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Chemical Composition and Acaricidal Activity of *Thymus algeriensis* Essential Oil against *Varroa destructor*

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The aim of the present study was to determine the chemical composition and evaluate the acaricidal activity of *Thymus algeriensis* essential oil (TAEO) against *Varroa destructor.* This ectoparasitic mite is a pest of the honey bee *Apis mellifera*. The essential oil from the aerial parts of *T. algeriensis*, obtained by hydrodistillation, was obtained in a yield of 2.8± 0.2%, w/w. The TAEO was analyzed by GC and GC/MS. Thirty-four compounds were identified, representing 99.3% of the oil. The main constituents were carvacrol (48.4%), γ-terpinene (14.9%), *p*-cymene (14.7%), and thymol (5.6%). Four lots were constituted at the level of an apiary in order to study the dynamics of the *Varroa destructor* and its host, *Apis mellifera*. After diagnosis by the biological method "install of diapers", the lots were treated at different doses of TAEO (0.1, 0.3 and 0.5%). TAEO was sprayed on top of the hives. The results show that TAEO at 0.5% resulted in a decrease in the rate of infestation of *Varroa destructor*, causing a mortality rate of 32.6% without negative effect on the nesting of the queen. The essential oil of *T. algeriensis* could be used as a bioacaricidal agent.

Keywords: *Thymus algeriensis* L., *Varroa destructor*, *Apis mellifera*, Essential oils, Acaricidal activity.

The honey bee, *Apis mellifera* L., is critical for crop pollination and honey production. The ectoparasitic mite *Varroa destructor* is a pest of the honey bee. This mite, which feeds on haemolymph of brood and adult bees, causes colony disorder, weakness, decrease in brood and deformation of bees [1a]. It also reduces the ability of the colony to pollinate plants [1b]. The parasite destroys the mechanical protective barriers of the integument and impairs the immune system of the bees. The disease caused by the *Varroa* mites is called varroasis and is one of the main causes of economic damage to the beekeeping industry. Varroasis was reported for the first time in Umm Teboul (East of Algeria) in June 1981, in an apiary of a beekeeping cooperative. Currently, this parasite has spread rapidly throughout the country. Natural products having components with various modes of action might provide an effective solution to the problem of varroaosis [1c]. Essential oils might be an option to control *V. destructor*. Several authors report that these oils might be useful in reducing both mite infestation rates and hive contamination [1d-1e].

The genus *Thymus* L. (Lamiaceae) comprises more than 250 species growing wild throughout the world. This genus is represented by eleven species in the flora of Algeria, one of which is *T. algeriensis* Boiss. et Reut. *T. algeriensis* is the most endemic widespread North African species and well known as "*Zaatar*". The chemical composition of *T. algeriensis* essential oil has been extensively studied in different Maghreb countries [2a-2o], and the existence of several chemotypes has been revealed (Table 1). Therefore, the aims of this study were to analyze further the composition of the essential oil of wild *T. algeriensis* from Ain-Defla (Northern Algeria), and to determine its acaricidal activity against *V. destructor* and its host, *A. mellifera*. To the best of our knowledge, this study represents the first report on acaricidal activity of *T. algeriensis* essential oil.

Extraction afforded a yellow-red oil with a very strong and persistent odor of *Thymus*. The essential oil yield was 2.8 ± 0.2%, w/w, (n= 03). This plant can be assigned to one of the oil-rich species of the Lamiaceae. The percentages and retention indices of the identified oil components are listed in Table 2 in the order of their elution on the HP-5MS column. Thirty-four components were identified, accounting for 99.3% of the total oil. This was found to be rich in monoterpene phenols (carvacrol: 48.4% and thymol: 5.6%) and their corresponding monoterpene hydrocarbon precursors: *p*-cymene (14.7%) and γ-terpinene (14.9 %). Oxygenated monoterpenes were the predominent chemical group (58.4%), followed by monoterpenes (38.2%), whereas the sesquiterpenoid content was very low (2.7%). Chemical profiling of the TAEO sample revealed that it could belong to the carvacrol chemotype, as is quite common for Algerian samples of this species. The compositions of samples from North African countries showed that carvacrol and thymol were individually or jointly the predominent components. Thus, thymol (36.8%) and myrcene (20.2%) were reported as the major components for *T. algeriensis* from Libya [2c], while other authors cited thymol (14.4–65.1%) and carvacrol (22.8–70.3%) as the major compounds for Moroccan samples [2b].

According to the main compounds, several chemotypes were identified in wild plants growing in Tunisia [2h]. In Algeria, two chemotypes were found: thymol/ *p*-cymene/γ-terpinene (i) and

Table 1: Major compounds of *T. algeriensis* essential oils (%) from various countries.

Country	Ref [a]	Major compounds [b] (%)								
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Libya	[2c]		36.8	20.2						
	[2a]	4.7	38.5	8.9	7.2					
	[2i]		38.5	8.9	7.1					
	[2b]	22.8-70.3	14.4-65.1							
	[2m]	8.1	37.8	11.7	15.1					
Morocco	[2k]	80.4	3.4	5.0	2.0	1.8				
		49.3	0.8	2.6	0.9	0.8				
	[2j]								20.5	27.7
	[2h]						17.7		15.5	8.2
Algeria	[2g]		29.5	13	6.9					
				9.9			6.5	10.6	6.5	10.1
	[2e]		29.2	6.8		43.3				
	[2l]	4	71		3	0.5				
	[2d]		62.7			78.8				

[a] Ref: References; [b] Compound: **1**. Carvacrol; **2**. Thymol; **3.** *p*-Cymene; **4**. γ-Terpinene, **5**. Linalool; **6**. 1,8 –Cineole; **7**. 4-Terpineol; **8**. α-Pinene; 9. Camphor.

Table 2: Chemical composition of *Thymus algeriensis* essential oil from Northern Algeria (mean of triplicates).

Compound	RI[a]	RI[b]	Area (%)
α-Thujene	930	923	1.4±0.6
α-Pinene	939	930	1.1±0.4
Camphene	944	945	tr
Sabinene	975	970	tr
β-Pinene	979	973	tr
1-Octen-3 ol	977	977	0.2±0.1
β-Myrcene	991	988	2.7±0.9
α- Phellandrene	1003	1002	0.3±0.1
α-Terpinene	1017	1015	2.2±0.9
***p*-Cymene**	1025	**1023**	**14.7± 2.6**
Limonene	1029	1028	0.8±0.3
trans β-Ocimene	1050	1048	tr
γ-Terpinene	1060	**1059**	**14.9± 2.8**
cis-Sabinene hydrate	1070	1066	0.1±0.1
α-Terpinolene	1089	1088	0.1±0.1
Linalool	1097	1098	1.2±0.6
1-Octen-3- yl-acetate	1116	1113	0.4±0.1
Camphor	1146	1145	tr
Borneol	1175	1167	0.1±0.1
Terpinen -4-ol	1177	1177	1.5±0.7
trans-Dihydrocarvone	1201	1197	0.1±0.1
Carvacrol methyl ether	1245	1244	1±0.6
Thymol	1290	**1293**	**5.6±1.8**
Carvacrol	1299	**1306**	**48.4±4.2**
Terpinyl acetate	1347	1340	0.1±0.1
α-Gurjunene	1411	1413	0.7±0.2
β-Caryophyllene	1419	1423	0.1±0.1
Aromadendrene	1441	1443	0.2±0.1
Alloaromadendrene	1460	1465	0.1±0.1
Bicyclosesquiphellandrene	1473	1479	0.1±0.1
Ledene	1493	1499	0.2±0.1
γ-Cadinene	1506	1517	0.1±0.1
δ-Cadinene	1523	1526	1±0.4
Caryophyllene oxide	1583	1582	0.2±0.1

tr: traces (<0.1); RI[a], retention indices [4a]; RI[b], retention indices relative to C7–C30 on HP-5MS capillary column. Monoterpene hydrocarbons: 38.2%. Oxygen-containing monoterpenes: 58.4%; Sesquiterpene hydrocarbons: 2.5%; Oxygen-containing sesquiterpenes: 0.2%; Total identified: 99.3%

terpinyl acetate / nerolidol / α-pinene / borneol / bornyl acetate (ii) [2g]. The oil composition of two *T. algeriensis* samples collected from the same location was different with one thymol-rich and the other linalool-rich [2d]**.** TAEO showed a large variability and displayed different chemical profiles. This great variability and diversity observed in the chemical composition of the essential oils of *Thymus* can be attributed to many factors, including climatic and soil variations, stage of the vegetative cycle, seasonal variation, and the method of preservation and extraction.

Diagnosis of the presence of *Varroa destructor* mite in an apiary revealed the existence of the mite in all the hives. All colonies of the hive were parasitized by the *Varroa* mite and presented a degree of infestation variable from 1.4 to 34%. There is a correlation between the number of *Varroa* collected daily and the total population within the colony. The mite population can be estimated throughout the year by multiplying the daily mite drop by: 250 - 500 or 20 - 40 when the brood is either absent or present, respectively [3a].

In order to ensure the survival of the colony the following year, anti-*Varroa* treatment is imperative, and some authors propose critical periods of intervention [3b] to the apiculture industry. To reduce the infestation threshold to 50 mites, it is necessary that the acaricidal treatment is conducted in August before the breeding of the winter bee brood [3c].

In order to determine the effect of treatment doses on the nesting of the queen, calculation of the brood area is necessary. The essential oils were diluted with analytical grade ethanol to the following concentrations: 0.1, 0.3, and 0.5%, v/v. The results of the treatments are represented in Figure 1. Lot D presents a very high brood area (1147 cm^2), followed by lot B (913 cm^2), A (903 cm^2), and C (447.5 cm^2), respectively. The hives treated by TAEO (0.5%) represent the best brood area with an evolution from 925 - 1147 cm^2 corresponding to an increase of 225 cm^2. This means that this treatment had no adverse effect on the activity of the bee colony and the laying of the queen. As for the duration of application of treatment, the difference is not significant (p = 0.4123). Finally, the treatment did not disrupt the population of the hive; the brood remained compact and hatching continued normally, indicating the safety of the essential oils to the colony bees. The treatment with TAEO had no influence on the activity of the colony bees and the laying of the queen. Application of TAEO, in winter (from14/01 to 16/03) reduced the infestation of various lots infected by *V. destructor*. The results of the treatments at different concentrations (0.1, 0.3, and 0.5%) are represented in Figure 2. Mortality rates obtained were: A (4.1%), B (24.0%), C (32.4%) and D (32.6%), an average number of dead mites: A (173), B (435) C (1274) and D (1366). These results are consistent with those reported in the literature [3d]. Concerning the factor treatment dose, there is a clear significant difference (p<0.05) between the four lots. Statistical analysis shows that treatment of lots A and B had no effect on the mortality of *Varroa*; then, it is very significant for lots C and D, which shows the effectiveness of treatment by the TAEO concentration of 0.5%. There is a strong correlation between the number of mites killed and the concentrations of the oils tested. One possible explanation for this result is the presence of carvacrol and thymol as major components and the synergic effect with other monoterpenes, such as *p*-cymene, and γ-terpinene. Indeed, previous studies have found that carvacrol was acaricidal against several species of ticks, while carvacrol and γ-terpinene acted as miticides [3e]. Thymol and structurally related compounds like *p*-cymene are effective as acaricides; it has an acaricidal activity against *Varroa*, but while decreasing the laying of the queen [3f]. γ-Terpinene, another active substance of *Thymus* oil, has a very good varroacidal effect. However, the use of *Thymus* oil containing γ-terpinene at a concentration of 250 µg/L of air has proved to be very toxic for both *V. destructor* and bees [1c]. On the other hand, the third application of TAEO (0.5%) presented the most important rate of mortality (340 mites) during the treatment. The statistical analysis also reveals that this application (p= 0.012) corresponds to the stadium phoretic of the *Varroa* mite (Figure 2). During the period of development of the laying and nesting of the queen, the natural

mortality of *Varroa* decreases. This result is consistent with the literature [3g]. Also, it is slightly higher than that obtained by spraying oxalic acid at 0.56 g per colony on adult worker bees, where a mite mortality rate in the phoretic stage of 25.9% was observed [3h]. So, it is much higher than that obtained by applying, for 12 days, an extract of *Lantana camara*, when the infestation rate was reduced to 0.20% [3i]. Treatment with *Citrus aurantium* and *Cymbopagon flexuosus* reduced the average percentage of *Varroa* infestation by 100% after the fourth week [3j]. However, the spray of thyme oils resulted in the death of 65.9% of *Varroa* [3k]. In late autumn, the *Varroa* mite is very sensitive to essential oils due to the formation of a cluster and the absence of brood [3l]. When the external temperature range is from 15-20°C, some authors recommend the application of products containing thymol, because, beyond this range, outside temperature affects negatively the efficiency of the thymol and bee activity [1c]. These conditions are for our study and in the climatic conditions of Algeria in late winter with a temperature of 12-19° C. It follows that the application, in winter, of *T. algeriensis* essential oil by spraying inside the hives is very effective against infestation by *V. destructor* and for maintaining the activity of colony bees and the laying of the queen.

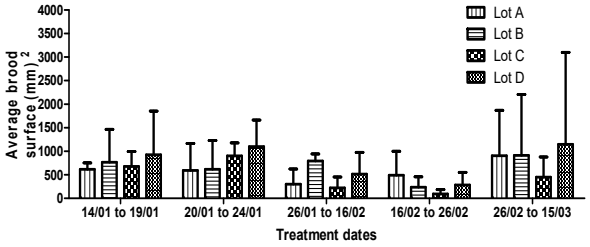

Figure 1: Average brood area during the spraying period of *T. algeriensis* essential oils (TAEO) at different doses. Lot A: control (untreated colonies), Lot B: TAEO (0.1%), Lot C: TAEO (0.3%), Lot D: TAEO (0.5%).

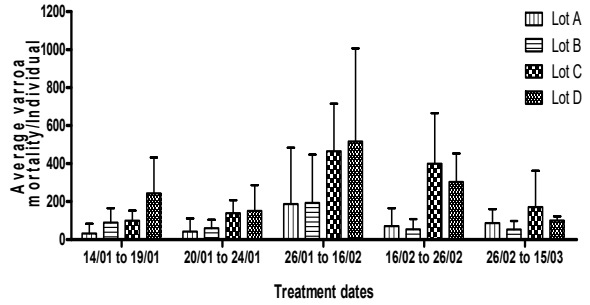

Figure 2: Average mortality of *Varroa* during the spraying period of *T. algeriensis* essential oils (TAEO) at different doses. Lot A: control (untreated colonies), Lot B: TAEO (0.1%), Lot C: TAEO (0.3%), Lot D: TAEO (0.5%).

The use of natural products as acaricides may represent an important alternative for the control of *V. destructor* since they are rich sources of bioactive compounds that are biodegradable. The present findings demonstrate that *T. algeriensis* essential oil may be used as a natural acaricide. Carvacrol, thymol, *p*-cymene, and γ-terpinene are the major compounds in *T. algeriensis* essential oil and may act synergistically to produce the observed acaricidal action against *V. destructor*. Treatment with TAEO (concentration 0.5%) applied in the climatic conditions of Algeria in late winter, is effective against the mite *V. destructor* and has no influence on the activity of the colony bees and the laying of the queen.

Experimental

Biological material: This study has been carried out in an apiary installed at Ain Defla (Algeria). Twenty hives were randomly distributed in 4 blocs (A, B, C, and D), each block containing 5 hives. Block A (hives witnesses) was left without applications. The other blocs were treated with different doses (0.1, 0.3, and 0.5%) of *T. algeriensis* essential oil in 96% ethanol.

Plant material and essential oil hydrodistillation: Aerial parts of wild *T. algeriensis* were collected in the first week of June 2013 in Mekhatria within the region of Ain-Defla located in northern Algeria (at 140 Km Northwest of Algiers - latitude: 36°25' N; longitude: 2° 21'7" E; Altitude: 365m). A voucher specimen was deposited in the Herbarium of the Agronomic Departement, Djilali Bounaama University of Khemis Miliana. Air-dried plants (50 g) with 600 mL distilled water (1:12, w/v) were separately subjected to hydrodistillation for 2 h using a Clevenger-type apparatus. All experiments were conducted in triplicate and results were expressed on the basis of dry weight.

Essential oil analysis: Ten mg of essential oil was dissolved in 5 mL of diethyl ether. The essential oils were analyzed by gas chromatography coupled to a flame ionization detector (GC-FID) and by gas chromatography coupled to a mass spectrometer (GC-MS).

GC-FID analysis: The analysis of the oil was carried out by means of an Agilent technology HP GC 6890 system with a flame ionization detector (FID), using a capillary column coated with 5 % phenyl-methyl siloxane (30 m x 0.25 mm x 0.25 µm film thickness Agilent Technologies, Hewlett-Packard, CA, USA). The temperature program was as follows: 40°C during1 min, then raised in a first ramp to 200°C at 6°C/min, followed by a second ramp to 280°C at 30°C/min, and finally kept at 280°C during 2 min. Injection was realized in splitless mode at 280°C; the volume injected was 1 µL of diluted oil (10 mg of oil/5 mL diethyl ether). Detector temperature was fixed at 300°C; Carrier gas was helium at 1 mL/min.

GC-MS analysis: GC/MS was performed with an Agilent HP 6890 GC system coupled with an Agilent HP 5973 Network Mass Selective Detector operated by HP Enhanced ChemStation software. Analytical conditions were fixed as follows: Agilent HP-5MS capillary column (30 m × 0.25 mm, df = 0.25 µm), a split-splitless injector at 250°C (splitless mode), temperature program: from 40°-250°C at 6°C/min, mobile phase: carrier gas helium at 1 mL/min. The mass spectra were recorded in EI mode (70 eV), scanned mass range: from 35 to 500 amu. Source and quadrupole temperatures were fixed at 230°C and 150°C, respectively. The identification of the components was performed on the basis of chromatographic retention indices and by comparison of the recorded spectra with computed spectral library (Wiley 275. L) [4a]. For sesquiterpene hydrocarbons, further confirmations were obtained by comparing the mass spectra with data from the literature [4a-4b]. Retention indices (RI) were calculated by means of a mixture of homologue *n*-alkanes (C_7-C_{30}) analyzed under the same chromatographic conditions used for the analysis of essential oils [4a].

Diagnosis of the presence of the* Varroa *mite before treatment by spraying: A diagnosis using the biological method "Installing the swaddling clothes" was performed before the treatment with *T. algeriensis* essential oil during the summer period. It allows the detection of the presence of the parasite and then to confirm and to assess the degree of infestation. Also, the diagnosis allowed us to establish a procedure to follow in order to preserve the bees in the best possible conditions [4c]. Among the biological methods for the diagnosis, the utilization of diapers or cover-bottoms was chosen. These swaddling clothes were smeared with fat (Vaseline)

supported by a grid on the floor of the hive. The swaddling clothes were then removed and examined carefully using a magnifying glass to detect dead *Varroa* mites. This method lasted for 30 days during which the swaddling clothes were replaced in the morning once every 3 days.

Treatment of the* Varroa *mites by spraying: The technique used was the spraying of TAEO at different doses (0.1, 0.3, and 0.5% in 96% ethanol). It was applied to the top of the frames of the hive using sprayers to ensure contact of the treatment with the *Varroa* mites. The treatments were applied all 6 days during the winter period from 14/01/2012 to 26/02/2012. Then, dead *Varroa* mites were counted on the dashboard greased all 3 days with the aid of a magnifying glass. The surface of the brood was calculated using the equation of an ellipse.

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