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EFFECT OF NANO-IRON CHELATE ON CHEMICAL COMPOSITION AND ANTIMICROBIAL PROPERTIES OF *CARUM COPTICUM* L. ESSENTIAL OIL AND ITS MAIN TERPENES FROM IRAN

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Abstract

The effect of exogenous application of nano-iron chelate (100 and 200 mg/l) in early flowering stage on components of essential oils (EOs) of *Carum copticum* was evaluated. The EO extracted by hydrodistillation from Iranian *C. copticum* was characterized by means of GC/MS. The nano-iron chelate application increased γ- terpinene and thymol and decreased α-pinene, β-pinene and p-cymene concentration. The oil was also subjected to antimicrobial activity. *C. copticum* oil was found to inhibit *Bacillus cereus* and *Candida albicans* with the lowest MIC and MBC/MFC values. Thymol and carvacrol possessed the highest antimicrobial activity among the major components. These results clearly show the antimicrobial effects of the essential oil *C. copticum*.

Introduction

The presence and growth of microorganisms in food may cause spoilage and result in a reduction in quality and quantity. Food poisoning is still a concern for both consumers and the food industry despite the use of various preservation methods. The problem of preserving food products is becoming more complex, due to the fact that the new products being introduced in the market require an ever longer shelf life and a higher degree of protection against pathogenic microorganisms (Marino *et al.* 2001). Aromatic and medicinal plants are known to produce certain bioactive molecules which react with other organisms in the environment, inhibiting bacterial or fungal growth (Sengul *et al.* 2009). Indeed, natural crude extracts and biologically active compounds from plant species used in traditional medicine may represent valuable sources for such new preservatives (Al-Fatimi *et al.* 2007). Application of plant materials as dietary regimens and preservatives is mainly due to their antioxidant, antimicrobial and other biological potentials. Antioxidants can inhibit or delay the oxidation of oxidizable substrates and this appears to be very important in the prevention of oxidative stress which is suggested as the leading cause of many oxidation related diseases (Halliwell *et al.* 1992). *Carum copticum* L. is an aromatic, grassy, annual plant grown in Iran. The seeds of *C. copticum* have several therapeutic effects, including diuretic, antivomiting, analgesic, antiasthma, and antidyspnea effects. Mohagheghzadeh *et al.* (2007) showed that *C. copticum* has two chemotypes, thymol and carvacrol. The main objectives of this study were to evaluate the chemical composition and antimicrobial properties of the essential oils (EO) from *C. copticum* seeds and to determine the compounds that contributed to the effects.

Materials and Methods

The plant was identified by Dr. Esmaili and a voucher specimen has been deposited at private herbarium of Mr F Esmaili (Voucher No. 121). Two separate sets of experiments were conducted

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in a completely randomized design. In the first set, the effect of exogenous application of nano iron chelate (100 and 200 mg/L) in early flowering stage on components of Eos of *C. copticum* was evaluated. The second set included investigating the effect of nano iron chelate at 200 mg/L on antimicrobial and anti-inflammatory activities.

Seeds of *C. 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 100 and 200 mg/ L. All spray solution were sprayed to the point of run off. The experiment was arranged in completely randomized block design with 4 replications, each consisting of 3 pots with each pot containing one plant. The temperature conditions were 24 ± 5°C and 15 ± 4°C, during days and nights, respectively with relative humidity of 70%. At seed stage *C. copticum* were harvested and air dried at ambient temperature in the shade.

The *C. copticum* seeds (500 g) 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 (1975). The obtained essential oils were dried over anhydrous sodium sulphate and after filtration stored at +4ºC 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 50ºC as an initial temperature, holding for 6 min, with 3ºC increase per minute to the temperature of 240ºC, followed by a temperature enhancement of 15ºC per minute up to 300ºC, 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 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 library spectra (2001).

Total phenolic contents in seeds *C. copticum* were determined by Folin–Ciocalteu method (Jimoh *et al.* 2007). The total phenolic content was expressed as gallic acid equivalents (GAE) (mg/g).

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

Gram-positive bacteria - *Bacillus cereus* (ATCC 10876), *Enterococcus faecalis* (ATCC 49452), *Staphylococcus aureus* (ATCC 25923) ; Gram-negative bacteria - *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Proteus mirabilis* (ATCC 35659), *Salmonella typhimurium* (ATCC 13311), *Citrobacter freundii* (ATCC 8090); Fungal strains: *Candida albicans* (ATCC 10231), *Candida parapsilosis* (ATCC 90018) and *Aspergillus fumigatus* (ATCC 46645). The bacterial species were maintained in Mueller Hinton Agar and Tryptic Soy Agar media. Strains of *Candida* spp. and *Aspergillus* spp. were maintained on Sabourand dextrose agar.

Minimum inhibitory (MIC) and minimum bactericidal/fungicidal (MBC/MFC) concentrations were determined by microdilution method in 96 well microtitre plates as described by Douk *et al.* (1995) and EUCAST (2002). Briefly, fresh over night cultures of bacteria were adjusted with sterile saline to a concentration of 1.0×10^5 CFU per well, and 1.0×10^4 CFU per well for fungi. EOs were added in TSB medium for bacteria, and SDB medium for fungi. The microplates were incubated for 24 h at 37°C for bacteria, and 48 h at 37°C for fungi. The MIC was defined as the lowest concentration of EO inhibiting the visible growth of the test strain. However, the MIC/MBC values for bacteria and fungi were detected following the addition of 40 μL of

piodonitrotetrazoliumviolet (INT) 0.2 mg/mL and incubation at 37°C for 30 min (Tsukatani *et al.* 2012). The MBCs/MFCs were determined by serial subcultivations of 10 µL into microtiter plates containing100 µL of broth per well and further incubation for 24 h at 37°C. The lowest concentration with no visible growth was defined as the MFC, indicating 99.5% killing of the original inoculum. Following positive controls were used in both experiments: antibiotics (Streptomycin) and mycotic (Fluconazole). Each test was carried out in triplicates and repeated three times.

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

Results and Discussion

Hydrodistillation showed that *C. copticum* seeds contained 1.2% (v/w) EO. Results of GC/MS analysis of the EO (Table 1) indicate that γ-terpinene was the main monoterpene hydrocarbon, with a content of 33.12%. The GC/MS analysis of *C. copticum* oil revealed 14 compounds representing 84.79% of the total oil; γ-terpinene was the main constituent (33.12%) followed by p-cymene (21.00 %), thymol (15.87%), α-pinene (5.90%), β-phellendrene (3.54%), β-myrcene (0.90%), β-fenchyl alcohol (0.76%), β-pinene (0.70%), 4-terpineol (0.63%), α-thujene

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

	Components	[a]*C. copticum* EO (%)			[b]Retention index	Identification methods
		Control (%)	Nano-iron chelate (100 mg/l) (%)	Nano-iron chelate (200 mg/l) (%)		
1	α-thujene	0.60	-	-	850	MS, RI
2	α-pinene	5.90	1.50	1.00	855	,,
3	β-pinene	0.70	0.30	0.10	190	,,
4	β-myrcene	0.90	_	_	920	,,
5	*P*-cymene	21.00	15.00	10.00	950	,,
6	β-phellendrene	3.54	_	_	954	,,
7	limonene	0.51	1.00	1.32	960	,,
8	γ- terpinene	33.12	46.12	40.65	980	,,
9	4-terpineol	0.63	-	-	1.63	,,
10	*cis* limonene oxide	-	0.51	1.65	1085	,,
11	dodecane	_	1.12	1.50	1110	,,
12	β-fenchyl alcohol	0.76	_	_	1126	,,
13	thymol	15.87	19.06	30.43	1208	,,
14	ethylene methacrylate	0.54	0.49	0.26	1235	,,
15	pentadecane	_	0.20	1.00	1264	,,
16	hexadecane	0.41	-	-	1285	,,
17	nonadecane	0.31	_	-	1293	,,
18	carvacrol	_	3.64	10.87	1306	,,
	Total	84.79	85.30	87.91		
	Yield	1.20%	1.84%	2.61%		

[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.

(0.60%), ethylene methacrylate (0.54%), limonene (0.51), hexadecane (0.41%) and nonadecane (0.31 %). The constituents of the obtained EOs of *C. copticum* treated with nano-iron chelate are presented in Table 1. Fourteen components were identified in untreated plants and 11 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 *C. copticum* EO. Decrease in the proportion of α-pinene, β-pinene, p-cymene and ethylene methacrylate have been found according to the concentration of nanoiron chelate. Some compounds such as α-thujene, β-myrcene, 4-terpineol, β-fenchyl alcohol, hexadecane and nonadecane were only detected in the sample control (Table 1). Limonene, γ- terpinene and thymol were increased with nano iron chelate - treatment (Table 1). The yield of the *C. copticum* oil was 1.20% in control, 1.84 % (100 mg/l) and 2.61% (200 mg/l). The nano-iron chelate significantly increased the yield of EO (Table 1). 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 restricting factor of yield and extremely decrease crop yield quality.

Table 2. Effect of nano-iron chelate on extraction yields, total phenolic oils and total flavonoid contents of *C. copticum*.

	Extract	Extraction yield[a]	Total phenolic[b]	Total flavonoid[c]
1	Control	98.44±04	261.11±59	106.45±81
2	Nano-iron chelate (100 mg/l)	100.31±70	298.10±16	118.98±43
3	Nano-iron chelate (200 mg/l)	125.81±83	318.46±63	156.34±05

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.

As shown in Table 2, the extraction yield of *C. copticum* ranged from lowest 98.44 ± 04 mg/g (control) to highest 125.81 ± 83 mg/g (nano-iron chelate (200 mg/l)). Among the three *C. copticum* extracts, *C. copticum* treated with nano-iron chelate at 200 mg/l the highest total phenolics (318.46±63 mgg^{-1}) and showed the highest total flavonoids (156.34 ± 05 mg/g^{-1}). Furthermore, the total phenolic and total flavonoid contents exhibited the descending order among: *C. copticum* extract (treated with nano iron chelate 200 mg/l) > *C. copticum* extract (treated with nano-iron chelate 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.

According to the results given in Tables 3 and 4, *C. copticum* EO and its main components exhibited significant antimicrobial activity against all tested strains. Results obtained from MIC and MBC/MFC indicated that *B. cereus* and *C. albicans* were the most sensitive microorganisms tested, with the lowest MIC and MBC/MFC values (Table 3) in the presence of the oil isolated from *C. copticum*. The results of antibacterial activity of essential oil components are presented in Table 4. α-Pinene and p-cymene showed the lowest antibacterial activity among the tested components. In the present study, most of the antimicrobial activity in EOs from *C. copticum* appears to be associated with γ- terpinene, thymol and carvacrol (Table 4).

Table 3. Antimicrobial activity of the EOs (µg/ml) from *C. copticum* using minimum inhibitory (MIC) and minimum bactericidal/fungicidal (MBC/MFC) test.

Microorganisms	EO			Microorganisms	EO		
	Control	N-N-Ch 200 mg/l	Antibiotic (Streptomycin)		Control	N-N-Ch 200 mg/l	mycotic (Fluconazole)
	MIC	MIC	MIC		MIC	MIC	MIC
	MBC	MBC	MBC		MFC	MFC	MFC
Bacillus cereus	2.5±0.55	1±0.00	10±0.70	*Candida albicans*	2.5±0.5	1.5±0.32	4.5±0.00
	2.5±0.19	1±0.00	15±0.32		3±0.0	1.5±0.30	5±0.00
Enterococcus faecalis	5.5±0.32	3±0.56	10±0.59	*Candida parapsilosis*	5.5±0.0	4±0.43	5±0.9
	5.5±0.0	3±0.21	10±0.52		5.5±0.0	3.5±0.87	3.5±0.5
Staphylococcus aureus	15±0.05	6±0.45	250±0.0	*Aspergillus fumigatus*	3±0.54	2.5±0.5	3±0.5
	10±0.05	6.5±0.06	250±0.0		23±0.41	2.5±0.32	4.5±0.06
Escherichia coli	3.5±0.00	2±0.00	25±0.5				
	3.5±0.00	2±0.00	25±0.5				
Pseudomonas aeruginosa	8±0.12	6.5±0.35	100±0.34				
	6±0.65	6±0.5	150±0.87				
Proteus mirabilis	6.5±0.5	5.5±0.29	20±0.54				
	4.5±0.5	3.5±0.76	15±0.5				
Salmonella typhimurium	3.5±0.5	2.2±0.19	15±0.05				
	3.5±0.5	2±0.90	15±0.02				
Citrobacter freundii	6±0.00	6±0.07	15±0.87				
	6±0.00	5.5±0.02	20±0.56				

Control: Plants treated with distilled water. EOs: Essential oils. N-N-Ch 200: Plants treated with nano-iron chelate at 200 mg/l. All tests were performed in triplicate, repeated three times twice (means ± Sd). Statistically, the differences were significant at $p \leq 0.01$ or at least at $p \leq 0.05$ (in few cases), using DMRT.

According to these results, there is a relationship between total phenolic contents and antimicrobial activity. Considering the promising inhibitory and bactericidal activity of the examined EOs, it might be used as a natural food preservative as well as antimicrobial substance in nosocomial infections. However, further studies are still required to investigate its application in medicine and food industries.

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