2 also increased considerably at 10 µM (p=0.029), 20 µM (p=0.004) and 25 µM (p=0.008) concentrations compared to the group treated with 15 µM thymoquinone (Fig. 3).

The Effect of Thymoquinone on Gene Expression Levels of mTOR, PI3K, and AKT Genes in HL-60 Cells

PI3K/AKT/mTOR pathway is one of the main signaling pathways activated in cancer cells (Liu *et al.* 2018). Therefore, the influence of thymoquinone on PI3K/AKT/mTOR pathway was explored. The results of different treatment doses of thymoquinone on gene expression levels of mTOR, PI3K, and AKT genes in HL-60 cells are shown in Fig. 4. It was determined that the mTOR and PI3K gene expression levels were diminished in the cell groups treated with thymoquinone compared to the control group (p <0.001, p <0.001, p <0.001, p <0.001). There was a decrease in PI3K gene expression in thymoquinone groups treated with 15 µM, 20 µM, and 25 µM concentrations compared to the group treated with 10 µM (p=0.009, p <0.001, p <0.001) thymoquinone. When AKT gene expression levels were compared, a statistically significant reduction was found for 15 µM thymoquinone treated cells compared to the control group (p=0.025).

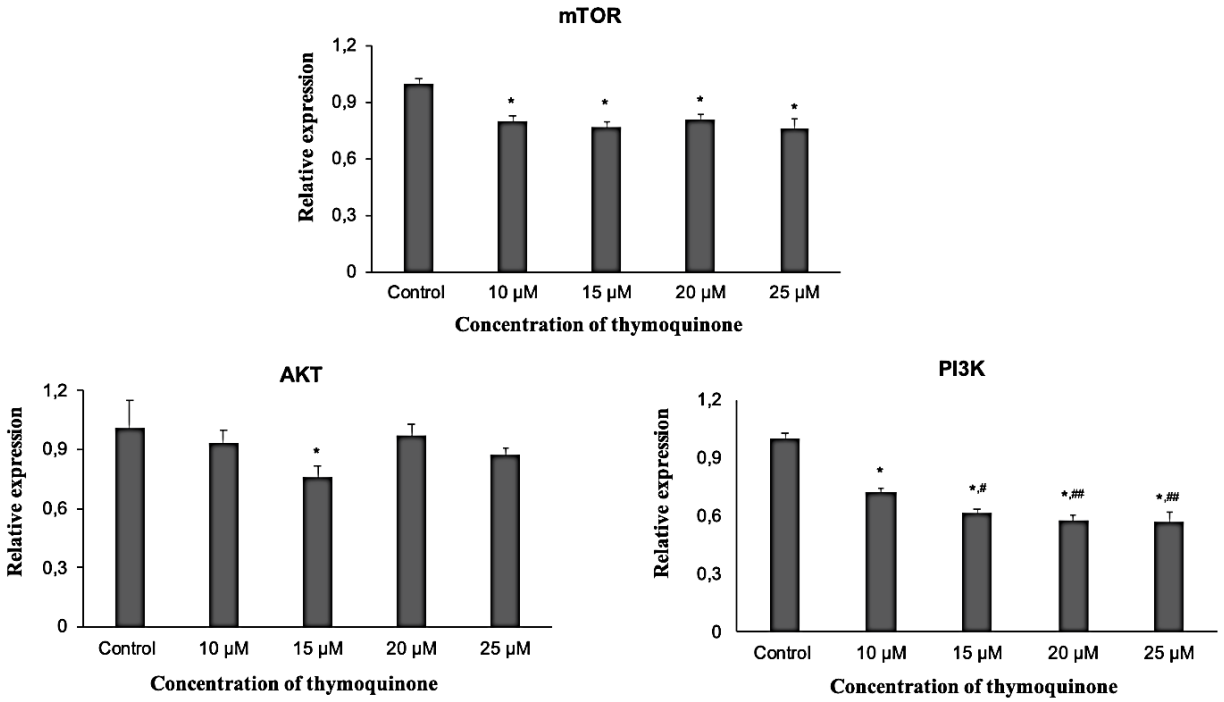

Fig. 4. The effects of thymoquinone on expression levels of mTOR, AKT, and PI3K genes in HL-60 cells. The values of each group are given as mean±standard deviation (SD). *p <0.001 significant difference compared to the control, **p <0.05 significant difference compared to the control, #p <0.01 significant difference compared to 10 µM treatment group, ##p <0.001 significant difference compared to 10 µM treatment group.

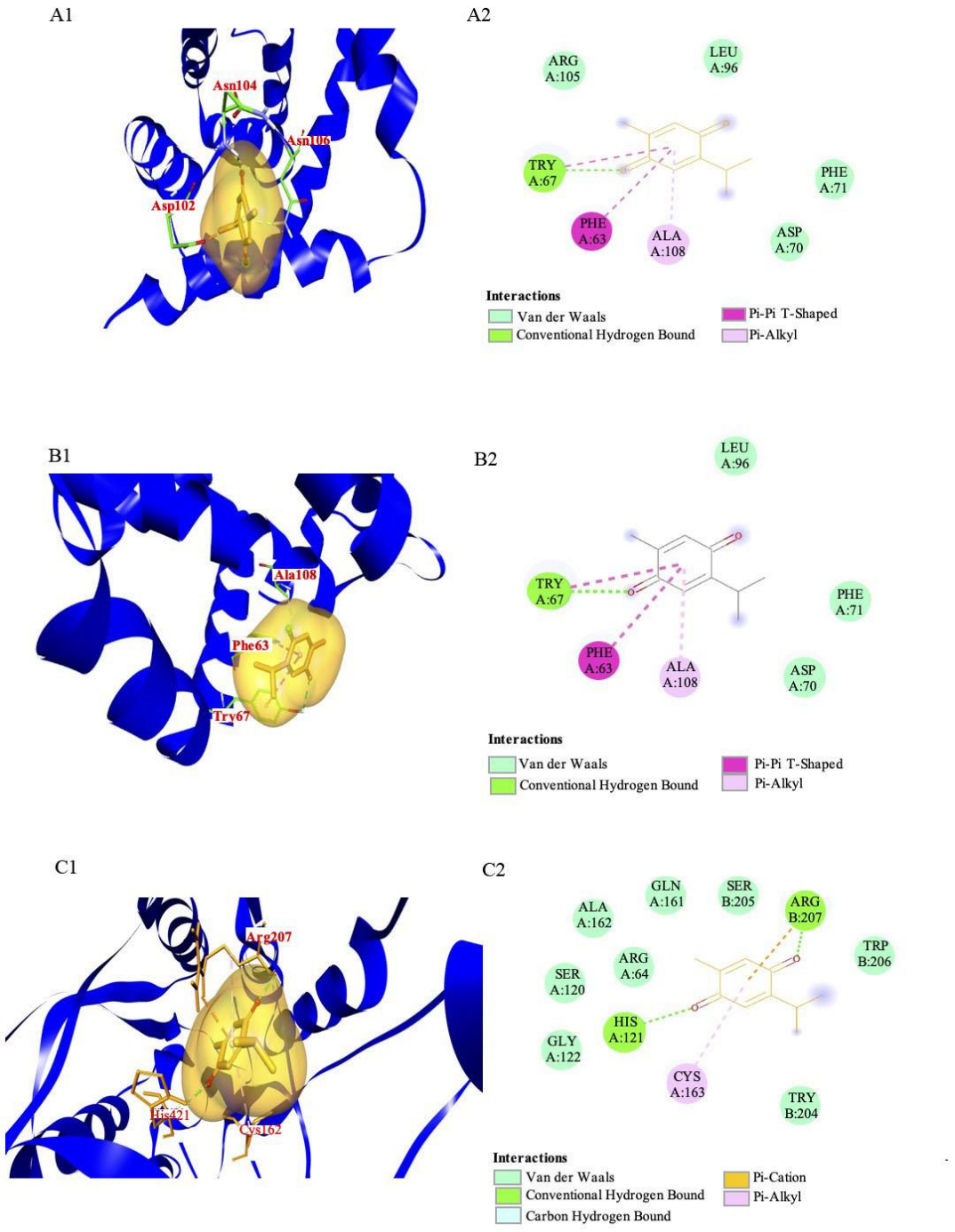

Fig. 5. Docking results of thymoquinone in the active sites of Bax, Bcl-2, and caspase 3, proteins. A1: Docking pose of thymoquinone in the active site of Bax; A2: Interactions of thymoquinone in the active site of Bax; B1: Docking pose of thymoquinone in the active site of Bcl-2; B2: Interactions of thymoquinone in the active site of Bcl-2; C1: Docking pose of thymoquinone in the active site of caspase 3; C2: Interactions of thymoquinone in the active site of caspase 3.

Molecular Docking Studies

Molecular docking simulations were implemented to reveal the potential binding modes of thymoquinone to the active sites of Bax, Bcl-2, caspase 3, PI3K, AKT, and mTOR (March 25, 2021). The docking results of thymoquinone specified that hydrophobic interactions and H bonds were responsible for the detected affinity (Figs 5, 6). Bax, Bcl-2, caspase 3, PI3K, AKT, and mTOR docked with thymoquinone with scores -4.3, -5.2, -5.2, -6.5, -7.5, and -5.9 kcal/mol, respectively (Table 3). However, the table ensures information for interacting residues with respective atomic contacts. All proteins had high affinity and strongly bonded with polar contacts (Table 3). The polar and nonpolar amino acid residues interacted with thymoquinone. Table 3 shows that Bax contains ASN104, ASN106, and ASP102; Bcl-2 contains TYR67, PHE63, TYR67, and ALA108; caspase 3 contains HIS121, ARG207, HIS121, ARG207, CYS163, and ARG207; PI3K contains ASP964, ILE879, and ILE963; AKT contains ALA230, LEU15, and ALA77; mTOR contains VAL2240, MET2345, MET2345, TRP2239, and TRP2239.

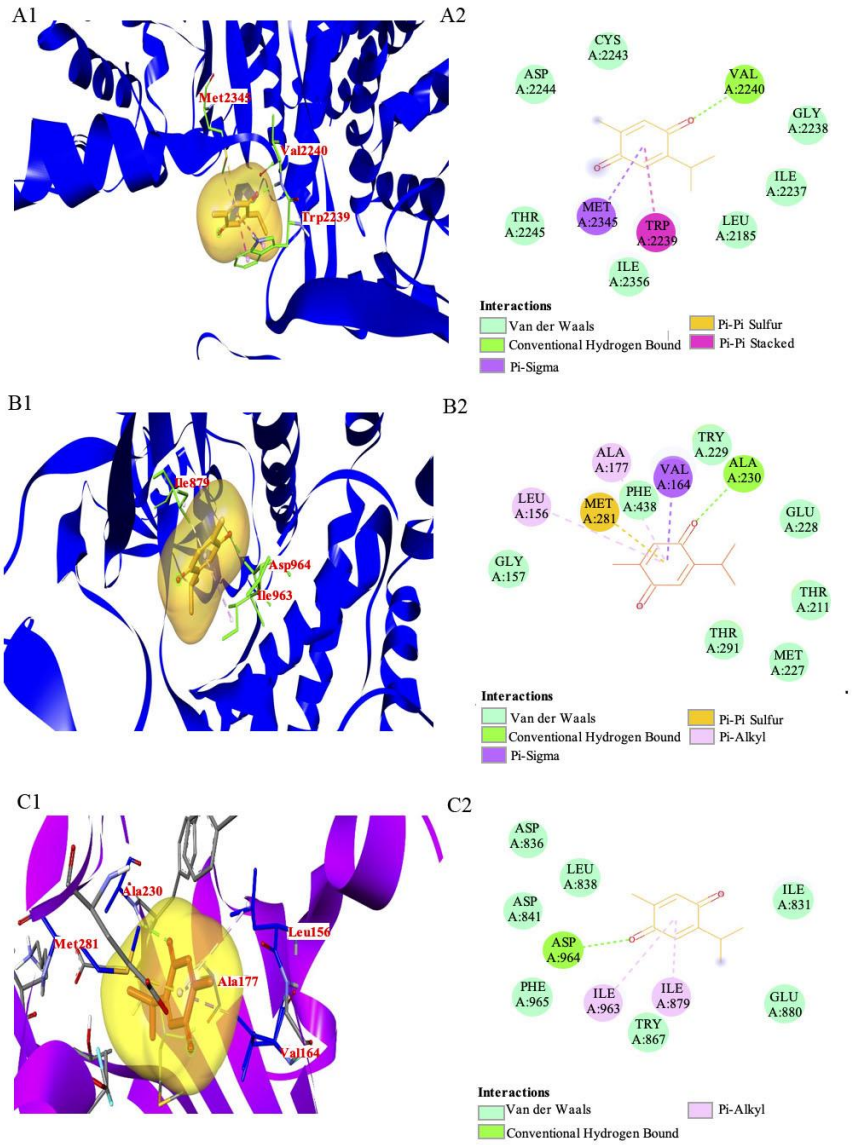

Fig. 6. Docking results of thymoquinone in the active site of mTOR, PI3K, and AKT proteins A1: Docking pose of thymoquinone in the active site of mTOR; A2: Interactions of thymoquinone in the active site of mTOR; B1: Docking pose of thymoquinone in the active site of PI3K; B2: Interactions of thymoquinone in the active site of PI3K; C1: Docking pose of thymoquinone in the active site of AKT; C2: Interactions of thymoquinone in the active site of AKT.

Table 3. Binding energy values and the important amino acids in the active sites of the Bax, Bcl-2, caspase 3, PI3K, AKT, and mTOR proteins which are responsible for interactions.

Receptors	**Compound name**	**Parameters**					
		Binding energy (kcal/mol)	Number of hydrogen bonds	Hydrogen Bonding with Amino acid residues	Number of hydrophobic interactions	Hydrophobic Bonding with Amino acid residues	Distance (Å)
Bax	Thymoquinone	-4.3	2	ASN104-ASN106	1	ASP102	2.5802
Bcl-2		-5.2	1	TYR67	3	PHE63-TYR67-ALA108	2.51399
caspase 3		-5.2	3	HIS121-ARG207-HIS121	3	ARG207-CYS163-ARG207	2.08891
PI3K		-6.5	1	ASP964	2	ILE879-ILE963	2.12918
AKT		-7.5	1	ALA230	4	VAL164-MET281-LEU15-ALA177-	2.23519
mTOR		-5.9	1	VAL2240	4	MET2345-MET2345-TRP2239-TRP2239	2.04166

Discussion

Cancer is one of the world's greatest health problems, so the identification of new antitumor drugs is of paramount importance. Pharmaceutical plants are good sources of biologically active compounds that contain antioxidant and immunomodulatory features, making them potential candidates as anticancer agents (Majoloab *et al.* 2019). In addition to being edible (Samarghandian *et al.* 2019), natural compounds are also gaining attention as cancer therapeutic agents due to their more effective but less toxic profile (Gupta *et al.* 2013).

The precise mechanisms underlying thymoquinone's anticancer effects have not been fully discovered. There are not enough studies investigating the apoptotic and autophagic effects of thymoquinone in AML HL-60 cells. Therefore, apoptotic and autophagic mechanisms were evaluated by looking at the effect of thymoquinone on Bax, Bcl-2, caspase 3, mTOR, AKT and PI3K gene expressions in HL-60 cells in the present study.

Thymoquinone has a low overall toxicity as well as promising anti-tumor activity (Effenberger-Neidnicht & Schobert 2011). Studies showed that thymoquinone can prevent the proliferation of human promyelocytic leukemia HL-60 cells through apoptotic cell death (Racoma *et al.* 2013). According to the results of MTT test, it was determined that thymoquinone prevents the proliferation rate of HL-60 cells in a dose-dependent way. Such an inhibitory effect may be related to the apoptotic properties of thymoquinone. After this finding, the focus was shifted to apoptotic and autophagic gene markers to explain the influence of thymoquinone on cell death mechanisms.

Apoptotic induction induced movement of Bax from cytosol to mitochondria, leading to multimerization, integration and cytochrome c release, caspase activation, and apoptotic cell death (Aykaç *et al.* 2015). While Bcl-2 defends cells from apoptotic cell death, the over expression of Bax can stimulate apoptosis (Salim *et al.* 2014). In the current study, a reduction in Bcl-2 gene expression levels was detected in cells treated with 10 μM, 15 μM, 20 μM, and 25μM thymoquinone compared to the the control. A higher level of Bcl-2 gene expression was detected in cells applied with 15 μM of thymoquinone compared to other treatment groups. This result suggests that the anticancer effects of thymoquinone may possibly be associated with the inhibition of antiapoptotic gene expression of Bcl-2. In HL-60 cell line, there was no alteration in Bax expression level in the groups applied with thymoquinone compared to the control. Bax/Bcl-2 ratio instead of Bcl-2 alone is important for the occurrence of drug-induced apoptosis in leukemia cell lines (Salim *et al.* 2014). Bax/Bcl-2 ratio determines the sensitivity or resistance of cells to apoptotic inducements, containing cure with natural compounds (Christodoulou *et al.* 2014). Although Bax ratio did not change in the present study, it was found that Bax/Bcl-2 proportion was higher in the thymoquinone applied groups compared to the control. The rise in Bax/Bcl-2 proportion determined in the study was consistent with recent studies using MCF-7 (Woo *et al.* 2011), HL-60 (El-Mahdy *et al.* 2005) and HCT116 (Gali-Muhtasib *et al.* 2004b) cancer cells. The increase in the proportion of total Bax/Bcl-2 proposes that it may be one of the thymoquinone induced apoptosis mechanisms.

Induction of apoptotic cell death is an important mechanism for cancer prevention (Zhang *et al.* 2012). Apoptogenic factors release and activate caspases, which are the promoters of apoptosis (Chen *et al.* 2017). Caspase 3 is the most significant mediator of apoptosis and is often used to determine apoptotic activity (Huang *et al.* 2010). Caspase 3 is the driving factor of apoptotic cell death, as it activates endonucleases to break down nuclear DNA, resulting in DNA fragmentation leading to cell death (Chen *et al.* 2017). Resistance to apoptotic stimuli, including chemotherapeutic agents, in leukemia is known to be due to insufficient expression of caspase 3 (Fricdrich *et al.* 2001). In many reports using thymoquinone, the role of the caspase pathway in apoptosis has been determined (Paramasivam *et al.* 2012). El-Mahdy et al. (2005) stated that thymoquinone stimulated apoptotic cell death in HL-60 cells was due to caspase activation. In the present study, a reduction in caspase 3 expression levels was determined in HL-60 cell line in the groups treated with 10 μM, 15 μM, and 25 μM thymoquinone compared to the control. The decrease in caspase 3 expression level was an unexpected finding. However, the decrease in caspase 3 level with selected doses of thymoquinone therapy proposes that cancer cells downregulate caspase 3 expression to escape apoptosis. It means that down-regulation of caspases may indicate a resistance mechanism of cancer cells to thymoquinone stimulated apoptosis. However, this result suggests that thymoquinone induced cell death may be due to the activation of a different pathway that occurs independently of caspase 3. Therefore, genes in the autophagic pathway were further analyzed in the present study.

The role of autophagy in tumors continuously alters with the development of the tumors. Autophagy suppresses the onset of cancer by removing genome imbalance, inflammation, and tissue injury in the early stages of tumor formation. mTOR is a serine / threonine protein kinase that regulates autophagic progression (Meng *et al.* 2020). It has been reported that mTOR is a negative regulator of autophagy, and mTOR inhibition can trigger autophagy (Fan *et al.* 2018). mTOR is regulated by a kinase cascade that includes class I phosphatidylinositol 3-kinase (PI3K) and serine / threonine kinase (AKT). PI3K activates AKT, which then activates mTOR (Meng *et al.* 2020). Studies showed that autophagy can be mediated in a variety of ways, containing PI3K/AKT/mTOR (Zhang *et al.* 2018b). PI3K/AKT/mTOR pathway modulates various cellular events that are also important for carcinogenesis, such as cell metabolism, cell cycle progression, cell proliferation,

angiogenesis, autophagy and apoptosis. Recently, intensive endeavors have been taken on inhibitors of PI3K/AKT/mTOR pathway to cure cancers and discover new inhibitors, due to the significantly altered expression of PI3K/AKT/mTOR proteins in various tumors (Pathania *et al.* 2013). This pathway can be manipulated to induce autophagy in cancer cells as cancer cells continuously initiate PI3K/AKT/mTOR, which negatively regulates autophagy (Kuttikrishnan *et al.* 2019). Upregulation of mTOR pathway has been stated in many oncogenic disorders, including acute and chronic leukemias. mTOR activation is usually induced by PI3K/AKT (Paramasivam *et al.* 2019). While studies showed that thymoquinone can stimulate autophagy in drug-resistant colon cancer cells and squamous carcinoma cells (Zhang *et al.* 2018b), the influence of thymoquinone on autophagy in HL60 cell lines has not been studied. However, in studies of Iskender *et al.* (2016a, b) on bladder cancer, thymoquinone was reported to weaken mTOR activity and inhibit PI3K/AKT signal. In the present study, it was determined that four different thymoquinone concentrations (10 μM, 15 μM, 20 μM, and 25 mM) decreased mTOR and PI3K gene expressions compared to the control group. Several other studies have suggested that thymoquinone may exert its inhibitory effects on cell proliferation through regulating the activity of AKT, a known positive modulator of cell survival (Majdalawieh *et al.* 2017). It was determined that AKT gene expression level decreased in the group treated with 15 μM of thymoquinone compared to the control. AKT inhibition may be responsible for the decrease in mTOR activity.

Today, the use of *in silico* methods help scientists in the discovery of medical agents and play a very important role in structure-based drug design. As cancer cells are more prone to apoptosis, a comprehensive understanding of the molecular pathways that regulate apoptosis may aid in the search for new cancer chemotherapeutic targets. This will open up new opportunities for drug discovery and development (Sitheek et al. 2020). The docking explorations in the present study were done with the purpose of defining possible anticancer action mechanisms of thymoquinone. The binding energy, hydrogen bond interaction and hydrophobic interaction between the thymoquinone and apoptotic and autophagic proteins were documented for further consideration. The results showed that there is a presence of binding site between Bax, Bcl-2, caspase 3, mTOR, PI3K, and AKT proteins and thymoquinone. The docking study is valid especially for the formation of hydrogen bond among thymoquinone and PI3K, AKT, and mTOR. Thymoquinone showed H-bond interactions with residues that were advised to be necessary for inhibitory activity. The interactions showed optimal binding features for further consideration of thymoquinone as a possible drug candidate in cancer exploration. Therefore, PI3K/AKT/mTOR pathway can be an encouraging goal for anticancer drug design.

In the present study, it was determined that thymoquinone application affects the expression levels of mTOR, AKT, and PI3K genes that promote cell viability, proliferation and progression in cancer cells. PI3K, AKT, and mTOR gene expressions were down-regulated in response to treatment with thymoquinone. This study is the first to emphasize that thymoquinone can significantly prevent the proliferation of HL-60 cells by preventing the PI3K/AKT/mTOR pathway and inducing apoptosis. These observations suggest that it may lead to the development of new therapeutic approaches for human cancers targeting PI3K/AKT/mTOR signaling pathway. These data demonstrate the new and imperative function of thymoquinone in PI3K/AKT/mTOR signaling pathway in the cell death mechanism of HL-60 cell lines.

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