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Effects of treatments on yield and quality of essential oil of cultivated Numidian thyme (*Thymus numidicus* Poiret)

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ABSTRACT

This study aimed to optimize yield and to improve the quality of volatile biomolecules in cultivated Numidian thyme (*Thymus numidicus*). Numidian thyme plants were cultivated in greenhouse with following biological treatments: common nettle manure (*Urtica dioica*) (T1), algal extract (*Ulva lactuca*) (T2), conidial suspensions of two fungal strains of *Trichoderma asperellum* (T3, T4) and control irrigated with tap water (T0). The measured growth parameters were: stem length and fresh and dry plant biomass. The essential oils (EOs) were extracted by hydrodistillation and their yields, chemical composition was determined by GC-MS. T1 did not stimulate the vegetative growth, but significantly increased the oil yield (1.8 %) and its volatile compounds. T2 and T3 stimulated the synthesis of geraniol, thymol, terpinene, pinene and carvacrol. The T1, T2 and T4 improved the antioxidant potency. In conclusion, bio-inputs improved the chemical composition of Numidian thyme essential oil as well as its antioxidant efficacy. It is thus recommended to apply these amendments in field for better industrial value of Thyme.

Keywords: Antioxidant, bio-inputs, essential oil, GC-MS, Numidian thyme, *Thymus numidicus*, *Trichoderma asperellum*, *Urtica dioica*, *Ulva lactuca*

INTRODUCTION

Numidian thyme, (*Thymus numidicus* Poiret), (Family: Lamiaceae) is endemic to Algeria and Northern Tunisia (25). It is vegetatively propagated, thrives in poor calcareous soils (6). This plant is in great demand for its plant products/compounds (4). Its essential oils are used in medical, agricultural and food production (5). It varies in chemical polymorphism, due to complex genetic and environmental factors, which affects the quality

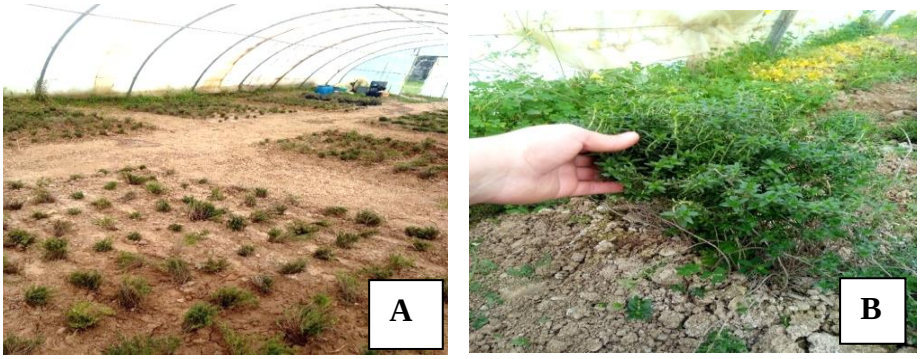

Figure 1. Experimental site of the Numidian thyme cultivation under the effect of biological inputs (A), and morphology of aerial parts of the cultivated Numidian thyme at vegetative stage (B).

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and quantity of its oil (5). So, there is need to improve its cultivation, production and quality of essential oils (22). This study aimed to increase its oil yield and quality of its volatile biomolecules.

MATERIAL AND METHODS

Plant material

The tufts of *Thymus numidicus* young seedlings of same size were selected and uprooted in November 2019, from the mountain of Adkar, wilaya of Bejaï [longitude : 4.66667, latitude : 36.6833, mean height above sea level : 859 m, annual rainfall : 739 mm, while, maximum and minimum temperatures were 10 °C and 25 °C, respectively.

Experimental Treatments

The Experimental Treatments of this study are given below.

Details of experimental treatments

Treatments	Treatment details	Conc. (%)	References
T0	Control (Distilled water)	-	-
T1	Manure of nettle (*Urtica dioica*)	10	13
T2	Algal extract sea weed (*Ulva lactuca*)	20	17
T3	Conidial suspensions of fungi (*Trichoderma asperellum*)	10^9	21
T4	Conidial suspensions of fungi (*Trichoderma asperellum*)	10^9	21

Cultivation

Young Numidian thyme seedlings were transplanted in tunnel greenhouse in Blida in December 2019 (Longitude : 2°28 12, latitude : 36°49 39, mean height above sea level : 229 m, annual rainfall : 421.7 mm; maximum and minimum temperatures were 33 °C and 4 °C, respectively). The plot size was 20 m x 4 m and spacing between plants was 30 cm. The young seedlings were irrigated every 3 days. After 15 days, treatments were applied through adding 200 ml treatment solution per plant every 10 days for 180 days. The treatments were replicated thrice in complete randomised blocks design. The plants were harvested at the vegetative stage in end May, 2020. The plant samples were collected separately treatment-wise and dried in shade to extract volatile molecules.

Growth parameters

The plant height, fresh and dry weight of shoots were recorded at harvest. From each treatment, the plant height was measured from 5-plants, while, fresh and dry weights of 30 plants were recorded. Dry weight was recorded after oven drying at 45 ^{o}C.

Extraction of essential oil

The dried aerial parts of cultivated plants were subjected to hydrodistillation using a Clevenger type apparatus for 2 h. Thirty g dry powdered material was placed in 1 L flask with 700 ml distilled water (9). The essential oil yield (%) for each sample was determined as under (5):

$$R\ (\%) = (Ph/Pv) \times 100$$

Where, R : Oil, Ph: Quantity of essential oil recovered, Pv: Amount of dried plant material.

Chemical analysis of essential oil

The chemical analysis of essential oil samples was done as per Ben *et al.* (7). Gas Chromatography Mass Spectrometry analysis was carried out at Air Force Research and Development Centre, Algiers, using a Gas Chromatograph coupled to a Mass Spectrometer (GC-MS QP5050A, Shimadzu). The fragmentation was carried out by electronic impact under a field of 70 eV. The column used was an Rtx-5MS capillary column (30m X 0.25mm ID X 0.25µm). The carrier gas used was helium, set at a flow rate of 0.8 ml/min. The oven temperature program varied between 60 °C and 240 °C, with an increase of 4 °C/min. The injector temperature was set at 250 °C, and that of the interface between the column and the mass spectrometer at 250 °C. The temperature of the ionization source was 250 °C. The injection volume was 1µl in split mode (split ratio: 50). The identification of the compounds was carried out according to their retention times using databases NIST05S and NIST107. The relative percentages of the compounds were calculated based on the areas of the chromatographic peaks, the relative content of each component was calculated by peak area normalization. Indices of kovats were confirmed according to (1) and (3).

Antioxidant activity of essential oil

The antioxidant activity of essential oil samples was evaluated by measuring the radical scavenging activity of the DPPH radical (1,1-diphenyl-2-picrylhydrazyl), as per Brutis (8) with some modifications. Fifty µl of each methanolic solutions of essential oils were mixed with 5 ml methanolic solution of DPPH (0.0045 %) and tested at different concentrations (25-100 μg/ml). After 30 min incubation at room temperature, the absorbance was recorded at 517 nm. Ascorbic acid was used as positive control. The inhibition of free radical of DPPH (%) was calculated as under (25).

$$I\ \% = [(AB - AA) / AB] \times 100$$

Where, I: Inhibition of DPPH (%), AB: Absorbance of blank and AA: Absorbance of test compound.

The scavenging activity of each essential oil was represented by the IC_{50}, defined as the concentration of antioxidant required to decrease the initial DPPH concentration by 50 %. All the tests were repeated thrice.

Statistical analysis

Each experiment was carried out in triplicate, and the results were presented by a mean value. Statistical analysis was carried out using ANOVA followed by Tukey's test using MINITAB software, at ($P \leqslant 0.05$) (24).

RESULTS AND DISCUSSION

Crop growth

(i). Stem length: The applied treatments did not increase thyme plants growth (stem length, fresh biomass, dry biomass) than control, rather the stem length was significantly decreased than control. For example, decrease by nettle manure (T1) was 24.5 %, the extract prepared from algae (*U. lactuca*) (T2: decrease 27.3 %), and the conidial suspensions of two strains of (*T. asperellum*) (T4: decrease 36.9 % and T3: decrease 47 %) (Figure 2 stem height).

(ii). Fresh weight: Similar to stem length, all treatments decreased the fresh biomass of Numidian thyme. The highest fresh biomass was recorded in control plants (T0). There was 8.8 %, 39.8 %, 34 %, 61.7 % and 60 % decrease in the fresh biomass of Numidian thyme by nettle manure (T1), the seaweed extract (T2), nettle manure, and conidial suspensions of the two strains of *T. asperellum* (T3 and T4), respectively (Figure 2 fresh weight).

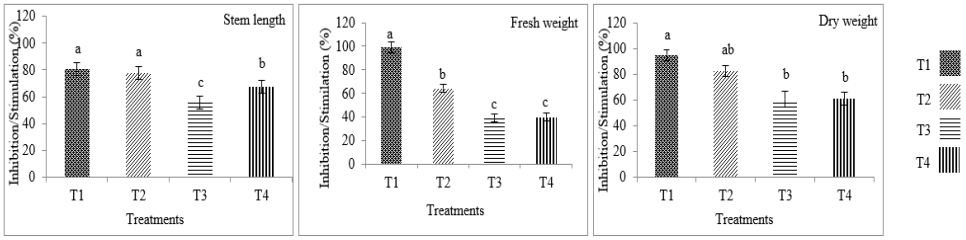

Figure 2. Effects of treatments on stem length, fresh and dry biomass of Numidian thyme. T1: Manure of nettle (*Urtica dioica*), T2: Algal extract (*Ulva lactuca*),T3 & T4: Conidial suspensions of fungi *Trichoderma asperellum*. Bars topped with different letters indicate significant difference among treatments at $P < 0.05$, as calculated by ANOVA followed by Tukey's test using Minitab computer software.

(iii). Dry weight: Similar to fresh weight, dry weight was higher in control (T0) than all treatments except the nettle manure (T1). The dry biomass of cultivated plants of Numidian thyme under the effects of nettle manure (T1) was statistically non significant when compared with control (T0). In all other treatments, the dry biomass of Numidian thyme was significantly lower than control (T0). There was a significant decrease of 14.8 %, 6 %, 52.7 % and 47 % in the dry biomass of Numidian thyme when treated with (T2), nettle manure, and conidial suspensions of the two strains of *T. asperellum* (T3 and T4), respectively (Figure 2).

The applied treatments did not improve the growth of Numidian thyme compared with control (T0). In control, the stem length, fresh and dry biomass of thyme were higher as compared with treatments. The previous literature did not report any similar work, particularly on medicinal plants. However, work has been reported on other crops and these studies depict the biostimulating effect of some bioinputs. For example, Khan *et al* (18) reported that foliar spray of algae (*Ascophyllum nodosum*) extract at different concentrations stimulated the tomato plants growth and increased the chlorophyll levels even at the lowest dose. Similarly, soil incorporation of *Trichoderma atroviride* improved the growth of young plants, the vegetative yield and the nutritional quality of Italian winter wheat grains (protein content and mineral composition) (10). Moreover, in another study, the application of *Trichoderma harzianum* and arbuscular mycorrhizal fungi improved the growth and productivity of *Arabidopsis thaliana* and *Brassica napus* (23).

Essential oil yields

The treatments significantly influenced the yield of essential oils (EO) extracted from cultivated plants of Numidian thyme. Only T1 significantly increased the EO by 10.4 % compared to control plants (T0). On the other hand, all other treatments decreased the EO yields. The plants treated with algae extract (T2) decreased the yield of EO (18.4 %) as compared to control. Similarly, the conidial suspensions of *T. asperellum* significantly reduced EO yield by 26.4 % and 36.8 % in T3 and T4, respectively (Figure 3).

A variability has been recorded in the EO yield of this Numidian thyme, under different treatments. In another study in Bejaia, its EO yield was 1.83 % and EO yield was slightly higher (1.92 %) in Annaba region (19). Whereas, Djeddi *et al.* (14) and Messara *et al.* (20) reported lower yields for Numidian thyme in regions of Tizi Ouzou (1.58 %) and Annaba (1.02 %). The highest EO yield recorded in T1, in the present study agrees with that recorded by (2) in the Béjaia region. The yields recorded in control and other bio-inputs are closer to Tizi ouzou and Annaba regions (14,20).

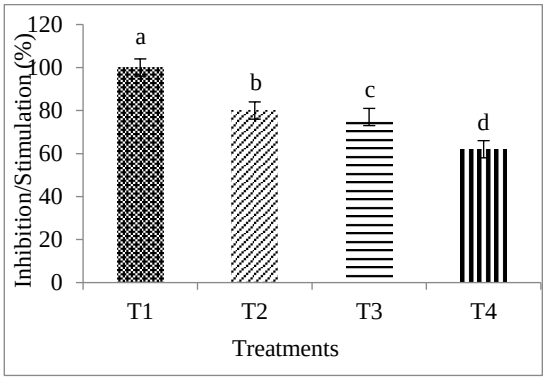

Figure 3. Effects of treatments on Essential oil yield (%) of cultivated plants of Numidian thyme. T1: Manure of nettle (*Urtica dioica*), T2: Algal extract (*Ulva lactuca*)), T3 & T4: Conidial suspensions of fungi *Trichoderma asperellum*. Data are means ± SD of three replicates. Bars topped with different letters indicate significant difference among treatments at $P < 0.05$, as calculated by ANOVA followed by Tukey's test using Minitab computer software.

Chemical composition of essential oil

Four chemical groups (hydrocarbon monoterpenes, oxygenated monoterpenes, hydrocarbons sesquiterpenes and oxygenated sesquiterpenes) were identified in EO samples. All treatments increased the levels of hydrocarbon monoterpenes (13.95-45.06 %) than control (11.89 %). Levels of oxygenated monoterpenes were higher in plants treated with nettle manure (67.62 %) compared to control (61.89 %). The chemical composition of the various essential oil samples showed variability under the effects of treatments and in contents of α-terpineol and δ-cadinene. Indeed, different essential oil chemotypes depend on the application of biological inputs on cultivated plants of *T. numidicus.* The EO extracted from the control plants was a linalool chemotype (55.2 %), in applied nettle manure it was of geraniol chemotype (28.8 %) and associated with other important compounds [linalool (22.7 %), thymol (12.37 %) and y-terpinene (4.08 %). EO with thymol/linalool chemotype (18.07 % : 16.74 %)] was associated with compounds [γ-terpinene (11.31 %), p-cymene (10.79 %), B-myrcene (6.77 %), α-pinene (5.77 %), geraniol (5.79 %) and thymol methyl ether (5.7 %)] was identified from plants treated with the extract of green alga *U. lactuca*. The applied conidial strains of *Trichoderma* in T4 and T5 induced two chemotypes, one with thymol/linalool (16.08 % : 16.85 %) and the other with linalool (17.11 %), respectively, also exhibiting the presence of γ-terpinene (10.77 %), geraniol (9.08 %), p-cymene (7.86

%), B-myrcene (7.5 %), α-pinene (5.38 %), thymol methyl ether (4.95 %)), thymol (14.09 %), γ-terpinene (10.61 %), p-cymene (10.3 %), geraniol (8.18 %), α-pinene (7.18 %), thymol methyl ether (5.47 %), and β-myrcene (4.62 %). In addition to these results, all treatments except control (T0) induced geraniol in plants (Table 1).

Table 1. Effects of treatments on Gas Chromatography-Mass Spectrometry (GC-MS) analysis of essential oil samples obtained by hydro distillation from cultivated plants of Numidian thyme

S. N.	Compounds	RT (mn)	RI (Lit)	Area (%)			
				T0	T1	T2	T3	T4
1	α-Thujene	5.87	924[1]	0.23±0.030^{b}	0.5±0.200^{b}	1.38±0.180^{a}	1.15±0.030^{a}	1.57±0.070^{a}
2	α-Pinene	6.08	932[1]	1.16±0.160^{b}	1.14±0.240^{b}	5.77±1.440^{a}	5.38±0.200^{a}	7.18±0.190^{a}
3	Camphene	6.47	946[1]	–	–	0.15±0.060^{ab}	0.14±0.060^{ab}	0.23±0.040^{a}
4	1-Octen-3-ol	7.175	974[1]	2.71±0.710^{a}	–	3.25±0.650^{a}	–	–
5	β-myrcene	7.52	988[1]	3.6±0.600^{c}	3.04±0.140^{c}	6.77±2.270^{ab}	7.5±0.700^{abc}	4.62±0.630^{abc}
6	α-phelandrene	7.97	1002[3]	0.15±0.050^{a}	0.15±0.060^{a}	0.38±0.200^{a}	0.44±0.060^{a}	0.4±0.060^{a}
7	δ-3-Carene	8.36	1008[1]	0.21±0.060^{b}	0.84±0.180^{b}	2.39±0.240^{a}	2.3±0.400^{a}	2.49±0.350^{a}
8	P-Cymene	8.64	1020[1]	0.85±0.350^{b}	2.69±0.700^{b}	10.79±1.890^{a}	7.86±0.980^{a}	10.3±1.290^{a}
9	Limonene	8.73	1024[1]	0.65±0.150^{b}	0.58±0.230^{b}	2.36±0.400^{a}	2.25±0.370^{a}	2.3±0.160^{a}
10	Cis-β-Ocimene	8.92	1032[1]	0.59±0.110^{a}	0.29±0.060^{b}	0.08±0.020^{c}	0.11±0.040^{bc}	0.12±0.020^{bc}
11	Trans-β-Ocimene	9.25	1044[1]	0.91±0.210^{a}	0.43±0.080^{ab}	0.13±0.470^{b}	0.18±0.100^{ab}	0.18±0.080^{ab}
12	γ-Terpinene	9.71	1054[1]	1.25±0.150^{c}	4.08±0.580^{bc}	11.31±2.810^{a}	10.77±1.890^{ab}	10.61±1.860^{a}
13	α-Terpinolene	10.65	1086[1]	0.17±0.090^{a}	0.21±0.100^{a}	0.3±0.110^{a}	0.28±0.100^{a}	0.28±0.080^{a}
14	Linalool	11.3	1095[1]	55.2±10.30^{a}	22.7±1.40^{b}	16.74±4.240^{b}	16.85±2.180^{b}	17.11±0.240^{b}
15	Terpinene-4-ol	13.84	1174[1]	–	0.18±0.100^{ab}	0.27±0.060^{a}	0.27±0.100^{ab}	0.28±0.080^{a}
16	α-Terpineol	14.34	1186[1]	–	0.25±0.100^{ab}	0.40±0.100^{a}	0.39±0.110^{a}	0.36±0.070^{a}
17	Thymol methyl ether	15.84	1232[1]	1.49±0.260^{c}	3.23±0.210^{ab}	5.7±0.900^{a}	4.95±1.150^{ab}	5.47±0.480^{a}
18	Geraniol	16.78	1249[1]	–	28.8±3.50^{a}	5.79±0.890^{b}	9.08±1.240^{b}	8.18±0.640^{b}
19	Geranial	17.34	1264[1]	1.04±0.140^{a}	–	0.09±0.030^{bc}	0.2±0.060^{bc}	0.24±0.040^{b}
20	Thymol	18.17	1289[1]	2.37±0.380^{c}	12.37±1.270^{b}	18.07±0.570^{a}	16.08±1.300^{ab}	14.09±1.490^{b}
21	Carvacrol	18.32	1298[1]	0.25±0.100^{c}	0.09±0.060^{c}	1.65±0.320^{ab}	2.42±0.430^{ab}	1.36±0.370^{b}
22	Geranyl acetate	21.1	1379[1]	1.54±0.030^{ab}	–	0.35±0.090^{bc}	2.24±0.090^{a}	2.84±0.950^{a}
23	β-Caryophyllene	22.47	1417[1]	1.47±0.150^{b}	3.36±1.210^{a}	1.22±0.110^{b}	1.66±0.130^{ab}	1.65±0.110^{b}
24	Germacrene-D	24.52	1484[1]	1.68±0.160^{ab}	1.86±0.970^{a}	0.52±0.080^{ab}	0.72±0.010^{ab}	0.45±0.190^{b}
25	β-Bisabolene	25.31	1505[1]	0.23±0.030^{ab}	0.39±0.100^{a}	0.16±0.070^{bc}	0.32±0.020^{ab}	–
26	δ-Cadinene	25.88	1522[1]	0.32±0.170^{a}	0.38±0.090^{a}	0.21±0.080^{a}	0.27±0.070^{a}	0.23±0.030^{a}
	Hydrocarbon monoterpenes			11.89	13.95	45.06	38.36	40.16
	Oxygenated monoterpenes			61.89	67.62	49.06	52.48	49.93
	Hydrocarbons sesquiterpenes			3.15	5.22	1.74	2.38	2.1
	Oxygenated sesquiterpenes			0.55	0.77	0.37	0.59	0.23

Taking into account the domestication of Numidian thyme, the results obtained in this study agreed with previous research on plants of Lamiaceae family and other species of thyme. The nitrogen fertilization decreased the contents of chemical compounds in EO extracted from cultivated plants of *Origanum vulgare* L (11). Likewise, the pre-domestication of *Satureja montana* in Spain increased the yield of EO and the contents of certain volatile compounds (β-myrcene, γ-terpinene, p-cymene, thymol and β-bisabolene) with decrease of α-thujene and carvacrol (22). Schmidt *et al.* (26) reported that three chemotypes of *Thymus vulgaris* cultivated in north France contained geraniol (26 %), thymol (38.8 %) and linalool (68.5 %).

Antioxidant activity of essential oil

Methanolic extracts of leaves of Numidian thyme exhibited significant antioxidant activity and the IC_{50} values showed significant variability among different treatments. The lowest IC_{50} values were recorded in the EO extracted from plants treated with conidial suspension of strain 1 of *T. asperellum* (T3) (Figure 4). The IC_{50} values in nettle manure (T1) and algal extract (T2) were significantly lower than control (T0) and were statistically similar to each other (IC_{50}, 0.07 and 0.06 mg/ml, respectively) and their IC_{50} values were also statistically non-significant, when compared with ascorbic acid control. The antioxidant potential was significant in the EO extracted from cultivated plants treated with conidial suspension of strain 1 of *T. asperellum* (T3) (IC_{50} 0.03 mg / ml), and this value was significantly higher than ascorbic acid, used as control. In this experiment, the IC_{50} values recorded in all treatments were significantly lower than control plants (T0).

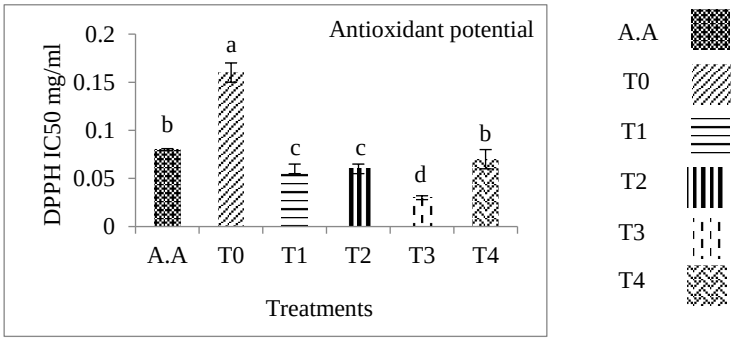

Figure 4. Effects of treatments on the antioxidant potential of essential oil samples of cultivated Numidian thyme compared to ascorbic acid. A.A: Ascorbic acid, T1: Manure of common nettle (*Urtica dioica*), T2: Extract of seaweed (*Ulva lactuca*), T3 &T4: Conidial suspensions of two endemic strains of *Trichoderma asperellum*. Data are means ± SD of three replicates. Different letters indicate significant difference among treatments at $P < 0.05$.

The antioxidant potential depends on the presence of adequate proportions of biomolecules with antioxidant activity The applied algae extracts (T2) and conidial suspensions of two strains of *T. asperellum* (T3 and T4) induced the thymol, γ-Terpinene, p-Cymene and α-Pinene. Similarly, carvacrol and β-myrcene were produced under the influence of strain 1 of *T. asperellum* (T3), which also reflected the synergistic antioxidant effects between the different chemical compounds. In this context, numerous researches

based on the antioxidant activity of essential oils of different species of thyme are consistent with our results. There was significant antioxidant potential in two new chemotypes of *Thymus algeriensis* (IC_{50}: 2.2 and 3.3 g / ml) as well as in *Thymus dreatensis* (IC_{50}: 3.3 g / ml) and *Thymus pallescens*, (IC_{50}: 2.3 g / ml) (16). The antioxidant activity of these essential oils was found superior than the benchmark antioxidant, the mannitol. This antioxidant potential can be attributed to the main phenolic compounds such as thymol and / or carvacrol, however, other compounds, such as p-cymene and γ-terpinene also seem to be involved. A very low IC_{50} (156.53 µg / ml) was recorded in the EO of Numidian thyme (2) and it was believed that this excellent antioxidant activity was due to thymol, the main compound of the EO. There was less antioxidant activity (52 %) at 4 mg/ml concentration (29), despite the dominance and the high percentage of thymol (40.40 %), which explains the synergistic effects of chemical compounds. In 3-species of *T. algeriensis*, collected from different regions of Tunisia, those from region of Dj. Jdidi had the best antioxidant activity (IC_{50}: 4.31mg/ml) compared to very low synthetic antioxidant potential BHA (Butyl hydroxy anisole) and other two oils. This low anti-free radical capacity in the latter could be attributed to the low content (0.3 %), as well as the absence of carvacrol in these oils, nevertheless the fairly high levels of certain compounds [camphor (14.8 %), linalool (14.5 %) and linalyl acetate (6.4 %)] in the species of Dj. Jdidi were responsible for the increased antioxidant activity of this EO, compared to remaining essential oils. In this context, the antioxidant potential of a compound or the number of compounds in EOs are difficult to confirm with certainty, since compounds even in low concentrations are also likely to make a significant contribution to the activity of essential oils (28).

CONCLUSIONS

All treatments significantly influenced the Numidian thyme essential oil yield as well as its contents of volatile compounds. The treatments also produced new chemotypes of essential oils having industrial importance, of which, the most important were geraniol, thymol and carvacrol. The treatments also stimulated the antioxidant potential of certain essential oils, in particular those extracted from plants cultivated under the influence of two strains of *Trichoderma asperellum* and those treated with *Ulva lactuca* and nettle manure, all exhibited more antioxidant potential than ascorbic acid, used as control.

ACKNOWLEDGEMENTS

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DECLARATION

We declare that all authors of this Ms. have made substantial contributions. We did not exclude any author who substantially contributed to this Ms. We have followed our ethical norms established by our respective institutions.

CONFLICT OF INTEREST

The authors announce that they have no conflict of interest.

ETHICAL APPROVAL

The authors declare that the study was carried out following scientific ethics and conduct. However, this study did not involve any use of animals, hence no ethical approval has been obtained from the concerned committee.

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