Original Contribution

EFFICACY OF CHEMICALLY CHARACTERIZED *CARUM COPTICUM* ESSENTIAL OIL AS AN ANTIOXIDANT AND LIPID PEROXIDATION INHIBITION

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ABSTRACT

The study reports potential of *Carum copticum* seed essential oil (EO) as a plant based shelf life enhancer against contamination and lipid peroxidation. Also, the effect of exogenous application of nano iron chelate on components of essential oils (Eos) of *Ca.copticum* was evaluated. The nano iron chelate application increased γ- terpinene and thymol concentration. Thymol exhibit a higher activity in each antioxidant system with a special attention for b-carotene bleaching test (IC50: 11.30± 0.45 µg/ml), lipid peroxidation inhibition (60.11± 0.27 µg/ml) and reducing power (EC50: 9.24 ± 0.12 µg/ml). These results indicate that *Ca.copticum* EO and its main compounds might be applicable in natural medicine and healthy food.

Key words: *Carum copticum*; essential oil; lipid peroxidation; antioxidant.

INTRODUCTION

Oxidation of lipid during processing and storage affect food quality causing qualitative deterioration of the treated commodities because of chemical spoilage, rancidity, changing their color and texture (1). Free radicals or reactive oxygen species have been shown to be the causative agent in aging and several degenerative diseases such as cancer, atherogenesis, heart and neurodegenerative diseases (2). Antioxidant compounds, such as phenolics, organic acids, vitamin E, and carotenoids scavenge free radicals, thus inhibiting the oxidative mechanisms that may lead to degenerative illnesses (3). Phenolic compounds may serve this purpose by reducing or donating hydrogen to other compounds, scavenging free radicals, and quenching singlet oxygen (4). Generally, the EOs possessing the strongest antioxidant activities against oxidation of lipid contain a high percentage of phenolic compounds such as carvacrol, eugenol, isoeugenol and thymol.The present study was

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performed to investigate application of nano iron chelate on components of essential oils *Ca. copticum* and to evaluate the efficacy of chemically characterized *Ca. copticum* essential oil as antioxidant and lipid peroxidation inhibition agent.

MATERIALS AND METHODS

Two separate sets of experiments were conducted in a completely randomized design. In the first set, the effect of exogenous application of nano iron chelate (150 and 250 mgL^{-1}) in early flowering stage on components of Eos of *Ca. copticum* was evaluated. The second set included investigating the effect of nano iron chelat at 200 mgL^{-1} on antioxidant capacity and lipid peroxidation inhibition.

Plant materials and nano iron chelate treatments

Seeds of *Ca. copticum* were sown in Jefe pot in experimental greenhouse of Ilam, Iran. Plants at flowering stage (2013-2014) were sprayed with distilled water as a control, and nano iron chelate at 150 and 250 mg L^{-1}. All sprays solution were sprayed to the point of run off. The experiment was arranged in completely randomized block design with three replications for each treatment. At seed stage of *Ca. copticum* were harvested

and air dried at ambient temperature in the shade.

Oil isolation and identification of the oil components

The *Ca. copticum* seeds were ground and the resulting powder was subjected to hydrodistillation for 3 hours in an all glass Clevenger-type apparatus according to the method recommended by the European Pharmacopoeia (5). The obtained essential oils were dried over anhydrous sodium sulphate and after filtration, stored at +4 ºC until tested and analysed. The GC/MS analyses were executed on a Hewlett–Packard 5973N gas chromatograph equipped with a column HP-5MS (30 m length × 0.25 mm i.d., film thickness 0.25 lm) coupled with a Hewlett–Packard 5973N mass spectrometer. The column temperature was programmed at 50 ºC as an initial temperature, holding for 6 min, with 3 ºC increases per minute to the temperature of 240 ºC, followed by a temperature enhancement of 15 ºC per minute up to 300 ºC, holding at the mentioned temperature for 3 min. Injector port temperature was 290 ºC and helium used as carrier gas at a flow rate 1.5 ml/min. Ionization voltage of mass spectrometer in the EI-mode was equal to 70 eV and ionization source temperature was 250 ºC. Linear retention indices for all components were determined by coinjection of the samples with a solution containing homologous series of C8-C22 *n*-alkanes and comparing them and their mass spectra with those of authentic samples or with available library data of the GC/MS system (WILEY 2001 data software) and Adams libraries spectra (6).

Total phenolic determination

Total phenolic contents in seeds *Ca. copticum* were determined by Folin–Ciocalteu method (7). The total phenolic content was expressed as gallic acid equivalents (GAE) (mg g^{-1}).

Total flavonoid determination

Total flavonoid contents in seeds *Ca. copticum* were measured as described previously (8). The total flavonoid content was calculated as rutin equivalents (mg g^{-1}).

Antioxidant activity

The efficacy of the essential oils to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals was evaluated using a spectrophotometry method (9,10). On basis of bleaching of the bluish-red or purple colour of DPPH solution as a reagent. Briefly, a 50 µl volume of various dilutions of each samples was mixed with 5 ml of 0.004%

methanol solutions of DPPH followed by 30 min incubation at ambient temperature. Thereafter, the sample absorbance was recorded against control at 517 nm. The inhibition percentages were measured using Eq. (1). The antioxidants activity of the test samples in concentration providing 50% inhibition, were considered as IC50 (µg/ml).

$$\text{Inhibition percent} = \mathbf{Abs_{control}\text{-}Abs_{sample}/Abs_{control}} \times \mathbf{100} \quad (1)$$

Butylhydroxyanisole (BHA) and ascorbic acid were used as positive controls. All experiments were repeated three times and the average results and standard deviations calculated.

Rapid screening for antioxidants

For screening of antioxidant compounds in *Ca. copticum* essential oil, the TLC-bioautography method was carried out (11,12). The diluted oil (1:20 in methanol) was spotted on silica gel sheets (silica gel 60 F254 TLC plates) and developed in n-hexane-ethyl acetate (9:1). Plates were sprayed with the methanolic solution of DPPH• (0.2%). The active constituents were detected as yellow spots on a violet background. Only zones where their color turned from violet to yellow within the first 30 min (after spraying) were taken as positive results.

Activity guided fractionation of the essential oil for antioxidants

For the isolation and identification of the active compounds in the essential oil, TLC was performed using the conditions previously described (12). The regions showing DPPH• scavenging activity were scrapped off then, they were eluted with chloroform. All resulting constituents were analyzed by GC/MS and also tested for their antioxidant activities.

b-Carotene-linoleic acid model system (-CLAMS)

The b-CLAMS method by the peroxides generated during the oxidation of linoleic acid at elevated temperature (13). The antioxidant activity (AA) of the extracts was evaluated in term of b-carotene blanching using the following formula: AA (%)= $[(A_0- A_1)/A_0]*100$. where A_0 is the absorbance of the control at 0 min, and A1 is the absorbance of the sample at 120 min. The results are expressed as IC50 values (µg/ml). All samples were prepared and analyzed in triplicate.

Reducing power and lipid peroxidation inhibition

The ability of the extracts to reduce Fe $^{3+}$ was assayed by the method of Oyaizu (14). 1 ml of

Ca. copticum essential oil were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% K₃Fe (CN) 6. After incubation at 50 ^{0}C for 25 min, 2.5 ml of 10% trichloroacetic acid was added and the mixture was centrifuged at 650 g for 10 min. Finally, 2.5 ml of the upper layer was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% aqueous $FeCl_3$. The absorbance was measured at 700 nm. The mean of absorbance values were plotted against concentration and a linear regression analysis was carried out. Increase absorbance of the reaction mixture indicated increased reducing power. EC50 value (µg/ml) is the effective concentration at which the absorbance was 0.5 for reducing power. Ascorbic acid was used as positive control. Lipid peroxidation inhibition was determined by Shirwaikar *et al.,* (15). Ascorbic acid and trolox were used for comparison.

Statistical analysis
The results are presented as mean±S.D and statistically analyzed by oneway analysis of variance (ANOVA) followed by Duncan test.

RESULTS AND DISCUSSION
Effect of nano iron chelate on chemical composition of Ca.copticum EO
The GC/MS analysis of *Ca. copticum* oil revealed 14 compounds representing 79.25% of the total oil; γ-terpinene was the main constituent (28.74%.), followed by p-cymene (20.21 %), thymol (14.35%) and α-pinene (6.21 %). The constituents of the obtained Eos of *Ca. copticum* treated with nano iron chelate are presented in **Table 1**. Fourteen components were identified in untreated plants and eleven components in nano iron chelate -treated plants (**Table 1**). The differences were supposed to be the effects of nano iron chelate on chemical composition of *Ca.copticum* EO. Limonene, γ- terpinene and thymol were increased with nano iron chelate-treatment (**Table 1**). The yield of the *Ca. copticum* oil was 1.30% in control, 2.00 % (150 mgL^{-1}) and 2.81% (250 mgL^{-1}). Nano iron chelate significantly increased the yield of EO (**Table 1**).

Table 1. *Effect of nano iron chelate on chemical composition of Ca.copticum essential oil.*

	Components	^{a} *Ca.copticum* EO (%)			^{b}Retention Index	Identification Methods
		Control (%)	Nano iron chelate (150 mgL^{-1}) (%)	Nano iron chelate (250 mgL^{-1}) (%)		
1	α-thujene	0.41	-_	_	850	MS, RI
2	α-pinene	6.21	3.21	2.14	855	MS, RI
3	β-pinene	1.00	0.41	0.22	190	MS, RI
4	β-myrcene	1	_	_	920	MS, RI
5	*P*-cymene	20.21	16.54	13.24	950	MS, RI
6	β-phellendrene	3.54	-_	_-	954	MS, RI
7	limonene	0.12	3.14	3.48	960	MS, RI
8	γ– terpinene	28.74	38.47	42.25	980	MS, RI
9	4-terpineol	2.14	1.54	1.00	1.63	MS, RI
10	cis limonene oxide	-	-	0.28	1085	MS, RI
11	dodecane	-_	0.58	1.25	1110	MS, RI
12	β-fenchyl alcohol	0.11	-_	-_	1126	MS, RI
13	thymol	14.35	20.14	23.61	1208	MS, RI
14	Ethylene methacrylate	1.00	0.22	0.10	1235	MS, RI
15	pentadecane	_-	0.57	0.74	1264	MS, RI
16	hexadecane	0.11	-_	_-	1285	MS, RI
17	nonadecane	0.31	_-	-_	1293	MS, RI
18	carvacrol	-_	5.12	8.24	1306	MS, RI
	Total	79.25	89.94	96.55		
	Yield	1.30%	2.00%	2.81%		

^{a} Percentage composition determined on column HP 5. ^{b} The retention Kovats indices were determined on HP 5 capillary column in reference to *n*-alkanes. MS= Mass Spectroscopy, RI= Retention Index.

Lu *et al.,* (16) have shown that application of nano fertilizers could increase the nitrate reductase enzyme in soybean (*Glycine max* L.), increase its abilities of absorbing and utilizing water and fertilizer, promote its antioxidant system, and, in fact, accelerate its germination and growth.

Effect of nano iron chelate on extraction yield, total phenolic contents and total flavonoid contents
It is well-known that phenolic compounds contribute to quality and nutritional value in terms of modifying color, taste, aroma, and flavor and also in providing health-benefical effects (17). As shown in **Table 2**, the extraction yield of *Ca. copticum* ranged from lowest 85.21±22 mgg^{-1} (control) to highest 131.12±07

mgg^{-1} (nano iron chelate (250 mg L^{-1})) (P≤0.05). Among the three *Ca. copticum* extracts, *Ca. copticum* treated with nano iron chelate at 250 mg L^{-1} showed the highest total phenolic content (300.15±47 mgg^{-1}) and showed the highest total flavonoid content (187.33±07 mg g^{-1}) (P≤0.05). Furthermore, the total phenolic and total flavonoid contents exhibited the descending order among: *Ca. copticum* extract (treated with nano iron chelate 250 mg L^{-1}) > *Ca. copticum* extract (treated with nano iron chelate 150 mg L^{-1}) > *Ca. copticum* extract (treated with control) (P≤0.05). These results showed that the total phenolic and total flavonoid contents have an obvious variation in various concentrations.

Table 2. *Effect of nano iron chelate on extraction yields, total phenolic contents and total flavonoid contents of Ca.copticum extracts.*

	Extract	Extraction yield[a]	Total phenolic[b]	Total flavonoid[c]
1	Control	85.21±22	200.07±37	94.07±29
2	Nano iron chelate (150 mgL^{-1})	110.14±74	241.01±35	110.47±65
3	Nano iron chelate (250 mgL^{-1})	131.12±07	300.15±47	187.33±07

The data are expressed as mean±SD. [a]Expressed as mg of extract per gr dry material. [b]Expressed as mg of gallic acid per gr dry extract. [c]Expressed as mg of rutin per gr dry extract. Statistically, the differences were significant at p≤0.05 using Duncan test.

Antioxidant activity
The results presented in **Table 3** revealed that *Ca. copticum* EO and its main constituents exhibited a remarkable activity. In particular, thymol exhibited clearly a higher activity (9.6± 0.12 µg/ml) followed by *Ca. copticum* EO (11.89± 0.21 µg/ml), γ- terpinene (11.78± 0.02 µg/ml) and carvacrol (11.68± 0.07 µg/ml) (**Table 3**) (P≤0.05), while the activities of other terpenoids were weak (α-pinene and p-cymene) .The positive controls BHT and ascorbic acid exhibited IC50 values equal to 12.11± 0.09 µg/ml and 12.38± 0.11 µg/ml, respectively (P≤0.05). The monoterpene hydrocarbons, p-cymene and b-pinene were inactive (**Table 3**), despite previous reports of their in vitro antioxidant activities (18). **Table 3** depicts the inhibition of b-carotene bleaching by the *Ca. copticum* EO. The IC$_{50}$ value was 10.12± 0.21 µg/ml. **Table 3**, the reducing power of *Ca. copticum* EO, expressed as CE$_{50}$, was clearly more significant than that of the positive control BHA (P≤0.05). Because of high antioxidant and free radical-scavenging activities of *Ca. copticum* EO, further investigation was carried out to identify its active constituents. Therefore,

a preliminary screening was initially carried out using the dot-blot DPPH˙ stainingmethod on TLC. As the EO presented a significant antioxidant activity in the assays and bioautography test, it was subjected to the TLC for isolation of the active compounds. Components identified and their antioxidant activity relative percentages have been shown in **Table 4**. The major compound found in the active band was thymol (50± 0.21%) (**Table 4**). Many aroma components of essential oils, such as terpenes and terpenoids, were proposed to contribute to the antioxidant activity of essential oils ; including thymol and eugenol, linalool and 1,8-cineole. According to these results, there is a relationship between total phenolic contents and antioxidant activity.

Lipid peroxidation inhibition
According to the results obtained, *Ca. copticum* EO and its main component significantly inhibited the formation of TBARS in brain homogenates in a concentration-dependent manner (**Table 5**). The suppressive power on the lipid peroxidation of *Ca. copticum* EO and were thymol found to be the most potent (55.31± 0.14

and 51.24± 0.34 µg/ml), followed by γ-terpinene (60.11± 0.21 µg/ml) and carvacrol (60.33± 0.04 µg/ml) (P≤0.05). Ascorbic acid and trolox showed significant suppressive power on lipid peroxidation in mice brain homogenate with IC50 value of 60.38± 0.47 and 59.35± 0.18 (**Table 5**) (P≤0.05). Phenolics are important components of the human diet due to their potential antioxidant activity, their capacity to diminish oxidative stress induced tissue damage resulted from chronic diseases and their potentially important properties such as anticancer activities (19).

Table 3. *Antioxidant activity of EO extract from Ca. copticum: scavenging activity (expressed as IC50 values: µg/ml), and b-carotene bleaching test. Reducing power was expressed as EC50 values (µg/ml). Butylhydroxyanisole (BHA) and ascorbic acid were used as positive controls.*

Tested compounds	Control IC50 (µg/ml)	N-N-Ch 250 mg L^{-1} IC50 (µg/ml)
Ca. copticum EO	18± 0.54 µg/ml	11.89± 0.21 µg/ml
α-pinene	20± 0.43 µg/ml	15.21± 0.07 µg/ml
P-cymene	16.87± 0.89 µg/ml	13.54± 0.74 µg/ml
γ− terpinene	15.32± 0.28 µg/ml	11.78± 0.02 µg/ml
thymol	13.11± 0.60 µg/ml	9.6± 0.12 µg/ml
carvacrol	15.83± 0.24 µg/ml	11.68± 0.07 µg/ml
Ca. copticum EO (b-Carotenes IC50 µg/ml)	14.12± 0.55 µg/ml	10.12± 0.21 µg/ml
Ca. copticum EO (PR EC50 µg/ml)	14.12 ± 0.34 µg/ml	10.01 ± 0.07 µg/ml
BHA	14± 0.05 µg/ml	12.11± 0.09 µg/ml
AA	14± 0.75 µg/ml	12.38± 0.11 µg/ml

Control: Plants treated with distilled water. EO: Essential oil. N-N-Ch 250: Plants treated nano iron chelate 250 mg L^{-1}. Values are mean±S.D. of three replications.* IC50 values have been presented with their respective 95% confidence limits. Statistically, the differences were significant at p≤0.05 using Duncan test.

Table 4. *Components identified and their antioxidant activity relative percentages.*

compounds	Control %	N-N-Ch 250 mg L^{-1} %
α-pinene	10± 0.43	4± 0.51
P-cymene	12± 0.87	5± 0.21
γ− terpinene	10± 0.11	20± 0.41
carvacrol	10± 0.45	13± 0.20
thymol	30± 0.1	50± 0.21

Control: Plants treated with distilled water. EO: Essential oil. N-N-Ch 250: Plants treated nano iron chelate 250 mg L^{-1}. Results are expressed as a percentage of antioxidant activity relative. Experiments were carried out in triplicate. Statistically, the differences were significant at p≤0.05 using Duncan test

Table 5. *Lipid peroxidation inhibition of EO extract from Ca. opticum and its main component (expressed as IC50 values: µg/ml). Trolox and ascorbic acid (AA) were used as positive controls.*

Tested compounds	Control IC50 (µg/ml)	N-N-Ch 250 mg L^{-1} IC50 (µg/ml)
Ca. copticum EO	71.34± 0.34 µg/ml	55.31± 0.14 µg/ml
α-pinene	130.34± 0.15 µg/ml	88.24± 0.34 µg/ml
P-cymene	132.21± 0.56 µg/ml	90.00± 0.47 µg/ml
γ− terpinene	70.54± 0.87 µg/ml	60.11± 0.21 µg/ml
thymol	68.47± 0.34 µg/ml	51.24± 0.34 µg/ml
carvacrol	78.29± 0.70 µg/ml	60.33± 0.04 µg/ml
trolox	62.21± 0.05 µg/ml	59.35± 0.18 µg/ml
AA	89.54± 0.12 µg/ml	60.38± 0.47 µg/ml

Experiments were carried out in triplicate and the results are expressed as mean±S.D. Statistically, the differences were significant at p≤0.05 using Duncan test

CONCLUSION

There was a good correlation between total phenol content, lipid peroxidation inhibition and antioxidant capacity of the extracts. In conclusion, *Ca. copticum* extracts appear to contain compounds with lipid peroxidation inhibition and antioxidant activities.

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