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Nonenzymatic reduction of thymoquinone in physiological conditions

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Abstract
Thymoquinone (TQ) is the bioactive constituent of the volatile oil of *Nigella sativa* L. and has been shown to exert antioxidant antineoplastic and anti-inflammatory effects. During the study of its possible mechanism of action, we found that TQ reacts chemically (i.e. nonenzymatically) with glutathione (GSH), NADH and NADPH. A combination of liquid chromatography/UV–Vis spectrophotometry/Mass spectrometry analyses was used to identify the products of these reactions. The reaction that occur in physiological conditions indicates the formation of only two products, glutathionyl–dihydrothymoquinone after rapid reaction with GSH, and dihydrothymoquinone (DHTQ) after slow reaction time with NADH and NADPH. Measurement of the antioxidant activity of reduced compounds against organic radicals such as 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)(ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) also revealed a potential scavenging activity for glutathionyl–dihydrothymoquinone similar to that of DHTQ. Under our experimental conditions, TQ shows lower scavenging activities than glutathionyl–dihydrothymoquinone and DHTQ; it is very interesting to observe that the reduced compounds apparently show an antioxidant capacity equivalent to Trolox. The results indicate a possible intracellular nonenzymatic metabolic activation of TQ dependent on GSH, NADH or NADPH that may represent a "cellular switch" able to modulate cellular antioxidant defences.

Keywords: *Thymoquinone, dihydrothymoquinone, reactive oxygen species, medicinal plants, radical scavenging activity,* Nigella sativa *L*

Abbreviations: *TQ, Thymoquinone; DHTQ, dihydrothymoquinone; TOH, Thymol; DTQ, dithymoquinone; DHTQ–GS, glutathionyl–dihydrothymoquinone;* H_2O_2*, hydrogen peroxide; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid);* $ABTS^{\cdot+}$*, ABTS cation radical; DTPA, diethylene triaminepentaacetic acid; DPPH, 1,1-diphenyl-2- picrylhydrazyl; GSH, Glutathione; DTNB, dithionitrobenzene; NADH, Nicotinamide adenine dinucleotide reduced form; NADPH, nicotinamide adenine dinucleotide phosphate reduced form*

Introduction

Recent pharmacological investigations on *Nigella sativa* L, commonly known as black cumin seed, used in folk (herbal) medicine all over the world, have revealed a wide spectrum of activities [1–10] that have been attributed to active components contained in the seeds and the essential volatile oil obtained from them. The most active of these compounds is thymoquinone (TQ), which has been demonstrated to possess strong antioxidant properties, as do other minor constituents such as thymol (TOH) and thymoquinone dimer (DTQ) [10,11]. TQ shows a potential radical scavenging activity towards different radical systems, but the possible mechanism of its pharmacological actions have yet to be clarified. Typical administration of crude extracts of the seeds, essential oil prepared from seed, or TQ are characterized by a very low degree of toxicity and the beneficial effects observed from their use might be related to their cytoprotective and antioxidant action in inflammation [12]. Moreover, recent evidence indicates that these compounds

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protect against nephrotoxicity and hepatoxicity, and show anti-inflammatory, analgesic, antipyretic, antimicrobial and antineoplastic activity [12]. *N. sativa* seeds contain 36–38% fixed oil and low concentrations of some unusual unsaturated fatty acids [13]. Different components were characterized from the oil: the major ones were TQ (28–57%) and cymene (7–15%), while low concentrations of the dimeric form of TQ (dithymoquinone), TOH, and only a minimal quantity of dihydrothymoquinone (DHTQ) were detected in the oil [14]. DHTQ could be formed in the body after TQ ingestion, following the action of reductases, as reported for DT-diaphorase, which catalyzes reduction of TQ to DHTQ in different organs [15]. Several authors reported the possibility that the *in vivo* protection of different organs against oxidative damage exerted by TQ may be due to the combined antioxidant effects of TQ and DHTQ against free radical generation [16,17]. Generally, endogenous TQ may act as quinone (TQ), DHTQ or dithymoquinone (DTQ) forms (Figure 1). However, it is not clear which form is endowed with greater antioxidant activity (AA). The mechanism of the metabolic activation of TQ *in vivo* still remain an open problem, as are the possible redox reactions that might account for its action as antioxidant and pro-oxidant. In particular, the reduction of TQ could be catalyzed by different endogenous enzymatic and non enzymatic cellular redox systems with mechanisms similar to those reported for the activation of other important quinones, such as menadione or CoenzymeQ, that have a reducing potential very close to that of TQ. The three most important non enzymatic redox systems that we commonly find within the cell are

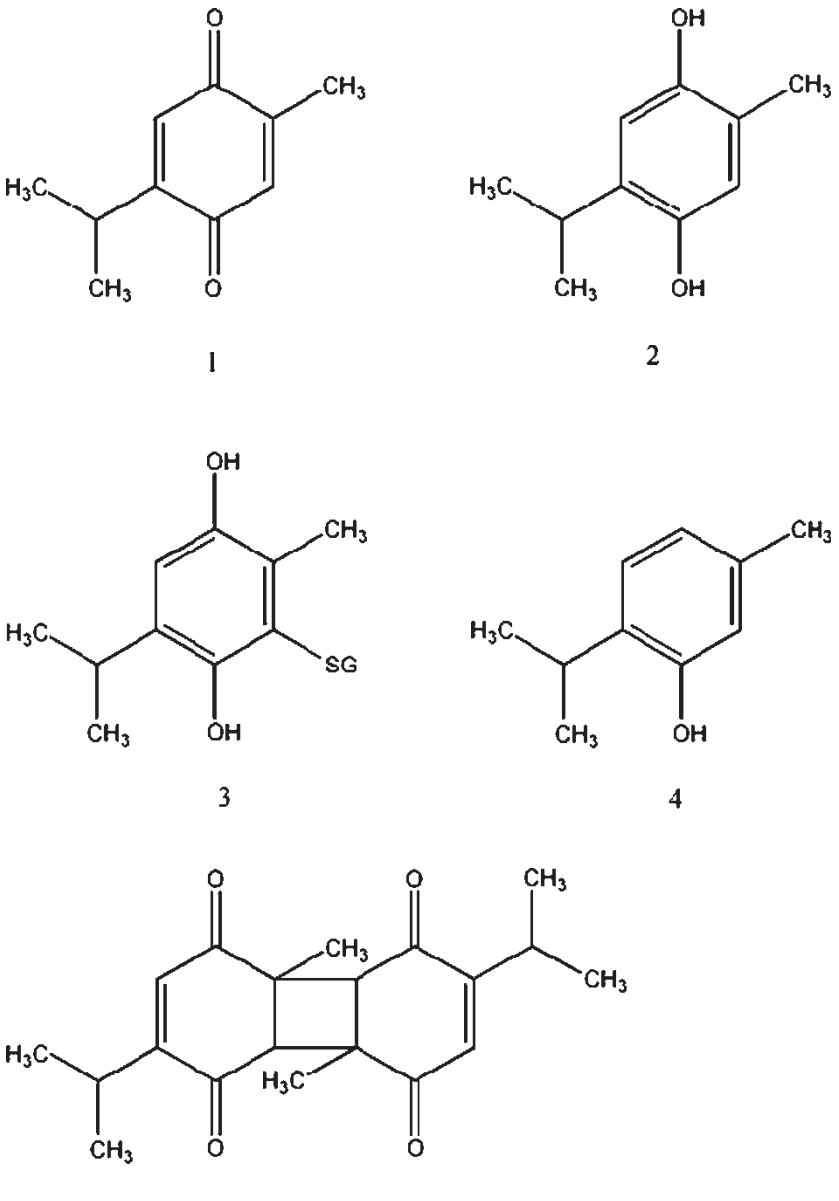


Figure 1. Structures of TQ and its derivatives: (1) TQ; (2) DHTQ: (3) Glutathionyl–dihydrothymoquinone; (4) TOH; (5) DTQ.

NADPH/NADP+, thioredoxin (TRX_{red}/TRX_{ox}) and glutathione (GSH/GSSG). Among these, the last is the most important since the concentration of GSH is about 500/1000-fold higher than that of thioredoxin and NADPH. GSH is essential for the activity of thiol-dependent enzymes, and is a cofactor for antioxidant enzymes such as GSH-peroxidase selenium dependent. In addition, it is important for copper cellular transport and modulates copper dangerous effects and toxicity [18–21]. Under physiological conditions GSH is from 10 to 100-fold higher than oxidize form and it could be able to reduce different compounds as lyophilic molecules that can freely cross the plasma membrane owing to their chemical nature. The aim of the present study was to determine how TQ fits into the antioxidant network. More specifically, the goal of this study is to characterize the potential reaction of TQ with different endogenous non-enzymatic redox systems such as GSH, NADH and NADP, in an attempt to better understand a possible alternative mechanism(s) through which TQ exerts its protective effects. The intracellular reduction of TQ mediated by reducing systems, may represent an additional "cellular switch" able to modulate cellular antioxidant defences.

Materials and methods

Materials

Chemicals: DTNB, Hydrogen peroxide, TQ, TOH and trolox were obtained from Aldrich. ABTS diammonium salt, DPPH, NADH, NADPH and GSH were purchase from Sigma Chem. Co. USA. Cis-parinaric acid was obtained from Molecular Probes (Junction City, OR USA). DHTQ was prepared from TQ with reduction with sodium hydrosulphite according to the procedure of Smith and Tess [22]. The purity of DHTQ was checked by melting point and thin-layer chromatography as described previously [14]. DTQ was prepared to the method described in Ref. [23]. All the reagents were of the highest quality available.

Methods

Interaction of TQ and derivatives with DPPH and ABTS radicals. The stable nitrogen-centred free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) shows a strong absorption at 516 nm which decrease after addition of reducing equivalent [24]. A different concentrations of compounds (dissolved in 0.5 ml of ethanol) were added to a solution of DPPH (0.3 mM) dissolved in 1 ml of absolute ethanol and incubated for 30 min at 37°C [25]. The changes in absorbance were measured at $\lambda = 516$ nm (A_{516}) using a Cary 1 spectro- photometer. Similar experiments were done using the monocation radical of ABTS ($ABTS^{\cdot+}$) generated by this probe's reaction with potassium persulfate (2.45 mM) as described in Ref. [26], and allowing the mixture to stand in the dark at room temperature for 12 h before use [26]. Assuming complete reaction with a stoichiometry of one mole of potassium persulfate per 2 moles of ABTS, the extinction coefficient for $ABTS^{\cdot+}$ at 734 nm was established to be 12,867 $M^{-1}\,cm^{-1}$. The extinction coefficient and UV/Vis spectrum of $ABTS^{\cdot+}$ are the same at pH values ranging from 3 to 7.4 [26]. This radical form was stable for more than two days. $ABTS^{\cdot+}$ radical was diluted in PBS buffer to an absorbance of 0.9 (± 0.03) at 734 nm and equilibrated at 30°C. Stock solutions of different antioxidants in ethanol were added to mixture and the absorbance reordered after 10 min of incubation at 30°C. Appropriate solvent blanks were run in each assay. All determinations were carried out at least in triplicate. The percentage of AA for DPPH and $ABTS^{\cdot+}$ was calculated using the following formula:

$$(AA\%) = 100 - \left\{\frac{[(Abs_{sample} - Abs_{blank}) \times 100]}{Abs_{control}}\right\}$$

The extent of the percentage of AA for DPPH and $ABTS^{\cdot+}$ was plotted as a function of concentration in order to determine the TEAC(trolox equivalent AA) values that were calculated by the ratio between the trolox concentration value and values of different concentrations of antioxidant necessary to reduce the 50% of absorbance of the solution of $ABTS^{\cdot+}$ or DPPH radicals.

Reaction between GSH, NADH, NADPH, and TQ. The reaction of TQ was performed at 25°C in PBS buffer pH 7.4 containing 30 μM TQ and the different reducing compound (GSH, NADH, NADPH) as indicated. Spectra were recorded and processed using a Varian Cary 1 spectrophotometer. The kinetics of the reaction between TQ and GSH were followed by monitoring the decay of at maximum TQ absorbance as a function of time. The kinetics of the reaction between TQ and NADH and NADPH were followed by monitoring the decay of absorption spectra of reducing compounds at 340 nm as a function of time. The observed pseudo-first order constant was calculated by fitting related curves.

HPLC separations of TQ derivatives. A C18 reversed-phase analytical column (150 × 4.6 mm, 3 μm particle size Supelco Supercosil LC-18T) connected to a LC-18 reversed phase guard column (20 mm) was used in all HPLC studies. An Altech HPLC system connected in series to a Linear UV–Vis 200 variable wavelength detector was utilized. The isocratic mobil phase was composed of water:metanol:2-propanol (50:45:5 v/v) and was filtered through a 0.45 mm Millipore filter and deareated before use. Analyses

were performed at room temperature. UV monitoring of the eluted solutions was carried out at 254 and 294 nm for all products analyzed. A flow rate of 1 ml/min was used. All samples before and after analysis were refrigerated and covered to protect from light and heat, in order to prevent radical formation.

MS detection of products for the reaction of GSH, NADH and NADPH. LC/MS analyses were performed on a HP 1100 series LC/MSD trap system with elecrospray ionization (with an ESI ionization source and quadrupole analyzer) interface equipped with an auto-sampler, and diode array. Samples (20 μl) formed by reaction between TQ and GSH, NADH and NADPH were directed to the elecrospray source after passing through a diode array multiple wavelength detector (acquisition range 190–600 nm) and were analyzed in negative ESI. Data on the samples were acquired over m/z range of 100–1000. Data analyses were performed using the LC/MSD Chemstation Rev. A.08.03 software.

TQ interaction with human erythrocytes. Human erythrocytes (hematocrit 20%) in PBS were incubated in a shaking bath at 37°C with different concentrations of TQ. At different intervals of incubation, aliquots (250 μl) were removed from different samples and vortexed with 2.5 ml of 30% trichloroacetic acid. A 100 μl aliquot was removed for a colorimetric determination of GSH concentrations using DTNB (125 mM) in a final 1 ml mixture containing 0.1 M tris–HCl/0.1 mM DTPA. Absorbance was detected using a Varian Cary 1.

Results

Reaction of thymoquinone with GSH

Figure 2 shows the changes in the UV spectrum during the reaction of 30 μM TQ in PBS at pH 7.4 with GSH. Addition of GSH (300 μM) resulted in a progressive decrease of the absorption at 257 nm with increase in absorption at 303 nm. In the spectrum, two isosbestic points can be observed at 234 and 278 nm, suggesting the conversion of TQ into one new single product (Figure 2(A)). The increase of absorbance at 303 nm represents the formation of a new product deriving from the direct reaction between GSH and TQ; the maximum of absorbance observed differs from that shown in a solution of the same concentration of DHTQ (Figure 2(B) line b). The spectrum of the new product shows an absorbance of about 260 nm with intensity similar to that shown at 303 nm (Figure 2(B) line c). The rate of reaction of TQ depends on the concentration of GSH as reported in Figure 3(A). The observed first-order rate constant

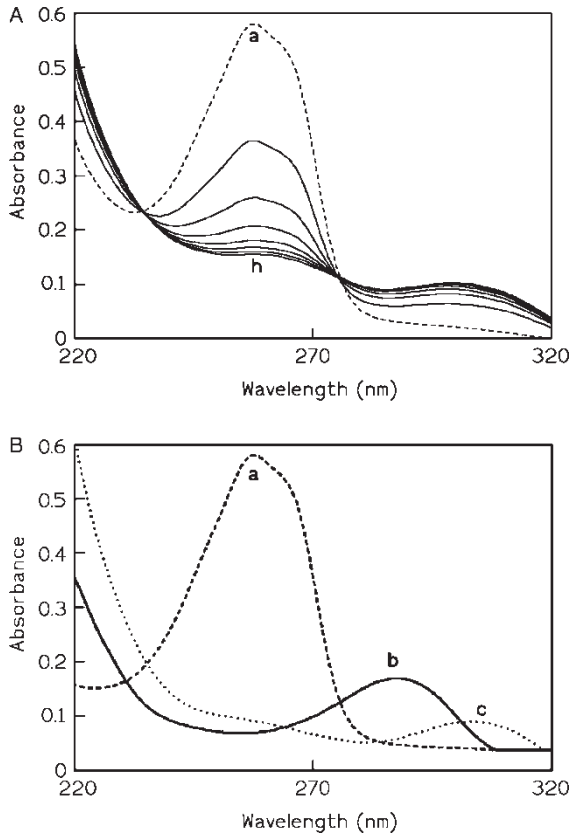


Figure 2. (A) UV-visible absorption spectra changes of TQ during GSH mediated reduction. To the reaction mixture (1 ml) containing 30 μM of TQ in 50 mM phosphate buffer pH 7.2, aliquots (5 μl) of a concentrated solution of GSH (final concentration 160 μM) were added, and the absorption spectra were recording every 2 min for 16 min. (B) UV absorption spectra of (a) TQ, (b) DHTQ, (c) reaction mixture after TQ+GSH reaction.

k_{obs} depends linearly on the concentration of GSH (Figure 3(B)), and the apparent second order rate constant ($4 \times 10^3\,M^{-1}\,min^{-1}$) for GSH can be calculated from the graph. The influence of pH on the value of k_{obs} is shown in Figure 3(C). The reaction rate (k_{obs}) measured in the pH interval 4.5–8.5 shows strong pH dependence, and in physiological conditions the presence of GSH causes an extensive reaction of TQ that increases with the increase of pH, depending on GSH ionization.

Thymoquinone reduction by NADH and NADPH

As shown in Figure 4, the non enzymatic reduction of TQ with NADH or NADPH proceeded very slowly and only after more than 24 h of incubation at room temperature could the 50% of product be reduced. In these conditions we did not calculate the apparent rate constants. Results were identical with either NADH or NADPH as reductant.

HPLC detection of GSH conjugates. The TQ/GSH reaction mixture, was fractionated by use of HPLC

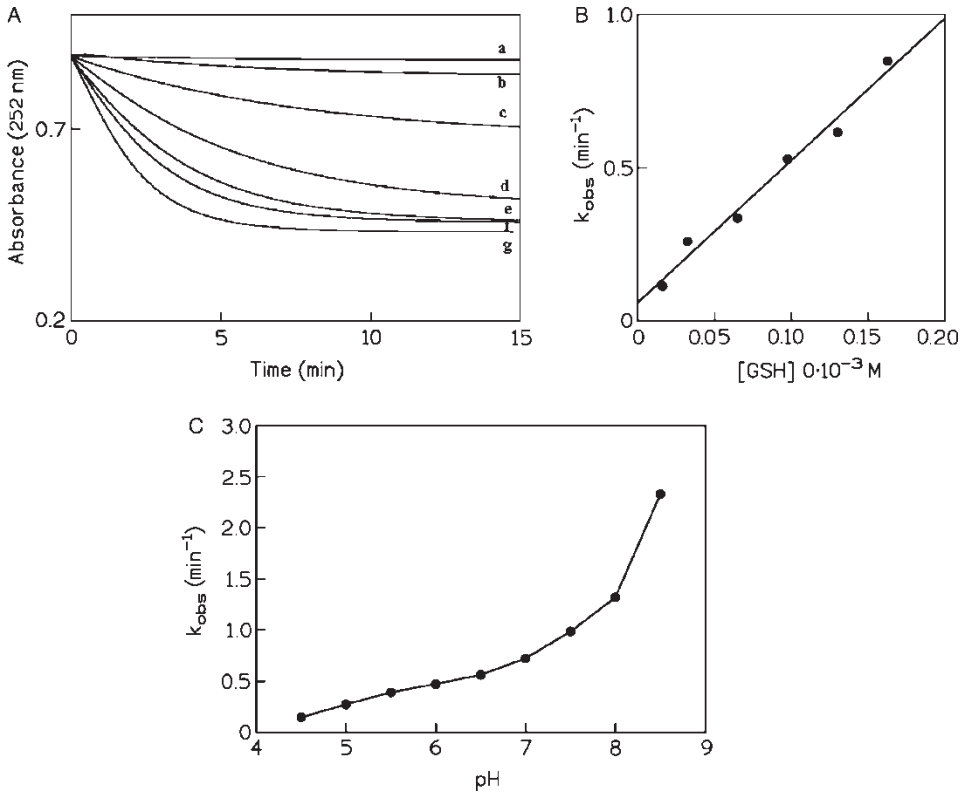


Figure 3. (A) Time profiles of absorption change showing the effect of various concentrations of GSH on the rate of reduction of TQ. To the different reaction mixtures (1 ml) containing 50 μM of TQ in 50 mM phosphate buffer pH 7.2, several concentrations of GSH were added to the solution and the decrease of absorbance at 252 nm was recorded (from (a) 0, to (b) 16.25,(c) 32.5, (d) 65, (e) 97.5, (f) 130, (g) 162.5 μM). (B) Dependence of k_{obs} on the concentration of GSH. (C) Dependence of k_{obs} on pH. The effect of GSH (130 μM) on the rate of\reaction with TQ(50 μM) were calculated as function of pH from 5 to 8. The value of kobs (DOD252/min) was calculated by fitting each curve and reported as function of pH.

and analyzed at two wavelength (254 and 294 nm) to detect all reaction products. HPLC analysis of mixture with detection at 294 nm shows the formation of a new product after reaction of TQ with GSH that eluted before the DHTQ in the same conditions used. Table I gives retention times (t_R) for the species observed. The HPLC chromatogram obtained after analysis of the TQ/GSH reaction mixture shows the formation of a new product detected also at 254 nm (with the same elution time shown in the same sample at 294 nm). The results obtained seem to indicate that the TQ is thoroughly transformed after incubation with all reducing compounds; no residual TQ is present in the mixture analyzed.

MS detection of TQ- GSH, NADH and NADPH reaction products. Data in Table I report the spectral data of $[M-H]^-$ ions obtained from different reaction mixtures. When TQ was incubated with GSH, the mass spectra of mixture, after incubation at 25°C for 15 min, displayed a peak at m/z 306 $[M-H]^-$ corresponding to free GSH and a peak at m/z 470 $[M-H]^-$ corresponding to a DTHQ-GS conjugate. No peak at 163 $[M-H]^-$ related to TQ was detected, proving a total conjugation of TQ by GSH (10-fold in excess). MS analysis of TQ/NADH and TQ/NADPH mixtures treated as previously reported for GSH displayed a peak at m/z 97 $[M-H]^-$ corresponding to phosphate, a peak at m/z 283 $[M-H]^-$ not identified,

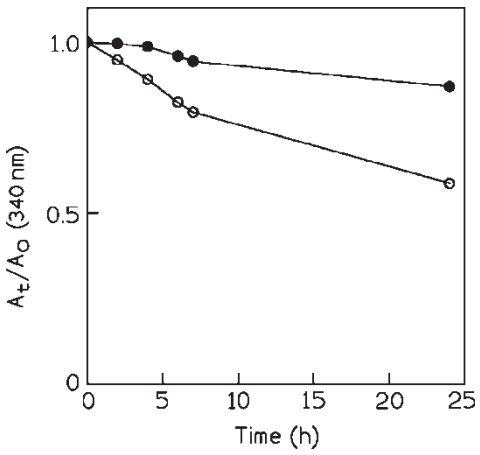


Figure 4. Time profiles of absorption change at 340 nm, showing the effect of NADH on the rate of reduction of TQ. To the reaction mixture (1 ml) containing 30 μM of TQ in 50 mM phosphate buffer pH 7.2, and NADH (final concentration 347.3 μM) was added and the absorption spectra was recorded as function of time. A_0 is the absorbance value at 340 nm before addition of TQ while A_t represents the values of absorbance (at the same wavelength) at different times.

Table I. Spectral data for the reaction of TQ with GSH, NADH and NADPH.

Compound	$t_{R(254)}$ min	$t_{R(294)}$ min	[M−H]
TQ	12.6	–	–
DHTQ	–	4.4	–
GSH + TQ	2.8	2.8	306[100]; 470[33]
NADH + TQ	–	4.3	97[100];165[43];283[89]
NADPH + TQ	–	4.3	97[25];165[27];283[100]

[] Abundances (normalized to the most abundant product ion) are given in parentheses.

and a peak at m/z 165 $[M-H]^-$ corresponding to DHTQ formed. In addition, in these mixtures, there were no peaks at m/z 163 $[M-H]^-$ related to free TQ.

Effect of TQ on GSH depletion in human erythrocytes. The reaction of TQ with GSH was proven from the study of red cell GSH depletion after ed cell treatment with TQ. Red cells were incubated for 150 min in the presence and absence of TQ at 37°C in PBS. At different time intervals, aliquots were withdrawn and analyzed for calculation of GSH as described in methods. Figure 5 shows that in the presence of TQ, the concentration of GSH in red cells decreases compared to the control, confirming the possible reaction of TQ with GSH. No haemolysis was observed in the two mixtures during the incubation.

Interaction of thymoquinone and derivatives with DPPH and ABTS radicals

To prove that the reduced form of DHTQ–GS have a large AA compared with that shown for DHTQ, the product was isolated after HPLC chromatography and its scavenging activity was measured by reaction with DPPH.

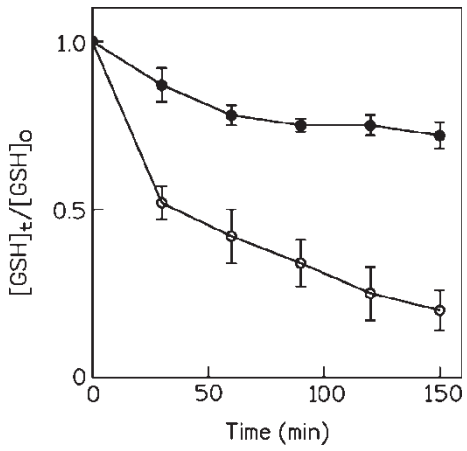


Figure 5. Effect of TQ on reduced GSH levels in human erythrocytes. Human erythrocytes were re-suspesed (hematocrit 20%) in PBS and incubated in a shaking bath at 37°C for 150 min in the presence (●...●) and in absence (○...○) of TQ(80 μM). GSH levels were measured as reported in materials and methods. All values are the means of three independent determinations. $[GSH]_0$ represent the value of concentration of GSH before the addition of TQ, while $[GSH]_t$ is the measure of concentration of GSH after addition of TQ at different times of incubation.

Two approaches were taken, namely, inhibition assays in which the extent of the scavenging electron-donation of a pre-formed free radical is the marker of AA, as well as assays involving the presence of an antioxidant system during the generation of the radicals. DPPH is a molecule containing a stable free radical and in the presence of an electron-donating anti-oxidant, the purple colour typical of the free DPPH radical shows a decrease in intensity, a change that can be measured spectrophotometrically at 517 nm [27]. This simple test provides information on the ability of the compound to donate an electron and on the mechanism of anti-oxidant action. The concentration-dependent scavenging activity of different compounds was measured as the decrease of DPPH absorbance when a solution of this radical was treated with different antioxidants. Preliminary data (not shown) demonstrated that after 30-min of incubation, the absorbance of reaction mixture did not change during the period of the experiments in the presence or absence of test samples, which suggests that 30 min of reaction time was enough to determine the AA of various samples in the pattern. TEAC(trolox equivalent AA) values were calculated and reported in Table II. The two forms, DHTQ and DHTQ–GS, showed powerful free radical scavenging activities compared to trolox, while lower scavenging activity was shown for TQ and DTQ. It could be hypothesized that DPPH as a free radical in the initial step reacts with reduced quinones by hydrogen abstraction and leads to the formation of one-electron oxidized form of the antioxidants, the phenoxyl-type radical $DHTQ^{\cdot}$ and $^{\cdot}DHTQ–GS$. These radicals can react with DPPH in excess, with formation of oxidation products through dimerization or disproportionation reactions involving resonant forms, with an mechanism analogous to that hypothesised for the reaction of DPPH with Trolox [28]. As reported, pure compounds such as TQ, TOH and DTQ derivatives, show little scavenging activity, while DHTQ, GS-DHTQ and trolox were the most effective antioxidants in scavenging DPPH radical with

Table II. ABTS and DPPH radical-scavenging activity of TQ and its derivatives.

	TEAC	
Compound	$ABTS^{\cdot+}$	DPPH
Trolox	1	1
DHTQ	1	1.04
DHTQ–GS	1	1.08
TQ	1.65	10.6
TOH	1.96	–
DTQ	>7.35	12.5

Trolox equivalent antioxidant activity (TEAC). See methods.

similar IC_{50} values of 33.7(±0.9)μM, 35.2(±0.6)μM and 36.3(±0.7)μM for trolox, GS–DHTQ and DHTQ, respectively.

AA is screened toward the pre-formed radical monocation of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)(ABTS) generated by oxidation of ABTS with potassium persulfate, and is reduced ion by presence of hydrogen-donating antioxidants. The extent of the reduction of the absorbance of the ABTS+ was plotted as a function of concentration in order to determine the TEAC(trolox equivalent AA). The TEAC values for the reaction of $ABTS^{\cdot+}$ reported in Table II showed high antioxidant power for DHTQ and DHTQ–GS similar to the data reported from the reaction with DPPH. Differences were observed in the reaction of $ABTS^{\cdot+}$ with other derivatives. A good radical scavenging activity was observed for TOH and TQ, while a lower value of activity was shown for DTQ. $ABTS^{\cdot+}$ reacts with different compounds through an electron transfer process with formation of an intermediate that eliminates H+ , formation of radicals, and successive oxidation. $ABTS^{\cdot+}$ probably reacts with TQ with formation of intermediates that eliminate H+ from the isopropyl group, and the resulting radical can be stabilized by conjugation with adjacent double bound and oxygen at position 1 of the molecule. The same mechanism could be hypothesised for DTQ and the different activity (seven times less) observed for this compound compared to TQ probably could be due to a decrease in $ABTS^{\cdot+}$ reactivity due to increase of steric hindrance on the DTQ moiety.

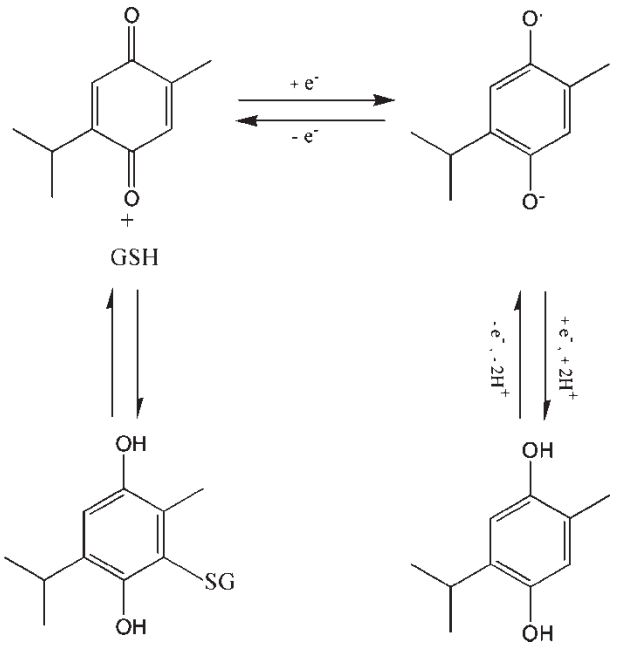
Discussion

The volatile oil of the black seed (*N. sativa* Linn) has a long history of folklore medicine for treatment of various diseases, and TQ, the main constituent of this seed's oil, has been the subject of a range of pharmacological investigations in recent years [29,30]. The exact antioxidant functions of TQ have not been conclusively established. In particular, it is still unclear whether it acts in the hydroquinone or quinone form. Similarly, the possible metabolic mechanism responsible for TQ reduction *in vivo* still remain an open problem. *In vivo*, different redox systems may react with TQ. The pathway of TQ reduction could be mediated by different reductases [15], which catalyze the redox conversion of quinones to hydroquinone in tissues with high efficiency. Different mechanisms have been described for other important quinines, such as CoQ10 and its synthetic derivatives (idebenone) [31,12] or other short-chain analogues [32,33], whose antioxidant interventions became effective after reduction of the quinone form to hydroquinone. In the same way, other redox systems could also be able to reduce TQ with different rates, making it suitable for redox coupling with endogen radicals. *In vivo*, most cells contain relatively high levels of GSH (up to 1 mM in erythrocytes and up 10 mM in hepatocytes [34,35]), and under physiological conditions GSH could be able to reduce different compounds. It has been reported that menadione (2-methyl 1,4,-naphtoquinone;vitamin K3) can react with GSH to form a menadione-GSH conjugate at position 3 [38] and this conjugation does not necessarily form less redox-active species [39].

As studied in this report, TQ can rapidly react with the cysteine moiety of GSH, forming thiolated adduct in which TQ is probably substituted in the 3-position of benzoquinone ring. Data seems to indicate the formation of only one product resembles glutathionyl–hydroxythymoquinone. Mass spectrometric analysis provided additional evidence for the identification of DHTQ–GS adduct. Data obtained show that reaction of TQ with GSH does not produce thiol 3,6-bisubstituted derivatives (DHTQ-GS2) of TQ. From MS spectra no peak at m/z 776 [$M-H^+$] related to the product DHTQ-$(GS)_2$ was detected. and it was not showed by HPLC analysis. The presence of an isopropyl group at carbon 5 of the TQ ring probably limits the substitution at the 6-ring carbon. As shown, the reaction occurs at physiological pH and GSH concentration, indicating that GSH could play a key role in the pharmacological effect of TQ *in vivo*. Reaction of thiol groups of GSH or of other critical cellular proteins could be significant in terms of the mechanism of toxicity exerted by some clinically important antitumor drugs [40–42], as well as in detoxification processes of natural quinones assumed as dietary supplements [43,44]. Moreover, the formation of GSH conjugate has also been reported for the action mechanism of menadione(vitamin K_3), where the reaction with GSH forms a thiol conjugate at the 3-position [45,46],suggesting that not only the menadione but also the menadione-GSH conjugate was responsible for the cellular redox cycling [47]. The observed reaction of TQ if, partially decrease the GSH content in the cells in the same way increase their antioxidant defence with enrichment of sequinone pool, thereby magnifying its effects. The possible transformation of TQ in the cells mediated by GSH also appears evident after incubation of human erythrocyte with TQ, where a decrease of cellular GSH concentration is detected (Figure 5). Erythrocyte exposure to TQ reduces the GSH pool but produces neither haemolysis nor met-Hb formation in the range of TQ concentration used. TQ can also be reduced from NADH and NADPH, but with an apparently very slow reaction rate, and the formation of the reaction product DHTQ can be detected by mass spectrometry. To estimate a putative correlation of the observed AA of DHTQ with DHTQ–GS, aliquots of this product were obtained through separation and purification by HPLC. Data obtained by measuring its AA revealed a potent scavenging activity similar to DHTQ (confirming that there were

no obvious differences in radical scavenging activity between the two reduced products, and apparently (as for DHTQ) showing the same Trolox equivalent antioxidant capacity). Exposing erythrocytes to TQ reduces the GSH pool but does not produce haemolysis in the range of TQ concentration used. The loss of intracellular GSH (of about 50% compared to the control), confirms that TQ crossed cell membrane and could be able to react with cytoplasmatic elements. Based on the products formed upon reaction with NADH, NADPH, and GSH, the proposed reduction and conjugation pathway for TQ in the presence of non-enzymatic reduction agents is offered in chart I. In cells, TQ could rapidly react with GSH to form a GSH conjugate, probably through addition of thiolate anion (GS-), or alternatively, (with a very slow process) it can react through the formation of a semiquinone to form DHTQ. At the moment we are unable to demonstrate the exact reaction mechanism, though we think it should resemble that proposed for the reaction between menadione and the same reducing agent [48]. We are performing ESR studies to establish the real reaction pathway.

In conclusion, the present findings suggest that reduced TQ or its conjugate forms could play a significant role in the activities reported for different *in vivo* treatments with TQ or *N. sativa* essential oil. In particular, the increase of antioxidant effects observed after their administration might be dependent on electron transfer mechanisms that involve the oxidation/reduction of TQ mediated by endogenous enzymatic and non-enzymatic reducing systems. The low toxicity and high reactivity of TQ with GSH in physiological conditions appear to be very interesting, related to possible *S*-thiolation reactions where TQ could participate in the binding with specific thiol groups in different proteins or react with GSH, decreasing the glutathione-*S*-thiolation processes. In fact, it has been recently demonstrated that the process of glutathione-*S*-thiolation is involved in the regulation of protein activity during free radical-mediated oxidative reactions [36]. Changes in the redox state of protein thiols may represent a "molecular switch" able to activate/deactivate protein function, a regulation mode that dramatically changes protein activity. Although none of these findings for TQ to date have been demonstrated in cellular systems, the GSH conjugates should be considered as possible species responsible for some physiological effects observed. Our results may suggest a novel role for therapeutic use of TQ and the essential oil from *N. sativa* in the interaction with cellular systems. The reduced forms of TQ should also be considered as an important lipid-soluble antioxidant, able to protect cell membranes or other cellular components, that might also play a role in protecting other systems such as lipoproteins against peroxidative damage. Further studies will be required to evaluate the exact mechanism(s) of the protective effect of TQ and the data presented here could therefore provide a molecular basis for further studies to elucidate the possible underlying reactions mediating the biological effects of these quinone compounds (Scheme 1).



Scheme 1. Proposed reduction and conjugation pathway for thymoquinone in the presence of GSH and NADH or NADPH.

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