Comparative Investigation of *Umbellularia californica* and *Laurus nobilis* Leaf Essential Oils and Identification of Constituents Active against *Aedes aegypti*

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ABSTRACT: *Umbellularia californica* (California bay laurel) and *Laurus nobilis* (Mediterranean bay laurel) leaves may be mistaken or used as a substitute on the market due to their morphological similarity. In this study, a comparison of anatomical and chemical features and biological activity of both plants is presented. *L. nobilis* essential oil biting deterrent and larvicidal activity were negligible. On the other hand, *U. californica* leaf oil showed biting deterrent activity against *Aedes aegypti*. The identified active repellents was thymol, along with (−)-umbellulone, 1,8-cineole, and (−)-α-terpineol. *U. californica* essential oil also demonstrated good larvicidal activity against 1-day-old *Ae. aegypti* larvae with a LD_{50} value of 52.6 ppm. Thymol (LD_{50} = 17.6 ppm), *p*-cymene, (−)-umbellulone, and methyleugenol were the primary larvicidal in this oil. Umbellulone was found as the principal compound (37%) of *U. californica* essential oil, but was not present in *L. nobilis* essential oil. Umbellulone mosquito activity is here reported for the first time.

KEYWORDS: *Umbellularia californica, Laurus nobilis, Aedes aegypti*, (−)-umbellulone, (−)-terpinen-4-ol, (−)-α-terpinenol, thymol, mosquito control, leaf anatomy

■ INTRODUCTION

Natural products from plants have the potential to provide a green alternative to conventional insecticides. In particular, plant species that have chemical defense mechanisms against micro-organisms and predators could represent a new source of control against a wide variety of insect vectors.[1,2] The synthetic repellents and insecticides commonly available on the market are raising concerns because of the toxic effects sometimes experienced by the users. They can cause nonspecific target effects as well as possible development of insecticide resistance.[1−3] Much effort has been focused recently on plant extracts/essential oils or phytochemicals as potential sources of mosquito-control agents or bioactive chemical compounds.[4−8] *Aedes aegypti* L. is one of the most common mosquito species and is often responsible for the transmission of both dengue fever and dengue hemorrhagic fever. Because there are no vaccines available for any disease carried by insects, personnel protection and mosquito control at the larval stages are important strategies to prevent vector-borne diseases.[3,4,7,8]

Umbellularia californica (Hook. & Arn.) Nutt. is a strongly aromatic shrub belonging to the monotypic genus *Umbellularia* (Lauraceae).[9] Native to southwestern Oregon and northern California, this plant has several names that evoke the shape and texture of its leaves (California myrtle, California laurel, California bay, myrtlewood, and sassafras laurel) and also its alleged headache-inducing properties (headache tree).[10] Traditional uses of *U. californica* are strongly related to the aromatic properties of the leaves. Native Americans used it as spice and for the treatment of rheumatism,[9] whereas the first European settlers used it as deer repellent and insecticide.[10−13] The leaves have been used by Costanoan Indians in California to repel fleas and to eliminate ground squirrel problems, and decoctions were traditionally used as a wash to treat poison oak dermatitis.[14] Even though the traditional use of the plant has a long history, the potential uses of *U. californica* as insecticide and repellent have yet to be scientifically proven. Earlier chemical studies reported volatile constituents,[12] alkaloids[15] and flavonoids,[16] as major constituents of California bay laurel. *U. californica* leaves are similar in appearance and flavor to the Mediterranean bay *Laurus nobilis* L. (Lauraceae) (sweet bay, Mediterranean bay), although the former has a stronger or more pungent odor. This similarity can lead to misidentification of *U. californica* with sweet bay, which is extensively used in the food industry.[17] The differentiation of both species is very important because of their chemical diversity, which includes the unique, irritant compound umbellulone present only in *U. californica*. Umbellulone is a volatile monoterpene that can cause intensive headache in some sensitive individuals.[18]

Recent interest in developing plant-based insecticides led us to a further and deeper investigation of the deterrent and larvicidal activity of *U. californica* essential oil against *Ae. aegypti*. The present study deals with (i) the comparison of the chemical composition of *U. californica* and *L. nobilis* essential

Table 1. Composition of *U. californica* and *L. nobilis* Leaf Essential Oils

no.	compound	RRI[a]	RRI[b]	U[c]%[d]	L[e]%[d]	identification[f]
1	α-pinene	1032	939	0.1	3.8	t_R, MS
2	α-thujene	1035			0.4	t_R, MS
3	β-pinene	1118	979	0.1	3.6	t_R, MS
4	sabinene	1132	975	0.1	5.7	t_R, MS
5	myrcene	1174	991	0.2		t_R, MS
6	α-terpinene	1188	1017	0.2	0.2	t_R, MS
7	dehydro-1,8-cineole	1195			0.9	MS
8	limonene	1203	1029	0.1	1.0	t_R, MS
9	1,8-cineole	1213	1031	19.5	57.4	t_R, NMR, MS
10	γ-terpinene	1255	1060	0.3	0.3	t_R, MS
11	p-cymene	1280	1025	2.1	2.2	t_R, MS
12	terpinolene	1290	1089	0.1	0.1	t_R, MS
13	α,p-dimethylstyrene	1452		0.1		MS
14	trans-sabinene hydrate	1474	1098	0.1	0.6	MS
15	camphor	1532		0.2		t_R, MS
16	linalool	1553	1097	0.4	0.3	t_R, MS
17	cis-sabinene hydrate	1556	1070	0.1	0.6	MS
18	trans-p-menth-2-en-1-ol	1571		0.1	0.2	MS
19	pinocarvone	1586			0.3	t_R, MS
20	bornyl acetate	1591			0.2	t_R, MS
21	terpinen-4-ol	1611	1177	6.6	4.0	t_R, MS
22	cis-p-menth-2-en-1-ol	1638		0.1		MS
23	trans-p-mentha-2,8-dien-1-ol	1639			0.5	MS
24	thuj-3-en-10-al	1642			0.5	MS
25	myrtenal	1648			0.8	MS
26	umbellulone	1657	1171	36.7		t_R, NMR, MS
27	trans-pinocarveol	1670			0.5	t_R, MS
28	δ-terpineol	1682		0.6	0.9	MS
29	α-terpineol	1706	1189	6.5	3.8	t_R, MS
30	α-terpinyl acetate	1709			7.0	t_R, MS
31	borneol	1719			0.1	t_R, MS
32	β-bisabolene	1741	1506	2.2		MS
33	phellandral	1744		0.1	0.4	MS
34	(E)-α-bisabolene	1784		0.5		MS
35	ar-curcumene	1786		0.1		MS
36	myrtenol	1804			0.3	t_R, MS
37	nerol	1808		0.1		t_R, MS
38	trans-p-mentha-1(7),8-dien-2-ol	1811			0.3	MS
39	p-mentha-1,5-dien-7-ol	1814			0.2	MS
40	2-tridecanone	1815		0.1		MS
41	trans-carveol	1845			0.1	t_R, MS
42	p-cymen-8-ol	1864		0.2	0.2	t_R, MS
43	cis-p-mentha-1(7),8-diene-2-ol	1896			0.3	MS
44	cuminyl acetate	1981			0.1	t_R, MS
45	caryophyllene oxide	2008	1583	tr	0.1	t_R, MS
46	methyleugenol	2030	1404	8.4	0.9	t_R, MS
47	(E)-nerolidol	2050	1563	0.3		t_R, MS
48	p-mentha-1,4-dien-7-ol	2073			0.3	MS
49	elemol	2096	1550	0.4		MS
50	cumin alcohol	2113		0.5	0.1	t_R, MS
51	cis-p-menth-3-en-1,2-diol	2184		tr		MS
52	eugenol	2186	1359	0.4	0.1	t_R, MS
53	γ-eudesmol	2185		0.2		MS
54	thymol	2198	1290	7.8		t_R, MS
55	carvacrol	2239	1299	tr		t_R, MS
56	elemicine	2245	1557	0.1		MS
57	α-eudesmol	2250	1654	0.1		MS
58	β-eudesmol	2257	1651	0.2	0.3	MS
59	chavicol	2353		0.2		MS
60	dodecanoic acid	2503		0.3		t_R, MS
61	hexadecanoic acid	2931		0.1		t_R, MS
	total			97.1	98.9	

[a]RRI, relative retention indices calculated against *n*-alkanes on polar column. [b]RRI, relative retention indices calculated against *n*-alkanes on apolar column (Adams, 2001). [c]U, *Umbellularia californica*. [d]%, calculated from FID data for polar column; tr, trace (<0.1%). [e]L, *Laurus nobilis*. [f]Identification method: t_R, identification based on the retention times (t_R) of genuine compounds on the HP Innowax column; MS, identified on the basis of computer matching of the mass spectra with those of the Wiley and MassFinder libraries and comparison with literature data.

oils, (ii) the comparative leaf anatomy of *U. californica* and *L. nobilis*, (iii) the evaluation of both essential oils for mosquito bioassays, (iv) the follow-up bioassay-guided investigation of *U. californica* to identify active biting deterrent and larvicidal compounds in the essential oil, and (v) the determination of the chiral distribution of terpene enantiomers in *U. californica* essential oil.

■ MATERIALS AND METHODS

Chemicals. Thymol (CAS Registry No. 89-83-8), methyleugenol (CAS Registry No. 93-15-2), (−)-terpinen-4-ol (CAS Registry No. 20126-76-5), (+)-terpinen-4-ol (CAS Registry No. 2438-10-0), (+)-α-terpineol (CAS Registry No. 7785-53-7), and α-terpineol (CAS Registry No. 10482-56-1) were purchased from Sigma-Aldrich Co., St. Louis, MO, USA.

General Experimental Procedure. Reversed phase RP C_{18} silica (Polarbond, J. T. Baker) was used for fractionation and purification of umbellulone. Optical rotations were measured on a Rudolph Research Analytical digital polarimeter at 589 nm and 20 °C, using a 2 cm path length microcell. [1]H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded on American Varian Mercury plus 400 NMR spectrometers.

Plant Material. *U. californica* leaves were collected from the University of Genoa Botanical Garden, Italy. A voucher specimen (UC042011) was deposited at the DISCAFF, University of Eastern Piedmont, Novara, Italy. *L. nobilis* leaves were collected from Antalya, Turkey, and a voucher specimen (ESSE 14680) was deposited in the Herbarium at the Faculty of Pharmacy of Anadolu University in Eskisehir, Turkey.

Leaf Anatomy. Fresh leaves were collected from plants of *L. nobilis* grown in the Maynard W. Quimby Medicinal Plant Garden, University of Mississippi, and used for anatomical study. Fresh leaves of *U. californica* from their native region were purchased online. Voucher samples of both *L. nobilis* (NCNPR No. 15775) and *U. californica* (NCNPR No. 15780) were deposited in the Botanical Repository of National Center for Natural Products Research (NCNPR) at the University of Mississippi.

Freshly collected leaves were fixed in formalin−acetic acid−alcohol (FAA) for 2 days and washed in distilled water. The samples were

transferred to 30% alcohol solution and then to 50% alcohol before sections were taken. Hand-sectioning was done using razors, and the sections were clarified with chloral hydrate solution. The sections were stained with phloroglucinol/HCl to identify lignified tissues. A drop of 1% iodine solution was added to fresh sections to detect the presence of starch grains. The stained sections were mounted in glycerin on a glass slide, covered, and viewed and photographed using a Nikon Eclipse E600 microscope equipped with a Nikon DS-Fi1 camera system.

Isolation of The Essential Oils. Air-dried *U. californica* leaves (950 g) were water distilled for 10 h using a Clevenger-type apparatus. The distilled oil was diluted with 3 volumes of methylene chloride, and the organic phase was separated (three times). The collected organic phase was treated with Na_2SO_4 to remove traces of water. The solvent was evaporated (at temperature below 40 °C) under vacuum to obtain 50.2 g of a yellowish essential oil. *L. nobilis* leaf essential oil was provided by the repository from the Faculty of Pharmacy of Anadolu University in Eskişehir, Turkey.

Gas Chromatography and Gas Chromatography–Mass Spectrometry Analysis of Essential Oils. *U. californica* essential oil was analyzed by using an Agilent 6890N GC equipped with both a 5975 MSD and a flame ionization detector (FID) with a polar Innowax FSC column (60 m × 0.25 mm, 0.25 μm). The GC oven temperature were 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. The split ratio was 40:1, and the injector temperature was 250 °C. FID temperature was 300 °C.

An auxiliary Shimadzu QP2010 Plus GC-MS system was used with an apolar CPSil-5CB (25 m × 0.25 mm, 0.25 μm). Helium was used as the carrier gas (0.8 mL/min). The oven temperature was programmed from 60 to 260 °C at a rate of 5 °C/min and kept constant at 260 °C for 20 min. Split ratio was 50:1. The injector temperature was set at 200 °C. Mass spectra were recorded at 70 eV, and mass range was from m/z 35 to 450.

To obtain the same elution order with GC-MS, simultaneous autoinjection was done on a duplicate of the same column by applying the same operational conditions. Relative percentage amounts of the separated compounds were calculated from the FID chromatograms. The analysis results are given in Table 1. Identification of the *U. californica* essential oil components was confirmed by comparison of their relative retention times with those of authentic samples on polar (Innowax FSC) and apolar (CPSil-5CB) columns with their relative retention index (RRI). Identification was made by computer matching of the recorded mass spectra with those stored in the Wiley GC-MS Library, Adams Library, MassFinder 3 Library),[19−21] and in-house "Başer Library of Essential Oil Constituents" built from genuine compounds and components of known oils, as well as MS literature data.[22,23]

L. nobilis essential oil was analyzed using the same Agilent 6890N GC coupled with 5975 MSD instrumentation, methods, and columns (Table 1).

Fractionation and Purification of the Bioactive Compounds. Two grams of *U. californica* leaf essential oil was purified by isocratic elution on 40 g of RP-C_{18} silica gel CC (column diameter = 3 cm, 1:20 w/w) using 50% water in methanol. Collected fractions were joined according to their TLC profile to give four major fractions (A1−A4). The obtained fractions were extracted with 3 volumes of dichloromethane. The collected organic phase for each fraction was treated with Na_2SO_4 to remove traces of water and filtered, and the solvent was evaporated under vacuum (below 40 °C). The collected fractions were A1 (0.737 g), A2 (0.158 g), A3 (0.316 g), and A4 (0.395 g), respectively. All of the fractions were analyzed by GC-MS to identify their constituents. Fraction A1 contained umbellulone, whereas 1,8-cineole was found in fraction A2, and their NMR data were compared to the literature.[24,25]

Umbellulone: ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.32 (s, 1H), 2.15 (1H, hept, J = 6.9 Hz), 2.11 (3H, s), 2.07 (1H, dd, J = 7.0, 3.2 Hz), 1.36 (1H, dd, J = 7.0, 3.7), 1.23 (1H, t, J = 3.4 Hz), 1.03 (3H, d, J = 6.9 Hz), 0.96 (3H, d, J = 6.9 Hz); ^{13}C NMR (101 MHz, $CDCl_3$) δ 208.1,

177.8, 124.1, 40.7, 38.1, 29.0, 26.4, 20.3, 19.4, 18.6; MS m/z (relative intensity) 150 [M]$^+$ for $C_{10}H_{14}O$ (16), 135 (23), 122 (9), 121 (70), 115 (4), 109 (13), 108 (100), 107 (94), 105 (17), 94 (10), 91 (52), 80 (14), 79 (28), 77 (21), 67 (80), 65 (90), 53 (6), 51 (95), 41 (90), 39 (11); R_f = 0.22 (*n*-hexane/ethyl acetate, 90:10 v/v); UV absorbance at $\lambda_{254\ nm}$ revealed a yellow to orange spot with anisaldehyde stain solution; $[\alpha]_D^{20}$ = −29.4 (*c* 1.54 g/100 mL in CH_2Cl_2).

1,8-Cineole: ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.02 (2H, ddd, J = 11.2, 9.0, 4.2 Hz), 1.71−1.57 (2H, m), 1.55−1.35 (5H, m), 1.24 (6H, s) 1.05 (3H, s); ^{13}C NMR (101 MHz, $CDCl_3$) δ 73.8, 69.9, 33.1, 33.0, 31.6, 29.0 (2C), 27.7, 23.0, 22.9.

Gas Chromatography–Mass Spectrometry Analysis for Subfractions (A1–A4) and Isolated Compounds. The analyses were performed on a third GC-MS instrument. The system consisted of an Agilent 7890 GC instrument equipped with an Agilent 5975C mass selective detector and an Agilent 7693 autosampler. A fused silica capillary column (30 m, 0.25 mm i.d.) coated with a 0.25 μm film of cross-linked 5% phenyl methyl silicone (J&W HP-5MS) was used with helium as the carrier gas at a flow rate of 1 mL/min. The oven temperature was kept at 50 °C for 2 min and programmed to 100 °C at a rate of 2 °C/min and then programmed to 200 °C at a rate of 2 °C/min. The injector temperature was 250 °C. The split ratio was set to 25:1. Components of the subfractions (A1−A4) were identified by comparing the retention indices and mass spectra of each analyte with reference standards purchased from Sigma-Aldrich, Inc., and from National Center for Natural Products Research, University of Mississippi, repository and NIST library searches.

Chiral Separation of Umbellulone, Terpinen-4-ol, and α-Terpineol. Chiral separations were performed using the same Agilent 7890/5975 GC-MS system with an Agilent HP-Chiral-20B (30 m × 0.25 mm, i.d. 0.25 μm) (Agilent Technologies, Inc. New Castle, DE, USA) column. The chiral column was maintained at 50 °C for 2 min, programmed to 70 °C at 1 °C/min, to 160 °C at 2 °C/min, and then to 180 °C at 5 °C/min. The split flow was adjusted to 50:1. Mass spectra were recorded at 70 eV with the mass range m/z 35−400. The injection volume was 1 μL, and helium was used as the carrier gas (3.0 mL/min). The *U. californica* essential oil was compared with authentic standards of (+)-terpinen-4-ol, (−)-terpinen-4-ol, (+)-α-terpineol, and (−)-α-terpineol to confirm their enantiomeric identity. The identification of (−)-umbellulone was confirmed by optical rotation and NMR data.

Bioassays. *Ae. aegypti* used in larvicidal and biting deterrent bioassays originated from a laboratory colony maintained at the Mosquito and Fly Research Unit at the Center for Medical, Agricultural and Veterinary Entomology, U.S. Department of Agriculture, Agriculture Research Service, Gainesville, FL, since 1952 using standard procedures.[26]

Mosquito Biting Bioassays. Mosquitoes were reared to the adult stage by using the method of Ali et al.[27] Adult mosquitoes were fed from cotton pads moistened with 10% sucrose solution placed on the top of screens of 4 L cages. The 8−15-day-old mated females used in these bioassays were deprived of sucrose for 24 h prior to the test but had free access to water-soaked cotton. A six-celled Klun & Debboun (K&D) module bioassay system[28] was used to quantify the biting deterrence of *U. californica* essential oil, subfractions, pure compounds, and *L. nobilis* essential oil. The K&D system consisted of a six-well reservoir with each of the 4 × 3 cm wells containing 6 mL of feeding solution. As reported previously by Ali et al.,[27] a citrate−phosphate−dextrose−adenine (CPDA-1) plus ATP solution was used instead of human blood. CPDA-1 and ATP preparations were freshly made on the day of the test, and the mixture contained a red dye for verifying whether mosquitoes had imbibed the solution. DEET (97% purity *N,N*-diethyl-*m*-toluamide) was obtained from Sigma-Aldrich and used as a positive control. Molecular biology grade ethanol was obtained from Fisher Scientific Chemical Co. (Fair Lawn, NJ, USA). Stocks and dilutions of essential oils, subfractions, individual compounds, and DEET were prepared in ethanol. Stock solutions were kept in a refrigerator set at 3−4 °C. During the bioassay, the temperature of the solution in the reservoirs covered with a collagen membrane was maintained at 37.5 °C by circulating water through the reservoir with a

temperature-controlled circulatory bath. The test compounds and controls were randomly applied to six 4 × 3 cm marked portions of nylon organdy strip, which was positioned over the six, membrane-covered wells. A Teflon separator was placed between the treated cloth and the module. The K&D module containing five females of *Ae. aegypti* per cell was positioned over the six wells. Trap doors were opened and mosquitoes allowed access for 3 min, after which they were collected back into the module. Mosquitoes were squashed, and the presence of red dye in the gut was used as an indicator of feeding. A replicate consisted of six treatments: four treatments (samples), DEET (positive control), and 100% ethanol (solvent control). Five replicates were conducted per day using new batches of mosquitoes for each. Bioassays were conducted between 1:00 and 4:00 p.m., and 10 replications were conducted for each treatment.

Larval Bioassays. Bioassays were conducted using the system the described by Pridgeon et al.[26] to determine the larvicidal activity of individual compounds and essential oils against *Ae. aegypti*. Eggs were hatched, and larvae were held in a room maintained at a temperature of 27 ± 2 °C with 60 ± 10% relative humidity. Five 1-day-old larvae were transferred to individual wells of a 24-well tissue culture plates in a 30−40 μL droplet of water. Fifty microliters of larval diet of 2% slurry of 3:2 beef liver powder (Now Foods, Bloomingdale, IL, USA) and brewer's yeast (Lewis Laboratories Ltd., Westport, CT, USA) and 1 mL of deionized water were added to each well by using a Finnpipette stepper (Thermo Fisher, Vantaa, Finland). *U. californica* essential oil, subfractions, pure compounds, and *L. nobilis* essential oil were diluted in DMSO. Eleven microliters of the test chemical was added to the labeled wells, whereas 11 μL of DMSO was added to control treatments. After treatment application, the plates were swirled to ensure even mixing of the test compounds. Larval mortality was recorded 24 and 48 h post treatment. Larvae that showed no movement in the well after manual disturbance of water were recorded as dead. A series of five concentrations ranging between 250 and 6.25 ppm were used in each treatment to get a range of mortality between 0 and 100%. Treatments were replicated 10−20 times for each sample.

Statistical Analyses. Proportion not biting (PNB) was calculated using the following formula:

$$PNB = 1 - \left(\frac{\text{total no. of females biting}}{\text{total no. of females}} \right)$$

PNB data were analyzed using SAS Proc ANOVA,[29] and means were separated using the Ryan−Einot−Gabriel−Welsch multiple-range test. Control mortality was corrected by using Abbott's formula. LD_{50} values for larvicidal data were calculated by using SAS, Proc Probit.[29]

RESULTS AND DISCUSSION

U. californica leaf essential oil was analyzed by GC-FID and GC-MS using polar and apolar columns. Forty-seven compounds representing 97.1% of the oil were identified by using the polar column. Umbellulone (36.7%), 1,8-cineole (19.5%), methyleugenol (8.4%), thymol (7.8%), terpinen-4-ol (6.6%), and α-terpineol (6.5%) were found to be the major compounds (Table 1). The main constituent, umbellulone, was found as a distinguishing compound for *U. californica* essential oil, whereas it was not present in *L. nobilis*. Umbellulone has a strong, camphor-like odor, and it is one of the major compounds responsible for the strong scent of *U. californica* leaves after crushing. The percentage of umbellulone in the essential oil can vary from 30 to 70% depending on the geographical origin and time of collection.[30,31] Umbellulone can spontaneously convert to thymol in the presence of light;[32,33] thus, the presence and abundance of thymol is highly dependent on the initial content of umbellulone and the age of the oil. Kelsey reported umbellulone present as a minor compound (2.2%) in *U. californica* bark essential oil, whereas leaf oil contained umbellulone up to 41.0%.[31] Umbellulone was previously found to activate the trigeminovascular system by

acting on TRPA1 receptors[18] and can affect respiration, heartbeat, and blood circulation in laboratory animals.[34] Macgregor et al. reported that the acute oral toxicity of California bay oil (*U. californica*) in mice was higher than that of Mediterranean bay oil (*L. nobilis*). Subfractions containing primarily umbellulone demonstrated significant toxicity in mice.[13] Therefore, umbellulone content in the oil is an important parameter in the toxicological profile of the oil. In the current study, the absence of umbellulone in *L. nobilis* leaf essential oil was confirmed. Both *U. californica* and *L. nobilis* essential oils were analyzed using the same column and methods (Figure 1). Thirty-seven compounds were identified
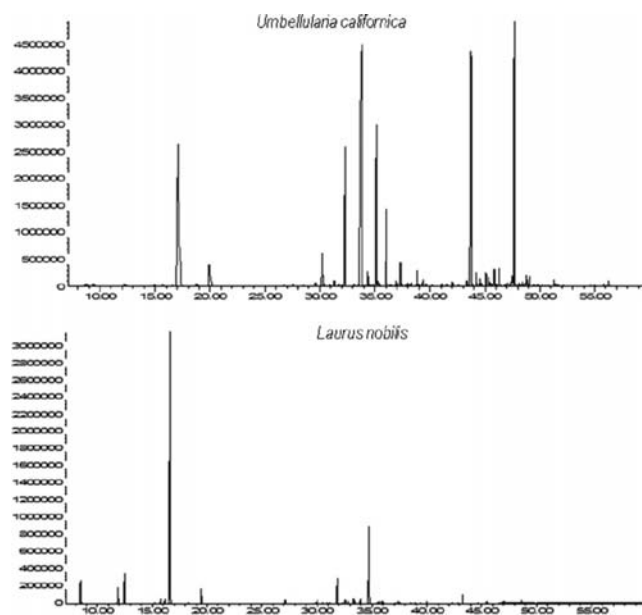

Figure 1. Comparison GC-FID chromatograms of *U. californica* and *L. nobilis* leaf essential oils.

in the oil of *L. nobilis*, representing 98.9% of the oil. 1,8-Cineole (57.4%), α-terpinyl acetate (7.0%), sabinene (5.7%), and terpinen-4-ol (4.0%) were characterized as the major compounds in *L. nobilis* oil (Table 1).

In the *U. californica* essential oil, a peak at 33.67 min was determined to represent umbellulone, and this compound was not detected in *L. nobilis* oil. These results are in agreement with previous studies.[12,31] Two compounds eluted at 33.46 and 33.54 min in *L. nobilis* oil; however, their mass spectra did not match that of umbellulone (Figure 2). The compounds in *L. nobilis* were found to be *trans*-pinocarveol and an unknown terpene.

We also compared the leaf anatomy of *U. californica* and *L. nobilis* (Table 2; Figure 3). The leaves of the two species show similarities in their basic anatomical features. The leaves are dorsiventral in cross section. Both adaxial and abaxial epidermises are unilayered, covered externally by a thick layer of cuticle. Stomata are partially sunken, present only in the abaxial epidermis. Mesophyll is made up of palisade and spongy tissues. Spherical oil cells are distributed in the mesophyll and often contain droplets of essential oil. Vascular bundles to veinlets traverse the lamina and are enclosed by an unilayered sheath that is connected to both the epidermises by sclerenchyma cells. A major portion of the midrib is occupied by a large vascular bundle, which is abutted above and below by groups of thick-walled sclerenchyma. Xylem is positioned

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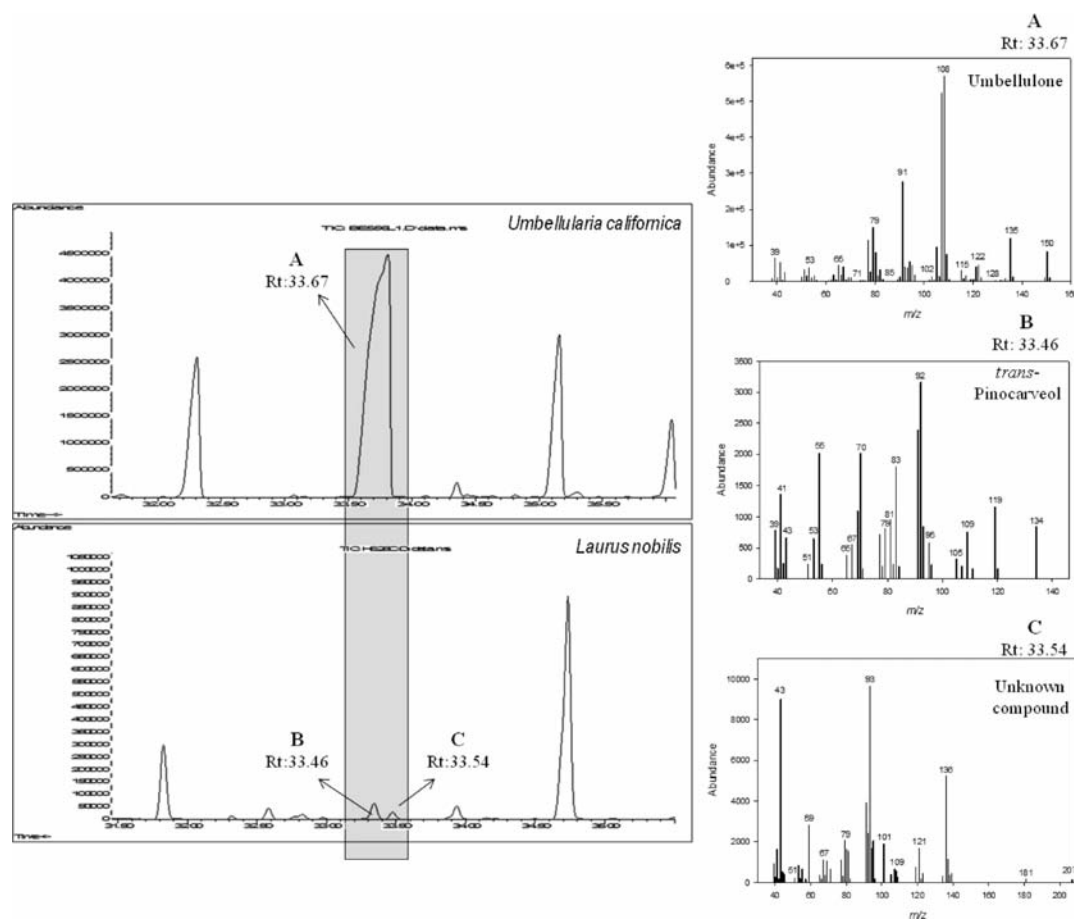
Figure 2. Chromatographic profiles of *U. californica* and *L. nobilis* essential oil on HP-Innowax column. The shaded areas represent region selected for marker compound (umbellulone) for identification of *U. californica* and *L. nobilis* leaf essential oils.

**Table 2. Differences in the Leaf Anatomy of *Umbellularia californica* and *Laurus nobilis***

leaf anatomical feature	U. californica	L. nobilis
lamina	200−**240**−300 μm thick	270−**310**−400 μm thick
oil cells in mesophyll	abundant, 44−55 μm in diameter	infrequent, 35−53 μm in diameter
palisade tissue	usually 1-, 2-, rarely 3-layered; outermost layer cells 30−42 × 8−16 μm	usually 2-,3-layered; outermost layer cells 38−70 × 9−19 μm
starch granules	occasional	abundant
cuticle	smooth or granular, 4−5 μm thick	striated, 4−10 μm thick
annular collenchyma in the midrib	many	few
midrib ground tissue	almost all the cells are moderately lignified	only some of the cells are thinly lignified
trichomes	unicellular trichomes are occasionally present on abaxial epidermis	not observed

toward the adaxial side of the leaf, and an arc-like phloem band occurs below xylem. Xylem vessels are arranged radially, often separated by rays of sclerenchyma cells. Small oil cells with yellow substance and sometimes with oil droplets are present in xylem as well as phloem. Groups of collenchyma cells present inner to both epidermises. Parenchyma cells of the ground tissue are circular with thick and reticulate-pitted walls. However, the leaves of the two species show differences in some of their anatomical features as given in Table 2.

In the search for environmentally safe and effective ways of controlling mosquitoes, *U. californica* and *L. nobilis* leaf essential oils were evaluated for their biting deterrent effects and larvicidal activity against 1-day-old *Ae. aegypti*. The *U. californica*

oil showed better activity than *L. nobilis* essential oil, which demonstrated only a weak biting deterrent activity (Figure 4), and *L. nobilis* oil failed in the prescreening larvicidal bioassays against 1-day-old *Ae. aegypti* larvae. Therefore, no further investigations were performed on *L. nobilis* essential oil in either biting deterrent or larvicidal bioassays. The *U. californica* oil showed higher biting deterrent activity at 10 μg/cm² than *L. nobilis* essential oil, and bioassay-guided isolation studies were performed on *U. californica* oil, which resulted in four major subfractions (A1−A4). All fractions were analyzed by GC-MS, and their pure compounds were tested for mosquito biting deterrent activity. Subfractions A1 and A2 were found to contain umbellulone and 1,8-cineole, respectively. Subfraction
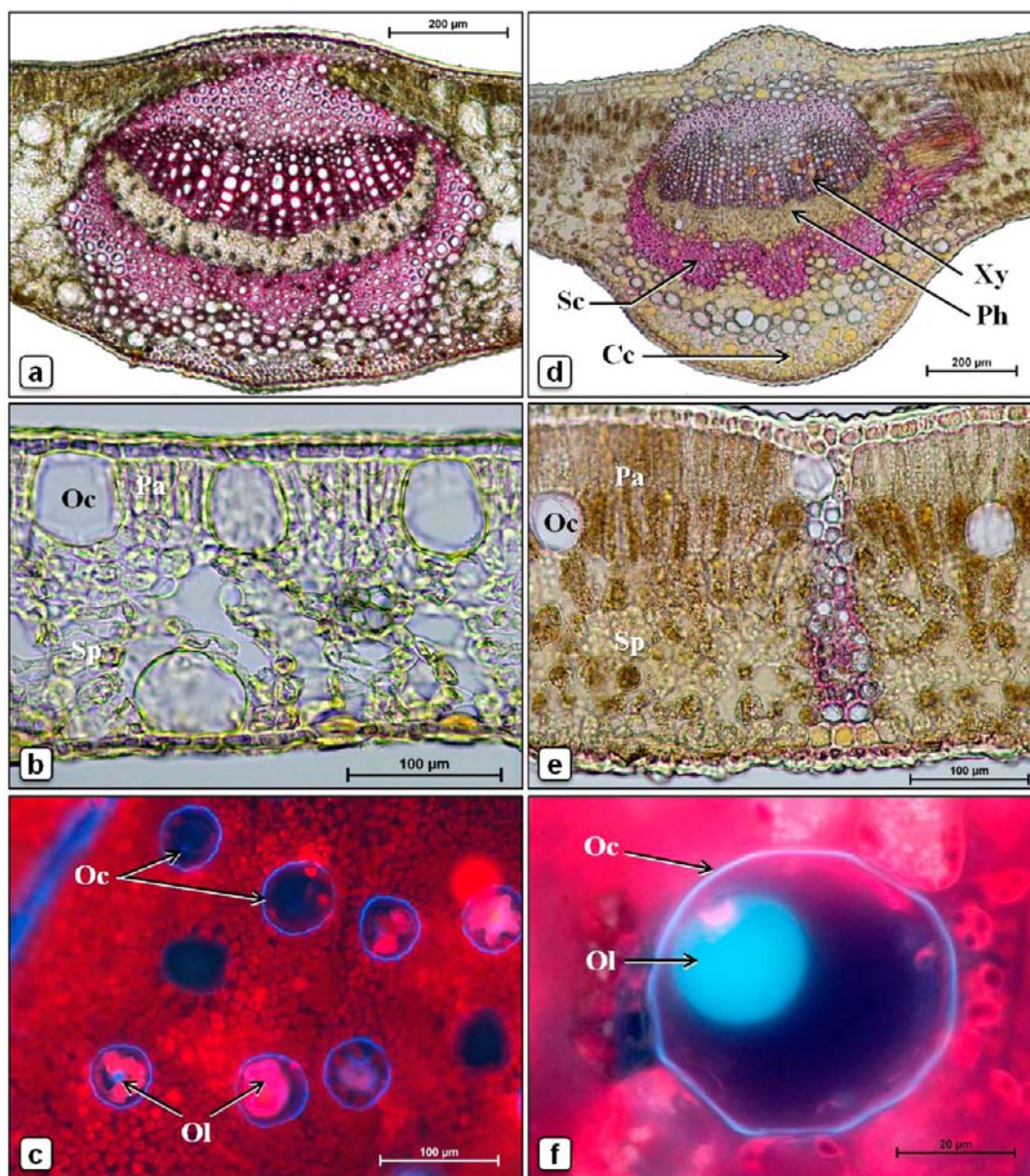
Figure 3. Comparative anatomy of the leaves of *U. californica* (a−c) and *L. nobilis* (d−f) [(a, b, d, e) light microscopy; (c, f) fluorescence microscopy, UV EX 450−490 and UV 330−380, respectively; (a, d, e) stained in phloroglucinol/HCl]: (a, d) transverse section (TS) of leaf through the midrib showing a single vascular bundle and a thick sheath of sclerenchyma; (b, e) TS of leaf through lamina showing oil cells in the mesophyll; (c) portion of leaf showing abundant oil cells in the mesophyll, containing essential oil; (f) enlarged view of oil cell containing oil droplet as seen in TS of lamina. Scale bars: (a, d) 200 μm; (b, c, e) 100 μm; (f) 20 μm. Cc, collenchyma; Oc, oil cell; Ol, oil droplet; Pa, palisade tissue; Ph, phloem; Sc, sclerenchyma; Sp, spongy tissue; Xy, xylem.

A3 contained mainly terpinen-4-ol, α-terpineol, thymol, and methyleugenol; A4 was rich in *p*-cymene and other minor monoterpene hydrocarbons. Subfractions A1, A2, and A3 showed biting deterrent activity; however, fraction A4 did not show any biting deterrent activity. Because the biological activities can depend on the enantiomeric purity of the compounds, the enantiomeric distributions of terpinen-4-ol and α-terpineol in subfraction A3 were determined by comparison of the authentic chiral compounds using the same chiral capillary column coated with β-cyclodextrin with the same GC parameters (Figure 5). The enantiomeric enrichment of (*R*)-(−)-terpinen-4-ol (73.74%) versus (*S*)-(+)-terpinen-4-ol (26.26%), as well as that of (*S*)-(−)-α-terpineol (87.78%) versus (*R*)-(+)-α-terpineol (12.22%), was

observed in chiral separation of *U. californica* oil. We confirmed (*R*)-(−)-umbellulone to be enantiomerically pure (100%) in the oil and verified by optical rotation measurements. In biting deterrent bioassays, all pure compounds were tested at 25 nmol/cm^2, including DEET as a positive control, and ethanol was used as a solvent control. The biting deterrent activity of *U. californica* essential oil and its individual compounds was significantly higher than the solvent control (*F* value = 51.8; df = 9,150; $P \leq 0.0001$), whereas the biting deterrent activity was lower than that of the positive control, DEET. Thymol showed the highest biting deterrent activity, followed by methyleugenol, (−)-terpinen-4-ol, 1,8-cineole, (−)-α-terpineol, and (−)-umbellulone. Neither active compound, thymol and umbellulone (which together account for >44% of *U. californica* oil), was
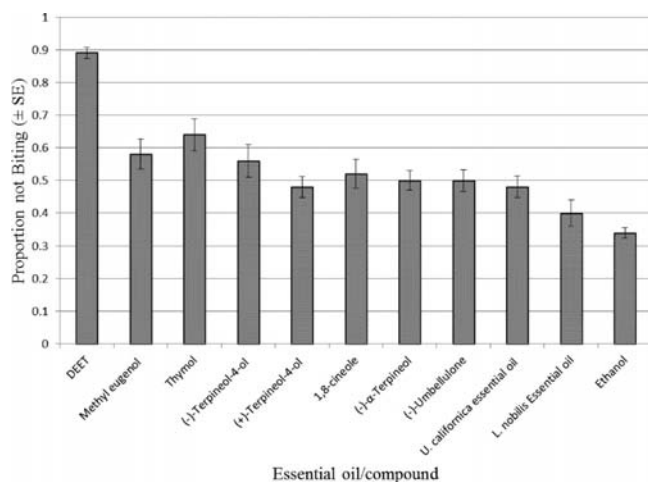
Figure 4. Proportion not biting values of *U. californica* and *L. nobilis* leaf essential oils and their individual pure compounds against female *Ae. aegypti*. All pure compounds including DEET were tested at 25 nmol/cm^2, and the essential oils were tested at 10 μg/cm^2. Ethanol was the solvent control, and DEET was used as positive control.
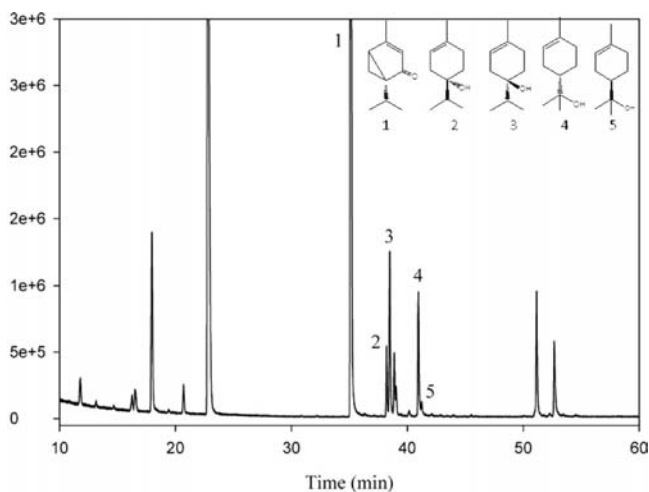
Figure 5. Enantiomeric separation of chiral compounds from *U. californica* essential oil using chiral column HP-Chiral-20B. Peaks: 1, (−)-umbellulone; 2, (+)-terpinen-4-ol; 3, (−)-terpinen-4-ol; 4, (−)-α-terpineol; 5, (+)-α-terpineol.

found in *L. nobilis* crude oil. It is also noteworthy to report that biting deterrent activities of (−)-terpinen-4-ol and (+)-terpinen-4-ol are not statistically different. *p*-Cymene did not show any activity at 25 nmol/cm^2 in screening bioassays.

In the search for new mosquito control agents from natural sources, *U. californica* oil, its subfractions, and pure compounds were screened against first-instar *Ae. aegypti* larvae. The essential oil showed good larvicidal activity with LD_{50} and LD_{90} values of 52.6 and 107.6 ppm, respectively, at 24 h post treatment (Table 3). Subfractions A1, A3, and A4 showed larvicidal activity, and their pure compounds were tested individually in larval bioassays. Of the pure compounds, thymol was the most active compound with an LD_{50} value of 17.5 ppm and followed with *p*-cymene (LD_{50} = 23.3 ppm), (−)-umbellulone (LD_{50} = 32.3 ppm), and methyleugenol (LD_{50} = 36.5 ppm) (Table 3). The monocyclic unsaturated monoterpene alcohols (+)- and (−)-terpinen-4-ol and (−)-α-terpineol and the bicyclic monoterpene etheroxide 1,8-cineole did not show any mortality in the prescreening larvicidal bioassays at a concentration of 100 ppm.

Thymol, as well as the majority of active compounds here reported, is occurring widely in several essential oils, especially *Thymus*, *Monarda*, and *Origanum* species, and it is well-known to contribute to the mosquito repellent and larvicidal activity of these oils.[7,35,36] On the other hand, the monoterpene umbellulone (which is the major constituent of *U. californica* essential oil) is less abundant in nature, and its biological activity as mosquito repellent and larvicidal activity are here reported for the first time. Umbellulone was found to act as a reversible Michael acceptor targeting the human and rat isoform of the nociceptive receptor TRPA1,[18,24] as well as other TRP receptors involved in chemosensation.[37] Phylogenetic analyses demonstrated that the TRPA1 receptor retained a high homology level through species, and both invertebrate and vertebrate TRPA1s share critical features, which may have played a crucial role in the evolution of these receptors as a sensorial sentinel for potential toxic compounds.[38] Several mosquito species, including *Ae. aegypti*, *Anopheles gambiae*,[39] and *Culex quinquefasciatus*, *Pediculus humanus corporis*, and *Drosophila melanogaster*[40] were found to express TRPA1 receptors as a sensor receptor. TRPA1 channels are activated by a wide variety of structurally diverse electrophiles, which act through a covalent bond to key cysteine residues on the receptor. The Michael acceptor property of these electrophiles has been demonstrated as an important feature of ligands for

Table 3. Toxicity of *U. californica* and *L. nobilis* Leaf Essential Oils and Pure Compounds against 1-Day-Old Larvae of *Ae. aegypti* at 24 h Post Treatment

essential oil/compound	LD_{50} (95% CI)[a]	LD_{90} (95% CI)	χ^2	DF
U. californica	52.6 (46.2−60.1)	107.6 (90.3−137.5)	86.3	48
L. nobilis	—[b]			
(−)-umbellulone	32.3 (29.4−35.5)	67.9 (59.7−80.1)	161.5	98
thymol	17.5 (15.7 −19.5)	36.5 (31.5−44.3)	130.9	87
methyleugenol	36.5 (30.2−45.1)	99.2 (74.5−153.7)	65.3	48
p-cymene	23.3 (20.5−26.5)	46.7 (39.4−59.4)	81.7	47
(−)-terpinen-4-ol	—[c]			
(+)-terpinen-4-ol	—[c]			
(−)-α-terpineol	—[c]			
1,8-cineole	—[c]			

[a]LD_{50} and LD_{90} values are given in ppm (95% confidence intervals). [b]No larvicidal activity at the highest dose of 125 ppm. [c]No larvicidal activity at the highest dose of 100 ppm.

TRPA1 receptors.[41] Several active constituents of *U. californica* essential oil, such as α-terpineol[42] and thymol, are also known to be TRPA1 agonists,[43] suggesting that the deterrent and activity of *U. californica* essential oil could be related to combined action at a molecular level. On the other side, the herein analyzed essential oil from *L. nobilis* is enriched in 1,8-cineole (>57.4% of the crude oil), which is a known TRPM8 agonist.[44] Further investigations are needed to assess the agonist activity and potency of *U. californica* constituents against mosquito isoform of TRPA1 and other sensorial TRP receptors, as this molecular approach could represent a useful strategy to investigate natural sources of mosquito repellents.

An important factor to be considered is the potential of toxicological side effects on humans because of irreversible target modifications.[45] Noncovalent and reversible interactions (like in the case of umbellulone)[24] could represent an important and safer solution to achieve efficacy while minimizing side effects, which are usually closely connected to irreversible binding. Because of the high homology between mosquitoes and human TRPA1 receptors, this fact is particularly important to identify new potential mosquito repellents with low human toxicity.

In conclusion, the present study aimed to investigate the differences between both laurel species based on their anatomies and the chemical characterization of their essential oils. Anatomical study of the leaves showed that the basic arrangement of tissues in the two species are comparable. However, they can be distinguished mainly by the thickness of the lamina and cuticle, size and abundance of the oil cells, nature of palisade tissue, and presence or absence of trichomes. The differentiation of the two species is very clear when the chemical distribution and abundance of both leaf essential oils are analyzed. Through the Deployed War-Fighter Protection (DWFP) Research Program, we have expanded our role in the exploration and identification of new natural compounds for mosquito repellent and larvicidal activity. In the current study, an investigation of the native American medicinal plant, *U. californica*, essential oil was performed for the first time for its mosquito biting deterrent and larvicidal activity. *U. californica* essential oil showed stronger larvicidal than biting deterrent activity. *U. californica* essential oil was a rich source of active compounds for larvicidal activity. New molecular insight about the occurrence of TRPA1 receptors in *Ae. aegypti* as well as in other mosquito species together with the identification of TRPA1 agonists as active repellents from *U. californica* essential oil encourages the investigation of this oil as a natural repellent for other arthropods of medical and veterinary importance and could lead to a new molecular-based approach to ethnopharmacological investigation of essential oils for pest control.

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