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Natural Products as Topoisomerase II Poisons: Effects of Thymoquinone on DNA Cleavage Mediated by Human Topoisomerase IIα

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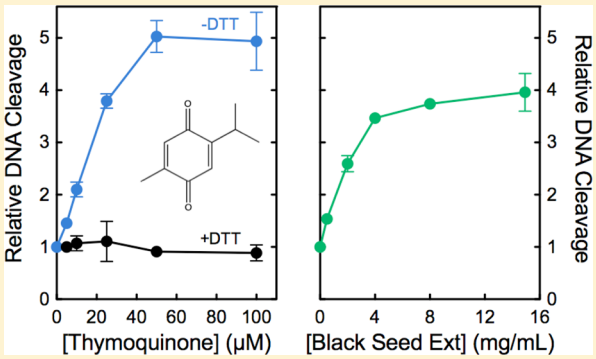
ABSTRACT: The seeds of *Nigella sativa* (often referred to as black seed) have long been utilized as a medicinal herb in Middle Eastern, Northern African, and Indian cultures. Historically, black seed has been used to treat a variety of illnesses associated with inflammation. More recent studies have found that it induces apoptosis and displays anticancer activity in animal and cellular models. The major bioactive compound of black seed is thymoquinone, which shares structural features with 1,4-benzoquinone and other covalent topoisomerase II poisons. Because a number of anticancer drugs target type II topoisomerases, we determined the effects of thymoquinone and a series of related quinones on human topoisomerase IIα. Thymoquinone enhanced enzyme-mediated DNA cleavage ~5-fold, which is similar to the increase seen with the anticancer drug etoposide. In order to enhance cleavage, compounds had to have at least two positions available for acylation. Furthermore, activity was decreased by the inclusion of electron-donating groups or bulky substituents. As predicted for a covalent topoisomerase II poison, the activity of thymoquinone (and related compounds) was abrogated by the addition of a reducing agent. Also, thymoquinone inhibited topoisomerase IIα activity when incubated with the enzyme prior to the addition of DNA. Cleavage complexes formed in the presence of the compound were stable for at least 8 h. Lastly, black seed extract and black seed oil both increased levels of enzyme-mediated DNA cleavage, suggesting that thymoquinone is active even in more complex herbal formulations. These findings indicate that thymoquinone can be added to the growing list of dietary and medicinal natural products with activity against human type II topoisomerases.



INTRODUCTION

Nigella sativa is an annual flowering plant that is indigenous to Mediterranean countries, India, and Pakistan.[1] The seeds of *N. sativa* (often referred to as black seed or black cumin) are used as a spice in Eastern cooking.[2,3] Furthermore, black seed has been utilized as a medicinal herb in Middle Eastern, Northern African, and Indian cultures for over 3000 years.[1,2,4] Seeds from *N. sativa* were found in Tutankhamun's tomb, indicating that their use in Egypt dates back at least to ca. 1325 B.C.[4] Historically, the herb has been used to treat a number of illnesses associated with inflammation, including asthma, bronchitis, fever, arthritis, and rheumatism.[5−7] More recently, it has been shown to have anticancer activity in animal and cellular models.[2,8−13]

The major and most well-studied bioactive compound in *N. sativa* is thymoquinone.[1] This compound is found in the essential oil, which comprises approximately 0.4% of the seed.[2] A high proportion of the essential oil (estimates range from 28−57%) is thymoquinone.

Since thymoquinone was first isolated in the 1960s, a number of studies have investigated its antioxidant and cellular effects.[14] The compound displays anti-inflammatory and pro-apoptotic properties.[1,14] In addition, it causes cell cycle arrest and inhibits the growth of cancer cells with minimal effects on non-malignant lines.[1,15−17]

Thymoquinone is similar in structure to 1,4-benzoquinone (Figure 1), a benzene metabolite that increases levels of DNA cleavage mediated by human type II topoisomerases.[18] These enzymes help modulate levels of torsional stress in the genetic material and remove knots and tangles from the genome.[19−25] They function by creating a transient double-strand break in one double helix and passing a separate intact DNA segment through the opening.[19−23] To maintain genomic integrity while the DNA is cleaved, type II topoisomerases covalently attach to the newly generated 5′ termini of the cleaved helix.[19−23] This covalent enzyme-cleaved DNA complex is known as the cleavage complex.[19] Compounds that increase the steady-state concentration of cleavage complexes are termed "topoisomerase II poisons" because they convert these enzymes to cellular toxins that generate permanent chromosomal breaks.[19−23] A number of well-characterized chemotherapeutic drugs as well as several natural products with anticancer or chemopreventive properties are topoisomerase II poisons.[19,20,22,23,26−36]

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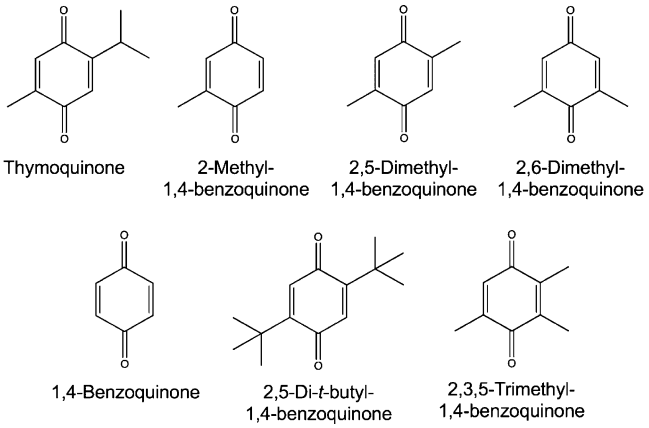

Figure 1. Structures of thymoquinone, 1,4-benzoquinone, and related compounds.

Because thymoquinone has anticancer properties and has structural similarities to known topoisomerase II poisons, the effects of the compound on the activity of human topoisomerase IIα were determined. Results indicate that purified thymoquinone, black seed extract, and black seed oil all increase levels of enzyme-mediated DNA cleavage. Thus, like several other dietary phytochemicals, thymoquinone is a topoisomerase II poison.

EXPERIMENTAL PROCEDURES

Enzymes and Materials. Recombinant human wild-type topoisomerase IIα and mutant topoisomerase $IIα^{C392A/C405A}$ were expressed in *Saccharomyces cerevisiae* and purified as described previously.[37−39] Negatively supercoiled pBR322 DNA was prepared from *Escherichia coli* using a plasmid mega kit (Qiagen) as described by the manufacturer. Thymoquinone, 2-methyl-1,4-benzoquinone, 2,6-dimethyl-1,4-benzoquinone, 2,5-di-*t*-butyl-1,4-benzoquinone, 2,3,5-trimethyl-1,4-benzoquinone, and etoposide were purchased from Sigma-Aldrich. 2,5-Dimethyl-1,4-benzoquinone was purchased from Santa Cruz Biotechnology. Compounds were prepared as 20 or 40 mM stock solutions in 100% DMSO and stored at 4 °C. In all cases, the activity of compounds in DMSO stock solutions was stable for over 6 months. Ground black seed and black seed oil were obtained from Amazing Herbs. The ground black seed was dissolved at 187 mg/mL in 50% DMSO. Insoluble components were removed by centrifugation, and the supernatant was stored at 4 °C. Black seed oil was stored at room temperature and added directly to reaction mixtures. Light mineral oil was obtained from Fisher, stored at room temperature, and added directly to reaction mixtures.

DNA Cleavage. DNA cleavage reactions were performed as described previously.[40] Reaction mixtures contained 150 nM human topoisomerase IIα and 10 nM negatively supercoiled pBR322 DNA in 20 μL of cleavage buffer [10 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 100 mM KCl, 0.1 mM EDTA, and 2.5% (v/v) glycerol]. Reactions were incubated for 6 min at 37 °C unless noted otherwise. Enzyme−DNA cleavage complexes were trapped by adding 2 μL of 5% SDS followed by 2 μL of 250 mM EDTA (pH 8.0). Proteinase K was added (2 μL of a 0.8 mg/mL solution), and reaction mixtures were incubated for 30 min at 45 °C to digest topoisomerase IIα. Samples were mixed with 2 μL of agarose loading dye [60% sucrose in 10 mM Tris-HCl (pH 7.9), 0.5% bromophenol blue, and 0.5% xylene cyanol FF], heated for 2 min at 45 °C, and subjected to electrophoresis in 1% agarose gels in 40 mM Tris-acetate (pH 8.3) and 2 mM EDTA containing 0.5 μg/mL ethidium bromide. DNA cleavage was monitored by the conversion of negatively supercoiled plasmid to linear molecules. DNA bands were visualized by ultraviolet light and quantified using an Alpha Innotech digital imaging system.

DNA cleavage reactions were carried out in the presence of 0−100 μM thymoquinone, 2-methyl-1,4-benzoquinone, 2,5-dimethyl-1,4-benzoquinone, 2,6-dimethyl-1,4-benzoquinone, 2,5-di-*t*-butyl-1,4-benzoquinone, 2,3,5-trimethyl-1,4-benzoquinone, or etoposide; 0−16 mg/mL black seed extract; or 2 μL of black seed oil or light mineral oil. Unless stated otherwise, compounds were added last to reaction mixtures. In some cases, reactions contained 250 μM ATP or 100 μM dithiothreitol (DTT). Alternatively, DTT was added after the 6 min cleavage reaction, and samples were incubated for an additional 5 min.

DNA Religation. DNA religation mediated by human topoisomerase IIα was monitored according to the procedure of Byl et al.[41] As described for the DNA cleavage assays, DNA cleavage/religation equilibria were established for 6 min at 37 °C in the presence of 0 or 50 μM thymoquinone, 2-methyl-1,4-benzoquinone, 2,5-dimethyl-1,4-benzoquinone, 2,6-dimethyl-1,4-benzoquinone, 2,5-di-*t*-butyl-1,4-benzoquinone, 2,3,5-trimethyl-1,4-benzoquinone, or etoposide. DNA religation was initiated by shifting samples from 37 to 0 °C. The shift to low temperature allows enzyme-mediated religation but prevents new rounds of DNA cleavage from occurring. Therefore, it results in a unidirectional sealing of the cleaved DNA. Reactions were stopped at time points up to 15 s by the addition of 2 μL of 5% SDS followed by 2 μL of 250 mM EDTA, pH 8.0. Samples were processed and analyzed as above. Religation was monitored by the loss of linear DNA.

Persistence of Cleavage Complexes. The persistence of topoisomerase IIα−DNA cleavage complexes was determined using the procedure of Gentry et al.[42] Initial reactions contained 50 nM DNA and 750 nM topoisomerase IIα in a total of 20 μL of DNA cleavage buffer. Reactions were carried out in the absence of compound or in the presence of 50 μM thymoquinone or 8 mg/mL black seed extract. Reactions were incubated at 37 °C for 6 min and then diluted 20-fold with DNA cleavage buffer at 37 °C. Samples (20 μL) were removed at times ranging from 0−8 h, and DNA cleavage was stopped with 2 μL of 5% SDS. Samples were processed as described above for plasmid cleavage assays. The persistence of cleavage complexes was determined by the decay of linear reaction product over time.

RESULTS AND DISCUSSION

Thymoquinone Is a Covalent Topoisomerase II Poison. Thymoquinone increased levels of DNA cleavage mediated by human topoisomerase IIα ∼5-fold in a dose-dependent manner (Figure 2, left). At all concentrations examined, cleavage induced by the compound was similar to or greater than that of etoposide, a commonly used anticancer drug. Maximal DNA scission was observed at ∼50 μM thymoquinone, and the cleavage-religation equilibrium was reached at ∼6 min (Figure 2, right).

Type II topoisomerases do not require ATP for DNA cleavage or religation (the above reactions did not contain ATP). However, because of the large conformational changes that accompany DNA strand passage, the cofactor is required to promote overall catalytic activity.[43,44] Therefore, the effects of thymoquinone on topoisomerase IIα activity were assessed in the presence of ATP (Figure 2, left, inset). Although the relative enhancement of DNA cleavage was somewhat lower in the presence of ATP, thymoquinone still poisoned the type II enzyme.

Topoisomerase II poisons can be categorized as interfacial or covalent.[19,36,45,46] Interfacial poisons, including several anticancer drugs and dietary compounds, interact noncovalently with both the enzyme and the DNA near the cleavage site.[19,45,46] After cleavage occurs, these compounds intercalate into the DNA at the scissile bond and prevent religation.[19,45,46]

Covalent topoisomerase II poisons contain protein-reactive groups such as quinones, isothiocyanates, and maleimides.[18,19,33,39,47,48] Unlike interfacial poisons, covalent poisons form adducts with the type II enzyme.[33,39,49] A number of

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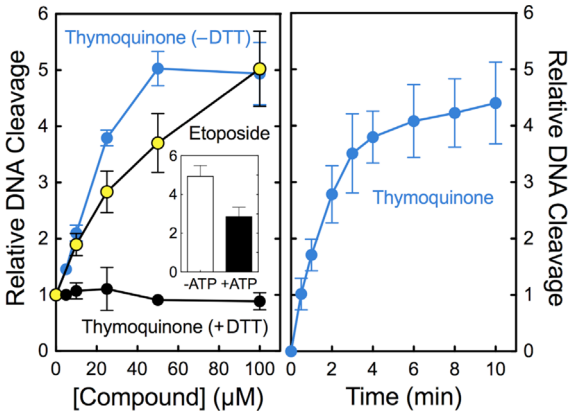

Figure 2. Thymoquinone enhances DNA cleavage mediated by human topoisomerase IIα. Left: Concentration dependence of thymoquinone-induced DNA cleavage in the absence (−DTT, blue) or presence (+DTT, black) of 100 μM DTT. Results for etoposide (yellow) are shown for comparison. Inset: Thymoquinone enhances topoisomerase IIα-mediated DNA cleavage in the presence of ATP. DNA cleavage induced by 100 μM thymoquinone is shown in the absence (white) or presence (black) of 250 μM ATP. Right: Time course of thymoquinone-induced DNA cleavage. DNA cleavage levels were calculated relative to a 6 min no drug control reaction. Error bars represent standard deviations for at least three independent experiments.

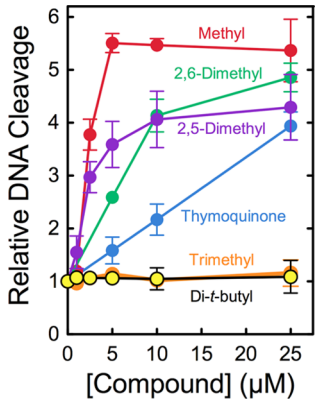

Figure 3. Effects of thymoquinone derivatives on DNA cleavage mediated by human topoisomerase IIα. Results are shown for 2-methyl-1,4-benzoquinone (red); 2,5-dimethyl-1,4-benzoquinone (purple); 2,6-dimethyl-1,4-benzoquinone (green); 2,3,5-trimethyl-1,4-benzoquinone (orange); and 2,5-di-*t*-butyl-1,4-benzoquinone (yellow). DNA cleavage levels were calculated relative to a no drug control reaction. Thymoquinone (blue) is shown for comparison. Error bars represent standard deviations for at least three independent experiments except for 2,3,5-trimethyl-1,4-benzoquinone and 2,5-di-*t*-butyl-1,4-benzoquinone, for which error bars represent the standard error of the mean of two independent replicates.

modified cysteine residues have been identified, all of which are distal to the DNA cleavage−religation active site.[33,39,48] It is believed that covalent poisons enhance topoisomerase II-mediated DNA cleavage, at least in part, by closing the N-terminal gate of the protein.[50,51]

Because the oxidation state of covalent poisons is critical for the adduction chemistry, reducing agents, such as DTT, prevent their activity against topoisomerase IIα.[18,47,49] Although covalent poisons increase DNA cleavage when added to the enzyme−DNA complex, they inhibit topoisomerase II activity when incubated with the enzyme prior to the addition of DNA.[18,49] This inhibition is a hallmark of covalent poisons.

Thymoquinone is structurally related to 1,4-benzoquinone, the archetypical covalent topoisomerase II poison (Figure 1).[18] This similarity suggests that thymoquinone also is a covalent poison. Therefore, several experiments were carried out to determine whether this hypothesis was correct.

First, a number of compounds related to thymoquinone (Figure 1) were tested for the ability to enhance DNA scission mediated by human topoisomerase IIα (Figure 3). If thymoquinone is a covalent poison, altering ring substituents should lead to predictable changes in cleavage activity. 2-Methyl-1,4-benzoquinone, which lacks the electron-donating isopropyl group of thymoquinone, should be more reactive than thymoquinone and therefore should be a more potent topoisomerase II poison. As seen in Figure 3, this was the case: 2-methyl-1,4-benzoquinone induced cleavage at much lower concentrations. Replacing the isopropyl moiety of thymoquinone with a methyl group, which is less electron donating, should also result in a compound that is more reactive than the parent compound (but less so than 2-methyl-1,4-benzoquinone). 2,5-Dimethyl-1,4-benzoquinone and 2,6-dimethyl-1,4-benzoquinone both displayed this predicted intermediate activity.

In contrast to the above, substitution of the methyl and isopropyl groups with tertiary butyl groups, which are more electron donating, should decrease the reactivity of the parent compound. 2,5-Di-*t*-butyl-1,4-benzoquinone, which contained these substitutions, displayed no ability to enhance enzyme-mediated DNA cleavage. Lastly, the ability of covalent poisons to induce DNA scission generally requires at least two available acylation sites on the compound.[39,49,50] Consistent with this requirement, 2,3,5-trimethyl-1,4-benzoquinone, which has only one available acylation site, displayed no activity against topoisomerase IIα.

Previous studies have shown that (within the guidelines discussed above) covalent poisons can accommodate a greater range of structural alterations than interfacial poisons.[18,33,34,49,50,52,53] This finding suggests that covalent poisons act more as chemical modification reagents than "ligands" that require specific binding pockets on topoisomerase IIα. The above notwithstanding, increased reactivity among the compounds examined also correlated with decreased substituent bulk (methyl > dimethyl > methyl + isopropyl > di-*t*-butyl). Thus, it is possible that changes in activity in this series are due to steric, rather than electronic, effects. If this were the case, the observed activity patterns would not be conclusive evidence that thymoquinone is a covalent poison.

Therefore, a second experiment was carried out in which the effects of DTT on thymoquinone and its derivatives were examined. DTT (100 μM) abrogated the ability of thymoquinone to increase DNA cleavage when added to reaction mixtures (Figure 2, left, and Figure 4). Similarly, the inclusion of DTT in reaction mixtures abolished the activity of 2-methyl-1,4-benzoquinone, 2,5-dimethyl-1,4-benzoquinone, and 2,6-dimethyl-1,4-benzoquinone (Figure 4).

Once covalent poisons have generated protein cross-links within topoisomerase II, their redox state no longer affects their activity. Consequently, the addition of reducing agents to assay mixtures after DNA cleavage−religation equilibria have been established with a covalent poison should not reverse the

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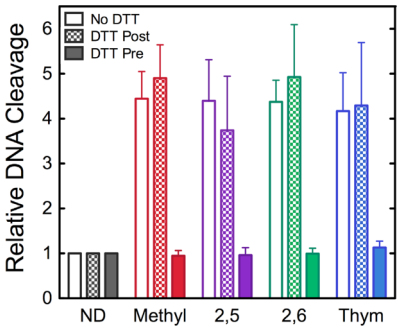

Figure 4. Effects of DTT on DNA cleavage enhancement by thymoquinone derivatives. Cleavage reactions were carried out in the absence of DTT (open bars), in the presence of 100 μM DTT added after establishment of cleavage−religation equilibria (checked bars), or in the presence of 100 μM DTT added at the start of the 6 min cleavage reaction (filled bars). Results are shown for 50 μM 2-methyl-1,4-benzoquinone (Methyl, red); 2,5-dimethyl-1,4-benzoquinone (2,5, purple); 2,6-dimethyl-1,4-benzoquinone (2,6, green); and thymoquinone (Thym, blue). DNA cleavage levels were calculated relative to a no drug control reaction. Error bars represent standard deviations for at least three independent experiments.

cleavage enhancement. As seen Figure 4, 100 μM DTT had no significant effect on the activity of thymoquinone, 2-methyl-1,4-benzoquinone, 2,5-dimethyl-1,4-benzoquinone, or 2,6-dimethyl-1,4-benzoquinone once adducts were formed.

In a third experiment, 50 μM thymoquinone was incubated with human topoisomerase IIα prior to the addition of DNA (Figure 5, left). As expected for a covalent poison, thymoquinone inactivated the enzyme ($t_{1/2} \approx 5$ min).

Lastly, in a fourth experiment, topoisomerase IIα was incubated with thymoquinone, and the resulting peptides generated by tryptic digestion were analyzed by MALDI mass spectrometry (data not shown). Mass changes in several peptides were observed following treatment with thymoquinone. Although this finding indicates that thymoquinone covalently modifies topoisomerase IIα, sites of adduction could not be assigned. This is most likely because thymoquinone has two sites for potential acylation and generates protein cross-links. A similar issue previously was reported for the analysis of topoisomerase IIα peptides following incubation with 1,4-benzoquinone.[39] In this latter case, sites of adduction were identified using plumbagin (a paraquinone that has only a single site for acylation) and were confirmed by mutagenesis studies.[39] This study established that quinones can adduct human topoisomerase IIα at Cys392 and Cys405 and that topoisomerase II$\alpha^{C392A/C405A}$ is partially (~40−50%) resistant to covalent poisons, such as 1,4-benzoquinone, PCB quinones, and curcumin oxidation products, but not to interfacial poisons.[34,39]

Therefore, the ability of thymoquinone to increase the level of DNA cleavage mediated by topoisomerase II$\alpha^{C392A/C405A}$ was compared to that of the wild-type enzyme. As seen in Figure 5 (right), levels of cleavage were ~40% lower with the mutant enzyme.

Taken together, the above results provide strong evidence that thymoquinone and related compounds are covalent topoisomerase II poisons.

Effects of Thymoquinone on DNA Religation Mediated by Topoisomerase IIα. Interfacial topoisomerase II poisons typically increase levels of DNA cleavage complexes by inhibiting the religation of cut strands.[19,45,46] In contrast, covalent poisons have varying abilities to inhibit the topoisomerase II DNA religation reaction. Thymoquinone displayed a modest effect on the rate of topoisomerase II religation (decreased by ~35−50%), whereas etoposide (an interfacial poison) inhibited the reaction by at least 10-fold (Figure 6). Thymoquinone derivatives 2-methyl-1,4-benzoquinone, 2,5-dimethyl-1,4-benzoquinone, and 2,6-dimethyl-1,4-benzoquinone displayed no appreciable ability to inhibit religation (Figure 6, inset).

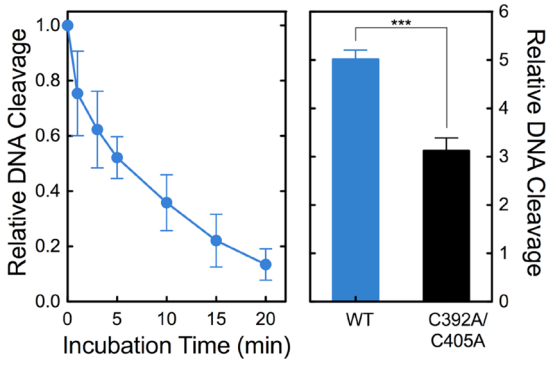

Figure 5. Thymoquinone is a covalent topoisomerase II poison. Left: Thymoquinone inactivates human topoisomerase IIα when incubated with the enzyme prior to the addition of DNA. Cleavage activity was monitored in the presence of 50 μM thymoquinone. DNA cleavage levels were calculated relative to cleavage induced when thymoquinone and the enzyme were not incubated prior to reaction initiation. Error bars represent standard deviations for at least three independent experiments. Right: Human topoisomerase II$\alpha^{C392A/C405A}$ is partially resistant to thymoquinone. Cleavage enhancement was monitored in the presence of 50 μM thymoquinone. DNA cleavage levels are shown for the wild-type enzyme (blue) and topoisomerase II$\alpha^{C392A/C405A}$ (black). Error bars represent standard deviations for at least three independent experiments. ***p = 0.0001.

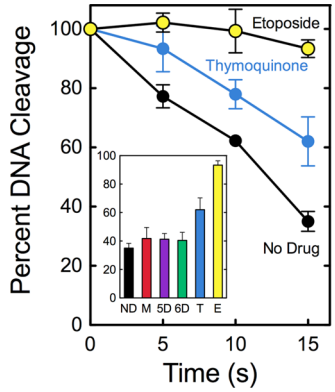

Figure 6. Effects of thymoquinone on religation mediated by human topoisomerase IIα. DNA cleavage reactions were initiated in the absence of compound (No Drug, black) or in the presence of 50 μM thymoquinone (blue). Results with 50 μM etoposide (yellow) are shown for comparison. DNA cleavage levels prior to the induction of religation were set to 100%. Inset: Levels of DNA cleavage remaining 15 s after the induction of religation are shown for reactions containing no drug (ND, black) or 50 μM 2-methyl-1,4-benzoquinone (M, red); 2,5-dimethyl-1,4-benzoquinone (5D, purple); 2,6-dimethyl-1,4-benzoquinone (6D, green); thymoquinone (T, blue); or etoposide (E, yellow). Error bars represent standard deviations for at least three independent experiments.

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Effects of Thymoquinone on the Stability of Topoisomerase IIα–DNA Cleavage Complexes. Upon dilution, DNA cleavage complexes formed with human topoisomerase IIα rapidly re-establish equilibria ($t_{1/2}$ < 1 min) in which levels of DNA cleavage are significantly decreased (Figure 7). Because

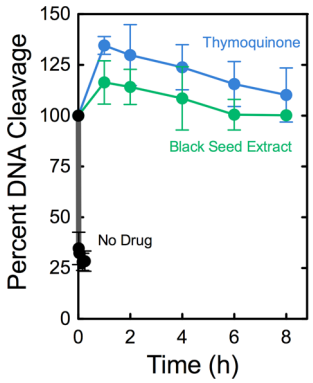

Figure 7. Effects of thymoquinone and black seed extract on the persistence of topoisomerase IIα–DNA cleavage complexes. Assays were carried out in the absence of compound (No Drug, black) or in the presence of 50 μM thymoquinone (blue) or 8 mg/mL black seed extract (green). DNA cleavage at time zero was set to 100%. Error bars represent standard deviations for at least three independent experiments.

covalent poisons trap the DNA within the annulus formed by the cross-linked N-terminal domains, the re-equilibration seen in the absence of the poison should not take place. As predicted, no significant decrease in thymoquinone-induced cleavage complexes was seen 8 h after dilution (Figure 7).

Black Seed Is a Topoisomerase II Poison. In culinary and medicinal applications, thymoquinone generally is consumed as either ground black seed or black seed oil.[1−4] Therefore, we determined the effects of both on the DNA cleavage activity of human topoisomerase IIα (Figure 8). Black seed extract increased enzyme-mediated DNA cleavage ~4-fold in a dose-dependent fashion. Levels of cleavage enhancement

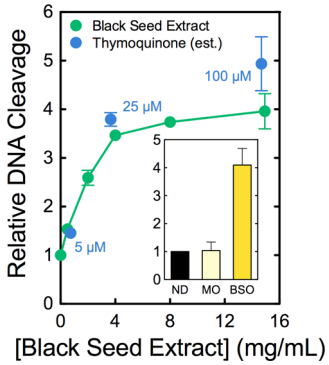

Figure 8. Effects of black seed extract on DNA cleavage mediated by human topoisomerase IIα (green). Cleavage results for 5, 25, and 100 μM thymoquinone (blue) are overlaid at concentrations of black seed extract estimated to contain the corresponding amounts of thymoquinone.[2] Inset: DNA cleavage is shown in the absence of compound (ND, black) or in the presence of 2 μL (in a total reaction volume of 20 μL) of light mineral oil (MO, light yellow) or black seed oil (BSO, yellow). DNA cleavage levels were calculated relative to a no drug control reaction. Error bars represent standard deviations for at least three independent experiments.

were consistent with the estimated thymoquinone concentration in the extract. The addition of 100 μM DTT abolished the activity of black seed extract (data not shown). Furthermore, as seen with thymoquinone, cleavage complexes formed in the presence of black seed extract remained stable for more than 8 h following dilution in persistence assays (Figure 7).

The inclusion of 2 μL of black seed oil in reaction mixtures also increased levels of DNA cleavage (~4-fold) mediated by human topoisomerase IIα (Figure 8, inset). In contrast, no cleavage enhancement was observed when light mineral oil was included instead.

Taken together, results with the black seed extract and oil imply that thymoquinone is a topoisomerase II poison even in its more complex natural formulation.

SUMMARY

Thymoquinone is the primary active compound in black seed, a Mediterranean plant with a rich history of use as a medicinal herb.[1,2,14] A number of topoisomerase II poisons derived from natural sources display chemotherapeutic or chemopreventive activity.[19,20,22,23,26−35] Given the structural similarity between thymoquinone and established topoisomerase II poisons, we examined the activity of the compound against human topoisomerase IIα. Results indicate that thymoquinone is a covalent topoisomerase II poison even in its herbal formulation. Thus, thymoquinone can be added to the growing list of dietary and medicinal natural products with activity against human type II topoisomerases.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

DTT, dithiothreitol

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