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Isolation and Identification of Mosquito Bite Deterrent Terpenoids from Leaves of American (*Callicarpa americana*) and Japanese (*Callicarpa japonica*) Beautyberry

C. L. CANTRELL,*,† J. A. KLUN,‡ C. T. BRYSON,§ M. KOBAISY,†,|| AND S. O. DUKE†

Natural Products Utilization Research Unit, Agricultural Research Service, U.S. Department of Agriculture, University, Mississippi 38677, Chemicals Affecting Insect Behavior Laboratory, Beltsville Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705, and Southern Weed Science Research Unit, Agricultural Research Service, U.S. Department of Agriculture, Stoneville, Mississippi 38776

Essential oil extracts from *Callicarpa americana* and *Callicarpa japonica* were investigated. Bioassay-guided fractionation of *C. americana* extracts using the yellow fever mosquito, *Aedes aegypti*, led to the isolation of α-humulene, humulene epoxide II, and intermedeol and a newly isolated terpenoid (callicarpenal). Similar work involving *C. japonica* resulted in the isolation of an additional compound, spathulenol, as well as the four compounds isolated from *C. americana*. Structure elucidation was performed on all isolated compounds using a combination of gas chromatography−mass spectrometry−electron ionization, high-resolution liquid chromatography−MS−electrospray ionization, and one- and two-dimensional NMR experiments. Heretofore, 13,14,15,16-tetranorclerodane, callicarpenal, has never been identified from natural sources. Complete ^{1}H and ^{13}C NMR assignment data are provided for this compound. In bite deterrent studies, spathulenol, intermedeol, and callicarpenal showed significant repellent activity against *A. aegypti* and *Anopheles stephensi*.

KEYWORDS: ***Callicarpa*; *americana*; *japonica*; Verbenaceae; spathulenol; intermedeol; α-humulene; callicarpenal; mosquito; repellent; deterrent; *Aedes aegypti*; *Anopheles stephensi***

INTRODUCTION

Decades ago, the grandfather of one of the authors (C.T.B.), residing in northeast Mississippi, used fresh crushed American beautyberry (*Callicarpa americana* L., Verbenaceae) leaves as a topical treatment for draft animals to repel flies and other biting insects. Specifically, the repellent was used as crushed leaves on or off the stems and partially placed under the harness where the animal's movement would continue to release the compound(s). Subsequently, this author (C.T.B.) has successfully used this quick preparation when on field-collecting trips as an insect repellent. This ethnobotanical information led us to investigate *C. americana* and its close relative, *Callicarpa japonica*, as sources of mosquito-repelling compounds.

Only two investigations have been conducted previously on the essential oils produced by *C. americana* (*1*, *2*). These studies were not directed toward the identification of mosquito repellent or bite-deterring constituents, and neither study reported on the purification of secondary metabolites or the biological activity of specific compounds.

C. japonica Thunb. (Japanese beautyberry, Verbenaceae) leaves were also examined for their bite-deterring constituents as an extension of this study. In contrast to that described above for *C. americana*, many investigations have examined the biological activities of secondary metabolites produced by *C. japonica* (*3*−*5*) as well as its essential oil composition (*2*, *6*). The biological activity of isolated methoxylated flavones from *C. japonica* has been demonstrated against herpes simplex virus type-1, and a piscicidal activity has been shown against *Oryzias latipes*. None of the investigations reported on the isolation of bite deterrent constituents.

Described in detail below is the *Aedes aegypti* bite-deterring bioassay-guided fractionation of the essential oils from these two *Callicarpa* species. A total of five isolated compounds with biological activities against *A. aegypti* and *Anopheles stephensi* are reported.

MATERIALS AND METHODS

General Procedures. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ on a Bruker Avance 400 MHz spectrometer. High-resolution mass spectra were obtained on either a JEOL AccuTOF (JMS-T100LC) or an Agilent LC/MSD TOF. Column chromatography was performed

* To whom correspondence should be addressed. Tel: +1-662-915-5898. Fax: +1-662-915-1035. E-mail: ccantrell@ars.usda.gov.
† Natural Products Utilization Research Unit.
‡ Beltsville Agricultural Research Center.
§ Southern Weed Science Research Unit.
|| Current address: The University of Mississippi, Thad Cochran Research Center, University, MS 38677.

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using a Biotage, Inc. Horizon Pump equipped with a Horizon Flash Collector and fixed wavelength (254 nm) detector.

Gas Chromatography−Mass Spectrometry (GC-MS) Analysis. Oil extracts of *C. americana* and *C. japonica* were analyzed by GC-MS on a Varian CP-3800 GC coupled to a Varian Saturn 2000 MS/MS. GC was equipped with a DB-5 column (30 m × 0.25 mm fused silica capillary column, film thickness of 0.25 μm) operated using the following conditions: injector temperature, 240 °C; column temperature, 60−240 °C at 3 °C/min and then held at 240 °C for 5 min; carrier gas, He; and injection volume, 1 μL (splitless). The MS ionization energy was set to 70 eV.

Plant Material. Leaves of indigenous *C. americana* were collected in July of 2004 from a single large plant (4 m tall × 5 m wide) growing in Lafayette County, Mississippi, at latitude 34° 20′ 25″ N and longitude 89° 40′ 17″ W. A voucher specimen was deposited in the Pullen Herbarium in Oxford, Mississippi, and assigned voucher number MISS #71,495.

Leaves of *C. japonica* were collected in August of 2004 from three separate cultivated plants growing in Lafayette County, Mississippi, at latitude 34° 20′ 25″ N and longitude 89° 40′ 16″ W. Cultivated plants were grown in full sun and averaged 1.2 m tall and 1.1 m wide. A voucher specimen was deposited in the Pullen Herbarium in Oxford, Mississippi, and assigned voucher number MISS #71,496.

Essential Oil Preparation. Steam distillations were conducted in a Nickerson−Likens (*7*) type apparatus. Fresh cut leaves of *C. americana* or *C. japonica* were immediately frozen in sealed plastic bags upon collection until needed. *C. americana* leaves (495 g fresh weight) were placed in a 2 L round bottom flask along with 1 L of H_2O. The distillate was continuously extracted during an 8 h distillation with 30 mL of pentane into a 50 mL pear-shaped flask heated in a water bath maintained at 70 °C. This process was repeated using 495 g of additional leaves to provide 937 mg of crude essential oils. In an identical manner, 440 g of *C. japonica* fresh leaves was extracted providing 382 mg of crude essential oil.

***C. americana* Oil Fractionation.** A portion (696 mg) of the *C. americana* essential oil was subjected to silica gel (25 mm × 150 mm, 60 Å, 40−63 μm) column chromatography. A hexane/EtOAc linear gradient was used consisting of the following steps: 100/0 to 90/10, 600 mL; 90/10 to 80/20, 408 mL; 80/20 to 50/50, 360 mL; and 50/50 to 0/100, 1008 mL. A total of 96 24 mL test tubes were collected and combined into seven fractions [Fr. A, 203 mg; Fr. B, 149 mg; Fr. C, 34 mg; Fr. D, 19 mg; Fr. E (intermedeol), 56 mg; Fr. F, 156 mg; and Fr. G, 24 mg] based on thin-layer chromatography (TLC) similarity. Fr. A was further purified using silica gel (25 mm × 150 mm, 60 Å, 40−63 μm) column chromatography with 1500 mL of hexane resulting in 25 mg of α-humulene. Fr. B was purified using a silica gel (25 mm × 150 mm, 60 Å, 40−63 μm) column chromatography gradient from 100% hexane to 20% EtOAc (1602 mL) resulting in Fr. B-1 (56 mg) and Fr. B-2 (57 mg, humulene epoxide II). Fr. B-1 was further purified using a silica gel (25 mm × 150 mm, 60 Å, 40−63 μm) column chromatography gradient from 100% hexane to 15% EtOAc (2001 mL) providing 38 mg of callicarpenal.

***C. japonica* Oil Fractionation.** A portion (270 mg) of the *C. japonica* essential oil was subjected to silica gel (25 mm × 150 mm, 60 Å, 40−63 μm) column chromatography. A hexane/EtOAc linear gradient was used consisting of the following steps: 100/0 to 90/10, 600 mL; 90/10 to 80/20, 408 mL; 80/20 to 50/50, 360 mL; and 50/50 to 0/100, 1008 mL. A total of 96 24 mL test tubes were collected and combined into seven fractions [Fr. A, 52 mg; Fr. B, 14 mg; Fr. C (humulene epoxide II), 18 mg; Fr. D, 13 mg; Fr. E, 36 mg; Fr. F, 29 mg; and Fr. G, 62 mg] based on TLC similarity. Fr. E and F were combined and purified using a silica gel (25 mm × 150 mm, 60 Å, 40−63 μm) column chromatography hexane/EtOAc linear gradient (100/0 to 80/20, 900 mL; 80/20 to 50/50, 402 mL; and 50/50 to 0/100, 309 mL) resulting in three fractions: Fr. EF-1, 4 mg (intermedeol); Fr. EF-2, 28 mg; and Fr. EF-3, 11 mg (spathulenol). Fr. EF-2 (28 mg) was further purified using a silica gel (25 mm × 150 mm, 60 Å, 40−63 μm) column chromatography hexane/Et_2O linear gradient (100/0 to 80/20, 1200 mL; 80/20 to 50/50, 402 mL; and 50/50 to 0/100, 309 mL) resulting in three fractions: Fr. 1, 11 mg (intermedeol); Fr. 2, 5 mg; and Fr. 3, 10 mg (spathulenol).

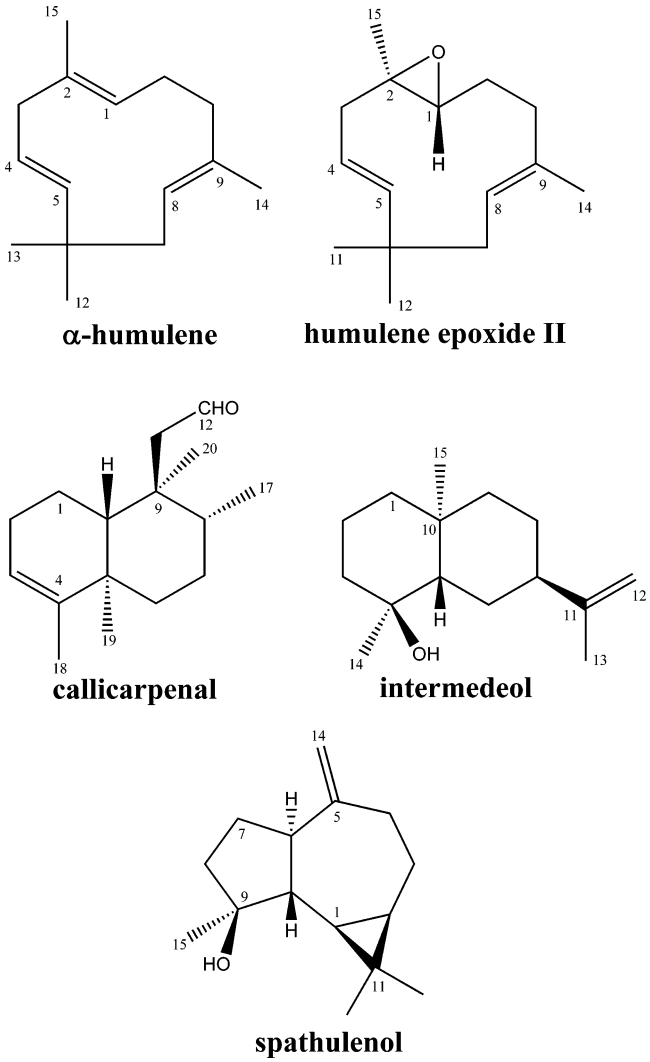

Figure 1. Compounds isolated from *C. americana* and *C. japonica*.

α-Humulene. The structure was assigned by comparison of ^{13}C NMR data with that reported in the literature (*8*).

Humulene Epoxide II, Intermedeol, and Spathulenol. MS, ^1H, and ^{13}C NMR data were in complete agreement with those previously reported in the literature for humulene epoxide II (*9, 10*), intermedeol (*11*), and spathulenol (*12, 13*).

Callicarpenal (13,14,15,16-Tetranor-3-cleroden-12-al) (Figure 1). For ^1H and ^{13}C NMR data, see **Table 2**. High-resolution APCI-MS: *m/z* 235.2063 $[M + H]^+$; calculated for $C_{16}H_{27}O$, 235.2062. $[\alpha]_D^{25} = -45.3$ (*c* 0.0053, benzene).

Insects. *A. aegypti* (L.) (red eye Liverpool strain) and *A. stephensi* Liston used in the study were from colonies maintained at the Walter Reed Army Institute of Research (Silver Spring, MD). The insects were reared (*14*) by feeding larvae ground tropical fish flakes (Tetramin Tropical Fish Flakes, Tetra Sales, Blacksburg, VA, www.tetra-fish.com). Adults were maintained in a photoperiod of 12:12 (L:D) h at 27 °C and 80% relative humidity with cotton pads moistened with 10% aqueous sucrose solution. Mated females were 5−15 days old when they were used in bioassays. *A. aegypti* females had access only to water 24 h and neither food nor water for another 24 h before testing. *A. stephensi* females were provided with water alone 24 h before testing. All tests were conducted 4−6h after the beginning of the photophase (1000 h).

Mosquito Bioassay Methods. Experiments were conducted by using a six-celled in vitro Klun & Debboun (K & D) module bioassay system developed by Klun et al. (*15*) for quantitative evaluation of bite deterrent properties of candidate compounds for human use. Dethier et al. (*16*) defined a repellent as a chemical that causes insects to make oriented movement away from its source, and a deterrent was defined as a

chemical that inhibits feeding or oviposition when present in a place where insects would, in its absence, feed or oviposit. The bioassay method that we used in this research specifically measured biting (feeding) deterrent properties of chemicals. Therefore, the compounds identified here are best defined as being deterrents and not repellents; although, in another bioassay mode, they might also exhibit a repellent effect. The assay system consists of a six well blood reservoir with each of the 3 cm × 4 cm wells containing 6 mL of human blood cells water bath-warmed (38 °C) reservoir and covered with a collagen membrane. The blood membrane unit simulates a human host for mosquito feeding. Antibiting activity of standard compounds, measured in the in vitro K & D module system, are known to be comparable to activities observed when tested on the skin of human volunteers (*15*).

Plant extracts, fractions, or isolated pure plant-derived compounds in 95% ethanol solution were each randomly applied to six 4 cm × 5 cm areas of organdy cloth and positioned over the membrane-covered blood. A replicate consisted of six treatments: four test chemicals or extract fractions, 95% ethanol-treated cloth as the control, and unfractionated plant extract or a standard bite detterent compound, (1*S*,2′*S*)-2-methylpiperidinyl-3-cyclohexen-1-carboxamide (SS-220) at 25 nmol/cm^2 cloth. The 25 nmol SS-220/cm^2 cloth dose was used as a standard, because it was known from human volunteer and in vitro assays to consistently suppress mosquito biting by 80% or more as compared to controls in replicated assays (*15*). Klun et al. (*17*) demonstrated that SS-220 possesses bite deterrent activity against *A. stephensi* equivalent to bench mark compounds Deet and Bayrepel, and against *A. aegypti*, SS-220 was more effective than Bayrepel and as effective as Deet. Routinely, a six-celled K & D module containing five mosquitoes/cell was positioned over cloth treatments covering the six blood membrane wells, and trap doors of the K & D modules were opened to expose the treatments to the sets of mosquitoes. After a 3 min exposure, the number of mosquitoes biting through cloth treatments in each cell was recorded and mosquitoes were prodded back into the cells. In experiment 8, all mosquitoes were retained in their respective K & D module cells, each fitted with a water-moist piece of cotton, and observed for total toxic knock down at 6 min, 1 h, and 24 h posttreatment exposure. Experiments were repeated 12, 18, 32, or 56 times. Thus, 60, 90, 160, or 280 mosquitoes were tested against each treatment depending on the experiment, and the proportion of mosquitoes not biting for each treatment was calculated. For each experiment, conducted in different time periods, a logistic regression approach (*17*, *18*) was used to model the proportion of nonbiting mosquitoes jointly for each group of compounds (including the control). Rather than make all possible comparisons of compound pairs (with a subsequent loss of power), two sets of one degree of freedom contrasts (*t*-tests) were made with the control and with the proved deterrent, SS-220. In other words, our statistical testing was done in a logistic regression framework using dummy variables (contrasts with the control or contrasts with SS-220). Nonbiting proportions were converted to logits [$\log(p/(1-p))$], and contrasts with the control and SS-220 were tested using *t*-tests. This statistical approach is more powerful than the more traditional way of converting the proportions using the arcsine transformation and then running an analysis of variance. Because the data were binomial (count of number of mosquitoes biting out of total number tested), a measure of variability came directly from the binomial distribution. As an example, a standard error for the proportion not biting is SE $= \sqrt{p(1-p)/n}$ where p is the proportion not biting and n is the number of mosquitoes tested. The level of significance was set at $P = 0.05$. In all, eight experiments were conducted. *A. aegypti* was used as the test mosquito in all experiments with the exception of experiment 7 where *A. stephensi* was used.

Experiments 1 and 2. These dose × response experiments compared *C. japonica* and *C. americana* essential oil preparations at 1, 10, and 100 μg oil/cm^2 cloth vs ethanol (95%) control and 25 nmol (5.18 μg) SS-220/cm^2 cloth for antibiting activity. Each experiment used 90 mosquitoes against each treatment.

Experiments 3−5. In each of these experiments, chromatographic fractions of essential oil at their respective percentage compositions of 100 μg essential oil/cm^2 cloth, 100 μg unfractionated essential oil/cm^2 cloth, 25 nmol SS-220/cm^2 cloth, and control were compared for mosquito antibiting activity. Ninety mosquitoes were tested/treatment.

Table 1. *C. japonica* and *C. americana* Dose × Response Essential Oil Dosage Preparations (Experiments 1 and 2) and Fractions (Fr.) (Experiments 3–5) against *A. aegypti*

exp.	treatment	concn (μg/cm^2)	proportion not biting (SE)
1	control		0.33 (0.05)[a]
	SS-220	5.18	0.81 (0.04)[b,c]
	C. japonica oil	1	0.34 (0.05)[a]
	C. japonica oil	10	0.68 (0.05)[b]
	C. japonica oil	100	0.83 (0.04)[b,c]
2	control		0.40 (0.05)[a]
	SS-220	5.18	0.90 (0.03)[b,c]
	C. americana oil	1	0.46 (0.05)[a]
	C. americana oil	10	0.69 (0.05)[b]
	C. americana oil	100	0.77 (0.04)[b]
3	control		0.10 (0.03)[a]
	SS-220	5.18	1.00 (0.00)[b,c]
	C. americana oil	100	0.98 (0.01)[b,c]
	C. americana Fr. A	36.9	0.13 (0.04)[a]
	C. americana Fr. B	8.4	0.97 (0.02)[b,c]
4	control		0.15 (0.04)[a]
	SS-220	5.18	0.92 (0.03)[b,c]
	C. americana oil	100	0.93 (0.03)[b,c]
	C. americana Fr. C	14.0	0.48 (0.05)[b]
	C. americana Fr. D	6.1	0.62 (0.05)[b]
	C. americana Fr. E	13.2	0.87 (0.04)[b,c]
5	control		0.22 (0.04)[a]
	SS-220	5.18	0.95 (0.02)[b,c]
	C. americana oil	100	1.00 (0.00)[b,c]
	C. americana Fr. F	14.5	0.87 (0.04)[b,c]
	C. americana Fr. G	6.9	0.45 (0.05)[b]

[a] Not different from control. [b] Significantly different from control. [c] Not different from SS-220. SE, standard error.

Experiments 6 and 7. These experiments compared the antibiting activities of SS-220 and plant-isolated callicarpenal, humulene epoxide II, intermedeol, and spathulenol at 25 nmol compound/cm^2 cloth vs control against *A. aegypti* and *A. stephensi*, respectively. Experiment 6 tested 160 mosquitoes/treatment, and experiment 7 had 280 mosquitoes/treatment.

Experiment 8. This experiment (60 mosquitoes/treatment) compared the antibiting activities of a mixture (25 nmol/cm^2 cloth) of 11.5% callicarpenal, 47.1% intermedeol, and 41.4% spathulenol. The percentage composition of the mixture was a normalized percentage active components in *C. japonica* essential oil based upon GC-MS analysis total ion area % analysis based on largest peaks. Other treatments in the experiment were SS-220, callicarpenal, intermedeol, and spathulenol each at 25 nmol/cm^2 cloth vs control. In addition to measurement of antibiting activity, the number of dead mosquitoes observed at three time intervals was recorded for the six treatments.

RESULTS AND DISCUSSION

The essential oil extracts from both *C. americana* and *C. japonica* were evaluated for their *A. aegypti* biting deterrent activities at 100, 10, and 1 μg/cm^2 cloth (experiments 1 and 2, **Table 1**). Both oils demonstrated significant biological activity as compared to control, and oil from *C. japonica* at 100 μg/cm^2 cloth exhibited an antibiting activity equal to 25 nmol SS-220. Because only small amounts of *C. japonica* oil were available, fractionation using silica gel was performed on *C. americana* essential oil. Fractions were screened at concentrations representing their weight percentages in the parent oil from the original fractionation. Analysis of the proportion of mosquitoes not biting in experiments 3, 4, and 5 (**Table 1**) showed that fractions B, E, and F contained the most active constituents and were therefore responsible for the activity of the crude oil. Consequently, all three fractions, as well as others, were thoroughly investigated.

Table 2. ^{1}H (400 MHz), ^{13}C (100 MHz), and HMBC NMR Assignment Data for Callicarpenal ($CDCl_3$)

position	δ_H mult (J in Hz)	δ_C mult[a]	HMBC (^{1}H to ^{13}C)
1		19.2 t	
2		26.8 t	
3	5.18 br s	120.8 d	1, 2, 5
4		143.8 s	
5		38.7 s	
6		36.7 t	
7		27.6 t	
8	1.61 m	39.3 d	
9		41.9 s	
10	1.43 m	49.6 d	2, 5, 9, 11, 19, 20
11	2.33 dd (3.6, 14.4), 2.46 dd (3.6, 14.4)	52.0 t	8, 9, 10, 12, 20
12	9.83 t (3.2)	203.8 d	11
17	0.94 d (6.4)	16.5 q	7, 8, 9
18	1.57 br s	18.1 q	3, 4, 5
19	1.01 s	20.1 q	4, 5, 6, 10
20	0.83 s	17.4 q	8, 9, 10, 11

[a] Carbon multiplicities deduced from DEPT NMR experiments.

Fraction B was further purified using silica gel column chromatography resulting in the isolation of a colorless oil with a molecular weight of *m*/*z* 220 by GC-MS. ^{1}H NMR spectral analysis suggested the presence of three olefinic methines (δ 5.28 m, δ 5.17 m, and δ 4.99 m), one oxygenated methine (δ 2.55 m), and four methyls, one of which appeared to be olefinic. DEPT (90 and 135°) and ^{13}C NMR analysis indicated the presence of four olefinic carbons, two oxygenated carbons (δ 63.5 s, 62.2 d), four aliphatic methylene carbons, four methyls, and one quaternary singlet (δ 36.7). The final identification of this compound as humulene epoxide II was accomplished by comparison of spectroscopic data with that previously reported in the literature (*9*, *10*).

A second purified compound (colorless oil) from fraction B gave a molecular ion at *m*/*z* 234 and an intense fragment at *m*/*z* 190 when analyzed by GC-MS. Analysis by positive ion high-resolution APCI-MS gave a molecular ion at *m*/*z* 235.2063 (calculated for $C_{16}H_{27}O$, 235.2062) corresponding to $[M + H]^+$. The above information suggested a molecular formula of $C_{16}H_{26}O$ and four sites of unsaturation. Initial inspection of the ^{1}H NMR spectrum indicated the presence of one aldehyde triplet (δ 9.83, $J = 3.2$ Hz, H-12), one olefinic proton (δ 5.18 br s, H-3), one olefinic methyl singlet (δ 1.57, H-18), one methyl doublet (δ 0.94, $J = 6.4$ Hz, H-17), and two methyl singlets (δ 1.01, H-19; δ 0.83, H-20) (**Table 3**). As expected from high-resolution MS data, ^{13}C NMR spectral analysis indicated a total of 16 carbons. The combination of 90° and 135° DEPT and ^{13}C NMR data indicated the presence of one carbonyl (δ 203.8 d), two olefinic (δ 120.8 d, δ 143.8 s), five aliphatic methylene, two aliphatic methine, two quartenary, and four methyl carbons (**Table 2**).

^{1}H−^{1}H COSY correlations were observed between the aldehyde triplet at δ 9.83 (H-12) and the methylene protons at δ 2.33 (H-11) and δ 2.46 (H-11), which were not further coupled, suggesting a vicinal quaternary center. HMBC correlations (**Table 2**) observed between H-11 methylene protons and δ 39.3 (C-8), δ 41.9 (C-9), δ 49.6 (C-10), and δ 17.4 (C-20) established the attachment of C-11 (δ 52.0 t) to C-9, which was further attached to carbons 8, 10, and 20. The methine proton at δ 1.61 (H-8) gave a strong COSY coupling to the methyl doublet at δ 0.94 (H-17). The HMBC correlations observed between the methine proton at δ 1.43 (H-10) and carbons at δ 26.8 (C-2), δ 38.7 (C-5), δ 41.9 (C-9), δ 52.0 (C-11), δ 20.1 (C-19), and δ 17.4 (C-20) as well as those observed between H-19 (δ 1.01 s) and δ 143.8 (C-4), δ 38.7 (C-5), δ 36.7 (C-6), and δ 49.6 (C-10) were critical to the establishment of an A−B ring structure consistent with that of a clerodane diterpenoid. HMBC correlations between H-3 (δ 5.18 br s) and those at δ 19.2 (C-1), δ 26.8 (C-2), and δ 38.7 (C-5) confirmed the location of the double bond within the A ring. Unambiguous ^{1}H, ^{13}C, and HMBC NMR spectral assignment data are reported in **Table 2** and firmly establish the structure as that drawn in **Figure 1** for which we have assigned the trivial name callicarpenal and systematic name 13,14,15,-16-tetranor-3-cleroden-12-al.

Upon further examination of the literature, it was determined that callicarpenal had been reported (*20*) as a synthetic intermediate during the total synthesis of the clerodane 16-hydroxycleroda-3,13(14)*Z*-dien-15,16-olide. ^{1}H NMR data had been reported in this paper; however, no spectral assignment data were provided. Additionally, both ^{1}H and ^{13}C NMR data for the enantiomer of callicarpenal had been reported previously, again with no spectral assignment data. Optical rotation data reported by Hagiwara et al. (*20*) during their enantioselective synthesis were in complete agreement with that observed for isolated callicarpenal confirming its absolute stereochemistry as that drawn in **Figure 1**. To the best of our knowledge, this is the first report on the isolation of a compound containing a 13,14,15,16-tetranorclerodane ring system. 13,14,15,16-Tetranorlabdanes have been reported previously, but very few reports exist (*19*).

Fraction E appeared to be a pure compound upon inspection by GC-MS, which indicated a molecular ion of *m*/*z* 222 $[M]^+$. ^{1}H NMR analysis indicated the presence of two olefinic protons (δ 4.92 s, δ 4.87 s), a single olefinic methyl (δ 1.75 s), and two aliphatic methyls. DEPT (90 and 135°) and ^{13}C NMR analysis revealed the presence of two olefinic carbons (δ 146.9 s, δ 110.8 t), one quaternary oxygenated carbon (δ 72.1 s), and 12 additional carbons, three methyls, six methylenes, two methines, and one quaternary carbon. Structural confirmation was ultimately provided by comparison of ^{1}H and ^{13}C NMR data with that reported in the literature (*11*) for intermedeol, providing unambiguous structural confirmation (**Figure 1**).

For completeness, fractions A and F were also investigated. Fraction F contained impure intermedeol while fraction A was further purified allowing for the isolation of a compound exhibiting a molecular ion at *m*/*z* 204 by GC-MS analysis. Structure confirmation was obtained by comparison of ^{1}H and ^{13}C NMR chemical shift data with that reported in the literature (*8*) for α-humulene allowing for its structure to be assigned that shown in **Figure 1**.

A similar approach to that described above for the investigation of *C. americana* was also performed on the essential oil extract of *C. japonica*. However, because of the small quantity of essential oil and raw material available, a bioassay-guided approach was not chosen. The initial fractionation of *C. japonica* oil was done in an identical manner to that for *C. americana* resulting in fractions A−G. Many of the same compounds isolated from *C. americana* were also isolated from *C. japonica* except for a compound present in fractions E and F. These fractions were combined due to similarities in their TLC and further purified using silica gel column chromatography resulting in the isolation of intermedeol and a compound giving a strong molecular ion of *m*/*z* 220 by GC-MS analysis. Initial inspection of ^{1}H NMR spectroscopic data indicated the presence of two olefinic protons (δ 4.68 s, δ 4.66 s) and three aliphatic methyls. ^{13}C NMR analysis revealed the presence of two olefinic carbons (δ 153.4, δ 106.3), one oxygenated carbon (δ 80.9), and 12

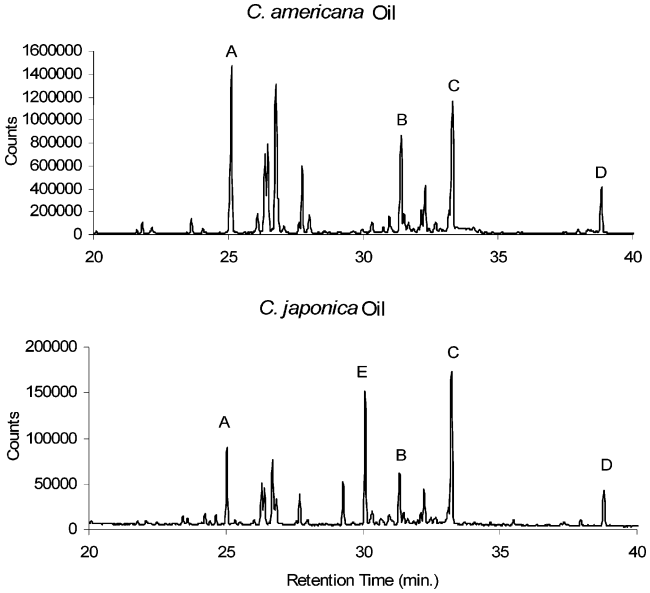

Figure 2. GC-MS total ion chromatograms for *C. americana* and *C. japonica* essential oil extracts (A, α-humulene; B, humulene epoxide II; C, intermedeol; D, callicarpenal; and E, spathulenol).

Table 3. Insect Deterrent Effects Using Isolated Compounds against *A. aegypti* (Experiment 6) and *A. stephensi* (Experiment 7)

exp.	treatment	proportion not biting (SE)
6	control	0.39 (0.04)[a]
	SS-220[d]	0.80 (0.03)[b,c]
	callicarpenal[d]	0.70 (0.04)[b,c]
	humulene epoxide II[d]	0.48 (0.04)[a]
	intermedeol[d]	0.70 (0.04)[b]
	spathulenol[d]	0.73 (0.04)[b,c]
7	control	0.42 (0.03)[a]
	SS-220[d]	0.78 (0.02)[b,c]
	callicarpenal[d]	0.75 (0.03)[b,c]
	humulene epoxide II[d]	0.58 (0.03)[a]
	intermedeol[d]	0.72 (0.03)[b,c]
	spathulenol[d]	0.75 (0.03)[b,c]

[a] Not different from control. [b] Significantly different from control. [c] Not different from SS-220. [d] 25 nmol compound/cm^2 cloth. SE, standard error.

additional carbons. The final structural confirmation was accomplished by comparison of ^{1}H and ^{13}C NMR spectral data with that reported in the literature (*12*, *13*) allowing for assignment of the structure to that of spathulenol (**Figure 1**).

GC-MS analysis of crude essential oils was performed for comparison of isolated constituents present in each species (**Figure 2**). Clearly, α-humulene, humulene epoxide II, intermedeol, and callicarpenal are all present in oils from both species. Further inspection of the chromatograms revealed a large amount of spathulenol in *C. japonica* oil and absence of it in *C. americana* oil.

Compounds isolated from bioactive fractions (humulene epoxide II, intermedeol, callicarpenal, and spathulenol) were tested for biting deterrent efficacy against both *A. aegypti* and *A. stephensi* in experiments 6 and 7 (**Table 3**), respectively.

Experiment 6 revealed that humulene epoxide II possessed no biting deterrent activity, and callicarpenal and intermedeol had significant activity and were only slightly less effective than spathulenol or SS-220, which were equally active against *A. aegypti*. Experiment 7 showed that callicarpenal, intermedeol, and spathulenol were as effective as SS-220 against *A. stephensi* and was the case with *A. aegypti*. Humulene epoxide II was not different from the control.

Table 4. Insect Deterrent Experiments and Knock Down Toxicity Test Using Isolated Compounds against *A. aegypti*

exp.	treatment	proportion not biting (SE)	total knock down 6 min	1 h	24 h
8	control	0.20 (0.05)[a]	0	1	2
	SS-220[d]	0.67(0.06)[b,c]	0	1	2
	mixture[d]	0.42 (0.06)[b]	0	3	6
	callicarpenal[d]	0.73 (0.06)[b,c]	0	1	5
	intermedeol[d]	0.62 (0.06)[b,c]	1	1	2
	spathulenol[d]	0.48 (0.06)[b]	1	2	0

[a] Not different from control. [b] Significantly different from control. [c] Not different from SS-220. [d] 25 nmol compound/cm^2 cloth. SE, standard error.

Moreover, experiments 6 and 7, taken together, showed a consistent trend that callicarpenal, intermedeol, and spathlenol were effective in fending off biting by *A. stephensi* and *A. aegypti*, and the three compounds were generally comparable to the highly potent SS-220. Humulene epoxide II was uniformly ineffective against either species of mosquito.

The "proportion not biting" data in experiment 8 (**Table 4**) against *A. aegypti* were similar to the results observed in experiment 6 inasmuch as callicarpenal and intermedeol expressed high biting deterrent activity that was comparable to SS-220. Spathulenol did not perform as well as it had in experiment 6. We believe the apparent diminished comparative efficacy of spathulenol in experiment 8 is due to the fact that statistical power for treatment resolution in experiment, made up of only 12 replicates, was significantly less than that of experiment 6 where 32 replicate observations were made. The 25 nmol mixture/cm^2 cloth, like spathulenol alone, was significantly different from control. The data also show that presenting a sesquiterpene mixture to *A. aegypti* did not result in a synergistic effect. More importantly, experiment 8 unambiguously showed that none of sequiterepenes alone or as a mixture possessed knock down toxic activity.

Clearly, this study progressed from an observed folklore use of a plant to the isolation of the mosquito biting deterrent constituents (intermedeol and callicarpenal) produced by the plant, *C. americana*. In addition, analysis of a second species from the same genus, *C. japonica*, led to the isolation of yet another bioactive compound, spathulenol. Callicarpenal, intermedeol, and spathulenol proved to be highly effective biting deterrents against *A. stephensi* and *A. aegypti*. These compounds and other terpenoids may represent useful alternatives to conventional, so-called synthetic insect repellents currently on the market. At least, they represent lead compounds suitable for synthetic modifications and synthesis of structural analogues in a search for new terpenoid compounds with enhanced and optimized mosquito bite deterrent properties.

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