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Nigella sativa Extract as a Potent Antioxidant for Petrochemical-Induced Oxidative Stress

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Abstract

Various beneficial properties has been attributed to Nigella sativa, including its antioxidant potential. Previously, it was reported that supercritical fluid extraction (SFE) could be used to obtain N. sativa extract rich in antioxidants. In the present study, N. sativa extracts prepared using the previously optimized SFE as well as the traditional Soxhlet extraction approaches were analyzed for various known antioxidants. N. sativa extracts were found to prevent protein carbonyl formation as well as depletion of intracellular glutathione (GSH) in fibroblasts exposed to toluene. Furthermore, partially purified SFE and Soxhlet fractions could prevent loss of hepatic GSH in toluene-induced oxidative stressed Wistar rats as well as in L929 fibroblasts. The results showed that SFE-produced *N. sativa* extract is richer in antioxidants than the Soxhlet approach. It was also shown using preparative silica gel and reverse phase chromatography that different fractions of SFEextracted or Soxhlet-extracted N. sativa had different levels of protective effects with regards to GSH depletion in vivo as well as in cell culture. Although fractions rich in thymoquinone were found to be most potent in terms of antioxidant capacity, the data indicates that the protective effects of *N. sativa* may not only be due to thymoquinone, but perhaps other antioxidants.

Introduction

The black seed (*Nigella sativa*) extract, commonly known as Habbat El Baraka in the Arab world, has been in use for generations in various parts of the world, including most of the Arab population. Recent investigations of black seed and many other herbs used for culinary as well as medical purposes have shown that they contain high levels of antioxidants (1,2). In fact, many potent antioxidants have actually been isolated from some natural herbs (3). Thus it is hypothesized that the beneficial effects of black seed and other herbs are most likely due to their protection against cellular damage caused by oxidative stress. The antioxidant properties of black seed oil are recently reviewed (2). It has been shown that some of the compounds isolated from black seed have appreciable free radical scavenging properties (4). This antioxidant property of black seed has also been reported by other investigators (5–7). However, no work has been done to examine the antioxidant effects of black seed oil and its components on petrochemical pollutant induced oxidative stress in vivo and in vitro. Pharmacologically active principles (thymoquinone, dithymoquinone, thymo-hydroquinone, and thymol) of black seed oil have been isolated by solid phase extraction (SPE) and high-performance liquid chromatography (HPLC) separation methods (8). In addition, four novel alkaloids namely nigellicine, nigellidine, nigellimine, and nigellimine Noxide have also been isolated from black seed (9). The essential components of black seed have been analyzed by gas chromatography-mass spectrometry (GC-MS) (4). Thymoguinone content of black seed oil has been estimated by GC (10). One of extraction approaches that has recently gained popularity is the supercritical fluid extraction (SFE) method, as this technology uses supercritical carbon dioxide (SC CO2), and thus no solvent residues are left behind in the product (11–14). This technique has the added advantage of recovering the volatile compounds and does not alter the delicate balance of components in natural products.

One of the areas that has been a focus of great interest by researchers in the life sciences is the field of reactive oxygen species (ROS). It has been hypothesized for some time that the harmful effects of many xenobiotics and petrochemical pollutants and their metabolites are due to their ability to generate ROS (15–17). There is a growing list of reports showing that benzene, toluene, and other petrochemical byproducts, when ingested, inhaled or absorbed, are metabolized and transformed into chemicals that lead to the generation of reactive oxygen species (18,19). These ROS, when present in high concentrations, can overwhelm cellular antioxidants, and lead to a condition termed "oxidative stress" (20). Cells exposed to oxidative stress undergo extensive cellular damage due to oxy-radical induced DNA-breakage, lipid (membrane) oxidation, and extensive protein damage (21). Recently, it has been shown that diverse environmental pollutants including xylene, redoxcycling metals, and UV radiation can cause oxidative stress in skin fibroblasts, leading to GSH depletion and causing S-thiola-

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tion of intracellular proteins (22). Not surprisingly, a large number of disease states have been linked to oxidative stress, including cancer, diabetes, Parkinson's disease, Alzheimer's disease, and heart disease (21,23). In fact, it was recently shown that petrochemical workers are under significant oxidative stress due to their exposure to petrochemical pollutants and byproducts (24).

In the present study, the antioxidant capacity of *N. sativa* was tested in a model system involving toluene-induced oxidative stress. Furthermore, it was desired to characterize and analyze silica-gel fractions of SFE-extracted *N. sativa* and compare it with the Soxhlet-extracted fractions. We show that both extraction approaches were successful in producing antioxidant-rich *N. sativa* extracts which showed strong protective effects against oxyradicals in both cultured hepatocytes as well as in whole animal models. Interestingly, it was shown that the antioxidant properties of *N. sativa* are probably due to other antioxidants in addition to its more potent component Thymoquinone.

Materials and Methods

SFE

The SFE was performed as described by Rao et al. (14). Briefly, the experimental apparatus consisted of a 260 mL capacity syringe pump and controller system (ISCO 260D), and an ISCO series 2000 SCF Extraction system (SFX 220) consisting of a dual-chamber extraction module with two 10-mL stainless steel vessels. Temperature and pressure within the vessels were measured and could be independently adjusted. The 10 mL stainless steel cell was filled with about 5 g of ground black seed. The cell was pressurized and heated to the desired pressure (100–400 bar) and temperature (30–70°C) and kept for 15 min to reach equilibrium. A known volume of SC CO2 (100–300 mL) was passed through the cell at a flow rate of 1 mL/min. The extract was collected in a cold trap after depressurization of the gas. The collected sample was stored at -18° C until analysis.

Soxhlet extraction

Approximately 20 g of finely ground powder of *N. sativa* was placed in a thimble and extracted in an all glass Soxhlet extractor

Table I. HPLC Characterization of Active Principles in the SFE and Solvent extracts and Their Fractions

LC System: Agilent 1100 Column: Symmetry C₁₈, 5 μm, 4.6 × 250 mm Detector: Agilent 1100 PDA (254 & 288 nm) & FLD (λEx = 300 nm, λEm = 360 nm)

Time (min)	Flow Rate (mL/min.)	Methanol (A)	Water-2-Propanol (9:1) (B)
0	1.0	20	80
20	1.0	60	40
30	1.0	60	40
35	1.0	20	80
40	1.0	20	80

for 8 h using hexane and methanol as solvents. Solvent was removed by rotary evaporation at 40°C under vacuum and the last traces of solvent in the extract were removed under a stream of nitrogen. The extract was stored at -18° C until analysis.

Fractionation of *N. sativa* Extracts:

Silica gel column fractionation: The extract obtained by Soxhlet extraction technique was subjected to fractionation on silica gel. A 250 g of activated silica gel was loaded to a column and cleaned with about 100 ml of hexane (HPLC grade). About 10 g of the extract was loaded on to the column. The compounds were eluted successively with 500 mL each of hexane, 15% diethyl ether in hexane, diethyl ether, and methanol. Solvent in the fractions was removed by rotary evaporation at 40oC under vacuum. A known quantity of each fractionation (100 mg) was made up to 10 mL with methanol and subjected to HPLC analysis.

Preparative HPLC fractionation

Fractionation of SFE extract was carried out on Knauer preparative HPLC consisting of Knauer K-1800 isocratic pump and K-2001 UV detector. Black seed oil extracted using above SFE conditions was dissolved in acetonitrile-methanol (30:70) and subjected to preparative HPLC fractionation on a C-18 column (Eurospher 100, 20 mm i.d.) using acetonitrile-methanol (30:70) as mobile phase at a flow rate of 5 mL/min. The elution of compounds was monitored using a UV detector at 254 nm. One milliliter of the sample solution was injected each time and fractions at time intervals of 0–18 min. 18-25 min, 25-30 min, 35-48 min, and 48-60 min. were collected successively into 500 mL rotary evaporation flasks. After repeated injections and collection of fractions, solvent in each fraction was removed by rotary evaporation at 40°C under vacuum. A known quantity of each SFE Fraction (100 mg) was made up to 10 mL with methanol and subjected to HPLC analysis under the conditions described in Table I.

A 20 µL of sample solution was injected and compounds in the extracts as well as fractions were identified with retention times. The results are shown in Table I. The concentrations of t-Anethole, thymoquinone, and carvacrol in the soxhlet and SFE extracts as well as their fractions (Tables II and III) were quantified by injecting known concentrations of analytical standards of t-anethole, thymoquinone and carvacrol to the HPLC with fluorescence detection, following our previously published method (14).

Table II. HPLC Characterization of Soxhlet Oil Fractions Obtained by Silica Gel Column Chromatography mg/g Fraction Sample t-Anethole Thymoquinone Carvacrol Unknown Hexane (Soxhlet, 8 h) 15 248 16 721 Hexane Fraction 29 368 19 555 15% Diethyl ether in Hexane 5 658 2 335 Diethyl ether fraction Tr* 288 Tr 712 Methanol fraction Tr 42 Tr 958 *Tr = traces.

Continuous cell line culture

Media for cell-culture was prepared by adding 86 mL of EMEM + 10 mL FBS (Bovine serum) + 2 mL of streptomycin/penicillin + 1 mL of fungizone + 1 mL of L-glutamine, which was stored in unscrewed bottles in a humidified 5% CO2 37°C incubator over night before experiment. Continuous cell lines of mouse skin fibroblasts L929 human hepatocytes HUH7 were cultured by slowly defrosting frozen vials and gently mixing them before placing the cells at a concentration of 2×105 cells per plate by placing 25 µL of the cells in 35 mm plate containing 2 mL of the media and then incubate them in a CO2 incubator. Plates with 70–90% confluency were then pre-incubated with 10 uL of N. sativa overnight and then treated with 10 mM of toluene (in dimethyl sulfoxide) for 3 h. Similarly, other sets of similar pre incubated plates were treated with hydrogen peroxide for 3 h. After 3 h of treatment, media was decanted and cells were lysed by freeze-thaw and scraping in 0.5 mL of 50 mM Tris + 10 mM EDTA buffer, pH 8.8. The lysate was then centrifuged at 4000 rpm to remove cellular debris and the supernatant was used for glutathione (GSH) assay.

In vivo studies

Male Wistar rats weighing between 200–250 g were housed in polyethylene cages at a room temperature of 22 ± 2 °C on a 12–12 h light-dark cycle.

A standard pellet diet and tap water were given ad libitum. Groups of animals (5 in each group) were treated once daily for 3 days as follows:

Group 1. No treatment, received dimethyl sulfoxide alone (equivalent to the concentration in "toluene-treated" group) as intraperitoneal (i.p.) injection;

Table III LIDIC Characterization of CEE Oil Fractions Obtained

		mg/g Fraction				
Sample	t-Anethole	Thymoquinone	Carvacrol	Unknown		
SFE oil	58	426	25	491		
Fraction 1	104	328	56	511		
Fraction 2	10	856	14	119		
Fraction 3	8	684	28	280		
Fraction 4	Tr	428	16	556		
Fraction 5	Tr	36	Tr	964		

Table IV. Experimental Conditions and Yield Obtained DuringSFE and Solvent Extraction Methods

Run #	Temp. (°C)	Pressure (bar)	CO ₂ volume (mL)	Yield (%)	GSH Recovery (%)
1	40	300	100	28.4	12
2	50	300	150	29.0	30
3	50	100	200	0.84	75
4	60	400	100	30.1	9.0
5	70	400	250	30.3	32
Soxhlet Extraction (Hexane) 28.1 –					
Soxhlet Extraction (Methanol) 29.2 –					-

Group 2. Toluene-treated, received i.p. injection of toluene 250 mg/kg body weight in dimethyl sulfoxide;

Group 3. Toluene + extract (or SFE fraction), received toluene i.p. and + 100μ l, p.o. (oral administration) black seed extract (or SFE fractions) 1 h later; and

Group 4. Toluene + known antioxidants, received toluene, i.p. + 20μ M thymoquinone (or carvacrol, thymol, t-anethol and p-cymene) i.p., 1 h later.

At the end of the three days and 1 h after the last injection, the animals were then sacrificed and a piece of liver was taken out and frozen immediately until estimation of GSH was carried out.

Protein estimation and GSH assay

Protein concentration of the liver tissues was estimated using the Bio-Rad Bradford protein assay and expressed as nmol/mg wet weight of tissue. Intracellular GSH was measured by using the micro-titer assay as published by Coleman et al. (25). Briefly, GSH standard or cellular lysate was mixed with DTNB in a micro-titer plate. After 5 min of incubation, the plate was read at 405 nm. Although this assay essentially measures free thiol groups (of both, proteins and low-molecular weight thiols), it is generally accepted that low molecular weight thiols (of which GSH comprises ~ 90%) are present in large excess (approximately 10-fold) than protein thiols. Hence, one could use this assay to get an approximate quantization of intracellular GSH levels.

Protein carbonyl determination

Protein carbonyls were detected using a kit form Cayman Chemical (Cat #10005020, Protein Carbonyl Assay Kit) which measures the reaction between protein carbonyls and 2,4-dintirophenylhydrazine (2,4-DNPH). The results shown are the mean and standard deviations from triplicate determinations normalized by total protein (Bradford Assay).

Results

Extraction and characterization of N. sativa

It was previously determined that supercritical fluid extraction (SFE) could be used to produce high quality extracts Nigella sativa that were rich in antioxidants (14). In the present study, the focus was further characterizing the biological (antioxidant) activities of N. sativa extracts. Table IV shows the summary of five different SFE extracts and their ability to prevent loss of glutathione in HUH-7 hepatocytes exposed to 10 mM H2O2. As can be seen from the table, the yields of the extract ranged from 0.84% to 30.3% under different conditions, however SFE condition #3 (temperature, pressure and CO2 volume: 50°C, 100 bar, 200 mL) which gave lowest yield, had the maximum antioxidant activity (as measured by prevention of loss of intracellular GSH defined as "GSH recovery" henceforth). It was interpreted that majority of fats and lipids normally present in N. sativa are not extracted under this condition (#3) and that high concentrations of antioxidants are present in this extract. Therefore, all the subsequent SFE of N. sativa reported here was done with this set of conditions.

To further confirm that this SFE-extracted *N. sativa* was rich in antioxidants, the ability of this extract to protect L929 cells against toluene-induced oxidative damage was tested. It had previously been shown that metabolism of toluene and other petrochemicals can lead to production of oxyradical, eventually causing oxidative stress and damage to cells. As can be seen from Figure 1, exposure of L929 cells to 10 mM toluene resulted in profound loss of intracellular GSH as well as increase in protein carbonyls (measure of protein damage). This is not surprising, as it is well established that metabolism of cellular detoxification of toluene leads to oxyradical production and can lead to oxidative stress (22,24). As expected, this oxidative damage could be readily prevented by pre-incubating the cells with *N. sativa* oil.

There was also an intereste in examining the extraction of *N*. *sativa* using the traditional hexane (Soxhlet) extraction and comparing it with that of the optimum SFE approach. Figure 2 shows the HPLC trace of *N*. *sativa* oil as extracted by SFE (using



Figure 1. Prevention of loss of intracellular GSH as well as protein carbonyl formation (PC) by *N. sativa* (SFE Extract). HUH-7 hepatocytes that were either pre-incubated overnight with N. sativa extract or nothing were exposed to 10 mM Toluene for 3 h and then GSH and PC levels were determined as described under "Materials and Methods". Results shown are the mean (\pm SD) of three replicates.



Figure 2. HPLC traces showing the individual components of *N. sativa* oil extracted using the Soxhlet (Hexane) as well as SFE approaches. Peaks labeled "1", "2", and "3", refer to retention times for t-Anethole, Thymoquinone, and Carvacrol standards.

CO2) and Soxhlet (using hexane). As was from the trace, there were significant differences in the number of peaks as well as their relative percentages. Although Soxhlet (hexane) extraction trace showed a lot more peaks than the SFE trace, they both seemed to be able to prevent loss of intracellular GSH in L929 cells exposed to toluene (Figures 3 and 4).

In order to better characterize the various components as well as their antioxidant properties, we carried out partial purification of both the hexane and the SFE-extracted N. sativa oils. SFEextracted oil was fractionated using a C18 reverse-phase column, whereas the hexane-extracted (Soxhlet) oil was subjected to silica gel chromatography, as explained under the experimental section. Tables II and III show the relative proportions of three of the identifiable antioxidants (t-anethole, thymoguinone, and carvacrol) in the fractions obtained from the partial purification of the two extracts. Fraction 1 of the SFE partial purification appears to have the highest enrichment of t-anethole and carvacrol, whereas fractions 2 and 3 had the highest concentration of thymoguinone (Table II). Fraction 5 was found to be devoid of t-anethole, thymoquinone and carvacrol, but highly enriched in lipids and fats. Partial purification of the hexane (Soxhlet) extracted N. sativa oil using silica gel chromatography did not lead to as nice separation as the C18 chromatography of SFE oil, but as can be seen in Table 3, 15% diethyl ether in hexane fraction and the hexane elution fraction were found to be most enriched in thymoguinone.

In vitro testing of N. sativa in L929 cells

In order to confirm which fractions had the most antioxidant activity, we tested the ability of these fractions to prevent loss of intracellular glutathione (as a measure of preventing oxidative stress) in L929 fibroblasts exposed to toluene. As can be seen from Figure 3, exposure of 10 mM toluene to L929 cells resulted in about 45% depletion within 3 hours, most of which could be



Figure 3. Prevention of toluene-induced loss of GSH in L929 fibroblasts by SFE-extracted *N. sativa* oil and partially purified fractions. L929 fibroblasts that were either pre-incubated overnight with *N. sativa* extract, various SFE fractions, or nothing were exposed to 10 mM Toluene for 3 h and then intracellular GSH levels were determined as described under "Materials and Methods". Results shown are the mean (\pm SD) of three replicates.

prevented by pre-incubating the cells with SFE-extracted N. sativa oil. When the semi-purified SFE fractions were tested in the same assay, as expected fractions 2 and 3 (highest in thymoquinone) were most effective in preventing loss of intracellular GSH. Not surprisingly, fraction 5, which was mostly composed of lipids and fats was not very rich in antioxidants. Interestingly, fraction 1 which was relatively rich in t-anethole (a known antioxidant) did not show any significant protection against GSH depletion, suggesting that perhaps t-anethole does not play a significant role in conferring antioxidant protection to fibroblasts exposed to oxidative stress. It was also suprising to note that the fact that fraction 4, which had half the amount of thymoguinone as fraction 2, showed significant GSH recovery. This seems to imply that the anti-oxidative effects observed by SFE oil is mostlikely not due to thymoguinone alone and there may be additional compounds (perhaps in fraction 4) that may have significant antioxidant properties. This hypothesis is further supported by the results obtained from testing of silica gel chromatography fractions of Soxhlet (hexane)-extracted oil. As can be seen from Figure 4, the diethyl ether fraction, which had less than half the amount of thymoguinone (Table III), had almost the same protective effect (towards GSH recovery) as the 15% diethyl ether/hexane fraction (the "best" fraction). Also, as can be seen from Figure 4, the unpurified Soxhlet (hexane)-extracted N. sativa oil appears not to be as potent as the SFE-extracted oil in preventing loss of intracellular GSH in L929 cells exposed to toluene.

In vivo testing of N. sativa in Wistar rats

In order to confirm that the *N. sativa* antioxidant effect seen in L929 cells would also hold true for whole animal models, hepatic GSH levels of rats exposed to toluene in the presence or absence of *N. sativa* oil was measured. As can be seen in Figure 5, expo-



Figure 4. Prevention of toluene-induced loss of GSH in L929 fibroblasts by Soxhlet-extracted N. sativa oil and partially purified fractions. L929 fibroblasts that were either pre-incubated overnight with N. sativa extract, various fractions, or nothing were exposed to 10 mM Toluene for 3 h and then intracellular GSH levels were determined as described under "Materials and Methods". Results shown are the mean (\pm SD) of three replicates.

sure of 250 mg/Kg toluene to rats led to about 25% decrease in hepatic GSH levels. This is very much consistent with what we and others have reported for toluene and petrochemical pollutant exposures to humans as well (22,24). As shown in Figure 5, co-injection of SFE-extracted N. sativa oil greatly minimized the toluene-induced hepatic loss of GSH. Testing of the partially purified SFE fractions for their antioxidant effect towards toluene-induced oxidative stress in rats showed a pattern similar to that observed in L929 cells (Figure 3 vs. 5). SFE fraction 1 showed the least protective effect in vivo as it did in L929 cells, again, suggesting that the high proportion of t-anethole found in this fraction is perhaps not very effective against tolueneinduced oxidative stress. This was further supported by data showing that of the five commercially available phyto-antioxidants tested, t-anethol had the least protective effect (GSH recovery) (Figure 6).



Figure 5. Recovery of toluene-depleted hepatic GSH in rats treated by SFEextracted N. sativa oil and partially purified fractions.



Conclusion

In conclusion, the data presented here clearly shows that *N. sativa* extracts, either extracted using the traditional Soxhlet (hexane) approach or by SFE method, are rich in antioxidants. Furthermore, we demonstrate using in vitro and in vivo assays that *N. sativa* can be used to prevent oxidative damage caused by a known petrochemical pollutant, toluene. Studies with partial purification and fractionation of *N. sativa* oil showed that although fractions rich in thymoquinone were most potent in terms of their antioxidant capacity, the protective effects of *N. sativa* may not only be due to thymoquinone, but perhaps due to other antioxidants as well.

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