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Application of combined fertilizers improves biomass, essential oil yield, aroma profile, and antioxidant properties of *Thymus daenensis* Celak.

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ABSTRACT

Thymus daenensis Celak., also known as denaian thyme, is an endemic and endangered medicinal herb, having valuable therapeutic properties in the Iranian traditional medicine. In the present study, a two years' field experiment was conducted to study the influence of organic and inorganic fertilizers on biomass, essential oil yield, essential oil content and compositions and antioxidant activity of denaian thyme in southwest of Iran. The treatments were: no fertilizer (control), chemical fertilizer (CF; NPK at 100-150-100 kg ha^{-1}), cow manure, vermicompost (VC) and combined fertilizers (chemical fertilizer + cow manure + vermicompost). Application of combined fertilizers significantly increased the biomass and essential oil yield of denaian thyme as compared with control, in the first and second year, by 39.61% and 68.62%, respectively. Application of fertilizers, however, significantly affected the essential oil compositions of the plants in both growing years. The essential oil of *T. daenensis* was rich in the phenolic monoterpene thymol. The highest amount of thymol was obtained under application of combined fertilizers in both years. The antioxidant activity of *T. daenensis* methanolic extracts, evaluated by the DPPH assay, increased (10.41% compared with control) under application of combined fertilizers in the second year. In both years, the N content of the soil increased after treatment with combined fertilizers (around 43% higher over the control). Alike, soil fertility parameters such as P and K showed a marked improvement in all treatments over the control. Results of this study showed that the combined application of fertilizers enhanced biomass and essential oil yield and improved the soil characteristics, antioxidant properties and aroma profile.

1. Introduction

The management of plant nutrition is one of the most important strategies for agricultural development. Understanding the relationships between different nutrients, and the combination of mineral and organic fertilizers is of crucial importance to improve the crop yields and production in an environmentally sound manner (FAO, 2007). In arid and semi-arid areas of the world, such as Iran, the organic matter level of the soil is usually very low. Thus, conservation and improvement of the soil fertility is of pivotal importance for maintaining soil health and sustainability of farming (Keshavarz Afshar et al., 2014).

Nowadays, there is a challenge in Iran to increase the herb and essential oil productivity of *T. daenensis* through improvement of agricultural practices without increasing the cultivation area. Plant nutrition is one of the most important factors affecting the secondary metabolism including the terpenoid components giving the aroma profile to essential oil bearing plants (Pandey et al., 2015; Patel et al., 2015). In addition, it has been reported that nutrition plays a pivotal role in plant growth, yield and development (Keshavarz Afshar et al., 2014; Pandey and Patra, 2015). In the case of medicinal and aromatic plants (MAPs), application of fertilizers could enhance effectively the essential oil yield and content (Aziz et al., 2010; Jabbari et al., 2011). Actually, crops react significantly to the application of nitrogen (N) fertilizer (Singh et al., 2007). In recent decades, the usage of organic fertilizers in developing countries to increase the crop productivity has been intensified (Patel et al., 2015). However, the organic fertilizers

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give rise to potential environmental and human health concerns (Keshavarz Afshar et al., 2014). Generally, organic manure has been applied to enhance crop production for centuries (Pandey and Patra, 2015). It is nowadays recognized that using vermicompost as an organic amendment in crop production is commercially possible and profitable (Singh and Wasnik, 2013).

Organic fertilizers are extensively used to improve the agricultural production (Zandvakili et al., 2017). Although the effect of different organic and chemical fertilizers in increasing plant yield has been well documented (Pandey and Patra, 2015), there is little information regarding the combination effects given by the concurrent use of chemical and organic fertilizers on plant yield and essential oil production in MAPs (Pandey et al., 2015). Recently, MAPs have attracted attention for their potential use as source of antioxidants able to neutralize toxic free radicals (Ghasemi Pirbalouti, 2009). Therefore, efforts in the search and enrichment of natural antioxidants in MAPs have been increased (Ghasemi Pirbalouti et al., 2012).

The genus *Thymus* L. (thyme), belonging to the Lamiaceae family, encompasses perennial herbs and small shrubs distributed mainly in the Mediterranean area (Cronquist, 1988). In Iran, the genus consists of 14 species which are distributed in different climatic areas. Among them, *T. daenensis* Celak subsp. *daenensis* Celak, *T. daenensis* Celak subsp. *lancifolius* (Celak.) Jalas, *T. carmanicus* Jalas, *T. persicus* (Roniger ex Reach. F.) and *T. trautvetteri* Klokov and Desj-Shost are recognized as endemisms (Rechinger, 1963-1998; Mozaffarian, 2012). *Thymus* species are well-known for their pleasant aroma and flavor as well as sources of bioactive compounds so that they are very popular as medicinal and flavoring agents (Nickavar et al., 2005; Ghasemi Pirbalouti et al., 2012; Dall'Acqua et al., 2017).

Thymus daenensis Celak., commonly known as denaian thyme, is an economically important culinary herb in Iran (Mozaffarian, 2012). Indeed, it is widely used as a flavoring agent (condiments and spices) in herbal teas, and also as a medicine (Nickavar et al., 2005; Ghasemi Pirbalouti et al., 2012). *Thymus* species are well-known for their strong antimicrobial and antioxidant activities (Ghasemi Pirbalouti et al., 2014). In the Iranian traditional medicine denaian thyme is used as carminative, digestive, antispasmodic, anti-inflammatory and expectorant agent (Nickavar et al., 2005; Ghasemi Pirbalouti, 2009). Essential oil of *T. daenensis* has different pharmacological properties, including anti-viral (Saderi and Abbasi, 2011), anti-bacterial (Ghasemi Pirbalouti et al., 2011), anti-fungal (Ghasemi Pirbalouti et al., 2012), antioxidant (Amiri, 2012), insecticidal (Gavadi Elmi et al., 2007) and immunomodulatory (Ghasemi Pirbalouti et al., 2011). The aromatic profile of this species is characterized by phenols, aromatic and non aromatic monoterpenes such as thymol and carvacrol and their biosynthetic precursors *p*-cymene and γ-terpinene, respectively. These components not only are responsible for the aroma and flavor of the herb but also significantly contribute to its biological effects (Ghasemi Pirbalouti et al., 2014; Vitali et al., 2016).

In the present study, a field experiment was conducted to determine the effects of (1) different chemical fertilizers (N, P, K); (2) organic manure (cow manure and vermicompost) and (3) integrated fertilizers (N, P, K, cow manure, vermicompost) on biomass yield, essential oil quality and antioxidant activity of *T. daenensis*.

2. Materials and methods

2.1. Plant materials and growth conditions

Thymus daenensis seeds were obtained from the Pakan Seed Co., Isfahan, Iran. A voucher specimen was collected at flowering stage, and deposited in the herbarium of Esfahan Agriculture and Natural Resources Research Center, Iran, with voucher number of 6268. The identification was performed by Mohammad Taghi Feyzi (a plant taxonomist). The seeds were germinated in a coco peat: perlite mix (70:30, w:w) contained in plastic germination trays in greenhouses at the experimental farm of Islamic Azad University of Shahrekord, Iran (N 32°20′, E 50° 51′, 2061 m a.s.l.). The climate of the experimental site is cold and semi-arid with annual average temperature of 12 °C. Eight weeks after seeding, seedlings with uniform size were transplanted into the main experimental plots containing silty clay soil in May 2014. The soil pH was 7.6 (Table 1).

Table 1
Physico-chemical properties of field soil (depth of 0–30 cm), cow manure and vermicompost.

Parameter	Field soil	Cow manure	Vermicompost
EC (dS m^{-1})	0.04	1.0	0.9
pH	7.6	8.3	8.0
Organic carbon	0.42%	36.46%	29.9%
Total N	0.02 (%)	2.03%	3.2%
Available P	51×10^{-4} (%)	0.3%	0.3%
Available K	0.02 (%)	0.5%	0.4%

2.2. Treatments details

A field experiment was conducted in two growing seasons (2014 and 2015) based on randomized block design with five treatments and three replications. The treatments were: control (without fertilizer), CF (chemical fertilizers 100-150-100 kg ha^{-1} N, P, K, respectively), CM (cow manure 20 t ha^{-1}), VC (vermicompost 10 t ha^{-1}), and CF + CM + VC (chemical fertilizers, 33.33-50-33.33 kg ha^{-1} N, P, K) + cow manure (6.6 t ha^{-1}) + vermicompost (3.33 t ha^{-1}). N, P and K were applied as urea ($CO\text{-}(NH_2)_2$), triple superphosphate ($Ca(H_2PO_4)_2$) H_2O and potassium sulphate (K_2SO_4), respectively. Cow manure, vermicompost, and chemical fertilizers were applied to the soil before transplanting. The ratio of CF and organic manures in different treatments was determined on the basis of optimum nutrient requirement of the plant, soil properties of the experimental site, and nutrient concentration of organic manures (Patra et al., 2000).

Plots were irrigated immediately after transplanting and throughout the growing seasons when needed. No pesticide was used during the experiment, and weeds were controlled manually. In both years, plants were harvested at full bloom stage and 5 cm above the soil surface. In the first and second year, the harvests were performed on 13 September and 26 June, respectively.

2.3. Soil nutrient analysis

After plant harvesting, soil samples were taken from three randomly selected sites in each plot from 0 to 30 cm of depth. The samples were homogenized, mixed and passed through a 2 mm filter for determination of soil chemical characteristics (Table 1). Total N was determined by the Kjeldahl method (Page, 1982). Available P content was determined spectrophotometrically after extraction in sodium bicarbonate as explained by Olsen et al. (1954). Potassium was extracted with ammonium acetate and analyzed by flame photometer (Black, 1965).

2.4. Essential oil extraction

The plants were harvested at 4 cm above the soil surface and then air-dried at room temperature for ten days. The dried plants were weighed and then ground into a powder using an electric grinder. Fifty g of each ground sample were subjected to hydrodistillation using a Clevenger-type apparatus for 3 h (Clevenger, 1928). Isolated essential oils were dried over anhydrous sodium sulphate, sealed in dark vials and kept at 4 °C until GC-FID and GC–MS analyses. Hydrodistillation was performed in triplicates (n = 3) and the essential oil content (%, v/w) and yield (g m^{-2}) were estimated according to the following equations:

Table 2
Effect of different fertilizers on herbage yield, essential oil yield and content and soil characteristics of *Thymus daenensis*in 2014 and 2015.

Year	Treatments	Herbage yield ($g\,m^{-2}$)	Oil content (mL/100 g dry matter)	Oil yield ($g\,m^{-2}$)	N (g/100 g)	P ($mg\,kg^{-1}$)	K ($mg\,kg^{-1}$)	Antioxidant activity (%)
2014	Control	135.6 ± 12.9	1.50 ± 0.10	2.05 ± 0.31	0.04 ± 0.00	9.53 ± 0.23	224.08 ± 21.19	61.73 ± 4.14
	CF	263.5 ± 20.4	2.00 ± 0.14	5.20 ± 0.67	0.04 ± 0.00	10.39 ± 0.39	297.25 ± 19.98	60.16 ± 2.90
	CM	179.4 ± 42.5	2.28 ± 0.09	4.27 ± 1.12	0.06 ± 0.01	11.01 ± 0.28	370.33 ± 40.93	70.24 ± 3.20
	VC	83.5 ± 24.5	1.41 ± 0.12	1.29 ± 0.44	0.04 ± 0.00	9.81 ± 0.47	224.67 ± 17.91	68.24 ± 3.20
	CF + CM + VC	202.3 ± 25.6	2.40 ± 0.15	5.03 ± 0.88	0.07 ± 0.01	11.46 ± 0.59	261.25 ± 25.43	62.58 ± 3.70
	LSD[a]	153.54	0.05	1.58	0.01	0.26	27.05	1.21
	ANOVA	p-value < 0.01	p-value < 0.01	p-value < 0.01	p-value < 0.01	p-value < 0.01	p-value < 0.01	p-value < 0.01
2015	Control	201.3 ± 18.6	1.72 ± 0.11	3.49 ± 0.53	0.034 ± 0.005	6.5 ± 1.67	235 ± 16.74	62.41 ± 0.16
	CF	282.6 ± 13.4	2.55 ± 0.19	7.24 ± 0.85	0.051 ± 0.004	8.33 ± 1.49	256.25 ± 9.30	65.83 ± 0.20
	CM	215.4 ± 22.5	2.43 ± 0.13	5.27 ± 0.83	0.036 ± 0.005	6.66 ± 1.49	236.66 ± 14.01	66.25 ± 0.13
	VC	291.6 ± 14.9	3.00 ± 0.21	8.77 ± 1.05	0.059 ± 0.004	9.33 ± 3.98	246.41 ± 15.19	67.20 ± 0.12
	CF + CM + VC	333.4 ± 13.4	3.33 ± 0.15	11.45 ± 0.92	0.07 ± 0.004	11.41 ± 1.55	274.5 ± 11.32	70.00 ± 0.17
	LSD	57.67	0.1	4.20	0.005	0.96	14.68	1.58
	ANOVA	p-value < 0.01	p-value < 0.01	p-value < 0.01	p-value < 0.01	p-value < 0.01	p-value < 0.01	p-value < 0.01

[a] Within each column means with the least significant difference (LSD test at 5% level) are not significantly different from each other.

$$\text{Essential oil content }\% = \left(\frac{\text{Extracted essential oil (mL)}}{\text{50 g of dried plant ground sample}}\right) \times 100 \quad (1)$$

$$\text{Essential oil yield g m}^{-2} = \text{Thyme biomass yield g m}^{-2} \times \text{Essential oil content }\% \quad (2)$$

2.5. Essential oil analysis

For gas chromatographic analysis a GC-2014, (Shimadzu, Japan) equipped with an RTX-5 capillary column (30 m length, 0.25 mm i.d., 0.25 μm film thickness) and flame ionization detector (FID) was used. To separate the essential oil components a ramp oven temperature was applied. The temperature was programmed at 60 °C for 5 min then raised to 260 °C at the rate of 4 °C min^{-1}. The injector and detector temperatures were set at 290 and 300 °C, respectively. The carrier gas was helium at a flow rate of 2 mL min^{-1}. Split mode ratio (1:20) was used for injector. For each sample, the essential oil was diluted in *n*-hexane (ratio 1:100) and then injected (1 μL) into the GC system. Quantification of the essential oil components was performed by peak area normalization without using correction factors (Morshedloo et al., 2018; Quassinti et al., 2013).

GC–MS analysis was performed on an Agilent 6890N gas chromatograph coupled to a 5973N mass spectrometer using a DB-5 MS capillary column (30 m length, 0.25 mm i.d., 0.25 μm film thicknesses). It was performed in the EI mode, electron energy of 70 eV, scan range of 50–550 amu, and transfer line temperature of 300 °C. Helium was used as the carrier gas with a flow rate of 2 mL/min, the injector temperature was set to 290 °C, the split ratio was 1:100, and the same oven temperature programme as GC-FID was applied. The essential oil constituents were identified by computer matching with the MS libraries (WILEY275, NIST 05, ADAMS) and a "homemade" library and also by co-injection with authentic standards (Sigma-Aldrich, USA) (Venditti et al., 2014). In addition, the calculation of temperature-programmed linear retention indices was carried out using a homologous series of *n*-alkanes (C_8–C_{40}, Sigma, USA), and compared with those reported in literature (Adams, 2007; NIST 05, 2005; Morshedloo et al., 2017a,b).

2.6. Plant extract and antioxidant activity

For each sample, 5 g of ground leaves were extracted by shaking with 50 mL of absolute methanol (Sigma-Aldrich) at room temperature for 72 h, and then filtered through a Whatman filter paper (No. 4). The residue was re-extracted by mentioned solvent and subsequently the extracts were evaporated under vacuum at room temperature to a stable weight and kept under vacuum until use. The extracts were redissolved in 2 mL of 50% methanol and evaluated for antioxidant activity.

The antioxidant activity of thyme extracts was determined by the reduction of 1,1-diphenyl-2-picrylhydrazyl (DPPH·) free radical according to the method described by Wang et al. (1998). For this assay 1000 μL of different concentrations of methanolic extracts (2.5–300 μg mL^{-1}) were added to 3.9 mL of DPPH solution (60 μM in methanol). The mixture was shaked and incubated for 30 min in the dark at room temperature. The absorbance of the samples (n = 3) at 517 nm was determined using the spectrophotometer UnicoSpectroQuest Model SQ2802 (Morshedloo et al., 2017b). The ability to scavenge the DPPH radical was calculated using the following equation:

$$\%\text{ Inhibition} = \left(\frac{AC(0) - AA(t)}{AC(0)}\right) \times 100 \quad (3)$$

where AC (0) is the absorbance of the control and AA(t) is the absorbance of sample. The EC_{50} value, which represents the concentration of extract that gives rise to a 50% reduction in DPPH· absorbance, was determined by linear regression analysis (Morshedloo et al., 2017b).

2.7. Statistical analysis

All data were subjected to analysis of variance (ANOVA) and significant difference test followed by the LSD test at p-value < 0.05 significance level by SAS version 9.1 (SAS Institute Inc., Cary, NC, USA). Mean values are presented with the standard errors. Pearson's rank correlation coefficient was calculated between biomass yield, essential oil content, yield and N, P, K contents using SPSS v. 22 software package (SPSS Inc., Chicago, USA).

3. Results and discussion

3.1. Soil chemical properties and biomass yield

3.1.1. Effect of different fertilizers on N, P and K contents of the soil

Application of different fertilizers induced a significant increase (p-value < 0.01) in N content and other available nutrients of the soil when compared with control conditions (Table 2). The highest and lowest amounts of N content was achieved in combined fertilizer treatments (0.07 g/100 g) and control conditions (0.04 g/100 g), respectively. Total P content followed a similar pattern as N. However, total P ranged between 6.5–11.50 mg kg^{-1} under different treatments. Higher levels of K were observed in cow manure treatment (370 mg kg^{-1}) in 2014. In the second year the highest K content

(274 mg kg^{-1}) was achieved in combined fertilizer applications (Table 2). According to previous studies, application of combined fertilizers improved remarkably the soil fertility (Pandey et al., 2015). The improvement of soil quality in the case of combined fertilizer treatments could be due to the presence of easily available water soluble organic carbon, which acts as a source of energy for soil micro-organisms (Manna and Ganguly, 1997). Our findings are in agreement with those reported by Adeniyan and Ojeniyi (2005) who explained that the organic manures in combination with chemical fertilizers gave greater residual soil fertility in relation to the increase in organic carbon content and the availability of N, P, and K, in the two years of cropping cycles. The variations in the soil nutrients under application of different inorganic and organic sources may be related to their capacity to supply these nutrients during the growth influencing the nutrients uptake by crops (Keshavarz Afshar et al., 2014; Pandey and Patra, 2015). The presence of other nutrients also played a fundamental role as a co-factor activating metal-ligand enzyme complexes (Pandey et al., 2015).

3.1.2. Correlation among soil nutrients and plant growth and essential oil production

Pearson's correlation coefficient analyses demonstrated positive correlations between biomass yield, essential oil content and essential oil yield with soil N content (r_s = 0.37, 0.60 and 0.51, respectively, (p-value < 0.01) (Table 3)). As a result, any factor that increases biomass yield and essential oil content could improve the essential oil yield (Singh et al., 2007).

3.1.3. Effect of different fertilizers on biomass yield

Results indicated that there was a significant difference in thyme biomass yield under different treatments (Table 2). In the first year, the highest biomass yield (263.5 g m^{-2}) was obtained under application of chemical fertilizers. Interestingly, in the second year, the highest biomass yield (333.4 g m^{-2}) was observed when combination of fertilizers was applied. The lowest biomass yields, however, were obtained using vermicompost without fertilizers in the first and second years, respectively (Table 2). The results also indicated that the biomass yield of denaian thyme in the second year was significantly higher than in the first year. Higher production of denaian thyme achieved with the use of different fertilizers can be explained by the fact that the combination of organic and chemical fertilizers induces a higher production of biomass (Emami Bistgani et al., 2016; Dadrasan et al., 2015). In fact, this may be due to the different nutrients supplied to the plants that improve the plant growth (Edmeades, 2003). Also Pandey et al. (2016) reported that the application of combined fertilizers could strengthen photosynthesis, thus enhancing the plant growth and crop yield. It should be noted that N and Mg are the essential constituents of the chlorophyll molecule (Taiz and Zeiger, 2006). The higher levels of N and Mg resulted in enhanced chlorophyll concentration leading to higher photosynthetic activity and biomass production (Pandey et al., 2015).

The obtained results are in agreement with those previously reported by Patra et al. (2000) and Anwar et al. (2005) who found that the biomass yield could significantly increase when combination of organic and inorganic fertilizers is adopted.

3.2. Effect of different fertilizers on essential oil content and yield and compositions

3.2.1. Essential oil content

Results in relation to the thyme essential oil content showed significant differences among fertilizer treatments in both years. The highest essential oil content (2.4–3.33 (mL/100 g dry matter)) was recorded with the combined use of fertilizers which showed approximately 41 and 48 (%) increase over the control in the first and second year, respectively (Table 2). On the other hand, the lowest essential oil content was observed in samples treated with vermicompost (1.41 mL/100 g dry matter) and with no fertilizers (1.72 mL/100 g dry matter) in 2014 and 2015, respectively. Overall, the essential oil content in the second year was higher than that in the first year. This may be related to the established plant and growth period in the second year (Emami Bistgani et al., 2017a,b). Our results were in agreement with those of Croteau et al. (1972), who noted that the combined application of organic manures and chemical fertilizers produced a significantly higher amount of essential oil compared with organic manure alone. Moreover, nitrogen, as a key nutrient, could promote development and division of cells and subsequently increase glandular trichome density (Agena, 1994). Actually, it is suggested to combine organic manure with chemical fertilizers to increase basil essential oil production (Pandey et al., 2016).

3.2.2. Essential oil yield

Our results demonstrated that the fertilizer treatments had a significant influence on the thyme essential oil yield in both years. In the first year, the highest (5.20 g m^{-2}) and lowest (1.29 g m^{-2}) essential oil yields were obtained under application of chemical fertilizers and combined fertilizers, respectively (Table 2). In the second year, however, the highest essential oil yields were obtained with combined fertilizers (11.45 g m^{-2}) whereas the lowest (3.49 g m^{-2}) were attained in the control plants. Interestingly, in the second year the plants produced approximately 56% more essential oil than in the first year.

Pearson's correlation coefficient analyses indicated that total biomass yield per plot is directly and positively associated with the total essential oil yield per plot (r = 0.94, p-value < 0.01) (Table 3). These results were in agreement with an earlier report of Pandey and Patra (2015) in *Pelargonium graveolens* and that of Pandey et al. (2015) in *Tagetes minuta*. The mentioned studies indicated that availability of nutrients such as N, P and Mg could increase the essential oil content and yield in the plants. On the other hand, Rajeswara Rao (2001) demonstrated that the essential oil yield of *Cymbopogon martini* (Roxb.) W. Watson increased by 10.3% when farm yard manure was applied. Tabrizi et al. (2011) reported that the essential oil yield was not affected by cattle manure in Khorasan thyme (*Thymus transcaspicus* Klokov). Bajeli et al. (2016) indicated that among the sole application treatments, the maximum oil yield (21.73 kg ha^{-1}) was obtained through application of poultry manure (7.5 kg ha^{-1}). We expected to see a significant influence of cow manure or vermicompost (alone) on biomass and essential oil yield, as previously reported (Keshavarz Afshar et al., 2014; Xu et al., 2016; Tabrizi et al., 2011) However, we

Table 3
Correlation among N, P, K, herbage yield, essential oil yield and content in *Thymus daenensis*.

	N	P	K	Herbage yield	Essential oil content	Essential oil Yield	Antioxidant activity
N	–						
P	0.68**	–					
K	0.40**	0.44**	–				
Herbage yield	0.37**	0.08^{ns}	0.15^{ns}	–			
Essential oil content	0.60**	0.21^{ns}	0.20^{ns}	0.85**	–		
Essential oil Yield	0.51**	0.19^{ns}	0.14^{ns}	0.94**	0.95**	–	
Antioxidant activity	0.27*	0.16^{ns}	0.21^{ns}	0.06^{ns}	0.38**	0.27*	–

* and **, significant difference at 5 and 1%, respectively.

Table 4
Effect of different fertilizers on main essential oil compositions of *Thymus daenensis*.

Compounds	RI[a]	LIT RI[b]	Year	Treatments Control (%)	CF (%)	CM (%)	VC (%)	CF + CM + VC (%)	LSD[c]	ID[d]
α-Terpinene	1016	1014	2014	0.92 ± 0.19	1.34 ± 0.77	1.98 ± 0.44	0.37 ± 0.03	1.86 ± 0.8	0.09	RI, MS
			2015	1.04 ± 0.26	1.24 ± 0.31	1.19 ± 0.35	1.50 ± 0.93	1.73 ± 0.68	0.31	
p-Cymene	1024	1020	2014	4.31 ± 0.37	4.22 ± 0.21	5.00 ± 0.29	3.49 ± 0.35	4.50 ± 0.24	0.16	Std
			2015	0.91 ± 0.30	2.25 ± 0.88	2.08 ± 0.60	2.30 ± 0.10	1.48 ± 1.68	0.27	
γ-Terpinene	1056	1054	2014	1.49 ± 0.92	1.69 ± 1.33	3.68 ± 1.02	1.34 ± 0.63	2.98 ± 0.64	0.62	Std
			2015	1.32 ± 0.76	1.76 ± 1.61	1.42 ± 0.19	2.06 ± 1.18	2.41 ± 0.93	0.38	
1,8-Cineole	1030	1026	2014	1.26 ± 0.22	1.26 ± 0.14	2.67 ± 0.24	0.80 ± 0.13	2.57 ± 0.26	0.13	RI, MS
			2015	1.04 ± 0.18	1.45 ± 0.14	1.45 ± 0.13	1.56 ± 0.09	1.70 ± 0.07	1.70	
Thymol	1291	1289	2014	57.27 ± 6.99	68.58 ± 4.52	69.91 ± 5.51	64.25 ± 5.25	72.75 ± 4.40	5.33	Std
			2015	65.66 ± 2.96	73.39 ± 4.60	71.32 ± 4.66	74.75 ± 2.87	76.83 ± 3.43	5.36	
Carvacrol	1297	1298	2014	3.11 ± 0.85	3.38 ± 0.59	4.01 ± 0.78	2.03 ± 0.49	3.32 ± 0.65	0.67	Std
			2015	3.44 ± 1.21	3.95 ± 0.75	3.68 ± 1.20	4 ± 1.14	4.05 ± 1.37	0.94	
(*E*)-Caryophyllene	1417	1417	2014	2.92 ± 0.13	2.97 ± 0.13	3.46 ± 0.33	2.84 ± 0.24	3.84 ± 0.32	0.17	Std
			2015	1.18 ± 0.33	1.30 ± 0.23	0.78 ± 0.010	1.34 ± 0.23	1.45 ± 0.30	0.14	

[a] RI, linear retention indices on DB-5MS column, experimentally determined using homologue series of *n*-alkanes.
[b] Relative retention indices taken from Adams.
[c] Within each row means with the least significant difference (LSD test at 5% level) haven't significantly different from each other.
[d] Identification methods: MS, by comparison of the mass spectrum with those of the computer mass libraries Wiley, Adams and NIST 05; RI, by comparison of retention index with those reported in literature; Std, by comparison of the retention time and mass spectrum of available authentic standard. Data are mean ± SE (n = 3).

found that cow manure had no positive influence on biomass and essential oil yield of thyme plants.

The increased essential oil yield of thyme under combined fertilizer treatments is probably due to the increase of availability of basic elements such as nitrogen and/or to that of cation exchange capacity (CEC) of the soil that may enhance the accumulation of nutrients in plant, leading to higher biomass and secondary metabolites production (Anwar et al., 2005; Patra et al., 2000; Agena, 1994). The results of Agena (1994) indicated that the availability of nutrients under application of fertilizers significantly increased the number of oil glands in chamomile. It is therefore recognized that combination of different fertilizers can be considered as a complete source of nutrients in sustainable agricultural systems (Singh and Guleria, 2013; Keshavarz Afshar et al., 2014; Singh et al., 2010).

3.2.3. Chemical profiles of essential oils

The essential oil compositions obtained from the aerial parts of denaian thyme are presented in Table 4. The main components determining the quality of *T. daenensis* essential oil are monoterpenes such as thymol, carvacrol, α-terpinene, *p*-cymene, γ-terpinene, 1,8-cineole, and sesquiterpenes like (*E*)-caryophyllene. The analysis of the essential oils obtained from aerial parts of the experimentally cultivated plants showed that the percentage of the mentioned compounds were affected by the fertilizer treatments (p-value < 0.01). As a matter of fact, the essential oil quality, expressed as the percentages of the marker compounds, was improved by the application of fertilizers over the control conditions. The phenolic monoterpene thymol was the major essential oil component in plants subjected to all treatments (57.2–76.8%). The variation in chemical composition of essential oils is dependent on the physiology of the whole plant (Tawfeeq et al., 2016). It seems that the combined fertilizers have positive effects on thymol as the major compound, due to the fact these combinations contain different minerals which affect the secondary metabolism of plants (Pandey and Patra, 2015; Pandey et al., 2015). These minerals will positively affect cellular metabolism and biomass production. As a consequence, an enhanced vegetative growth along with an increase of glandular trichomes is obtained (Patel et al., 2015; Pandey and Patra, 2015; Pandey et al., 2015, 2016).

Alike, Emami Bistgani et al. (2017a,b) reported that thymol, is one of the main flavoring components of the essential oils from denaiamn thyme. In combined fertilizer treatments, thymol increased by about 21 and 14% compared with control in the first and second year, respectively. Indeed, the highest amount of this component was achieved in combined fertilizer treatments reaching percentage levels of 72.7 and 76.8% in the first and second year, respectively (Table 4). Previous studies showed that different fertilizers such as chemical and organic manure can influence the aroma profile of MAPs (Singh et al., 2007; Tanu et al., 2004). Interestingly, in all treatments the amount of thymol was increased in the second harvest year. This may be due to the increase of oil glands and enhancement of terpene biosynthesis (Bahreininejad et al., 2013). In accordance with our results, Bahreininejad et al. (2013) reported that the thymol content in denaian thyme increased steadily during the second growth season. On the other hand, the amount of the sesquiterpene (*E*)-caryophyllene decreased significantly in the second year. This pattern can primarily be explained by the higher efficiency or activity of the methylerythritol 4-phosphate (MEP) pathway in *T. daenensis* (Morshedloo et al., 2017a).

In the first year, the highest percentages of the monoterpenes *p*-cymene (5%), α-terpinene (1.98%), γ-terpinene (3.68%), 1,8-cineole (2.6%) and carvacrol (4.0%), were obtained in plants treated with cow manure, whereas in the second year the highest amount of α-terpinene, γ-terpinene, 1,8-cineole and carvacrol were obtained in plants treated with combined fertilizers. This increase was of 9.93%, 45%, 38% and 17%, respectively, compared with the control plants (Table 4). Pandey et al. (2015) reported that application of fertilizers affects the essential oil composition of *T. minuta*. They indicated that the amounts of limonene, (*E*)-ocimenone, (*Z*)-ocimenone, (*Z*)-*β*-ocimene and (*Z*)-tagetone were higher in plants exposed to combined fertilizers compared with control plants. As a matter of fact, several authors (Jha et al., 2011; Pandey et al., 2015) showed that application of fertilizers changed significantly the aroma profile of the essential oils. Thus, organic manure and mineral fertilizers play a fundamental role in determining the quali-quantative production of secondary metabolites.

3.3. Antioxidant activities

Application of different fertilizers influenced considerably the antioxidant activity of denaian thyme (Table 2). Methanolic extracts from plants grown in the first year under cow manure displayed 14% higher activity against DPPH· compared with control plants. In the second year, however, combined fertilizers significantly improved the antioxidant capacity of the methanolic extracts by 10.41% (Table 2).

A possible factor contributing to the improvement of the antioxidant activity may be the enhancement of carvacrol and thymol production, as shown in *Lippia origanoides* Kunth (Teles et al., 2014). These essential oil constituents are generally recognized as excellent bioactive molecules. For instance, Emami Bistgani et al. (2017b) showed that carvacrol and thymol display excellent antioxidant effects. Other researchers also indicated that the increase of the antioxidant capacity may be due to differences in physico-chemical properties, composition of organic and chemical fertilizers and their effect on soil biota and plant metabolism (Emami Bistgani et al., 2016; Salama et al., 2015). The antioxidant activity of the extracts is mainly due to the presence of phenolic compounds such as rosmarinic acid and flavonool glycosides (Bendif et al., 2017; Bendif et al., 2018a,b). In fact, recent studies showed that many flavonoids and related polyphenols contribute to the total antioxidant activity in many medicinal plants (Bourgou et al., 2008; Bounatirou et al., 2007; Miguel et al., 2004).

4. Conclusion

The present study showed that the application of combined fertilizers (organic manure together with chemical fertilizers) in *T. daenensis* gives higher biomass yields, and improves the quality and quantity of essential oil as well as the antioxidant activity of polar extracts. These data also support conclusions that *T. daenensis* is a notable source of antioxidant compounds to be exploited on an industrial level. Finally, this study demonstrated that the combination of cow manure and chemical fertilizers is the most appropriated method to improve the soil properties and the economic productivity of this crop.

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