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Potential Cytotoxic, Antifungal, and Antioxidant Activity of Dithymoquinone and Thymoquinone

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Abstract: *Nigella sativa* is a well-known plant with various applications in traditional medicine. The research goals of the current study were focused on discovering *Nigella sativa's* active substances with antifungal, antioxidant, and anticancer properties. The major compound of the plant's essential oil was found to be thymoquinone (TQ), which was used to synthesize dithymoquinone (DTQ) by the simple photodimerization method. Formation of the compound was confirmed by high-performance liquid chromatography (HPLC), HNMR, IR, and ESI. Both compounds (TQ and DTQ) were assayed for antifungal, antioxidant, and cytotoxic properties, and the results showed higher activity of TQ compared to DTQ. In conclusion, thymoquinone is a promising antifungal, antioxidant, and anticancer agent and is recommended for further pharmaceutical evaluation. To the best of our knowledge, this is the first study to evaluate the bioactivity potential of DTQ synthesized from thymoquinone as it is present in low quantities in the essential oil of *Nigella sativa*.

Keywords: cytotoxicity, antioxidant, thymoquinone, dithymoquinone.

二百里醌和百里醌的潜在细胞毒性、抗真菌和抗氧化活性

摘要：黑种草是一种众所周知的植物，在传统医学中具有多种应用。当前研究的研究目标集中在发现具有抗真菌、抗氧化和抗癌特性的黑种草活性物质。发现该植物精油的主要化合物是百里醌（质量保证），它被用于通过简单的光二聚法合成二百里醌（DTQ）。通过高效液相色谱(高效液相色谱)、核磁共振、红外线和
ESI确认了化合物的形成。测试了两种化合物（质量保证和DTQ）的抗真菌、抗氧化和细胞毒性特性，结果表明与DTQ相比，质量保证的活性更高。总之，百里醌是一种很有前途的抗真菌、抗氧化剂和抗癌剂，推荐用于进一步的药物评估。据我们所知，这是第一项评估由百里醌合成的DTQ生物活性潜力的研究，因为它在黑种草精油中的含量很低。

关键词：细胞毒性，抗氧化剂，百里醌，二百里醌。

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1. Introduction

Scientific interest in medicinal plants and their ingredients has increased because of the high efficiency of herb-derived drugs and increasing interest in natural products. The seeds of *Nigella sativa* (from the Ranunculaceae family) have been used for thousands of years as a condiment and food preservative. TQ, as the bioactive component of the fugacious oil of the plant's black seeds, has demonstrated potent medicinal effects in traditional medicine [1]. The sudden emergence of multidrug-resistant pathogens and the rapid spread of new diseases has prompted health institutions and pharmaceutical companies around the world to change their policies regarding avoidance of the production of antibiotics based on traditional ingredients to combat multidrug-resistant pathogens [2].

Antibiotic resistance in pathogenic fungi is a major international issue of great concern due to the various diseases that pathogenic fungi cause. Furthermore, mutant resistant fungi have been documented all over the world, both in the lab and in the clinic, that show resistance to a wide range of antibiotics other than those originally used for treatment. This problem is exacerbated by the insufficient number of antifungal medications available [3].

Accordingly, the quest for plant-based medications and dietary supplements has intensified in recent years. Microbiologists, botanists, natural products chemists, and even ethnopharmacologists are scouring the globe for phytochemicals and "leads" that could be formulated as new antimicrobial drugs with innovative mechanisms different from ineffective antibiotics [4].

In this study, the essential oil content of *N. sativa* L. was determined to be 0.4–0.5% (w/w). This extract was analyzed by gas chromatography-mass spectrometry, which revealed the presence of the following major components: 62.17% TQ, 16.84% carvacrol, 8.29% 2-methyl-5-Prop-2-enyldihydroquinone, 6.99% dihydrothymoquinone, 2.07% terpini-4-en-1-ol, and 3.11% monoterpene [5]. Ozer et al. [6] investigated the protective effects of TQ on survival, vascular reactivity, mesenteric artery blood flow, oxidative, and inflammatory responses in rats. TQ has been suggested as a potential agent for fighting chemotherapy-induced nephrotoxicity [7].

TQ has demonstrated histopathological protective effects on multiple organ injury [8]. It also exerts antioxidant effects via strengthening of the oxidant scavenging system. Thus, it also has antitoxic properties. In addition, TQ has shown potential anti-inflammatory effects [9, 10]. Hanieh [11] indicated protective features of TQ against inflammation and oxidative stress in renal disorders. Likewise, TQ has shown anti-inflammatory and antioxidant properties in animal and in vitro models of several renal diseases caused by inflammation and oxidative stress. [11].

The main constituents of *Nigella sativa's* black seeds are three benzoquinone compounds: (1) thymoquinone, (2) thymohydroquinone, and (3) dithymoquinone [12]. The first two compounds are available for purchase from Sigma-Aldrich, while the third compound is not available. Thus, the present study aimed to synthesize dithymoquinone by photodimerization of thymoquinone and investigate the cytotoxic, antioxidant, and antifungal activities of both compounds.

2. Materials and Methods

2.1. Raw Materials

Thymoquinone (acetone, ethanol [C_2H_5OH], and HPLC-grade methanol) was purchased from Merck. De-ionized water was used throughout this study. The characterization methods and typical conditions for dithymoquinone are listed in Table 1.

Table 1 List of characterization methods for dithymoquinone

Analysis	Instruments
TLC	Silica TLC plates
FT-IR spectra	Brüker Tensor 27with ATR configuration.
^{1}H NMR	BrükerAvance 400 NMR spectrometer
UV-Vis's spectra	Varian Cary® 50 Scan spectrometer in a 1.0 cm square cuvette.
. ESIMS	LC-MS(/MS)

2.2. Synthesis of Dithymoquinone (DTQ)

TQ (0.50 g,) was dissolved in acetone (5.0 mL) in a 500 mL glass beaker. The bright yellow solution was gently rotated along the inner surface of the beaker until complete evaporation to a thin, crystalline layer. The resulting thin layer (solid state) of TQ was exposed to an ultraviolet lamp (345 ƛ max) in a fume hood at room temperature. The reaction was found to be > 99% complete after eight hours. The photodimerization reaction was monitored by TLC. Crude product was dissolved in a small amount of DCM, loaded on silica gel, and then purified by column chromatography using silica gel for the stationary phase and hexane and ethyl acetate (9:1) for the mobile phase.

Thymohydroquinone was dissolved in a minimal volume of ethyl acetate, transferred to a smaller Erlenmeyer flask, and then evaporated to dryness over gentle heat. Crystallization of thymohydroquinone into DTQ was performed using ethanol to render fine, pale yellow needle-like crystals. Ultra-pure water and cold 2-propanol were re-centrifuged and lyophilized overnight to dryness [13]: thymohydroquinone (110 mg, 22% yield, m.p. 200.5°C); UV_{max} 250 nm and UV_{min} 380 nm; IR

(solid state): 3060 (vinylic C = C-H stretch); 2969–2872 (C-H stretch of aliphatic groups); H^1NMR (600 MHz); δ 6.70 (s ,2H); δ 3. 01(s 2H); δ 3.12-3.06 (septet, j = 6.6,2 H); δ 1.22 (s, 6H); δ 1.16-1.13 (2d, j = 7.2, 6.6, 12 H); ESIMS: 329 [M + 1].

2.3. Microorganisms and Inoculum Preparation

The experiments were conducted at the University of Assiut Mycological Center, Egypt in November 2020. Six strains of yeast and fungi were tested: *Aspergillus flavus* AUMC 1276, *Candida albicans* AUMC 1299, *Fusarium oxysporum* AUMC 215, *Geotrichum candidum* AUMC 226, *Scopulariopsis brevicaulis* AUMC 1653, and *Trichophyton rubrum* AUMC 1804. Microorganisms were sub-cultured on Sabouraud Dextrose agar (SDA; Himedia, India), and, before conducting each antimicrobial test, a single colony from fresh microbial pure cultures was transferred to Sabouraud Dextrose broth (SDA; Himedia, India). The microbial cultures were then incubated at 37°C for 24 to 48 hours to ensure a fresh culture was used.

2.4. In Vitro Antimicrobial Bioassay

The well diffusion method was used for the antimicrobial assays as mentioned in Abdel Gawwad et al. [14] with minor modification. Sterile plates containing nutrient agar were prepared according to the manufacturer's instructions and allowed to solidify. The agar was then punched with a sterile cork borer (size 6 mm). Subsequently, the agar plates were seeded with fungal and yeasts strains adjusted to the McFarland solution using a sterile cotton swap, and a dose of 50 µl test solution was loaded into wells. Standard antifungal drug (Clotrimazole 10 mg/ml) was served as positive control and was launched into a separate well on the agar plates. All the seeded plates were incubated for 24–48 h at 37 °C for yeasts or for 48–72 h at 30–35°C for fungi. Inhibition zones were measured using a transparent ruler in the nearest millimeters [14].

2.5. Minimum Inhibitory Concentration Test

The negative control consisted of a broth medium without inoculum and antimicrobial agent, and the positive growth control contained pure microbial culture without an antimicrobial agent. The microtiter plates were incubated aerobically at 35°C for 48 h. The lowest concentration of extracts that did not show visible growth was recorded as the MIC. A broth medium without inoculum or antimicrobial agent served as the negative control, while pure microbial culture served as the positive growth control. The microtiter plates were incubated aerobically for 48 h at 35°C in the Sabouraud Dextrose broth. The MIC was determined as the lowest concentration of extracts that did not display visible growth [15].

2.6. 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Radical Scavenging Capacity

The antioxidant potential was evaluated by the DPPH radical scavenging activity as described in [16]. Ascorbic acid was used as a positive control, and the radical scavenging activity was expressed as % DPPH inhibition, calculated from the graph of inhibition percentage plotted.

DPPH radical scavenging activity = $(A_0\text{-}A_1) \setminus A_0 \times 100$,

where A_0 is control absorption (DPPH), and A_1 is the sample absorption.

Percentage radical activity was plotted against the concentration of the corresponding antioxidant substance to obtain the IC_{50} value.

2.7. Cytotoxicity

Cell viability was assessed with WST-1 assay using Abcam® kit (Proliferation Reagent). Aliquots of 50 µL cell suspension (3x103 cells) were seeded in 96-well plates and incubated in complete media for 24 h. Cells were treated with another aliquot of 50 µL media containing drugs at serial concentrations. After 48 h of drug exposure, cells were treated with 10 µL WST-1 reagent, and the absorbance was measured after 1 h at 450 nm using a BMG LABTECH®- FLUOstar [17].

2.8. Statistical Analysis

Quantitative data were expressed as the mean ± standard error of means. A paired sample t-test was employed to determine if there were any significant differences between each tested compound with the referenced antifungal drug. The program used was SPSS-Statistical Package, version 11.

3. Results and Discussion

Solid state photodimerization of 1 to 2 had been previously shown to proceed via a [2 + 2] cycloaddition reaction. Dithymoquinone (DTQ) was synthesized by a simple photodimerization reaction of compound 1 TQ (thymoquinone), which was adopted from Sigma-Aldrich. The synthetic pathway was outlined in Scheme 1. The prepared compound 2 DTQ was monitored by TLC [18] and confirmed by spectral analysis (UV, IR, HNMR, and ESIMS). HPLC detected the purity of the compound, which is further discussed in the experimental part.

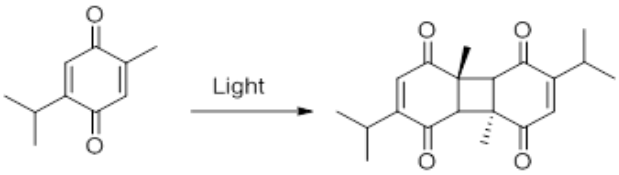


Fig. 1 Scheme for the synthesis of compound 2 (DTQ) from compound 1(TQ)

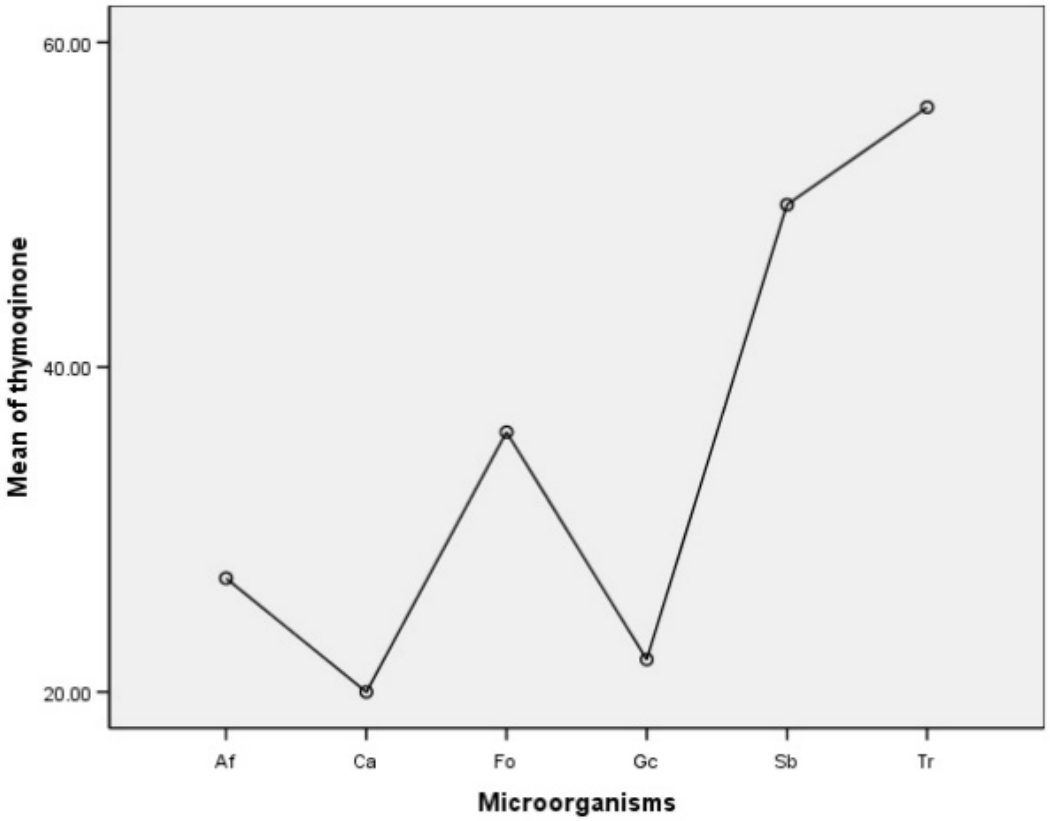
3.1. FTIR of Thymoquinone and Dimmer

In the experimental spectra of both thymoquinone and dimer, medium bands were detected at a range of 2974-2875 cm^{-1}, which can be associated with the alkane molecular stretching (νCH_2) vibration. The strong band appeared at 1646 cm^{-1} due to stretching vibrations of ν(C = O) of conjugated ketone. At a wavenumber of ≈ 1606 cm^{-1}, the strong band attributed to stretching vibrations of ν(C = C) was of α, β-unsaturated ketone. The obtained FTIR spectra of the bending δ(C-H) vibration resulted in the absorption band [19]. The strong band appeared at 1231cm^{-1} due to stretching vibrations of ν(C-O) of aromatic ether. The two medium absorption bands at 1124 cm^{-1} and 900 cm^{-1} attributed to bending vibration of δ(C = C) of α,β-unsaturated alkene.

3.2. ^{1}HNMR Spectra

NMR spectra are consistent with forming a single isomer of photodimerization reaction, with only one set of peaks in 1H NMR spectra. Furthermore, the olefinic methyl (δ 2.03 ppm, s) and proton of the same double bond (δ 6.18 ppm, s) of thymoquinone not shown, disappear upon irradiation and are accompanied by the appearance in the aliphatic region of the spectrum two new singlets (1.21 and 3.29). This result identifies the double bond involved in dimerization. ESI of the compound proved and confirmed the formation of dimmer. There is a peak at m/z, 351 [M+ Na], corresponding to the molecular weight of Compound 2 DTQ. The absence of a peak at m/z 165 [M+H] relayed to TQ confirmed the complete reaction; purity was followed by HPLC.

Results of the antifungal potential of the dimmer of thymoquinone and thymoquinone are shown in Table 2. The results were very interesting; both compounds showed noticeable antifungal activity. The dimmer of thymoquinone isolated from the black seeds showed moderate to high antifungal activity. The zones of inhibitions of the most susceptible microorganism w*ere* *Trichophyton rubrum* (44.0 mm), *Scopulariopsis brevicaulis* (34.0 mm), *Fusarium oxysporum* (22.0 mm), *Aspergillus flavus* (20.0 mm), *Geotrichum candidum* (18.0 mm), and *Candida albicans* (12.0 mm). On the other side, the thymoquinone isolated from black seeds showed higher antifungal activity than the dimmer of thymoquinone. The highest inhibition zones made by tested microorganisms were *Trichophyton rubrum (56.0 mm)*, *Scopulariopsis brevicaulis* (50.0 mm), *Fusarium oxysporum* (36.0 mm), *Aspergillus flavus (27.0 mm)*, *Geotrichum candidum* (22.0 mm), and *Candida albicans (20.0 mm)*. However, the thymoquinone recorded significant activity when we statistically compared each isolated compound with the referenced antifungal drug (Clotrimazole). In contrast, the dimmer of thymoquinone revealed non-significant effects at $P < 0.05$ (Fig. 2). This amazing result means that thymoquinone isolated from black seeds has a comparable antifungal effect and could be used as an effective antifungal drug. To the best of our knowledge, this is the first study recommending this compound as a novel antifungal drug. The MIC results support this claim, with the results ranging between 12.5 to 0.78 mg/ml for the dimmer of thymoquinone, and between 12.5 to 1.56 mg/ml for thymoquinone isolated from black seeds. Previous studies mentioned that the essential oil and various extracts of black seeds, particularly thymoquinone, have high antifungal activity against three pathogenic dermatophyte strains, namely Trichophyton mentagrophytes, Microsporumcanis, and Microsporumgypseum, and the study suggests the use of thymoquinone as an anti-dermatophyte drug [20]. Fixed oil of black seeds (Nigella sativa) showed good antifungal efficacy against Candida parapsilosis ATCC 22019 (13.33 mm) and Candida glabata ATCC 90030 (12 mm) with minimal MIC values [21]. The essential oils of the black seeds exhibited potent antifungal activity against eight seed borne fungi, namely Fusarium oxysporum, F. moniliforme, F. nivale, F. semitectum, Aspergillus niger, A. flavus, Alternaria alternata, and Drechslerahawiinesis [22]. Another in vivo study showed that the *Nigella sativa* seeds have a remarkable inhibitory effect against candidiasis induced in experimental mice [23]. Therefore, we highly recommend the development of an antifungal drug based on thymoquinone from black seeds.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Mean of thymoqinone
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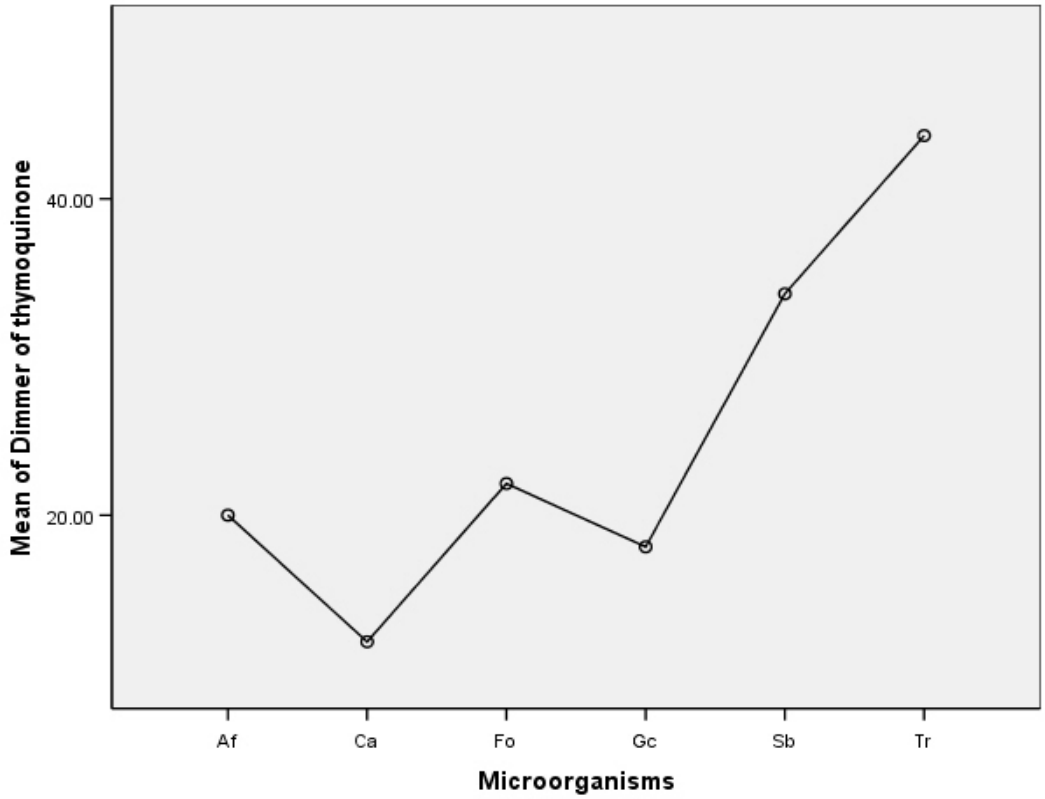
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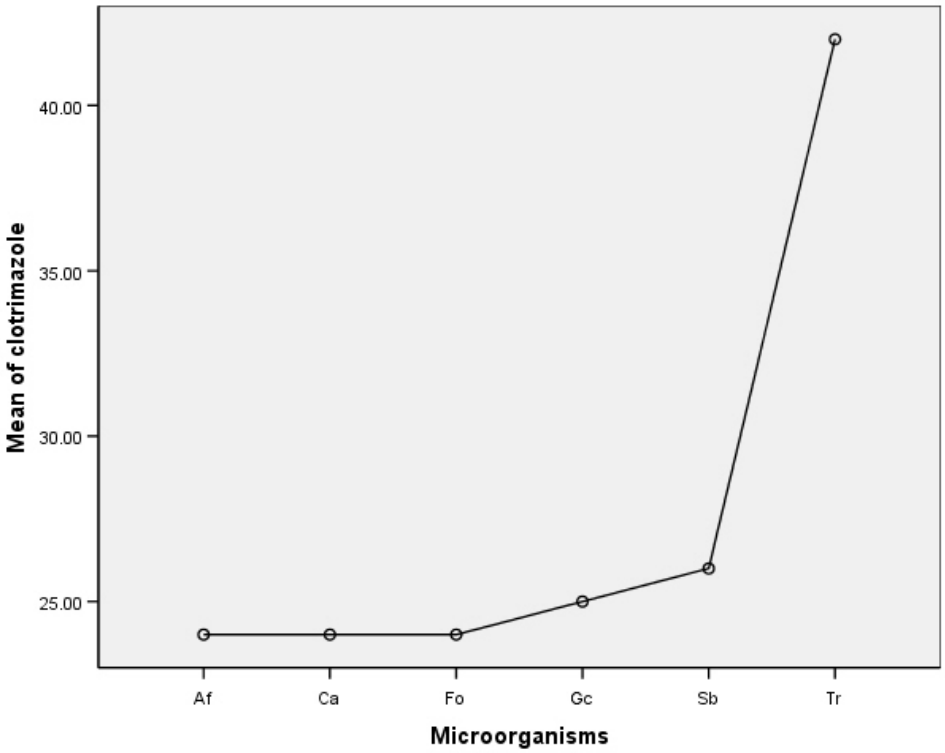

Fig. 2 Mean plots of the antifungal potential of the isolated compounds compared to the referenced antifungal drug

Table 2 Antifungal activity of tested compounds (inhibition zone and MIC) *

Tested compounds	Af	Ca	Fo	Gc	Sb	Tr
	ZI(MIC)	ZI(MIC)	ZI(MIC)	ZI(MIC)	ZI(MIC)	ZI(MIC)
Dimmer of Thymoquinone	20(12.5)	12(1.56)	22(6.25)	18(0.78)	34(6.25)	44(3.125)
Thymoquinone	27(12.5)**	20(6.25)**	36(3.125)**	22(1.56)**	50(1.56)**	56(1.56)**
Clotrimazole	27(0.039)	24(0.156)	24(0.039)	25(0.0039)	26(0.312)	42(0.019)

* Inhibition zone in mm, MIC in mg/ml, Af - *Aspergillus flavus*, Ca - *Candida albicans*, Fo - *Fusarium oxysporum*, Gc - *Geotrichum candidum*, Sb - *Scopulariopsis brevicaulis*, Tr - *Trichophyton rubrum*, ** - Significant at $P < 0.05$

3.3. Antioxidant Activity

Nigella sativa is a natural source of many active compounds used to overcome the effects of oxidative stress underlying pathogenic disease. The DPPH radical scavenging activity of two major compound of plant N. sativa, TQ and DTQ, were assayed by DPPH radical scavenging methods [19], with the results illustrated in Table 3. The results demonstrate the variation in antioxidant activity of the compounds, with TQ showing high antioxidant activity with IC50 (78.38 ± 0.01 mg\ml) compared to the activity of Ascorbic acid, while DTQ shows the lowest DPPH antioxidant activity with IC50 (95.95 ± 0.01 mg/ml).

Table 3 DPPH scavenging activity of compounds (thymoqinone and dithemiquinone)

Test	TQ	DTQ	AC
IC50 (mg/ml)	78.38 ± 0.01	95.95 ± 0.01	66.33 ± 0.001

Note: Ac - Ascorbic acid, TQ - thymoquinone, DTQ - dithymoquinone

TQ is a major compound of N. sativa that, in a previous study, exhibited high antioxidant activity, and is recommended as a substitute for synthetic drugs to handle oxidative damage caused to DNA, lipids, and proteins [24]. Antioxidant activity of TQ varies depending on extraction procedure [25]. These previous studies agree with the current study which also recommends the use of TQ as an antioxidant agent.

The scavenging activity increases as concentration increases. Similar studies in petroleum ether, distilled water, and methanol extract of N. sativa revealed a dose dependent increase in scavenging activity [26].

3.4. Cytotoxicity of Compounds Isolated from Nigella Sativa

Cell viability of compounds TQ and DTQ was assayed by the WAST-1 method [17] against three cell lines (MCF-7, MDA-MB-231 breast cancer cell line, and A549 lung cancer cell line), with the results indicating a higher activity of thymoquinone compound against MCF-7 and MDA-MB-231 with IC50 (7.9 ug/ml and 28.1mg/ml) than dimmer of thymoquinone (DTQ), while

DTQ shows good cytotoxic activity with IC50 (35.5 ug/ml) against the lung cancer cell line (Table 4).

Table 4 Cytotoxicity of TQ and DTQ on different cell line

Tested compounds	MCF-7 ug IC50 ug/ml	MDA-MB 231 IC50 ug/ml	A549 IC 50 ug/ml
Dimmer of Thymoquinone	36.7	42	35.5
Thymoquinone	7.9	28.1	39.8
Doxorubicin	26.12	32.22	28.32

Thymoquinone (TQ) is a naturally occurring compound drawing great attention as an anti-cancer agent and chemo modulator for chemotherapies.

In the current study WST-1 assay was used to assess the viability of TQ and DTQ against three different cell lines over concentration range 0.01- 100 ug. Treatment with TQ and DTQ induced a significant dose-dependent cytotoxic effect on MCF-7, MDA-MB 231 breast cancer, and A549 (Fig. 2). Thymoquinone isolated from N sativa exerted a closely dependent antiproliferative effect on the hella cancer cell line. Many previous studies confirmed the antiproliferative effect against different cell lines [27, 28, 29], but there are no previous studies concerned with the cytotoxicity of DTQ.

4. Conclusion

N. sativa seeds (Ranunculaceae family) have been used for thousands of years as a condiment and food preservative. The revival of traditional medicine using modern technologies could lead to noticeable drug development against some serious ailments. TQ and DTQ as the bioactive component of fugacious oil of the black seed has indicated potent medicinal effects in traditional medicine. DTQ was synthesized by photodimerization. Two compounds were studied for antifungal, antioxidant, and cytotoxic activity. TQ showed higher cytotoxic activity against MCF-7 and MDA-MB 231 cell lines with IC 50 (7.9 and 28.1) than dimmer of thymoquinone (DTQ); both compounds showed noticeable antifungal activity. The results recommended the use of thymoquinone as an anticancer drug against breast cancer cell lines. To the best of our knowledge, this is the first study on TQ and DTQ together, and the DTQ represented remarkable bioactivities. However, this study has some potential limitations: the promising antifungal, antioxidant, and anticancer activities are based on the *in vitro* assays. Accordingly, we recommend further *in vivo* studies in parallel with other pharmacological evaluations that could lead to the development of interesting drugs from this plant product.

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