SUPPLEMENTARY MATERIAL

Toxicity of essential oils obtained from *Juniperus thurifera* var. *africana* and *Mentha suaveolens* subsp. *timija* chemotypes against pre-adult stages of *Hyalomma aegyptium* tick (Acari: Ixodidae)

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Abstract

This experiment was undertaken to compare the acaricidal activity of two essential oil chemotypes obtained from Mint Timija (*Mentha suaveolens* subsp. *timija* (Briq.) Harley) and Incense Juniper (*Juniperus thurifera* var. *africana* Maire) against immature stages of *Hyalomma aegyptium* ticks. The results showed that both chemotypes obtained from the two species presented interesting acaricidal activity. The pulegone and menthone-rich chemotype of Mint Timija (CM1) presented the highest toxic activity, particularly against eggs (LD_{50} = 17.93 and LD_{90} = 36.54 ppm) and larvae (LD_{50} = 0.03 and LD_{90} = 2.29 ppm). While the piperitone-rich Mint Timija EO (CM2) presented the weakest activity (LD_{50} = 51.13 and LD_{90} = 83.66 ppm for eggs, LD_{50} = 7.84 and LD_{90} = 21.03 ppm for larvae). Regarding Incense Juniper, the two chemotypes presented relatively moderate activity, with that of sabinene-rich oil (CJ2) being the most effective against eggs (LD_{50} = 22.29 and LD_{90} = 53.11 ppm).

Keywords: *Mentha suaveolens*, *Juniperus thurifera*, Essential oils, Ticks, Acaricide activity.

Experimental

1. Ticks

Hyalomma aegyptium ticks were collected from wild spur-thighed tortoise; *T. graeca* population naturally infested in the central Jbilets, 25 km in the north of Marrakech (31° 49' N, 7 ° 59' W, 540 m above sea level) from April to June 2018. To avoid any negative interference, the hosts of ticks did not receive any treatment prior to the beginning of the study. A total of 113 tortoises were captured by hand and each isolated in cloth bags for measurements and collection of ticks. A total of 1936 ticks were isolated using a fine forceps. Engorged female and sampled nymphs were stored in cooled plastic tubes (≈ 15 °C) to reduce their activity and immediately transported to the laboratory for identification and biological tests. The tick species collected, their stage and sex were determined according to the key of Walker et al. (2003) and Estrada-Peña et al. (2004).

2. Plant materials and isolation of essential oils

The aerial parts of the two chemotypes of Mint Timija and Incense Juniper were harvested from two different locations in center of Morocco (Supplementary Table S1) based on what has been previously described by Achak et al. (2008) and Kasrati et al. (2015). The plants were collected by hand, stored in the bags and transported to the laboratory. The plant species were identified and voucher specimens were deposited at the Laboratory of Biodiversity and Ecosystem Dynamic (BioDEcos). Plant materials were dried in the shade at room temperature (≈ 25 °C) and subjected to hydro-distillation, using a Clevenger-type apparatus (Borosil 3451029 Clevenger Apparatus) for 3h until total recovery of oil. The isolation of the essential oils was performed three times (3×100 g) and EOs obtained were dried over anhydrous sodium sulfate and stored at 4 °C in the dark until used. The yield was calculated in % (v/w) of dry plant material. Qualitative and quantitative analysis of the EO chemical profiles were performed using gas chromatography/mass spectrometry (GC/MS)

3. Gas chromatography/mass spectrometry (GC/MS) analyses

The GC/MS analysis of EOs was carried out on an Agilent GC-MSD system (Agilent Technologies 6890/5973) with helium (high purity) as the carrier gas at a constant linear velocity of 37 cm/s. The transfer, source and quadrupole temperatures were 280°C, 230°C, and 150°C, respectively, operating at 70 eV ionisation energy and scanning the m/z range 41–450. The column used was an Agilent DB5 ms capillary column (30.0 m × 0.25 mm ID × 0.25

μm film thickness; Model Number: 122-5532) programmed from 60°C to 246°C at 3°C/min. EO samples (60 μL) were diluted with acetone (2 mL). The injection volume was 1.0 μL, the split ratio was 1:50, and the injector temperature was 260°C. Identification of the individual components was based on (i) comparison with the mass spectra of authentic reference compounds where possible and by reference to WILEY275, NBS75K, and Adams terpene library: (Adams 2007); (ii) comparison of their retention indices (RI) on a DB5 (apolar, 5% phenyl polysilphenylene-siloxane), calculated relative to the retention times of a series of C-9 to C-24 n-alkanes, with linear interpolation, with those of authentic compounds or published data. For semi-quantitative purposes, the normalized peak area of each compound was used without any correction factors to establish abundance.

4. Bioassays

The eggs used in these assays were obtained from engorged females kept under controlled conditions (in an incubator with temperature 27 ± 1°C and relative humidity (RH) 80±10%). Before putting the eggs in filter paper envelopes (2×2 cm), these latter were treated with concentrations of 0, 0.5, 1, 1.5 and 2 μL of the four EO chemotypes mixed with 1 mL of acetone. Acetone was used to dilute the EOs and also as negative control. After evaporation of the solvent, 200 eggs were placed inside the envelopes and sealed. The envelopes of each concentration of the same EO were placed in Petri dishes (9 cm in diameter). The Petri dishes was maintained in an incubator under controlled conditions (27 ± 1°C and 80 ± 10% RH). After the hatching of the eggs (between 14 and 16 days), we counted the number of the eggs hatching using a binocular stereomicroscope (40x). Each experiment was carried out in triplicate.

As for the eggs, the larvae were obtained from females retained in the laboratory after hatching of eggs under controlled conditions (27 ± 1°C and 80 ± 10% RH). The biological assay with the larvae was carried out by placing 200 larvae of 15 days in filter paper envelopes (2×2 cm). These envelopes were treated previously with doses 0, 0.25, 0.5, 1 and 2 μL of two EO chemotypes of Incense Juniper (CJ1 and CJ2). For Mint Timija, the doses of 0, 0.02, 0.04, 0.06, 0.08 μL and 0, 0.05, 0.1, 0.2, 0.4 μL were used for EO chemotypes CM1 and CM2, respectively. Each concentration was diluted in 1 mL of acetone. After evaporation of the solvent and as for the eggs, the larvae were placed inside the envelopes, and were placed in Petri dishes (9 cm in diameter) in a controlled incubator (27 ± 1°C and 80±10% RH). Each Petri dish contains three sealed envelopes of the same concentration for each EO. After 24h of incubation, dead and alive larvae were counted. Larvae whose appendages did not move when

prodded with a fine pencil were recorded as dead. Each experiment was carried out in triplicate.

Nymphs are obtained from the infestation of a group of hosts as a nutrient source (rabbits and tortoises) by larvae of *H. aegyptium* in the laboratory conditions. Petri dishes (9 cm in diameter and 2 cm in height) were covered with a filter paper (9 cm) previously treated with 0, 0.5, 1, 1.5 and 2 µL of Incense Juniper EO chemotypes (CJ1 and CJ2). For Mint Timija EO chemotypes (CM1 and CM2), the filter paper was treated with 0, 0.2, 0.4, 0.6 and 1.6 µL. Each EO volume was diluted in 1 mL of acetone. After evaporation of the solvent, 10 nymphs were placed inside each Petri dish and the experiment was carried out in triplicate. The whole Petri dishes were kept in an incubator under controlled conditions of 27±1 °C and 80±10% RH for 24 hours. After the incubation period, the dead and live nymphs were recorded. The nymphs who did not move by stimulation were recorded as dead.

5. Data analysis

Probit analysis was conducted to estimate lethal doses (LD_{50} and LD_{90}) with their 95% confidence interval by SPSS 12.0 Statistical Software. LD values were considered significantly different when their respective 95% confidence interval did not overlap.

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species. Bioscience Reports, Edinburgh.

Table 1

Locality and harvesting time of the two populations of Incense Juniper and Mint Timija.

Species	Population code	Voucher specimen	Collection site	Collection period	Latitude/Longitude	Oil Yield (% (v/ w))
Incense juniper						
Population 1	CJ1	JT01	Oukaimeden	June 2014	N 31°11'/ W 07°53'	0.25 ± 0.02
Population 2	CJ2	JT02	Zaouiat Ahensal	June 2014	31°49'N / 06°06'W	0.53 ± 0.12
Mint timija						
Population 1	CM1	MST01	Ourika	February 2014	N 31° 21' / W 07° 47'	1.00 ± 0.20
Population 2	CM2	MST02	Ait Ourir	February 2014	N 31° 27'/ W 07° 32'	0.38 ± 0.08

Table 2

Chemical composition of different essential oil chemotypes obtained from the two species studied.

Compounds[a)]	RI[b)]		Incense juniper		Mint timija	
		RI[C)]	CJ1	CJ2	CM1	CM2
α-Thujene	928	923	4.2	3.8	-	-
α-Pinene	936	934	12.0	11.0	1.1	0.8
Camphene	951	953	-	-	1.1	0.8
Sabinene	976	976	35.2	49.9	0.5	1.1
β-Pinene	980	980	1.1	1.6	1.1	0.7
Myrcene	991	991	2.7	3.9	-	-
δ-2-Carene	1013	1001	5.3	1.1	-	-
δ-3-Carene	1019	1011	3.7	3.3	-	-
p-Cymene	1026	1026	3.4	-	-	-
Limonene	1030	1031	1.7	2.7	0.9	1.1
(*Z*)-β-Ocimene	1036	1050	-	-	0.2	1.1
1.8-Cineole	1059	1033	-	-	0.9	-
γ-Terpinene	1060	1062	6.0	5.1	-	-
cis-Sabinene hydrate	1068	1097	0.6	-	0.4	3.1
Terpinolene	1091	1088	2.4	2.0	-	-
Linalool	1100	1098	1.2	0.9	0.3	1.3
α-Thujone	1118	1102	-	-	-	-
Menthone	1156	1154	-	-	46.8	2.1
Isomenthone	1166	1164	-	-	7.7	2.3
Borneol	1168	1165	-	-	2.3	4.8
cis-iso-Pulegone	1177	-	-	-	1.6	-
Terpinen-4-ol	1179	1177	3.5	2.7	-	1.0
Pulegone	1242	1237	-	-	26.6	1.3
Piperitone	1255	1282	-	-	0.4	35.5
Linalyl acetate	1256	1257	4.7	3.7	-	-
Menthyl acetate	1272	1294	-	-	-	0.6

Table 2. (cont.)

Compounds[a)]	RI[b)]	RI[C)]	Incense juniper		Mint timija	
			CJ1	CJ2	CM1	CM2
Bornyl acetate	1288	1285	0.7	3.9	-	0.6
Thymol	1291	1290	-	-	-	0.6
2-Undecanone	1294	1291	1.5	-	-	-
Carvacrol	1302	1298	0.5	-	-	1.3
Piperitenone	1342	1342	-	-	0.3	9.6
Piperitenone oxide	1367	1366	-	-	0.5	9.4
Methyl eugenol	1375	1401	0.5	-	-	-
β-Bourbonene	1390	1384	-	-	-	0.6
β-Elemene	1417	1375	-	-	-	5.4
(E)-Caryophyllene	1426	1433	0.5	-	2.5	5.4
α-Humulene	1460	1452	0.5	-	0.3	0.8
Germacrene D	1487	1480	0.7	-	1.2	6.9
Bicyclogermacrene	1502	1502	-	-	0.2	1.9
γ-Cadinene	1519	1513	1.0	-	-	-
δ-Cadinene	1528	1524	1.4	-	-	-
Elemol	1530	1530	0.8	-	-	-
Spathulenol	1534	1576	-	-	0.6	-
Germacrene D-4-ol	1581	1574	-	-	-	0.2
Caryophyllene oxide	1589	1581	-	-	1.1	-
α-Cadinol	1645	1653	-	-	0.2	-
β-Eudesmol	1652	1649	0.4	-	-	-
α-Eudesmol	1656	1652	0.7	-	-	-
Monoterpne hydrocabons			79.2	84.4	4.9	5.5
Oxygenate monoterpenes			11.7	11.2	87.9	73.4
Sesquiterpene hydrocarbons			4.1	-	4.3	20.2
Oxygenate sesquiterpenes			1.9	-	1.9	0.2
Total (%)			96.9	95.6	99.0	99.3

[a]) Compounds listed in order of elution. [b]) RI (retention indices) measured relative to *n*-alkanes (C-9 to C-24) on the non polar DB-5 column. [c]) RI (retention indices) taken from literature of commercial sources

Table 3

LD_{50} and LD_{90} of different essential oil chemotypes on the eggs of *H. aegyptium* after 15 days of treatment.

Essential oils	LD_{50} (ppm) (95% LD)[a]	LD_{90} (ppm) (95% LD)	SE	χ^2	ddl[b]	P
Incense juniper (CJ1)	38.903 (35.321-44.136)	78.979 (68.403-95.899)	3.837236	0.513141	3	0.15
Incense juniper (CJ2)	22.293 (20.145-24.77)	53.112 (46.256- 64.251)	5.209216	2.879102	2	0.15
Mint timija (CM1)	17.931 (16.528-19.281)	36.547 (33.816-40.198)	5.609026	3.339287	2	0.15
Mint timija (CM2)	51.134 (43.278-66.919)	83.663 (67.634-116.579)	6.991815	1.594535	2	0.15

[a] 95% lower and upper confidence limits are shown in parenthesis. [b] Degree of freedom; SE: standard error. LD values are considered significantly different when 95% LD did not overlap.

Table 4

LD_{50} and LD_{90} of different essential oil chemotypes on the larvae of *H. aegyptium* after 24h of treatment.

Essential oils	LD_{50} (ppm) (95% LD)[a]	LD_{90} (ppm) (95% LD)	SE	$\chi 2$	Ddl [b]	P
Incense juniper (CJ1)	6,72 (0,33-10,77)	23,22 (17,64-39,04)	6.686	4.172	2	0.15
Incense juniper (CJ2)	9,28 (4,02-15,15)	18,79 (13,72-49,06)	11.170	8.856	2	0.15
Mint timija (CM1)	0.03 (0.013-0.85)	2.29 (1.43-8.50)	49.074	47.582	4	0.15
Mint timija (CM2)	7.84 (3.90-13.49)	21.03 (14.79-42.908)	6.357	41.836	4	0.15

[a] 95% lower and upper confidence limits are shown in parenthesis. [b] Degree of freedom; SE: standard error. LD values are considered significantly different when 95% LD did not overlap.

Table 5

LD_{50} and LD_{90} of different essential oil chemotypes on the nymphs of *H. aegyptium* after 24h of treatment.

Essential oils	LD_{50} (µl/cm^2) (95% LD)[a]	LD_{90} (µl/cm^2) (95% LD)	SE	χ^2	Ddl[b]	P
Incense juniper (CJ1)	0.0152 (0.0007- 0.0246)	0.0450 (0.0327- 0.1120)	16.02705	0.8073	3	0.15
Incense juniper (CJ2)	0.0209 (0.0111- 0.0294)	0.0451 (0.0345- 0.0823)	16.5969	1.5794	3	0.15
Mint timija (CM1)	0.0059 (0.0035- 0.0119)	0.0234 (0.0159- 0.0738)	29.2095	1.6929	2	0.15
Mint timija (CM2)	0.0066 (0.0012- 0.0126)	0.0238 (0.0162- 0.0747)	29.5885	0.4428	2	0.15

[a] 95% lower and upper confidence limits are shown in parenthesis. [b] Degree of freedom; SE: standard error. LD values are considered significantly different when 95% LD did not overlap.