SUPPLEMENTARY MATERIAL

Repellency of the *Origanum onites* L. Essential Oil and Constituents to the Lone Star Tick and Yellow Fever Mosquito

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Abstract

The oregano, *Origanum onites* L. essential oil (EO) was tested in laboratory behavioral bioassays for repellent activity against *Amblyomma americanum* (L.) and *Aedes aegypti* (L.). The *O. onites* EO was characterized using GC-FID and GC-MS. Carvacrol (75.70 %), linalool (9.0 %), *p*-cymene (4.33 %) and thymol (1.9%) were the most abundant compounds. At a concentration of 0.413 mg oil/cm^2 of filter paper, *O. onites* EO repelled 100% of the ticks tested, and at 0.103 mg oil/cm^2 of filter paper, 66.7% of the ticks were repelled. At 0.075 mg oil/cm^2 filter paper, thymol repelled 66.7% of the ticks compared to 28.7% by carvacrol at that same concentration. Against

Ae. aegypti, *O. onites* EO was repellent at the minimum effective dosage (MED) of 0.011 (± 0.00) mg/cm^2 in the cloth patch assay compared to the reference control, *N*,*N*-dimethyl-3-methylbenzamide (DEET) with a MED = 0.007± (0.003) mg/cm^2.

Keywords: *Origanum onites*, Thymol, Carvacrol, (-)-Linalool, Terpinolene, α-Humulene, Natural Repellency, *Amblyomma americanum*, *Aedes aegypti*

1. Experimental

1.1 *General and Chemicals*

p-Cymene (Cas# 99-87-6), (-)-linalool (Cas# 126-91-0), thymol (Cas# 89-83-8), carvacrol (Cas # 499-75-2), and DEET were purchased from (Sigma–Aldrich, St Louis, MO, USA) and steam distilled *Origanum onites* essential oil from aerial parts was obtained from Altes Ltd., Antalya, Turkey and the oil was kept at the Department of Pharmacognosy, Faculty of Pharmacy, Anadolu University, Eskisehir, 26470, Turkey. Optical rotation was recorded on Rudolph Research Analytical Autopol IV automatic polarimeter. Thin layer chromatography was performed on aluminum-backed cards, pre-coated with silica gel F_{254} (20 cm x 20 cm, 200 μm, 60 Å, Merck). Visualization was done by spraying with vanillin- H_2SO_4 reagent and followed by drying with a heat gun.

1.2. *GC-FID, GC-MS analysis and identification of components*

The GC-MS analysis was carried out with an Agilent 5975 GC-MSD system. An Innowax FSC column (60 m x 0.25 mm, 0.25 μm film thickness) was used with helium as the carrier gas (0.8 mL/min). The GC oven temperature was kept at 60 °C for 10 min and programmed to 220 °C at a rate of 4 °C/min, kept constant at 220 °C for 10 min, and then programmed to 240 °C at a rate of 1°C/min. Split ratio was adjusted at 40:1. The injector temperature was set at 250 °C. Mass spectra were recorded at 70 eV. Mass range was from *m/z* 35 to 450.

The GC-FID analysis was carried out using an Agilent 6890N GC system. FID detector temperature was 300 °C. To obtain the same elution order with GC-MS, simultaneous auto-injection was done on a duplicate of the same column applying the same operational conditions. Relative percentages of the separated compounds were calculated from integration of the peak areas in the GC-FID chromatograms.

The analysis results are expressed as mean percentage ± standard deviation (SD) (*n*= 3) as listed in Table S1.

Identification of the EO components was carried out by comparison of their relative retention times with those of authentic samples or by comparison of their relative retention index (RRI) to a series of *n*-alkanes. Computer matching against commercial (Wiley GC-MS Library, MassFinder 3 Library) (McLafferty and Stauffer 1999; Koenig et al. 2004), and in-house "Başer Library of EO Constituents" built up by genuine compounds and components of known oils as well as MS literature data (Joulain and Koenig 1998; ESO 2000, 1999) was used for the identification.

1.3. *Isolation of (-)-linalool*

O. onites EO (110 mg) was subjected to High Performance Flash Chromatography (HPFC) using a Biotage Inc. Horizon pump (Charlottesville, VA) instrument with a 12 M silica column (flow rate: 5.0 mL/min) and eluted with hexanes (100%) and hexanes–Et_2O mixtures (up to 10%). Portions of 3 mL volume were collected in 16 × 100 mm test tubes. Fractions with similar TLC profiles (hexanes: Et_2O 95:5, 90:10, 85:15, 80:20, 70:30 v/v) were combined to give 10 fractions. Fraction 7 yielded 4.2 mg of (-)-linalool [R_f = 0.32 in *n*-hexane-acetone (95:5, v/v)], $[\alpha]_D$= - 18.2 (*c* 1.00 g/100 mL, $CHCl_3$).

1.4. *Ticks*

Nymphal *Am. americanum* were obtained from colonies at Oklahoma State University and the Knipling-Bushland U. S. Livestock Insects Laboratory. The ticks were held at 23-24 °C, 97% RH, and a photoperiod of 16:8 h (L:D). The *Am. americanum* nymphs were tested 1-6 mo after molting.

Host-seeking ticks of many species climb when they encounter a vertical surface. This behavioral tendency was exploited to expose nymphs of *Am. americanum* to repellent treatments. The bioassay described by Carroll et al. (2011) used a 4 × 7 cm rectangle of Whatman No. 4 filter paper marked with a pencil into three zones (two 1 × 4 cm zones at the far ends and a central 4 × 5 cm zone). Using a pipettor, 165 µL of test solution (solvent ethanol) was applied evenly to both sides of the central zone of the filter paper. After the filter paper dried for 10-15 min, the filter paper was suspended from a bulldog clip hung from a slender horizontal dowel

held by an Aptex No. 10 double clip work holder (Aptex, Bethel, CT). A Petri dish (9 cm diameter) glued in the center of a 15 cm Petri dish created a circular moat when water was added between the dishes' walls (1.5 cm high). The moated Petri dishes were centered directly below the vertically suspended filter paper strip. When *Am. americanum* nymphs climbed to the rim of an open storage vial centered in a second set of moated petri dishes, the bulldog clip holding the filter paper strip was removed from the dowel and positioned so that ticks could transfer from the vial to the lower untreated zone of the filter paper. As soon as the tenth tick had climbed onto the filter paper, the paper was reaffixed to the work holder. The locations of the ticks were recorded at 1, 3, 5, 10, and 15 min after the tenth tick successfully grasped hold of the lower untreated zone of the filter paper. Ticks were considered repelled if they were in the lower untreated zone at 15 min after the tenth tick had crawled onto the filter paper or if they dropped from the filter paper without having crossed the upper boundary of the treated zone.

O. onites EO and four major constituent compounds (carvacrol, thymol, *p*-cymene, (-)-linalool) were evaluated for repellent activity with the vertical filter paper bioassay. An ethanol or acetone control was tested against 10 *Am. americanum* nymphs each day that the oil or a compound was tested. Ticks were tested in replicates of ten ticks per combination of concentration of the oil or compound. Twelve groups of nymphs were tested at 0 (ethanol control) mg compound/cm^2 filter paper, and 5, 3, 2, 3, and 3 groups were tested at 0.827, 0.413, 0.206, 0.103, and 0.052 mg oil/cm^2 filter paper, respectively. For thymol 4, 3, and 3 groups were tested at 0.30, 0.15, and 0.075 mg compound/cm^2 filter paper, respectively. For carvacrol 3 groups at 0.075 mg compound/cm^2 filter paper, and for *p*-cymene 3 groups were tested at 0.827 mg compound/cm^2 filter paper. An acetone solution (-)-linalool was tested on three dates at 0.413 mg compound/cm^2 filter paper along with an acetone control. Four groups of ticks were tested against DEET (0.827, 0.413, 0.106, and 0.103 mg compound/cm^2 filter paper) were tested against 2, 3, 2, and 3 groups of ticks. We analyzed the proportion of repelled ticks in a generalized linear mixed models framework using the lme4 R package (Bates et al. 2011), where date of trial was a blocking factor. As there was still evidence of over-dispersion, we redid the analysis, also included a random effect for each test of 10 ticks. The fixed effect was the compound-concentration combination. Means separation was done using the multcomp R package (Hothorn et al. 2008). We did two sets of a posteriori comparisons, *O. onites* EO at various concentrations to the ethyl alcohol and DEET controls, and a second set comparing

constituents of *O. onites* to each other, and to the DEET and ethyl alcohol controls. Concentrations where 100% of the ticks were repelled were not included in the analysis (there is zero variance for these trials, so confidence intervals cannot be constructed). Linalool was tested separately against an acetone control.

1.5. *Mosquitoes*

Pupae of *Ae. aegypti* (Orlando, 1952) from the Gainesville (CMAVE) colony were maintained in the laboratory at 28 ± 1 °C and 30-60% RH, and the resulting adults aged 5-9 d were used for repellent testing. Each sample was tested by application of a suitable amount to cloth to produce successive serials dilution of 1.500, 0.750, 0.375, 0.094, 0.047, 0.023, and 0.011 mg/cm^2. Each concentration was tested to determine the point where the repellent failed for each of the volunteers in the study; this concentration was averaged and reported. The test was conducted by having each volunteer affix the treated cloth onto a plastic sleeve to cover a 32 cm^2 window previously cut into the sleeve. Each of the volunteers wore this sleeve/cloth assembly above a nylon stocking that covered the arm with the hand of each volunteer protected by a glove. The arm with the sleeve/cloth assembly was inserted into a cage where approximately 500 female *Ae. aegypti* mosquitoes (age 7 days) had been preselected as host-seeking using a draw box. Failure of the repellent treatment is predetermined to be 1% bite through, i.e. the volunteer receives 5 bites through the cloth over the sleeve window in the 1 minute assay (Tabanca et al. 2016). Repellency was determined as the MED, which is the minimum threshold surface concentration necessary to prevent mosquitoes from biting through the treated surface. The MED data are reported as the mean (±SE) of all subjects for each compound. Each subject received all treatments; therefore, each subject acted statistically as his or her own control. There were three human volunteers in this study and all three provided written informed consent to participate in this study as part of a protocol (636–2005) approved by the University of Florida Human Use Institutional Review Board (IRB-01).

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Table S1. The chemical composition of *O. onites* essential oil

RRI *	**Compounds**	**%****	**I*****
1018	Methyl 2-methylbutyrate	0.10± 0.00	a
1032	α-Pinene	0.33± 0.05	a,b
1035	α -Thujene	0.10± 0.00	a
1076	Camphene	0.10± 0.00	a,b
1118	β-Pinene	0.10± 0.00	a,b
1174	Myrcene	0.43± 0.05	a,b

1176	α-Phellandrene	0.10± 0.00	a,b
1188	α-Terpinene	0.56±0.05	a,b
1203	Limonene	0.10± 0.00	a,b
1213	1,8-Cineole	0.40± 0.00	a,b
1255	γ-Terpinene	1.13± 0.09	a,b
1280	*p*-Cymene	4.33± 0.31	a,b
1290	Terpinolene	0.10± 0.00	a,b
1393	3-Octanol	0.10± 0.00	a
1452	1-Octen-3-ol	0.20± 0.00	a
1474	*trans*-Sabinene hydrate	0.40± 0.00	a
1478	*cis*-Linalool oxide (*Furanoid*)	0.10± 0.00	a
1553	Linalool	9.00± 0.14	a,b
1556	*cis*-Sabinene hydrate	0.10± 0.00	a
1611	Terpinen-4-ol	0.70± 0.00	a,b
1612	β-Caryophyllene	0.90± 0.00	a,b
1624	*trans*-Dihydrocarvone	0.10± 0.00	a
1628	Aromadendrene	0.26± 0.05	a
1638	*cis-p*-Menth-2-en-1-ol	0.10± 0.00	a
1645	*cis*-Isodihydrocarvone	0.10± 0.00	a
1687	α-Humulene	0.10± 0.00	a,b
1706	α-Terpineol	0.80± 0.00	a,b
1719	Borneol	0.50± 0.00	a,b
1751	Carvone	0.20± 0.00	a,b
1773	δ-Cadinene	0.10± 0.00	a
1776	γ-Cadinene	<0.1	a
1864	*p*-Cymen-8-ol	0.10± 0.00	a,b
1940	4-Isopropyl salicylaldehyde	0.10± 0.00	a
2008	Caryophyllene oxide	0.10± 0.00	a,b
2144	Spathulenol	0.10± 0.00	a,b
2181	Isothymol (*=2-Isopropyl-4-methyl*	<0.1	a,b

	phenol)		
2198	Thymol	1.90± 0.00	a,b
2221	Isocarvacrol (*=4-Isopropyl-2-methyl*		a,b
	phenol)	<0.1	
2239	Carvacrol	75.70± 0.65	a,b
	Total	99.63± 0.05	

*RRI Relative retention indices calculated against *n*-alkanes on the HP Innowax column;
** mean % calculated from Flame Ionization Detector (FID) data ± SD (n=3); I***=identification method; a= comparison of mass spectra with the Wiley and Mass Finder libraries and retention times; b= comparison with genuine compounds on the HP Innowax column.

Table S2. Minimum effective dosage (MED) of *O. onites* EO and its some of individual constituents tested against *Ae. aegypti*

Samples	**CAS #**	**Minimum Effective Dose (MED) mg/cm^2** ± SE
O. onites EO		0.011 ± 0.000
(-)-β-Pinene	18172-67-3	0.140 ± 0.047
Carvacrol	499-75-2	0.013 ± 0.005
β-Caryophyllene	87-44-5	Not Active up to 1.5
Caryophyllene oxide	1139-30-6	Not Active up to 1.5
1,8-Cineole	470-82-6	0.500 ± 0.217
p-Cymene	99-87-6	Not active up to 1.5
α-Humulene	6753-98-6	Not Active up to 1.5
(-)-Linalool	126-91-0	0.125 ± 0.054
α-Terpinene	99-86-5	1.5 ± 0.000
γ-Terpinene	99-85-4	1.5 ± 0.000
(-)-Terpinen-4-ol	20126-76-5	0.109 ± 0.041
(+)-Terpinen-4-ol	2438-10-0	0.086 ± 0.051
α-Terpineol	10482-56-1	0.039 ± 0.008
Terpinolene	586-62-9	0.023 ± 0.000
Thymol	89-83-8	0.031 ± 0.008
DEET (positive control)	134-62-3	0.007 ± 0.002

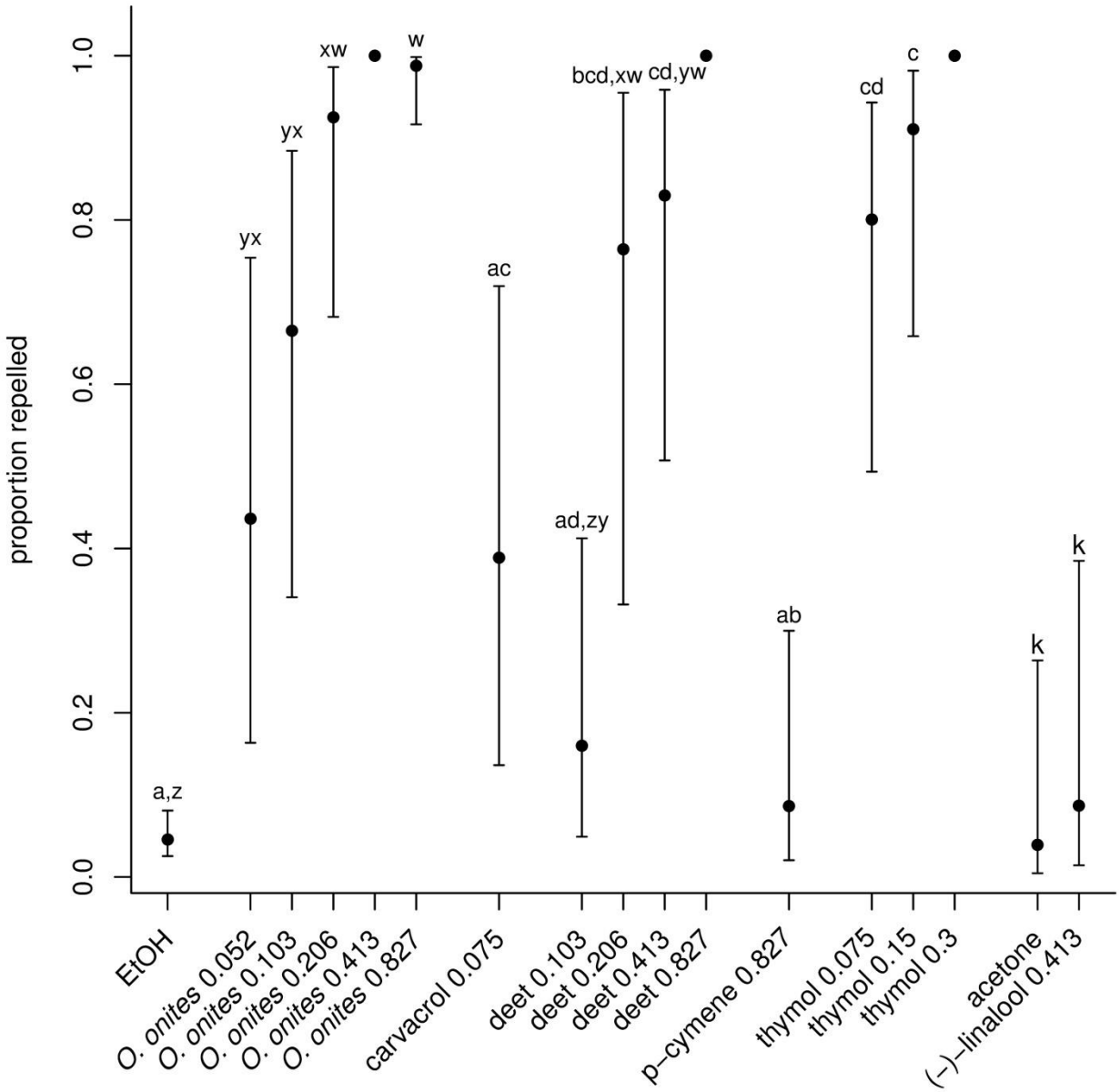

Figure S1. Responses of *Am. americanum* nymphs to *O. onites* EO, its major constituent compounds [carvacrol, (-)-linalool, *p*-cymene, thymol], DEET, and ethanol and acetone controls. Concentrations, as milligrams of oil or compound per centimeter squared of filter paper, of test solutions. We previously reported the (-)-linalool data in Tabanca et al. (2013). (-)-Linalool was tested in acetone solutions, and therefore paired with acetone on the extreme the right of the plot.