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Original Research Article

Effects of Nano- salicylate on Quality and Quantity of Essential Oil Components in *Trachyspermum copticum*

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ABSTRACT

The effect of application of nano- salicylate (2 and 3 mM) on components of essential oils (EOs) of *Trachyspermum copticum* was evaluated. The nano-salicylate increased α-pinene, γ-terpinene, thymol and carvacrol and decreased β-myrcene, *p*-cymene, β-phellendrene, 4-terpineol, *cis* limonene oxide, dodecane, β-fenchyl alcohol, ethylene methacrylate and pentadecane concentration. Among the three *T. copticum* extracts, *T. copticum* treated with 3 mM nano salicylate showed the highest total phenolic content (176.94±58 mg/g) and showed the highest total flavonoid content (200.76±34 mg/g).

Keywords: *Trachyspermum copticum*, Nano- salicylate, Essential oil

Nano-Salisilat'in *Trachyspermum copticum*'un Esansiyel Yağ Komponentlerinin Nitelik ve Niceliği Üzerine Etkileri

ÖZ

Nano-salisilat uygulamasının (2 ve 3 mM), *Trachyspermum copticum* esansiyel yağ bileşenlerinin üzerine etkisi incelenmiştir. Nano-salisilat α-pinen, γ-terpinen, timol ve karvakrol konsantrasyonunu artırıken β-myrcene, p-cymene, β-phellendrene, 4-terpineol, cis limonene oxide, dodecane, β-fenchyl alcohol, ethylene methacrylate ve pentadecane konsantrasyonunu azaltmıştır. Üç *T. copticum* ekstratı içerisinde 3 mM nano-salisilat uygulanan *T. copticum* en yüksek toplam fenolik (176.94±58 mg/g) ve flavonoid (200.76±34 mg/g) içeriğe sahip olmuştur.

Anahtar Kelimeler: *Trachyspermum copticum*, Nano-salisilat, Esansiyel yağ

INTRODUCTION

Use of nano fertilizers increases agricultural production and their quality. Moreover, they protect environment from pollution. Plants can absorb nano-composites completely and resolve needs and shortages. Aromatic plants are potential natural sources of novel antibiotics and particular interest has focused on their essential oils as main sources of potent antimicrobial and antifungal compounds classified as terpenoids, flavonoids and phenolics (Webster *et al.* 2008, Razzaghi-Abyaneh *et al.* 2009). *Trachyspermum copticum* is an aromatic plant belonging to the Apiaceae family (Mozaffarian 1996). Its fruit has been widely consumed as a food flavoring agent and spice. Carvacrol, γ-terpinene and *p*-cymene were reported as main components of Iranian and African ajowan oil (Shojaaddini *et al.* 2008), and thymol (97.9%) was the main component of south India ajowan oil (Minija *et al.* 2002). The main objectives of the present study were to evaluate the exogenous application of nano- salicylate on quality and quantity the essential oil from *T. copticum* seeds.

MATERIALS AND METHODS

Plant materials and nano salicylate treatments

Seeds of *T. 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 salicylate at 2 and 3 mM concentration. All spray solutions 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 *T. copticum* were harvested and air dried at ambient temperature in the shade.

Oil isolation and identification of the oil components

The *T. 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 (European Pharmacopoeia 1975). The obtained essential oils were dried over anhydrous sodium sulphate and after filtration, stored at +4 ºC until tested and analyzed. 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

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thickness 0.25 μm) 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 (2001).

Total phenolic determination

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

Total flavonoid determination

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

Antioxidant activity

The efficacy of the essential oils to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals was evaluated using a spectrophotometry method (Cuendet *et al.* 1997; Kirby and Schmidt 1997). 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 EC_{50} (μg/mL).

Inhibition percent = $\mathbf{Abs_{control} - Abs_{sample} / Abs_{control} \times 100}$ **(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 *T. 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 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 (Guleria *et al.* 2012). 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.

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

The *β*-CLAMS method by the peroxides generated during the oxidation of linoleic acid at elevated temperature (Koleva *et al.* 2002). The antioxidant activity (AA) of the extracts was evaluated in term of *β*-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 A_1 is the absorbance of the sample at 120 min. The results are expressed as EC_{50} 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 (1986). 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 % K_3 (Fe CN6) . After incubation at 67 ^{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 Fe Cl_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.* (2006). Ascorbic acid and trolox was 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 salicylate on chemical composition of T. copticum EO: The constituents of the obtained EOs of *T. copticum* treated with nano salicylate are presented in Table 1. Hydrodistillation showed that yield *T. copticum* treated with nano salicylate was 3.00 % (v/w) EO. The GC/MS analysis of *T. copticum* oil revealed 6 compounds representing 90.50 % of the total oil; γ-terpinene was the main constituent (35.98%.), followed by limonene (3.67%), thymol (30.98%) and carvacrol (16.98 %) (Table 1). Twelve components were identified in untreated plants and six components in 2 and 3 mM nano salicylate treated plants (Table 1). The differences were supposed to be the effects of nano salicylate on chemical composition of *T. copticum* EO. Decrease in the proportion of β-myrcene, *p*-cymene, β-phellendrene, 4-terpineol, *cis* limonene oxide, dodecane, β-fenchyl alcohol, ethylene methacrylate and pentadecane have been found. Some compounds such as β-myrcene, *p*-cymene, β-phellendrene only detected in control (Table 1). Limonene, γ- terpinene, carvacrol and thymol were increased with nano- salicylate treatment. The yield of the *T. copticum* oil was 1.00% in control, 1.67% (2 mM) and 3.00% (3 mM). Nano salicylate significantly increased the yield of EO (Table 1). We harvested plants materials in seeds stage. It may be affected the kind of oils, because it was showed 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%), c-terpinene (30.8%) and β-pinene (2.1%), but supercritical carbon dioxide extraction (SFE) of the EO revealed only three compounds (thymol, *p*-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 restricting factor of yield and extremely decrease crop yield quality. Lu *et al.* (2002) 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.

Table 1. Effect of nano salicylate on chemical composition of *T. copticum* essential oil.

	Components	[a] *T .copticum* EO (%)			[b]Retention Index	Identification Methods
		Control (%)	Nano- salicylate (2 mM) (%)	Nano salicylate (3 mM) (%)		
1	α-thujene	0	0.33	1.00_	850	MS, RI
2	α-pinene	1.12	1.54	1.89	855	MS, RI
3	β-myrcene	0.04	0	0	920	MS, RI
4	*P*-cymene	20.56	0	0	950	MS, RI
5	β-phellendrene	5.76	0	0	954	MS, RI
6	limonene	0	1.87	3.67	960	MS, RI
7	γ−terpinene	20.87	30.78	35.98	980	MS, RI
8	4-terpineol	1.05	0	0	1.63	MS, RI
9	cis limonene oxide	0.67	0	0	1085	MS, RI
10	dodecane	0.39	0	0	1110	MS, RI
11	β-fenchyl alcohol	0.84	0	0	1126	MS, RI
12	thymol	10.63	21.98	30.98	1208	MS, RI
13	Ethylene methacrylate	0.39	0	0	1235	MS, RI
14	pentadecane	1.98	0	0	1264	MS, RI
15	carvacrol	0	8.87	16.98	1306	MS, RI
Total		64.30	65.37	90.05		
Yield		1.00%	1.67%	3.00%		

[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.

Effect of nano salicylate on extraction yield, total phenolic contents and total flavonoid contents

As shown in Table 2, the extraction yield of *T. copticum* ranged from lowest 85.12 ± 76 mg/g (control) to highest 178.19 ± 67 mg/g (nano salicylate (3mM). Among the three *T. copticum* extracts, *T. copticum* treated with 3 mM nano salicylate showed the highest total phenolic content (176.94 ± 58 mg/g) and showed the highest total flavonoid content (200.76 ± 34 mg/g). Furthermore, the total phenolic and total flavonoid contents exhibited the descending order among: *T. copticum* extract (treated with nano salicylate 3mM) > *T. copticum* extract (treated with nano salicylate 3 mM) > *T. copticum* extract (treated with control). These results showed that the total phenolic and total flavonoid contents have an obvious variation in various concentrations. Nano-technology can present solution to increasing the value of agricultural products and environmental problems. With using of nano particles and nano-powders, we can produce controlled or delayed releasing fertilizers. Nano particles have high reactivity because of more specific surface area, more density of reactive areas, or increased reactivity of these areas on the particle surfaces. There are a few reviews about the effects of nano-particles on plants. Studies showed that the effect of nano particles on plants can be beneficial (seedling growth and development) or non-beneficial (to prevent root growth) (Zhu *et al.*, 2008). In coclusion, Use of nano fertilizers increases agricultural production and their quality. Moreover, they protect environment from pollution. Plants can absorb nano-composites completely and resolve needs and shortages. For this reason nanocomposites can be a good alternative to chemical fertilizers.

Table 2. Effect of nano salicylate on extraction yields, total phenolic contents and total flavonoid contents of *T. copticum* extracts.

	Extract	Extraction yield[a]	Total phenolic[b]	Total flavonoid[c]
1	Control	85.12±76	96.91±56	94.32±05
2	Nano salicylate (2 mM)	100.00±45	128.84±05	148.65 ±65
3	Nano salicylate (3 mM)	178.19±67	176.94±58	200.76±34

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.

Effect of nano salicylate on antioxidant activity

The results presented in Table 3 revealed that *T. copticum* EO and its main constituents exhibited a remarkable activity. In particular, γ-terpinene exhibited clearly a higher activity (11.00 ± 0.41 µg/mL) (Table 3). The positive controls BHT and ascorbic acid exhibited IC_{50} values equal to 13.93± 0.12 µg/ml and 12.43± 0.65 µg/mL, respectively. Table 3 depicts the inhibition of β-carotene bleaching by the *T. copticum* EO.The IC_{50} value was 12.01± 0.32 µg/mL. As shown in Table 3, the reducing power of *T. copticum* EO, expressed as EC_{50}, was clearly more significant than that of the positive control BHA and AA. Because of high antioxidant and free radical-scavenging activities of *T. 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 γ–terpinene (30.65± 0.11 µg/mL) (Table 4). According to these results, there is a relationship between total phenolic contents and antioxidant activity.In coclusion, Use of nano fertilizers increases agricultural production and their quality. Moreover, they protect environment from pollution. Plants can absorb nanocomposites completely and resolve needs and shortages. For this reason nanocomposites can be a good alternative to chemical fertilizers.

Table 3. Antioxidant activity of EO extract from *T. 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 were used as positive controls.

	Control	Nano salicylate (3mM)
Tested compounds	IC50 (µg/mL)	IC50 (µg/ mL l)
T.copticum EO	15.04± 0.24 µg/mL	11.74± 0.05 µg/mL
γ– terpinene	16.04± 0.34 µg/mL	12.00± 0.41 µg/mL
thymol	13.04± 0.54 µg/mL	10.00± 0.12 µg/mL
carvacrol	15.12± 0.29 µg/mL	13.05± 0.93 µg/mL
T.copticum EO (b-Carotenes IC_{50} µg/mL)	17.54± 0.11 µg/mL	12.01± 0.32 µg/mL
T.copticum EO (PR EC_{50} µg/mL)	16.12 ± 0.04 µg/mL	12.00 ± 0.02 µg/mL
BHA	13.93± 0.12 µg/mL	13.93± 0.12 µg/mL
AA	12.43± 0.65 µg/mL	12.43± 0.65 µg/mL

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

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

	Control	Nano salicylate (3mM)
compounds	%	%
γ– terpinene	10.32 ± 0.12	12.65 ± 0.11
carvacrol	5.34 ± 0.34	24.81 ± 0.32
thymol	21 ± 0.87	45.6 1± 0.82

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

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