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EFFECTS OF NANO-FERRIC OXIDE ON QUALITY, QUANTITY AND ANTIOXIDANT PROPERTIES OF ESSENTIAL OIL COMPONENTS IN *CARUM COPTICUM* (L.) LINK

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Abstract

The effect of exogenous application of nano-ferric oxide (50 and 100 mg/l) in early flowering stage on components of essential oils (EOs) of *Carum copticum* (L.) Link was evaluated. EO extracted by hydrodistillation from Iranian *C. copticum* was characterized by means of GC/MS. The nano-ferric oxide application increased limonene, γ- terpinene, *cis*-limonene oxide, thymol, carvacrol and decreased α-thujene, α-pinene, β-pinene and p-cymene concentration. The EO was also subjected to evaluation for antioxidant properties. Thymol, γ-terpinene and carvacrol possessed the highest antioxidant properties among the major components. *C. copticum* EO exhibited a higher activity in each antioxidant system with a special attention to β-carotene bleaching test and reducing power. The TLC-bioautography screening and fractionation resulted in the separation of the main antioxidant compounds which were identified as thymol, γ-terpinene and carvacrol.

Introduction

Nano-technology can provide solution to increasing the value of agricultural products and environmental problems. By using nano-particles and nano-powders, we can produce controlled or delayed releasing fertilizers. They have high reactivity because of more specific surface area, more density of reactivities, or increased reactivity of these areas on the particle surfaces. Antioxidants have been widely used as food additives to provide protection against oxidative degradation of foods by free radicals. Antioxidants are known as molecules capable of inhibiting oxidation process in the body to prevent the formation of free radicals. Recent research is now directed towards finding naturally occurring antioxidants of plant origin. Among these, the antioxidant properties of many aromatic and medicinal plants have shown to be effective in retarding the process of lipid peroxidation in oils and fatty foods and have gained interest of many research groups (Kulisic *et al.* 2004). The main objectives of the present study were to evaluate the exogenous application of nano-ferric oxide on quality, quantity and antioxidant properties of the essential oil from *Carum copticum* (L.) Link seeds and to find out which compounds can contribute to the antioxidant activities.

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-ferric oxide (50 and 100 mg/l) in early flowering stage on components of EOs of *C. copticum* was evaluated. The second set included investigating the effect of nano-ferric oxide at 100 mg/l on antioxidant properties.

The plant was identified by Dr. Esmaili, and the voucher specimen was deposited at private herbarium of Dr F. Esmaili (Voucher no. 121). Seeds of *C. copticum* were sown in Jefe pot in experimental greenhouse of Ilam, Iran. Plants at flowering stage (2013 - 2014) were sprayed with

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distilled water as control and nano ferric oxide at 50 and 100 mg/l concentration. All solutions were sprayed to the point of run off. The experiment was arranged in CRBD with three replications for each treatment. At seed stage of *C. copticum* plants were harvested and air dried at ambient temperature in the shade.

The *C. copticum* seeds were ground and the resulting powder was subjected to hydrodistillation for 3 hrs in an all glass Clevenger-type apparatus according to the method recommended by the European Pharmacopoeia (1975). The obtained essential oil was dried over anhydrous sodium sulphate and after filtration, stored at +4ºC until tested and analysed. 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 µm) coupled with a Hewlett–Packard 5973N mass spectrometer. The column temperature was programmed at 50ºC as an initial temperature, holding for 6 min with 3ºC increases per minute to 240ºC, followed by a temperature enhancement of 15ºC per minute up to 300ºC, then holding at the mentioned temperature for 3 min. Injector port temperature was 290ºC and helium was 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 250ºC. Linear retention indices for all components were determined by coinjection of the samples with a solution containing homologous series of C_8-C_{22} *n*-alkanes and comparing 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 (2001).

Total phenolic contents in seeds of *C. copticum* were determined by Folin-Ciocalteu method (Jimoh *et al.* 2007), was expressed as gallic acid equivalents (GAE) (mg/l).

Total flavonoid contents in seeds *C. copticum* were measured as described previously (Piccolella *et al.* 2008). The total flavonoid content was calculated as rutin equivalents (mg/l).

The efficacy of the essential oils to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals was evaluated by using a spectrophotometric method (Cuendet *et al.* 1997, Kirby and Schmidt 1997). The basis of bleaching of the bluish-red or purple colour of DPPH solution. 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 percentage inhibition was measured by using following equation. The antioxidants activity of the test samples in concentration providing 50% inhibition were considered as IC_{50} (µg/ml).

Inhibition percent = $Abs_{control} - Abs_{sample} / Abs_{control} \times 100$

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

For screening of antioxidant compounds in *C. copticum* essential oil, the TLC-bioautography method was carried out (Burits and Bucar 2000, Guleria *et al.* 2012). The diluted oil (1: 20 in methanol) was spotted on silica gel plates (silica gel 60 F_{254} TLC plates) and developed in *n*-hexane-ethyl acetate (9 : 1). Plates were sprayed with 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.

For the isolation and identification of the active compounds in the essential oil, TLC was performed using the conditions previously described (Guleria *et al.* 2012). The regions showing DPPH scavenging activity were scrapped off eluted with chloroform. All resulting constituents were analyzed by GC/MS and also tested for their antioxidant activities.

The b-CLAMS method of peroxides generation during the oxidation of linoleic acid at elevated temperature (Koleva *et al.* 2002) was utilized. The antioxidant activity (AA) of the extracts was evaluated in term of *β*-carotene 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 A_1 is the absorbance of the sample at 120 min. The results are expressed as IC_{50} values (µg/ml). All samples were prepared and analyzed in triplicate.

The ability of the extracts to reduce Fe^{3+} was by the method of Oyaizu (1986). Heres 1 ml of *C. copticum* essential oil was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% $K_3Fe(CN)_6$. After incubation at 50ºC 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 was plotted against concentration and a linear regression analysis was carried out. Increase in absorbance of the reaction mixture indicated increased reducing power. EC_{50} value (µg/ml), the effective concentration at which the absorbance was 0.5 for was determined as the reducing power. Ascorbic acid was used as positive control. Inhibition lipid peroxidation was determined by the method of Shirwaikar *et al.* (2006). Ascorbic acid and trolox was used for comparison.

The results are presented as mean ± Sd and statistically analyzed by ANOVA followed by DMRT.

Results and Discussion

The constituents of the essential oil obtained *C. copticum* treated with nano-ferric oxide are presented in Table 1. Hydrodistillation showed that yield of the seeds of *C. copticum* oil treated with nano-ferric oxide was 3.98% (v/w). The GC/MS analysis of *C. copticum* oil revealed 10 compounds representing 97.77% of the total oil; thymol was the main constituent (32.78%), followed by p-cymene (10.65%), limonene (5.98%), γ-terpinene (28.45%) and carvacrol (12.00%) (Table 1). Fifteen components were identified in the untreated plants and eleven components in 100 mg/l nano-ferric oxide-treated plants (Table 1). The differences were supposed to be the effects of nano-ferric oxide on chemical composition oil of *C.copticum*. Decrease in the proportion of α-thujene, α-pinene, β-pinene, p-cymene, β-myrcene, β-phellendrene, β-fenchyl alcohol, hexadecane and nonadecane have been found according to concentration of nano-ferric oxide. Some compounds such as α-thujene, β-pinene, β-myrcene, β-fenchyl alcohol, hexadecane and nonadecane was only detected in control (Table 1). On the other hand, limonene, γ-terpinene, carvacrol and thymol were increased with nano-ferric oxide-treatment (Table 1). The yield of the *C. copticum* oil was 1.14% in control, 2.00% (50 mg/l) and 3.98% (100 mg/l) in nano ferric oxide significantly increased the yield of EO (Table 1). We harvested plants materials at seeds stage. It may be affected the kind of oils, because it was observed that during the intensive growth period the precursor flow distributes between the cytoplasm (sites of sesquiterpene synthesis) and plastid (sites of monterpen synthesis) while after full development of the cell the majority is utilized in the plastids. Khajeh *et al.* (2004) showed that hydrodistilled oil of the plant contained eight main compounds, including thymol (49%), p-cymene (15.7%), γ-terpinene (30.8%) and β-pinene (2.1%), but supercritical carbon dioxide extraction (SFE) of the EO revealed only three compounds (thymol, q-cymene and c-terpinene), and the content of each depended on SFE conditions. Kobraee *et al.* (2011) reported that nano iron foliar application enhanced soybean yield by influencing number of seeds per plant and seed weight. Therefore, iron defficiency in soils could be a limiting factor of yield and extremely decreases crop yield. Lu *et al.* (2002) have shown that the 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 properties, and, in fact, accelerate its germination and growth.

Table 1. Effect of nano-ferric oxide on chemical composition of *C. copticum* essential oil.

	Components	[a]*C. copticum* essential oil (%)			[b]Retention index	Identification methods
		Control (%)	Nano-ferric oxide (50 mg/l) (%)	Nano-ferric oxide (100 mg/l) (%)		
1	α-thujene	0.11	0	0_	850	MS, RI
2	α-pinene	3.22	2.01	0	855	"
3	β-pinene	1.00	0	0	190	"
4	β-myrcene	1.03	0	0	920	"
5	*P*-cymene	15.45	12.76	10.65	950	"
6	β-phellendrene	3.54	0	0	954	"
7	limonene	0.51	2.87	5.98	960	"
8	γ-terpinene	21.02	23.65	28.45	980	"
9	4-terpineol	0	0.65	1.67	1.63	"
10	*cis* limonene oxide	1.78	1.98	2.87	1085	"
11	Dodecane	0	0.22	1.00	1110	"
12	β-fenchyl alcohol	0.76	0	0	1126	"
13	Thymol	18.00	20.12	32.78	1208	"
14	Ethylene methacrylate	0.54	0.49	0.26	1235	"
15	Pentadecane	0	0.35	2.11	1264	"
16	Hexadecane	0.11	0	0	1285	"
17	Nonadecane	0.02	0	0	1293	"
18	Carvacrol	1.43	5.77	12.00	1306	"
Total		68.52	70.87	97.77		
Yield		1.14	2.00	3.98		

[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.

As shown in Table 2, the extraction yield of *C. copticum* ranged from lowest 90.15±14 mg/g (control) to highest 150.66 ± 05 mg/g (nano-ferric oxide (100 mg/l). Among the three *C. copticum* extracts, *C. copticum* treated with nano-ferric oxide at 100 mg/l showed the highest total phenolics (306.87 ± 90 mg/g) and highest total flavonoid content (166.19 ± 39 mg/g). Furthermore, the total

Table 2. Effect of nano-ferric oxide on extraction yields, total phenolic contents and total flavonoid contents of *C.copticum* extracts.

	Extract	Extraction yield[a]	Total phenolic[b]	Total flavonoid[c]
1	Control	90.15±14	160.07±45	96.04±04
2	Nano ferric oxide (50 mg/l)	95.33±12	205.87±54	104.55±18
3	Nano ferric oxide (100 mg/l)	150.66±05	306.87±90	166.19±39

The data are expressed as mean ± Sd. [a]Expressed as mg of extract per g dry material. [b]Expressed as mg of gallic acid per g dry extract. [c]Expressed as mg of rutin per g dry extract.

phenolic and total flavonoid contents exhibited the descending order among: *C. copticum* extract (treated with nano-ferric oxide 100 mg/l) > *C. copticum* extract (treated with nano-ferric oxide 100

mg/l) > *C. copticum* extract (treated with control). These results showed that the total phenolic and total flavonoid contents have an obvious variation in various concentrations nano-ferric oxide.

Table 3. Antioxidant activity of essential oil component from *C. copticum*: scavenging activity (expressed as IC_{50} values: µg/ml), and β-carotene bleaching test. Reducing power was expressed as EC_{50} values (µg/ml). Butylhydroxyanisole (BHA) and ascorbic acid (AA) were used as positive controls.

Tested compounds	Control	Nano-ferric oxide (100 mg/l)
	IC_{50} (µg/ml)	IC_{50}
C. copticum essential oil	17.09± 0.03	13 ± 0.33
Limonene	25.78± 0.00	22.99 ± 0.01
P-cymene	23.06± 0.59	20.56 ± 0.19
γ-terpinene	19.36± 0.81	16.02 ± 0.14
Thymol	16.59± 0.05	12.00 ± 0.45
Carvacrol	18.45± 0.94	14.05 ± 0.48
C. copticum EO (β-carotenes IC_{50} µg/ml)	16.05± 0.16	10.00 ± 0.05
C. copticum EO (PR EC_{50} µg/ml)	15.48 ± 0.23	10.15 ± 0.11
BHA	13.8± 0.56	13.8 ± 0.56
AA	10.55± 0.10	10.55 ± 0.10

Values are mean ± Sd of three replications.*IC_{50} values have been presented with their respective 95% confidence limits.

Table 4. Components identified and their antioxidant activity.

Compounds	Control (%)	Nano-ferric oxide (100 mg/l)
Limonene	3.21± 0.77	4.9± 0.02
P-cymene	15.55± 0.04	5.78± 0.04
γ-terpinene	11.45± 0.34	15.87± 0.45
Carvacrol	3.11± 0.45	15.76± 0.33
Thymol	45± 0.1	55.60± 0.12

Results are expressed as a percentage of antioxidant activity relative. Experiments were carried out in triplicate.

The lower IC_{50} value indicates a stronger ability of the extract to act as a DPPH radicals scavenger while the higher IC_{50} value coorolate to a lower scavenging activity of the scavengers as more scavengers were required to achieve 50% scavenging reaction. The results presented in Table 3 revealed that *C. copticum* essential oil and its main constituents exhibited a remarkable activity. In particular, thymol exhibited clearly a higher activity (12.00 ± 0.45 µg/ml) followed by carvacrol (14.05 ± 0.48 µg/ml) and *C. copticum* essential oil (13 ± 0.33 µg/ml, Table 3), while the activities of other terpenoids were weak (limonene and p-cymene). The positive controls BHT and ascorbic acid exhibited IC_{50} values equal to 13.8 ± 0.56 µg/ml and 10.55 ± 0.10 µg/ml, respectively. The monoterpene hydrocarbons, p-cymene and β-pinene were inactive (Table 3) despite previous reports of their *in vitro* antioxidant activities (Ruberto and Baratta 2000). Table 3 depicts the inhibition of b-carotene bleaching by the *C. copticum* essential oil.The IC_{50} value was 10.00 ± 0.05 µg/ml. As shown in Table 3, the reducing power of *C. copticum*, expressed as CE_{50}, was clearly more significant than that of the positive control BHA and AA. Because of the high

antioxidant and free radical-scavenging activities of *C. copticum* essential oil, further investigation was carried out to identify its active constituents. Therefore, a preliminary screening was initially conducted by using the dot-blot DPPH staining method on TLC. As the essential oil presented a significant antioxidant activity in the assays and bioautography test, it was subjected to the TLC for isolation of the active compounds. The major compound found in the active band was thymol (55.60 ± 0.12%) (Table 4). Many aroma producer 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.

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