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ORIGINAL ARTICLE

Antioxidant and hepatoprotective activities of the oil fractions from wild carrot (*Daucus carota* ssp. *carota*)

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Abstract

Context: Wild carrot, *Daucus carota* L. ssp. *carota* (Apiaceae), is widely distributed throughout the world and has various uses in traditional medicine in Lebanon.

Objective: The present study aimed to fractionate and analyze the chemical composition of the *Daucus carota* oil extract (DCOE) fractions and to evaluate their antioxidant and hepatoprotective properties *in vitro* and *in vivo*.

Materials and methods: DCOE was chromatographed on silica gel column to produce four fractions: pentane (F1), 50:50 pentane:diethyl ether (F2), diethyl ether (F3), and 93:7 chloroform:methanol (F4). Qualitative and quantitative analyses of oil fractions were performed by GC-MS and HPLC techniques. The *in vitro* antioxidant properties were assessed using DPPH, FIC, and ferric-reducing antioxidant power (FRAP) assays. The hepatoprotective property was determined by examining the levels of serum markers (alanine transaminase (ALT) and aspartate transaminase (AST)) and hepatic antioxidant (superoxide dismutase (SOD), catalase (CAT), and glutathione-S-transferase (GST)) enzymes in CCl₄-intoxicated mice pretreated with intraperitoneal 50, 100, or 200 mg/kg b.w. of the oil fractions for 5 d.

Results: GCMS analysis of F2 revealed the presence of 2-himachalen-6-ol (61.4%) which is reported for the first time in *Daucus carota* species. F3 and F4 were rich in phenolics and flavonoids and demonstrated significant DPPH activity (IC₅₀ = 0.29 and 0.38 mg/ml, respectively) and high FRAP values (225.11 and 437.59 μmol FeSO₄/g, respectively). The sesquiterpene-rich fraction F1 had the highest FIC ability (IC₅₀ = 0.28 mg/ml). Pretreatment with F1 and F4 reversed the CCl₄-induced decrease in SOD, CAT, and GST levels and reduced significantly hepatic damage.

Discussion and conclusion: The current results suggested that wild carrot oil fractions exhibited a unique chemical composition and possessed significant antioxidant activities as well as hepatoprotective effects against CCl₄-induced hepatotoxicity.

Keywords

CAT, DPPH, FRAP, FIC, flavonoids, GST, phenolics, SOD

History

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Introduction

There is growing evidence that many human diseases are caused by oxidative stress, which partially results from an imbalance between the formation and the neutralization of pro-oxidants (Hazra et al., 2008). Oxidative stress is induced by free radicals, mainly reactive oxygen species (ROS), which include the superoxide radical (O₂^{•-}), hydrogen peroxide (H₂O₂), and hydroxyl radical (•OH). ROS molecules are highly reactive against macromolecules such as DNA, proteins, and membrane lipids in living cells. These changes are associated with the development of aging, cancer, cardiovascular, and inflammatory diseases (Braca et al., 2002; Hemnani & Parihar, 1998). The human body has natural defense mechanisms that protect against free radicals

and oxidative damage. These mechanisms are both enzymatic, such as the ones catalyzed by superoxide dismutase (SOD), catalase (CAT), and the glutathione system (glutathione, glutathione reductase, peroxidase, and transferase), and non-enzymatic, such as the ones led by low molecular weight antioxidants such as vitamins A, E, and C (Evans et al., 2004; Manda et al., 2009). Although ROS exist in all aerobic cells, they are normally in equilibrium with antioxidants to maintain lower free radicals levels (Waris & Ahsan, 2006). However, if the balance between free radicals and the antioxidant defense system is compromised, the need for antioxidant supplements becomes necessary to combat oxidative damage (Shukla et al., 2009). Over the last few decades, the literature has revealed the importance of natural products as a source of potent antioxidants (Mantle et al., 2000). Among the most described are phenolic acids, polyphenols, monoterpenes, sesquiterpenes, and phenylpropanoids which have been found to possess antioxidant activity (Medina-Holguín et al., 2008; Sibanda et al., 2004). These compounds have the capacity to

scavenge free radicals and to increase the levels of antioxidant enzymes thus protecting the body against damage from ROS (Tachakittirungrod et al., 2007; Terao, 2009).

Wild carrot, *Daucus carota* L. ssp. *carota* (Apiaceae), is a spiny-fruited herb that grows in moderate regions of Europe, Asia, Africa, and North and South America (Mitich, 1996). The plant is commonly consumed as a salad in the Mediterranean diet or used as an additive in some recipes (Nehir El & Karakaya, 2004). Its flower umbels are edible and usually French fried, and its seed oil, which is commercially available, is used to flavor beverages and food products (Leung, 1980). In Lebanon, people eat its young taproot cooked and use the plant in folk medicine for the treatment of cancer, gastric ulcer, diabetes, muscle pain, and to protect against hepatic diseases. Available literature on the therapeutic effects of wild carrot showed that the plant possesses antilithic, diuretic (Thomas et al., 2001; Wyk & Wink, 2004) antibacterial, and antifungal activities (Maxia et al., 2009; Rossi et al., 2007; Staniszewska et al., 2005). The oil extract of wild carrot has been shown to consist mainly of phenols, flavonoids, monoterpenes, sesquiterpenes, and phenylpropanoids (Gonny et al., 2004; Maxia et al., 2009; Mockute & Nivinskiene, 2004; Shebaby et al., 2013; Staniszewska et al., 2005). However, the percentage composition of the oil was shown to vary greatly with different geographical origins (Shebaby et al., 2013). Lately, we reported that the aqueous and methanolic extracts of wild carrot umbels exhibited anti-inflammatory, anti-ulcer (Wehbe et al., 2009), anticancer (Shebaby et al., 2013; Zeinab et al., 2011), and antioxidant activities (Wehbe et al., 2009). In the current study, we aimed at analyzing the chemical composition of the *Daucus carota* oil extract (DCOE) fractions, evaluating their *in vitro* and *in vivo* antioxidant activities as well as hepatoprotective properties.

Materials and methods

Chemicals

Folin–Ciocalteu reagent, 1,1-diphenyl-2-picryl hydrazyl (DPPH), 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ), quercetin, vanillic acid, chlorogenic acid, syringic acid, caffeic acid, ellagic acid, myricetin, luteolin, kaempferol, apigenin, ferrozine, gallic acid, trolox, pyrogallol, 1-chloro-2,4-dinitrobenzene (CDNB), dimethylsulfoxide (DMSO), and silica gel 40 (35–70) mesh were purchased from Sigma Aldrich, St. Louis, MO. All other chemicals used in the experiments were of analytical grade.

Sample collection and oil extraction

Daucus carota ssp. *carota* mature umbels were collected at the post-flowering season between August and September (2012) from Amioun, North Lebanon. The plant was identified according to the characteristics described in ‘‘Handbook of Medicinal Herbs’’ (Wyk & Wink, 2004) and confirmed by Dr. A. Houry, a Lebanese plant expert, Departmental of Natural Sciences at the Lebanese American University. A voucher specimen of the plant was deposited in the Departmental herbarium for further records. The extraction procedure was carried out according to the method described by Zeinab et al. (2011). Briefly, 1 kg of air-dried

umbels were cut into small pieces and soaked in 5 l of methanol/acetone (1:1) for 72 h. The extraction was repeated twice and the extracts were collected, filtered, and evaporated to dryness under reduced pressure. The residue was centrifuged at 500 rpm and the oil was removed and dried over anhydrous sodium sulfate yielding 34.7 g of crude oil extract, which was stored in a closed amber bottle at 4 °C until use.

Oil fractionation

DCOE (30 g) was chromatographed on a silica gel column (35–70 mesh). The first fraction (F1) was eluted with pentane (100%), the second fraction (F2) with pentane:diethyl ether (50:50), the third fraction (F3) with diethyl ether (100%), and the fourth fraction (F4) with chloroform:methanol (93:7). Fractions were analyzed by TLC using hexane:ethyl acetate (70:30) as a mobile phase and plates were stained with 2% anisaldehyde.

Gas chromatography and mass spectrometry analysis

The composition of the oil fractions was analyzed using gas chromatography and mass spectrometry (GC–MS) (Hewlett Packard, HP6890 series, Hewlett-Packard, Palo Alto, CA) fitted with a fused silica HP5-MS 5% phenyl methyl siloxane cap column (30 m × 0.25 mm i.d., film thickness 0.25) and directly coupled to the MS. The carrier gas was helium with splitless injection and the flow rate of 1.2 ml/min was applied. The temperature program was as follows: 2.0 min at 70 °C, from 70 °C to 130 °C at 8 °C/min and hold for 5 min, from 130 °C to 180 °C at 2 °C/min and hold for 10 min, from 180 °C to 220 °C at 15 °C/min and hold for 2 min and then from 220 °C to 280 °C at 15 °C/min and hold for 22 min. Identification of the components was performed by comparing their mass spectra with the literature (NIST11 and W9). Percentage composition was computed from GC peak areas.

HPLC analysis of oil fractions

The analysis of phenolic acids and flavonoids was performed on a Shimadzu HPLC system (Shimadzu Corp., Kyoto, Japan) consisting of LC 10-ADVP pump, SCL 10 A system controller coupled with a photo-diode array detector (SPD-M20A), FCV-10AL low-pressure gradient, rheodyne injector (Model 7125), DGU-20 A online degasser, Shim-pack VP-ODS column, 4.6 mm i.d. × 150 mm), pre-column (10 × 4.6 mm i.d. 5 μm) equipped with LC solution 1.23 SP1 software (Shimadzu, Kyoto, Japan). The column was operated at 25 °C. The mobile phase consisted of 10:2:88 – water:acetic acid:methanol v/v (solvent A) and 90:2:8 – water:acetic acid:methanol v/v (solvent B) at a flow rate of 1.5 ml/min. The gradient elution program was as follows: 0–15 min solvent A, 15–30 min solvent followed by washout period for 10 min, and the wavelength of detection was set at 280 nm. The phenolic acids and flavonoids were identified by matching the retention time and their spectral characteristics with those of the standard compounds (gallic acid, chlorogenic acid, vanillic acid, syringic acid, caffeic acid, ellagic acid, myricetin, quercetin, luteolin, kaempferol, and apigenin).

Total flavonoid content determination

Total flavonoid content of DCOE fractions was determined by the aluminum chloride colorimetric method (Chang et al., 2002). Briefly, 0.5 ml of each fraction (ranging from 0.2 to 5 mg/ml) in methanol was separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate, and 2.8 ml of distilled water. The absorbance of the reaction mixture was measured at 415 nm. Different concentrations of quercetin (12.5–200 µg/ml) in methanol were used as a standard curve.

Total phenolic content determination

Total phenolic content of DCOE fractions was determined according to the method described by Rossi et al. (2007) using gallic acid as a standard. About 0.3 ml of each fraction in methanol (0.5–5 mg) was mixed with the Folin–Ciocalteu reagent (2.5 ml, 1:10 dilution) for 5 min. Then 2 ml of aqueous Na₂CO₃ (7.5% w/v) was added and the mixture allowed to stand for 30 min at 25 °C. Absorption at 760 nm was measured spectrophotometrically. The results were expressed as mg gallic acid equivalent (GAE) per gram of oil fraction.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The DPPH assay was determined according to the method described by Mossa and Nawwar (2011). Samples (200 µl) of oil fractions of (0.025–1 mg/ml in DMSO) were mixed with 1.5 ml of 0.1 mM DPPH in methanol, and the final volume was adjusted to 3.5 ml with DMSO. The mixture was then shaken vigorously and allowed to stand for 30 min in the dark at room temperature. The absorbance was determined at 517 nm. Trolox was used as a reference compound. Absorbance of DPPH in DMSO was used as a control. The percent inhibition of DPPH free radical by the samples was calculated according to the following formula:

$$\% \text{ Inhibition} = [(A_c - A_s) / A_c] \times 100$$

where A_c and A_s are the absorbance of the control and the sample, respectively. The IC₅₀ value represented the concentration of the oil extract that caused 50% inhibition.

Ferrous ion-chelating ability assay

The ferrous ion-chelating (FIC) assay was carried out according to the method described by Dinis et al. (1994) with slight modifications. Aliquots of 1 ml of different concentrations of the oil fractions (0.25–4 mg/ml) were mixed with 1 ml FeCl₂·4H₂O (0.1 mM) and incubated for 5 min. The reaction was initiated by the addition of 1 ml ferrozine (0.25 mM) and the absorbance of the solution was measured at 562 nm. The percentage of the chelating ability of each fraction was calculated using the following formula:

$$\% \text{ Chelating ability} = [(A_0 - A_s) / A_0] \times 100$$

where A_0 is the absorbance of control sample (the control contains FeCl₂ and ferrozine) and A_s is the absorbance of a tested sample. Na₂EDTA was used as a positive control.

Ferric reducing antioxidant power assay

The total antioxidant power of the oil fractions was determined according to the method described by Benzie and Strain (1996). Briefly, the ferric reducing antioxidant power (FRAP) reagent was prepared by mixing 300 mM of acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl and 10 mM FeCl₃ at a ratio of 10:1:1. Aliquots (200 µl) of oil fractions (0.25–4 mg/ml) in methanol were added to 2.8 ml of the FRAP solution and the absorbance was recorded at 593 nm. Vitamin C was used as a positive control. The antioxidant power of the sample was calculated from the calibration curve of FeSO₄ solution and expressed as mM FeSO₄ equivalent per gram of sample.

CCl₄-induced hepatotoxicity model

BALB/c mice, weighing 27–30 g (Lebanese American University stock) were housed under stable conditions of temperature (20 ± 2 °C) and humidity (50 ± 5%), and an alternating cycle of light and dark. The animals were supplied with standard laboratory chow diet and water. All experimental protocols were approved by the Animal Ethical Committee of the Lebanese American University, which complies with the Guide for the Care and Use of Laboratory Animals (Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2010).

Liver damage was induced by intraperitoneal injection of CCl₄ in olive oil (1:1, v/v) at a dose of 2 ml/kg body weight (Coballase-Urrutia et al., 2011). The mice were divided randomly into 15 groups of six animals each. Group I served as a normal control and was given DMSO (2 ml/kg) for 5 d and olive oil on days 2 and 3. Group II served as a CCl₄ control and received DMSO (2 ml/kg) for 5 d and CCl₄/olive oil on days 2 and 3. Group III, positive control, was given vitamin E (100 mg/kg body weight) in DMSO (2 ml/kg) for 5 d and CCl₄/olive oil on days 2 and 3. Groups IV, V, and VI served as treated groups and were given 50, 100, and 200 mg/kg body weight of F1 fraction in DMSO (2 ml/kg), respectively, for 5 d and CCl₄/Olive oil on days 2 and 3. Similarly, Groups VII, VIII, and IX were given the F2 fraction, Groups X, XI, and XII were given the F3 fraction, and Groups XIII, XIV, and XV were given the F4 fraction. At the end of the experiment (day 6), blood was collected from overnight-fasted mice under mild ether anesthesia and the serum was analyzed for marker enzymes aspartate transaminase (AST) and alanine transaminase (ALT) using commercially available kits (SPINREACT) according to the manufacturer's instructions. The animals were later sacrificed by cervical dislocation under mild ether anesthesia and the liver was excised, washed, and homogenized in ice-cold phosphate buffer (0.1 M; pH = 7) and centrifuged at 19 000g for 15 min at 4 °C. The obtained supernatant was used to determine the total protein content (Bio-Rad Protein Assay, Bio-Rad Laboratories, Inc., Berkeley, CA) and evaluate CAT, SOD, and glutathione-S-transferase (GST) activities.

CAT assay

CAT activity was assayed on the basis of H₂O₂ disappearance at 240 nm (Pedraza-Chaverri et al., 2005). A 5 µl aliquot of

the 1:40 diluted supernatant was added to 720 μl of 30 mM H_2O_2 in 10 mM potassium phosphate solution. The decomposition of H_2O_2 by CAT contained in the sample was measured at 240 nm for a period of 15 s. The reaction follows first-order kinetics given by the equation $k = 2.3/t \log(A_0/A)$, where k is the first-order reaction rate constant, t is the time over which the decrease of H_2O_2 was measured, and A_0 and A are the optical densities at times 0 and 15 s, respectively. The results were expressed in k/mg protein.

SOD assay

SOD activity was assayed according to the method described by Naskar et al. (2009). Briefly, 50 μl of the homogenized liver supernatant was added to 2.8 ml Tris-EDTA (50 mM Tris, 1.2 mM EDTA; pH = 8.5) and 100 μl of 2 mM pyrogallol. The optical density (OD) of the mixture was read at zero and 3 min at 420 nm against the control which consisted of Tris-EDTA and pyrogallol. One unit of SOD is the amount of enzyme that inhibits the rate of auto-oxidation of pyrogallol by 50%. The results were calculated according to the following equations:

$$\begin{aligned} \text{Rate (R)} &= (\text{final OD} - \text{initial OD})/3 \\ \% \text{ Inhibition} &= [(R_{\text{control}} - R)/R_{\text{control}}] \times 100 \\ \text{Enzyme unit (U)} &= (\% \text{ inhibition}/50) \\ &\quad \times \text{dilution factor} \end{aligned}$$

GST assay

GST activity was assayed according to the method described by Al-Saffar et al. (2011). Two solutions were prepared (sample and reference) each of 3 ml from the following: 2.2 ml of 0.1 M potassium phosphate buffer (pH 6.5), 100 μl

of 30 mM CDNB (1-chloro-2,4-dinitrobenzene), and 100 μl of 30 mM glutathione reduced (GSH). The solutions were allowed to stand for 1 min at room temperature (25 °C), and 600 μl of the liver supernatant was added to the sample solution and mixed thoroughly. The absorbance was read at 340 nm after 1 min and the activity of GST was expressed in nmol of GSH-CDNB conjugates formed/min/mg protein by using molar extinction coefficient $9.6 \text{ mM}^{-1} \text{ cm}^{-1}$.

Statistical analysis

Data were analyzed for statistical significance using one-way analysis of variance (ANOVA). Values of the different tested parameters within each group are presented as mean \pm SEM. Significant main effect differences were tested using Tukey–Kramer's *post hoc* test for multiple comparisons. All data were analyzed with the statistical package SPSS 18 (SPSS Inc., Chicago, IL), and statistical significance was considered when $p < 0.05$.

Results

Chemical composition of oil fractions

Column chromatography of DCOE yielded four fractions based on TLC profiles. Table 1 shows the GC-MS analysis of the resulted fractions with their respective components. On one hand, the F1 fraction was predominantly composed of sesquiterpenes, mainly, α -humulene (28.2%), β -caryophyllene (17.2%), α -longipinene (7.18%), α -selinene (4.26%), γ -selinene (3.53%), β -selinene (2.86%), and β -himachalene (2.58%). The F2 fraction was markedly dominated by the sesquiterpene 2-himachalen-6-ol (61%) and noticeable amounts of three phenylpropanoids: elemicin (10.4%), (E)-methyl isoeugenol (11.8%), and methyl eugenol (2.22%). On the other hand,

Table 1. Main identified components (>0.5%) in the wild carrot oil fractions F1, F2, F3, and F4.

Retention time	Component	Percentage			
		F1	F2	F3	F4
11.88	(+)- α -Longipinene	7.18	–	5.60	8.18
12.38	Longicyclene	1.72	–	–	–
13.26	Methyl eugenol	–	2.28	–	–
13.33	10s,11a-Himachala-3(12),4-diene	6	–	–	0.59
13.88	β -Caryophyllene	17.2	–	–	0.78
15.08	1,1,4,8-Tetramethyl- <i>cis,cis</i> ,4,7,10-cycloundecatriene	–	–	–	1.25
15.14	α -Humulene	28.2	–	–	–
16.03	Aromadendrene	0.75	–	–	–
16.54	β -Selinene	2.86	–	2.38	5.33
16.70	γ -Selinene	–	–	0.56	1.86
16.87	α -Selinene	4.26	–	–	–
16.95	β -Himachalene	–	2.07	–	–
17.01	(E)- Methylisoeugenol	–	12.5	–	–
17.22	β -Bisabolene	–	–	0.62	2.59
17.47	2,3-Dicyano-6-ethyl-5,7-dimethyl-6H-1,4-diazepine	–	–	–	3.82
17.63	γ -Selinene	3.53	–	–	–
17.87	β -Himachalene	2.58	–	–	–
18.31	5-Benzyl-4-isopropyl-1H-imidazole	0.79	–	–	–
18.38	(–)- α -Panasinsen	1.08	–	–	–
19.55	Elemicine	–	10.9	–	–
20.50	Caryophyllene oxide	5.20	0.66	–	–
23.53	2-Himachalen-6-ol	–	61.4	0.79	1.67
25.22	β -Asarone	–	1.05	–	–
28.39	3,5,6,7,8,8 α -Hexahydro-4,8 α -dimethyl-6-(1-methylethenyl)-2(1H)-naphthalenone	1.92	–	–	–
61.05	Methanesulfonic acid, 9-oxabicyclo [3.3.1]non-3-ylmethyl ester	–	–	–	0.78

fewer compounds were identified in F3 and F4 fractions with α -longipinene and β -selinene being the major identified components (F3: 3.07% and 1.92%, respectively; F4: 8.18% and 5.33%, respectively).

On one hand, fractions F3 and F4 were further analyzed by HPLC and their chromatograms were compared with that of the standards presented in Figure 1(a). The chromatogram of F3 fraction (Figure 1b) showed several peaks, at retention times of 16.45, 31.81, 34.22, and 34.59 min, corresponding to caffeic acid, luteolin, kaempferol, and apigenin, respectively.

On the other hand, F4 fraction chromatogram (Figure 1c) presented five prominent peaks out of which four were identified at retention times of 30.5, 32.02, 34.35, and 34.89 min corresponding to quercetin, luteolin, kaempferol, and apigenin, respectively. The concentrations of the identified components were calculated by using the obtained calibration curves and are listed in Table 2. Luteolin (55.85 $\mu\text{g}/\text{mg}$) and apigenin (50.75 $\mu\text{g}/\text{mg}$) are the major components of F3, while kaempferol (103.00 $\mu\text{g}/\text{mg}$) and apigenin (118.00 $\mu\text{g}/\text{mg}$) are the major components of F4. In

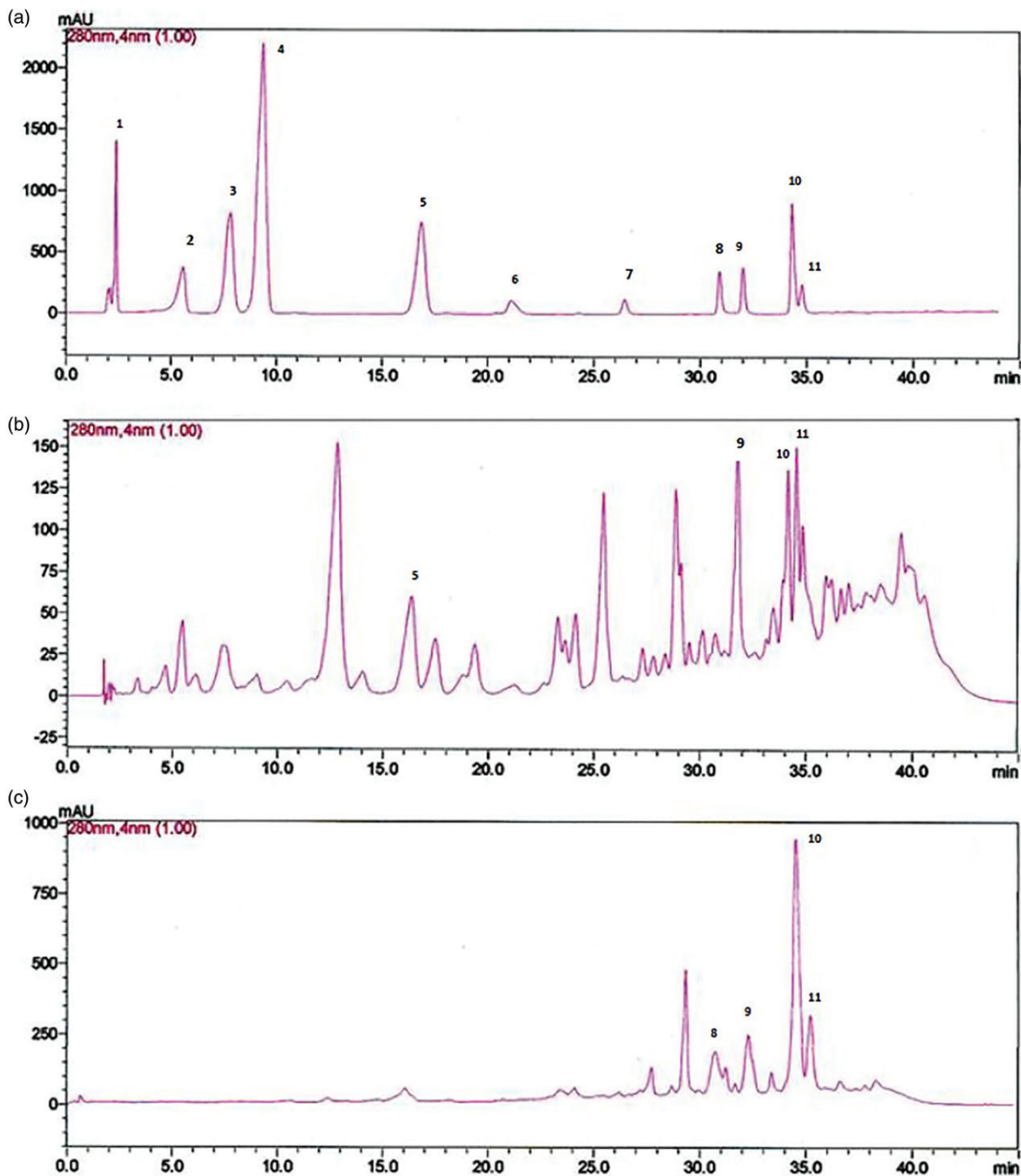


Figure 1. (a) HPLC chromatograms of a standard mixture of phenolic acids. Peaks: 1 = gallic acid; 2 = chlorogenic acid; 3 = vanillic acid; 4 = syringic acid; 5 = caffeic acid; 6 = ellagic acid; 7 = myricetin; 8 = quercetin; 9 = luteolin; 10 = kaempferol; 11 = apigenin. (b and c) HPLC chromatograms of F3 and F4 oil fractions of the wild carrot extract. HPLC conditions: mobile phase, water:acetic acid:methanol; 10:2:88 (solvent A) and water:acetic acid:methanol; 90:2:8 (solvent B), flow rate; 1.5 ml/min, detection; UV at 280 nm.

addition, the peak at 12.5 min in the chromatogram of F3 was tentatively assigned to *p*-coumaric acid based on the spectral data and the relative retention time (Rodríguez-Delgado et al., 2001)

Total phenolic and flavonoid contents

The total phenolic content of the oil fractions was measured using the Folin–Ciocalteu reagent and expressed in terms of GAE (mg GAE/g extract). F4 fraction showed the highest phenolic content, followed by F3, F1, and F2 (Table 3). The total flavonoid content was determined according to the AlCl₃ colorimetric method and reported in terms of quercetin equivalents (mg QE/g extract). Flavonoid content in the different fractions displayed a similar trend to phenolics (Table 3).

In vitro assays

DPPH radical-scavenging activity

The antioxidant activity of the oil fractions was evaluated using the DPPH radical scavenging assay. The scavenging activities of the fractions at various concentrations were determined and compared with that of the standard antioxidant Trolox (Figure 2a and Table 3). The F3 fraction revealed the strongest scavenging activity among all fractions with an IC₅₀ value of 0.29 ± 0.01 mg/ml while F1 fraction showed the lowest activity with an IC₅₀ value >2.5 ± 0.25 mg/ml.

Fe²⁺-chelating activity

The ferrous ion-chelating (FIC) assay relies on the complex formation between ferrozine and Fe²⁺ ions. In the presence of other chelating substances, the ferrozine complex formation is

disrupted resulting in a decrease in the red color of the complex. As shown in Figure 2(b), the F1 fraction demonstrated the highest FIC activity among all fractions with an IC₅₀ value = 0.28 ± 0.03 mg/ml and F4 showed the least FIC effect with an IC₅₀ value >7.25 ± 0.45 mg/ml (Table 3).

Ferric-reducing ability

The ferric-reducing antioxidant power (FRAP) measures the ability of a substance to reduce the ferric tripyridyltriazine (Fe³⁺–TPTZ) complex to the ferrous (Fe²⁺). The FRAP results (Table 3) showed that the F4 fraction had the highest FRAP value (437.59 ± 6.94 μmol FeSO₄/g), whereas the F1 fraction showed the lowest value (19.70 ± 0.92 μmol FeSO₄/g).

In vivo assays

In vivo antioxidant activity

The *in vivo* antioxidant properties of DCOE fractions were assessed by determining the CAT, SOD, and GST activities in the livers of the control and experimental mice (Figure 3). As expected, CCl₄ treatment caused a marked decrease in the enzymatic activity of the three liver enzymes when compared with the normal control group (*p* < 0.05). Groups pretreated with the four oil fractions had significantly (*p* < 0.05) increased GST activity when compared with the CCl₄-treated control group. On one hand, treatment with F1 (200 mg/kg) and F4 (50, 100, and 200 mg/kg) markedly (*p* < 0.05) elevated the CAT activity when compared with that of the CCl₄-treated control group. Similarly, the level of SOD activity in the liver homogenates was significantly (*p* < 0.01) improved after treatment with F1 at 200 mg/kg and F4 at all used doses. On the other hand, groups pretreated with the F2 and F3 fractions failed to restore the normal CAT and SOD activities.

Hepatoprotective activity

The activities of serum ALT and AST for the control and experimental groups are presented in Table 4. The results indicated that the levels of ALT and AST were significantly elevated in the CCl₄-treated control group compared with the normal control. Pretreated groups with F1 and F4 fractions exhibited significant (*p* < 0.01) hepatoprotective capacity and restored the liver enzyme activities to normal values. However, F2 and F3 fractions were less effective and showed moderate hepatoprotective activity.

Table 2. Concentrations of phenolic acids and flavonoids in F3 and F4 fractions of the wild carrot extract.

Component	Concentration (μg/mg)	
	F3 fraction	F4 fraction
Caffeic acid	9.13	ND
Quercetin	ND	69.70
Luteolin	55.85	83.10
Kaempferol	13.72	103.00
Apigenin	50.75	118.00

ND, not detected.

Table 3. Phenolic content, flavonoid content, DPPH, FRAP, and FIC assay of the different oil fractions of *Daucus carota* L. ssp. *carota*.

Sample	Phenolic content (mg GAE/g)	Flavonoid content (mg QE/g)	DPPH radical scavenging IC ₅₀ (mg/ml)	FRAP μM FeSO ₄ /g	Fe ²⁺ chelating activity IC ₅₀ (mg/ml)
F1-fraction	48.6 ± 2.87	3.5 ± 0.25	>2.50 ± 0.25 ^a	19.70 ± 0.92	0.28 ± 0.03 ^k
F2-fraction	40.10 ± 1.98	2.0 ± 0.18	>1.8 ± 0.30 ^b	38.5 ± 1.31 ^g	1.45 ± 0.15 ^l
F3-fraction	108.56 ± 2.94	35.5 ± 1.5	0.29 ± 0.01 ^c	225.11 ± 5.63 ^h	>5 ± 0.3 ^m
F4-fraction	147.14 ± 3.12	56 ± 2.3	0.38 ± 0.02 ^d	437.59 ± 6.94 ⁱ	>7.25 ± 0.45 ⁿ
Trolox	–	–	0.06 ± 0.01 ^e	–	–
Vitamin C	–	–	–	61 000 ± 1258 ^j	–
Na ₂ -EDTA	–	–	–	–	0.07 ± 0.012 ^o

The values are expressed as mean ± SEM from three experiments. Means followed by different letters are significantly different (*p* < 0.05).

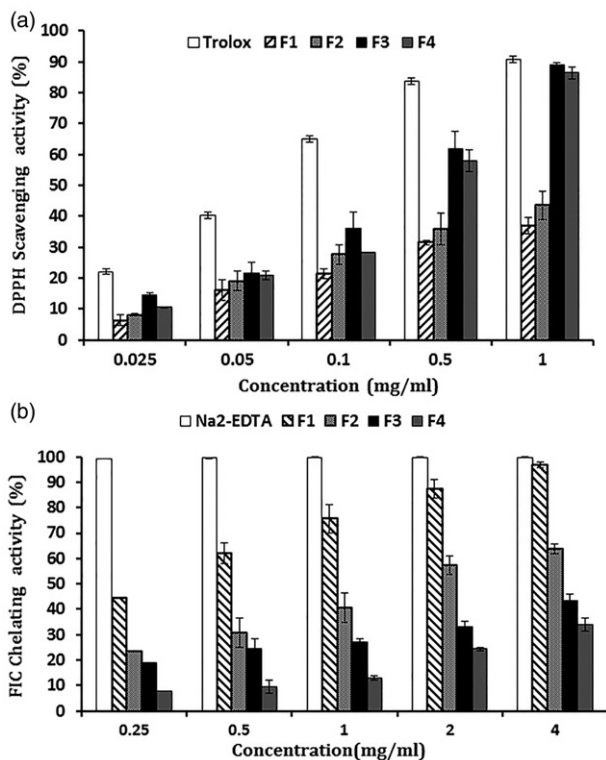


Figure 2. (a) Percentage DPPH scavenging activity of oil fractions (F1, F2, F3, and F4) and Trolox at different concentrations (25, 50, 100, 500, and 1000 μ g/ml) on DPPH radicals. (b) The percentage chelating activity of oil fractions (F1, F2, F3, and F4) and Na₂EDTA at different concentrations (0.25, 0.5, 1, 2, and 4 mg/ml) on ferrous ions. Data are presented as mean \pm SEM from three experiments.

Discussion and conclusion

On one hand, phytochemical analysis of the four DCOE fractions showed that sesquiterpenes were the major constituents of F1 and F2 with the latter containing phenylpropanoids as well. On the other hand, several phenolic and flavonoid compounds were detected in F3 and F4. The major sesquiterpene in F2 (61.4%) was identified as 2-himachalen-6-ol by GCMS (NIST Library 2011, 96% match). According to the available literature (Gonny et al., 2004; Maxia et al., 2009; Mockute & Nivinskiene, 2004; Rossi et al., 2007; Staniszewska et al., 2005), and to the best of our knowledge, this is the first time of reporting the presence of 2-himachalen-6-ol in wild carrot species. This sesquiterpene was first isolated from the heartwood of *Juniperus chinensis* Linn. var. *tsukusiensis* Masam in Taiwan (Shiu et al., 1999) and this was the only available literature related to 2-himachalen-6-ol. Additionally, the sesquiterpenes β -caryophyllene (17%; F1) and α -humelene (28%; F1) present in the native Lebanese *Daucus carota* were either detected in trace amounts or not found in DC species worldwide. On the contrary, α -longipinene and β -himachalene appear to be limited to the Lebanese and Italian wild carrot (Gonny et al., 2004; Maxia et al., 2009; Mockute & Nivinskiene, 2004; Rossi et al., 2007; Staniszewska et al., 2005). These findings stress the importance of the geographical location on the chemical composition of the plant.

Recent work in our lab showed that DCOE possesses important *in vitro* antioxidant activity (Shebaby et al., 2013),

and this has prompted us to investigate the *in vitro* and *in vivo* antioxidant properties of DCOE fractions. Out of the four fractions, F3 and F4 demonstrated the highest antioxidant activity in DPPH and FRAP assays. Such an effect could be partially attributed to the presence of phenolic compounds such as luteolin, kaempferol, apigenin, caffeic acid, and quercetin, all of which reported to possess antioxidant activity (Boots et al., 2008; Gülçin, 2006; Park et al., 2006; Škerget et al., 2005; Yin et al., 2008). Phenols, flavonoids, and terpenes are among natural non-enzymatic antioxidants, which play an important role in quenching and neutralizing free radicals and protecting against oxidative stress and tissue damage (Ajith & Janardhanan, 2001). The antioxidant capacity of the DCOE fractions evaluated by DPPH assay is strongly correlated with the phenolic ($r=0.93$) and flavonoid ($r=0.93$) contents. A high-positive correlation was also observed between FRAP antioxidant power and phenolic ($r=0.98$) and flavonoid ($r=0.99$) contents. Such an observation is in accordance with earlier studies which demonstrate a direct relationship between antioxidant capacity and total phenolic content in certain herbs, vegetables, and fruits (Sun et al., 2002; Velioglu et al., 1998; Zheng & Wang, 2001). The antioxidant activity of these phenolic compounds is believed to be attributed to the presence of hydrogen-donating hydroxyl groups on the aromatic rings (Heijnen et al., 2002; Kim & Lee, 2004). On the contrary, the ferrous ion-chelating effect of DCOE fractions did not correlate with their phenolic ($r=-0.81$) or flavonoid contents ($r=-0.82$). This is consistent with previous work that showed a similar relationship between total phenolic content and metal ion-chelating capacity of plant extracts (Wang et al., 2009). One possible explanation could be due to the diversity of the polyphenols' chemical structures with respect to the number and position of phenolic hydroxyl groups which could influence their Fe²⁺ chelating ability (Mladěnka et al., 2011).

The sesquiterpene-rich fraction F1 showed moderate DPPH-scavenging activity, which could be attributed to the presence of α -humelene and β -caryophyllene, the major constituents of the F1 fraction (Legault & Pichette, 2007; Mayachiew & Devahastin, 2008; Pérez Gutiérrez et al., 2006; Remya et al., 2013). Furthermore, the sesquiterpene α -longipinene, present in F1, F3, and F4, was also found in many plants that possess antioxidant activities (Kumar et al., 2012; Yang et al., 2010). Additionally, F1 and F2 fractions were shown to possess strong Fe²⁺ chelating activity. Metal ions such as Fe²⁺ play an essential role in producing hydroxyl radicals through the Fenton reaction which accelerates lipid peroxidation (Benedet & Shibamoto, 2008). Therefore, F1 and F2 may have an inhibitory effect on lipid peroxidation by stabilizing the Fe²⁺ metal ions. β -Caryophyllene, a major compound present in the F1 fraction, was reported to inhibit significantly lipid peroxidation by reacting with peroxy radicals (Khunkitti et al., 2012).

The *in vivo* antioxidant activity was also evaluated using CCl₄-induced hepatotoxicity in mice. CCl₄ is metabolized by the cytochrome P450 in the hepatic endoplasmic reticulum to produce the chemically reactive species, trichloromethyl (CCl₃[•]) and trichloromethyl peroxy (CCl₃O₂[•]) free radicals, which attack membrane lipids and proteins leading to lipid peroxidation and ultimately cell damage (Johnston &

Figure 3. Effect of wild carrot oil fractions (F1, F2, F3, and F4) and vitamin E on the activity of SOD, CAT, and GST antioxidant liver enzymes of CCl₄-intoxicated mice. Each column represents the mean \pm SEM, with $n = 6$ animals in each group. (a) SOD, superoxide dismutase; (b) CAT, catalase; (c) GST, glutathione-S-transferase. ^a $p < 0.05$ compared with the normal control group; ^b $p < 0.05$; ^c $p < 0.01$; ^d $p < 0.001$ compared with the CCl₄-control treated group.

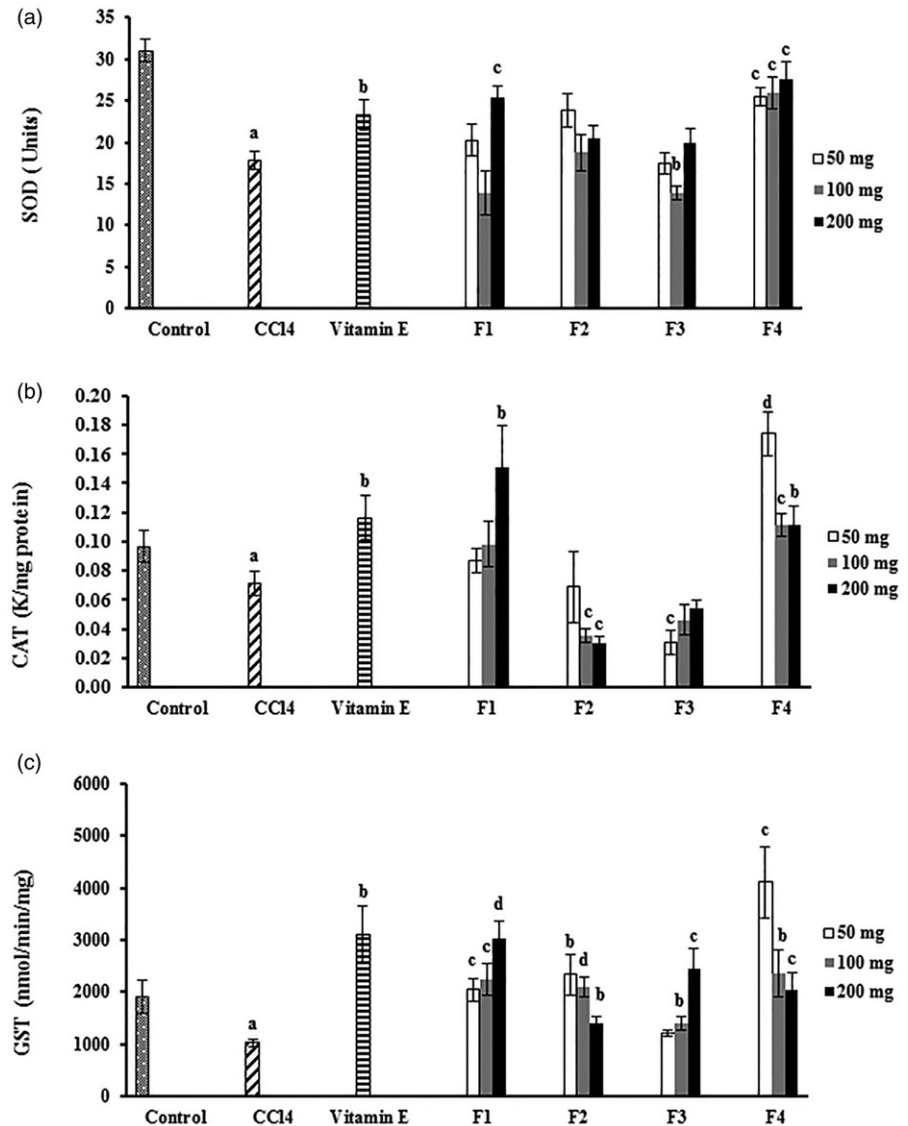


Table 4. Effect of DCOE fractions and vitamin E on AST and ALT in mice after CCl₄ application.

Groups	Dose (mg/kg)	Parameters	
		AST (U/l)	ALT (U/l)
Control		80.7 \pm 6.9	34.5 \pm 5.6
CCl ₄		200.8 \pm 22 ^a	184.2 \pm 19 ^a
Vitamin E		131 \pm 7.7 ^b	107 \pm 4.3 ^b
F1	50	95 \pm 11.1 ^c	89.3 \pm 3.4 ^c
	100	74.5 \pm 9.8 ^c	94.0 \pm 17.3 ^c
	200	70.2 \pm 3.4 ^d	73.6 \pm 6.1 ^d
F2	50	156.3 \pm 36.5 ^{ns}	174 \pm 25.2 ^{ns}
	100	163 \pm 33.9 ^{ns}	119.3 \pm 19.5 ^b
	200	179.2 \pm 40 ^{ns}	120.2 \pm 13.6 ^b
F3	50	170.7 \pm 23.9 ^{ns}	130.3 \pm 25.9 ^{ns}
	100	117.5 \pm 25.6 ^{ns}	182.3 \pm 48.3 ^{ns}
	200	148.5 \pm 29.8 ^{ns}	116 \pm 18.5 ^b
F4	50	126.7 \pm 4.8 ^b	75.0 \pm 4.0 ^c
	100	86.0 \pm 17.0 ^b	71.0 \pm 6.2 ^d
	200	82.3 \pm 5.2 ^c	78.3 \pm 4.9 ^c

The values are expressed as mean \pm SEM for $n = 6$. Where ns is non-significant compared with CCl₄, ^a $p < 0.0001$ compared with control, ^b $p < 0.05$ compared with CCl₄, ^c $p < 0.01$ compared with CCl₄, ^d $p < 0.0001$ compared with CCl₄.

Kroening, 1998; Shenoy et al., 2001). As expected, mice treated with CCl₄ developed significant hepatic damage marked by a substantial increase in serum levels of AST and ALT which are considered as specific markers of liver injury (Ferreira et al., 2010; Vozarova et al., 2002). However, pretreatment with F1 and F4 fractions significantly decreased the activities of these enzymes compared with the CCl₄-treated control, hence minimizing hepatic damage and exerting hepatoprotective activity. Additionally, the decrease in the activity of antioxidant liver enzymes SOD and CAT caused by CCl₄ treatment was reversed by F4 and to a lesser extent by F1 pretreatment. SOD is a key defense enzyme that catalyzes the conversion of O₂[•] to O₂ and H₂O₂. The latter is then metabolized by CAT into molecular oxygen and water, protecting cells against free radical induced damage (Lee et al., 2003). Therefore, inhibition or reduction in the activity of these protective enzymes may lead to excessive cellular damage due to the accumulation of free radicals. The antioxidant and hepatoprotective effects of DCOE fractions, mainly F1 and F4, can be attributed to the presence of sesquiterpenes in the F1 and flavonoids and phenolic compounds in the F4 fractions. These compounds may act

as primary antioxidants or as free radical scavengers, resulting in the protection of these defense enzymes as shown in several previous *in vivo* studies (He et al., 2012; Jain et al., 2012; Naskar et al., 2009; Oyedemi et al., 2010). Furthermore, the present data showed a significant increase in the activity of GST in the four pretreated groups compared with the CCl₄-treated control. GST is a phase-II metabolic enzyme that catalyzes the glutathione conjugation reaction involved in the detoxification of endogenous substances and xenobiotics providing protection against chemical toxicity and carcinogenesis (Yang et al., 2001).

In conclusion, the current study demonstrates the importance of DCOE fractions F3 and F4 as potential sources of antioxidants, and F1 and F4 as hepatoprotective remedies. Moreover, the results indicate that the chemical composition of the native Lebanese *Daucus carota* oil extract presents a unique profile from those of other origins.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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