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Composition and Antimicrobial Activity of *Anemopsis californica* Leaf Oil

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Isolation and characterization of leaf volatiles in *Anemopsis californica* (Nutt.) Hook. and Arn. (*A. californica*) was performed using steam distillation, solid-phase microextraction, and supercritical fluid extraction. Thirty-eight compounds were detected and identified by gas chromatography; elemicin was the major component of the leaf volatiles. While the composition of the leaf volatiles varied with method of extraction, α-pinene, sabinene, β-phellandrene, 1,8-cineole, piperitone, methyl eugenol, (*E*)-caryophyllene, and elemicin were usually present in readily detectable amounts. Greenhouse-reared clones of a wild population of *A. californica* had an identical leaf volatile composition with the parent plants. Steam-distilled oil had antimicrobial properties against 3 (*Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Geotrichim candidum)* of 11 microbial species tested. Some of this bioactivity could be accounted for by the α-pinene in the oil.

KEYWORDS: ***Anemopsis californica*, Yerba mansa, steam distillation, SPME, SFE, antimicrobial, elemicin, methyl eugenol**

INTRODUCTION

Anemopsis is one of five genera belonging to the Saururaceae family. The genus contains a single species, *A. californica* (Nutt.) Hook. and Arn. (=*Houttuynia californica* Benth. and Hook.), commonly known as yerba mansa. Native to the southwestern United States and northern Mexico, *A. californica* leaf and root preparations have been used medicinally to treat pain, inflammation, and infection (*1*−*3*). Sanvordeker and Chaubal (*4*) previously identified 12 volatiles isolated from the roots and rhizomes of *A. californica* collected in California. The most abundant compound in their extracts was methyl eugenol (57%). Other components detected in the root oil at greater than 1% (v/v) amounts were as follows: thymol, piperitone, thymol methyl ether, α-pinene, 1,8-cineole, methylchavicol, and *p*-cymene; leaf volatile oils were not examined.

A. californica is collected in New Mexico and used by local healers to treat a variety of ailments. Fresh or dry leaf or root samples are prepared as tinctures, decoctions, and teas for internal use; wilted leaves or root powder are used externally (*2*). Teas prepared from this plant are used to treat coughs, nausea, kidney problems, and menstrual cramps, to act as a diuretic, and to provide pain relief. Salves and poultices are used to prevent infection of burns and reduce swelling of bruises and are included in sitz baths and douches (*2, 3*). Efforts are underway to develop genetic selections of *A. californica* and to design management plans for this plant as a high-value crop for small farms in the state (*5*). We needed to establish a reliable and rapid method of extraction for this plant to compare the essential oil composition in *A. californica* grown in a variety of locations and under different environmental conditions. Two rapid methods of extraction, solid-phase microextraction (SPME) and super critical fluid extraction (SFE), have been used with varying results to characterize the volatile or essential oil composition of plant organs (*6*−*8*). These methods were compared with classic steam distillation to determine if the essential oil composition of *A. californica* leaves could be accurately described using either SPME or SFE. Additionally, we investigated the antimicrobial activity of *A. californica* leaf oil using bioassays against 11 microorganisms. To the best of our knowledge, this is the first publication describing the essential oil composition of *A. californica* leaves.

EXPERIMENTAL PROCEDURES

Plant Cultivation. *A. californica* from Dona Ana County, NM (elevation, 1182 m) was harvested and propagated by root division in August 1999. A voucher specimen was placed in the Range Science Herbarium at New Mexico State University in Las Cruces, NM (Collection number: Medina 6). Plants were greenhouse-cultivated in

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Metro Mix 360 (Greenhouse & Garden Supply Inc., Albuquerque, NM), fertilized with Osmocote 14-14-14, and watered daily using drip irrigation. All analyses were conducted on ground plant material using fully expanded leaves collected as indicated from either greenhouse-grown plants or plants from the Dona Ana county collection site; leaf material from three to five plants were pooled in a composite sample. For SPME and steam distillation, the leaf material was frozen before grinding; for SFE, the leaf material was dried at room temperature before grinding in a mortar and pestle.

Extraction Methods. Steam distillations were carried out in a Likens-Nickerson apparatus as previously described (*9*). Briefly, 20 g of ground leaf tissue was placed in a round-bottom flask with ~100 mL of distilled water; 12−15 mL of pentane was added to the U-tube of the Likens-Nickerson apparatus. A water bath under the pear-shaped flask was heated to boil the pentane, 60−70 °C; a heating mantle under the round-bottom flask was used to boil/reflux for 4−5 h. Pentane fractions were frozen to remove residual water rather than drying over anhydrous magnesium sulfate and filtering. This minimized the loss of oil and shortened the process. Duplicate steam distillations using 20 g of ground tissue were carried out as previously described (*9*), using pentane and water for 4−5 h.

SPME analyses were prepared by placing 0.5 g of ground tissue into 4 mL screw-top vials sealed with poly(tetrafluoroethylene) (PTFE)/silicon septa (Supelco). The vials were equilibrated at 30 °C for 2 h and then exposed to a 100 μm PDMS fiber (Supelco), 1 cm deep into the vial for 10 min. The fiber was immediately injected into the appropriate gas chromatograph inlet to a depth of 3 cm. The fiber remained in the injector for 5 min to remove residual volatiles, and blank runs were performed after each sample.

For SFE extraction, 0.5 g of ground leaf tissue was loaded in thimbles for extraction in ISCO SFX3560. The thimble was pressurized with CO_2 to a density of 0.72 g/mL (5150 psi, 100 °C), for 1 min (static extraction setting on instrument), and then the solubilized compounds were flushed from the thimble with 7 min dynamic extraction at a flow rate of 2.0 mL/min. The extraction is vented into a tube with 10 mL of methanol to trap the essential oil components as the CO_2 is bubbled off. The essential oil components in the methanol were quantified and identified by GC/MS (gas chromatography/mass spectrometry) or GC/FID (gas chromatography/flame ionization detection).

Gas Chromatography/Mass Spectrometry. Extracts were analyzed by GC/MS using a Varian model 3400 GC with a DB-5 column (30 m × 0.25 mm fused silica capillary, 0.25 μm film thickness), coupled to a Finnigan ion trap mass spectrometer (EI, 70 eV) or a Shimadzu GC8APF equipped with a flame ionization detector and a split/splitless injector. Helium carrier gas flowed at 1 mL/min, and injector and transfer line temperatures were 220 and 260 °C, respectively. The initial column temperature was 60 °C, with a linear gradient of 3 °C/min programmed into each 65 min run. Comparisons of mass spectra and Kováts retention indices (*10*) with literature data (*11*) or authentic standards were used to identify the peaks. Reference standards were obtained from Sigma-Aldrich, St. Louis, MO (borneol, camphene, camphor, (*E*)-caryophyllene, caryophyllene oxide, 1,8-cineole, *p*-cymene, linalool, methyl eugenol, myrcene, α-phellandrene, α-pinene, β-pinene, α-terpineol, terpinolene, thymol, tricyclene) and from Pfaltz & Bauer, Waterbury, CT (piperitone, sabinene). Elemicin was synthesized as described below. Two methods of quantitation of the essential oil components were employed, percent peak area or mass of analyte per gram dry weight of leaf. Percent peak areas were used for quantitation when we did not have calibration curves for all of the analytes of interest. Percent peak areas were calculated by dividing the ion counts for a particular peak (detected by FID or MS) by the total ion counts for the entire chromatogram and expressing this value as a percent. Calibration curves with authentic standards were used to quantify the abundance of α-pinene, 1,8-cineole, thymol, methyl eugenol, piperitone, and elemicin. In this case the peak area detected by FID or MS for each analyte shot at six or seven different concentrations between 0 and 1000 mg/L were plotted. The regression of this line was used to interpolate the abundance of individual essential oil components from the total ion count measured in their respective peaks. Dry matter percent of greenhouse tissue was determined using AOAC procedures (*12*).

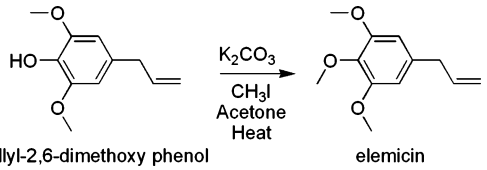

Figure 1. Synthesis of elemicin from 4-allyl-2,6-dimethoxyphenol.

Bioassays. Antimicrobial bioassays were conducted using the following organisms: *Candida keyfr* ATCC 44691, *Geotrichim candidum* (*G. candidum*) ATCC 48112, *Streptococcus pneumoniae* (*Strep. pneumoniae*) ATCC 6303, *Staphylococcus aureus* (*Staph. aureus*) ATCC 27661, *Enterobacter aerogenes* (*E. aerogenes*) ATCC 13048, *Enterobacter clocae* (*E. clocae*) ATCC 13047, *Shigella flexneri* (*Shig. flexneri*) ATCC 29903, *Klebsiella pneumoniae* (*K. pneumoniae*) ATCC 132, *Salmonella typhimurium* (*Sal. typhimurium*) ATCC 14028, *Chromobacterium violaceum* (*C. violaceum*) ATCC 12472, and *Neissera subflava* (*N. subflava*) ATCC 14799. Triplicate serial dilutions of *A. californica* leaf essential oil in nutrient broth ranging from 0.001 to 0.1% (v/v) were prepared in a 96-well microtiter plate. Controls included nutrient broth, uninoculated serial dilutions of oil, and nutrient broth inoculated with microorganisms minus essential oil. Cultures were incubated at 37 °C in a BioTek PowerwaveX Select-I spectrometer and agitated for 55 min before each reading. Optical density (600 nm) was recorded hourly for 48 h. Assays with α-pinene, elemicin, methyl eugenol, piperitone, 1,8-cineole, and sabinene were performed as above, at concentrations from 1×10^{-5} to 0.01% (v/v).

Synthesis of Elemicin. 4-Allyl-2,6-dimethoxyphenol (technical grade, 90%; 4.12 mL, 23.2 mmol, Sigma-Aldrich) and K_2CO_3 (9.61 g) were combined in acetone (125 mL). Iodomethane (4.3 mL, 46.4 mmol, Sigma-Aldrich) was then added, and the mixture was refluxed for 6 h (**Figure 1**), at which time GC/MS analysis of an aliquot indicated the reaction was complete. Then, the K_2CO_3 was filtered off and was rinsed with EtOAc (100 mL). The solvent was removed, and the mixture was loaded on a plug of silica (Merck, grade 9385, 230−400 mesh) which was then eluted with 1:1 EtOAc:hexane (150 mL total). The solvent was removed from the eluate, and the residue was distilled under vacuum (20 mmHg). Distillate fractions were analyzed by GC/MS for purity, and those with ≥90% purity, which were collected between 145 and 157 °C, were combined. The proton NMR spectrum of elemicin was also obtained (Bruker Avance 500 MHz).

RESULTS AND DISCUSSION

Synthesis of Elemicin. Elemicin is an abundant component of the essential oils of *A. californica* and *Myristica fragrans* (*M. fragrans*) but not commercially available. Therefore, to generate standard curves of this compound, it was necessary to synthesize elemicin (**Figure 1**). The synthesis of elemicin from 3.75 g of 4-allyl-2,6-dimethoxyphenol (a commercially available standard) yielded 2.3 g (11 mmol, 47%) of pure (>93%) elemicin. Sample purity was determined by spectrometry (GC/MS). The NMR spectrum of the elemicin product confirmed the identity predicted by GC/MS data. ^{1}H NMR (acetone-d_6): δ 3.32 (d of multiplets, J = 4 Hz, 2H), 3.67 (s, 3H), 3.79 (s, 6H), 5.01 (d of multiplets, J = 9 Hz, 1H), 5.09 (d of multiplets, J = 17 Hz), 5.9−6.0 (m, 1H), 6.50 (s, 2H).

Essential Oil and SPME Analysis. Distilled oil was prepared from two independent samples, and SPME was performed on two independent samples. All of the values for recovery and the abundance of essential oil components in these two extracts are expressed as the range of the value obtained for each of the duplicate samples, or as the midpoint between these values.

Dry matter accounted for 43.0% of the leaf fresh weight, and the leaf essential oil obtained by steam distillation accounted for 0.67−0.97% of the leaf dry matter. **Table 1** lists 38 compounds detected in either the steam distillate or the SPME sample of leaf tissue. Many of these compounds (21/38, 55%)

Table 1. Volatile Compounds Identified in *A. californica* Leaves: Comparison of Peak Area Percentages Detected for Steam-Distilled Oil and SPME Samples on the GC/FID

	compound[a]	std[b]	KI[c]	% peak area ($n = 2$) oil	SPME
1	2-(*E*)-hexenal	–	853	0.3–1.1	ND[d]
2	2,4-(*E*,*E*)-hexadienal	–	909	ND	trace
3	tricyclene	+	928	ND	0.0–0.3
4	α-pinene	+	940	0.4–3.4	10.3–13.1
5	camphene	+	955	0.0–0.1	0.5–0.5
6	sabinene	+	977	0.0–0.1	4.4–5.7
7	*β*-pinene	+	981	0.3–1.5	0.0–0.3
8	myrcene	+	992	0.1–0.3	0.8–1.0
9	α-phellandrene	+	1006	0.2–0.5	0.2–0.2
10	*p*-cymene	+	1027	0.0–0.2	1.5–2.2
11	*β*-phellandrene	–	1032	0.9–2.3	8.6–8.7
12	1,8-cineole	+	1035	1.5–3.5	10.0–10.1
13	(*Z*)-*β*-ocimene	–	1041	0.2–0.3	0.6–0.6
14	(*E*)-*β*-ocimene	–	1052	1.1–2.1	2.0–2.3
15	*cis*-sabinene hydrate	–	1069	ND	trace
16	terpinolene	+	1089	ND	trace
17	linalool	+	1099	0.8–0.8	0.9–1.0
18	*cis*-pinene hydrate	–	1143	ND	trace
19	camphor	+	1145	ND	trace
20	borneol	+	1168	0.3–0.3	0.3–0.3
21	α-terpineol	+	1190	0.4–0.5	0.3–0.3
22	methylchavicol	–	1196	0.7–0.8	1.2–1.3
23	thymol methyl ether	–	1236	ND	Trace
24	piperitone	+	1254	10.4–11.7	16.0–16.4
25	thymol	+	1292	0.1–0.2	trace
26	*δ*-elemene	–	1339	0.0–0.1	0.1–0.1
27	α-copaene	–	1377	ND	trace
28	*β*-elemene	–	1392	0.1–0.3	0.1–0.1
29	methyl eugenol	+	1403	6.5–7.3	5.0–5.2
30	(*E*)-caryophyllene	+	1420	4.2–5.0	8.2–8.3
31	*trans*-α-bergamotene	–	1437	ND	0.1–0.1
32	α-guaiene	–	1440	1.0–1.3	1.7–1.8
32	α-humulene	+	1454	0.8–1.1	1.4–1.4
34	(*E*)-*β*-farnesene	–	1458	0.2–0.5	0.3–0.3
35	germacrene D	–	1485	0.0–0.1	trace
36	*cis*-*β*-guaiene	–	1488	1.2–1.5	1.2–1.3
37	elemicin	+	1552	52.5–53.7	13.1–13.3
38	caryophyllene oxide	+	1582	ND	trace

[a] Compounds listed in order of elution from GC. [b] Std, compounds identified by comparison with reference standard. [c] KI, Kováts index. [d] ND, not detected.

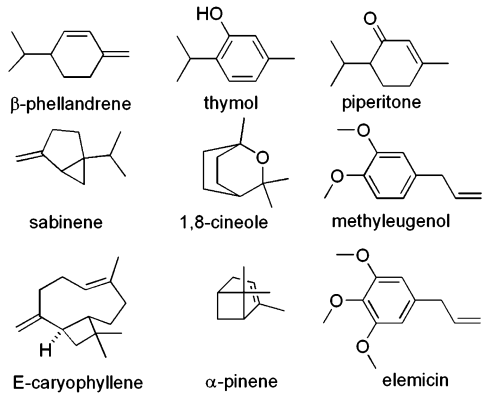

Figure 2. Selected components in essential oil from leaves of *A. californica*.

were identified on the basis of comparison with reference standards; the remaining 45% were tentatively identified on the basis of KI and mass spectra matches. Reference compounds were not readily available. The structures of several of the major components are shown in **Figure 2**. There were only four peaks that we were not able to identify; these compounds represented <1% of the total peak area. In the oil prepared by steam distillate, compounds comprising more than 5% of the FID peak areas were elemicin (53.1%), piperitone (11.1%), and methyl eugenol (6.9%). In SPME, a remarkably lower abundance of elemicin was observed; piperitone (16.2%), elemicin (13.2%), α-pinene (11.7%), 1,8-cineole (10.1%), *β*-phellandrene (8.7%), (*E*)-caryophyllene (8.3%), methyl eugenol (5.1%), and sabinene (5.1%) exceeded 5% of the total peak area on the FID chromatograms.

Yields for elemicin were remarkably high in the steam-distilled oil, and notably different from the SPME headspace composition. The initial SPME elemicin recovery with an equilibration temperature of 30 °C was much lower (13.2%) than the elemicin content in the oil (53.1%). To determine the role of extraction temperature in elemicin recovery, additional vials of leaf tissue were equilibrated at 45, 55, and 65 °C prior to SPME extraction. The resulting chromatograms revealed elemicin peak area percents of 22, 34, and 49%, respectively. Apparently, the low recovery of elemicin by SPME is due to its low volatility at 30 °C.

The *A. californica* root oil described by Sanvordeker and Chaubal (*4*) had three components present at >5%: methyl eugenol (57.0%), thymol (13.8%), and piperitone (8.0%). The leaf oil we describe has similar levels of piperitone, but much lower levels of thymol and methyl eugenol. Curiously, elemicin, which was not detected by Sanvordeker and Chaubal (*4*) in roots, comprised 53% of the leaf oil. Elemicin differs structurally from methyl eugenol by a single methoxy group. The compositional differences observed between studies are not surprising considering differences in tissue (root versus leaf, genetic variations, growth environments, and phenology). Studies examining roots of *A. californica* growing in New Mexico are underway.

SPME is especially useful for identifying compounds found at the trace level in essential oil (**Table 1**). Ten compounds are listed in **Table 1** as not detected in the oil-GC/FID profiles that were detected at trace or low levels in the SPME-GC/FID profiles. Variability between samples was lower in SPME extractions than in the steam-distilled oils. Examples of lower reproducibility in the oil extractions as compared with SPME can be seen in **Table 1** for α-pinene, *β*-pinene, *β*-phellandrene, 1,8-cineole, piperitone, and methyl eugenol. The range of extracted compounds, small tissue requirements, and the simplicity of the technique make SPME an attractive method for qualitative analysis of plant volatiles.

SFE Analysis. While the SPME samples were useful for the characterization of the leaf volatiles, there was no extract generated by this method to be used in tests for biological activity. In an effort to develop both an efficient method for chemical analysis as well as a source of material for bioassay, leaves from both greenhouse and wild plants were extracted using a supercritical fluid extractor. In this method we performed these extractions on three independent leaf samples.

Table 2 reports the abundances of leaf volatiles as a percent of total peak area for each of the three methods of extraction. A single value is reported for each volatile for each method. In the case of the oil and SPME samples, the value represents the midpoint of the two samples; for SFE it is the average of three samples. Only those compounds detected at 5% or greater in samples generated by any of the three extraction methods are listed in this table. All but one of these compounds were identified by comparison with authentic reference standards. The presence of *β*-phellandrene is tentative, on the basis of the KI and mass spectra match of this peak to values for *β*-phellandrene.

Table 2. Comparison of Abundances of Selected Essential Oil Components Detected by the Three Different Extraction Protocols on Leaf Tissues of *A. Californica* Cultured in the Greenhouse[a]

		avg % peak area		
compound[b]	std[c]	SPME ($n = 2$)	oil ($n = 2$)	SFE ($n = 3$)
α-pinene	+	11.7	1.9	
sabinene	+	5.0	0.1	
β-phellandrene	–	8.6	1.6	
1,8-cineole	+	10.1	2.5	0.3
piperitone	+	16.2	11.5	4.5
methyl eugenol	+	5.1	6.9	7.0
(*E*)-caryophyllene	+	8.3	4.6	2.1
elemicin	+	13.2	53.1	69.9

[a] Only those compounds detected at ≥5% by one or more extraction methods are listed. [b] Compounds listed in order of elution from GC. [c] Std, compounds identified by comparison with reference standard.

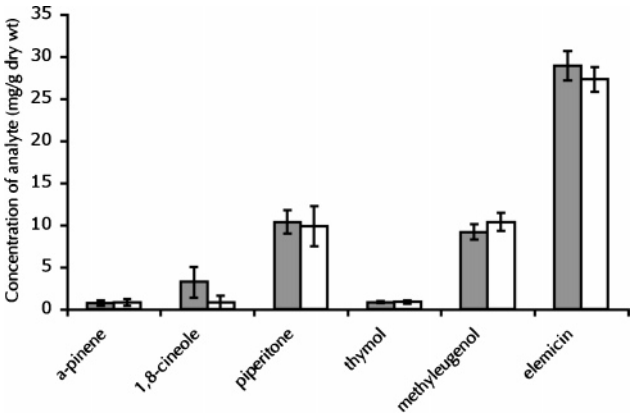

Figure 3. Comparison of greenhouse (open bars) and wild-grown (filled bars) *A. californica* leaf SFE extracts. Triplicate SFE extractions were characterized using GC/MS, and the abundances of the major essential oil components, quantified. Error bars represent the standard deviation around the mean ($n = 3$).

Of the three methods, the SFE extracted the fewest compounds, and SPME, the most. The SFE recovered detectable levels of all of the compounds present in the steam-distilled oil at 5% or higher. There were differences in the abundance of compounds recovered by each of the three extraction methods. Elemicin represented the most abundant, if not dominant, compound in the steam-distilled oil and the SFE sample. Only trace levels of α-pinene and β-phellandrene were recovered in SFE samples, yet these compounds were present at close to 2% of the steam-distilled oil. The SPME and steam-distilled preparations were performed on frozen leaves, while the SFE was performed on air-dried leaves. This difference in pretreatment might explain the lower levels of recovery of compounds with lower boiling points in the SFE sample, i.e., α-pinene, sabinene, and β-phellandrene. For bay leaves, the method of preparation (drying, freezing, or fresh tissue) had a significant impact on the recovery of specific volatile components (*7*).

There was little variability in leaf volatile composition in leaves from plants grown in the greenhouse and their clonal parent grown on the Rio Grande riverbank in Dona Ana county (**Figure 3**). The SFE method was reliable and produced low variability within the triplicate samples of either the field-grown or greenhouse-grown plant material. This extraction method is amenable to experiments comparing leaf or root volatile compositions within and between different populations or different environmental conditions. Steam distillation requires

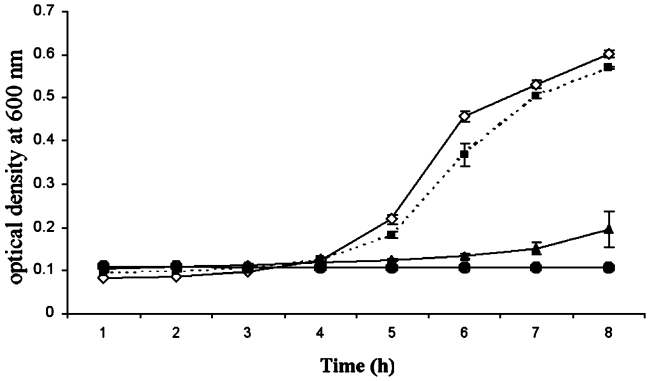

Figure 4. The effects of *A. californica* leaf essential oil and α-pinene on *Staph. aureus* growing in nutrient broth: ◇, media and bacteria; ▲, 0.1% (v/v) leaf essential oil; ■, 0.005% (v/v) α-pinene; ●, 0.01% (v/v) α-pinene.

20 g of tissue and 6−8 h for preparation and extraction. SFE requires 0.5 g of tissue and 1.5 h for preparation and extraction. We conclude that SFE is an effective method for the extraction of specific compounds in *A. californica* tissue, requiring minimal time and tissue for preparation and extraction and yielding extracted fractions that can be assayed for bioactivity.

Bioassays. All inhibition observed in bioassays was apparent in the first 8 h. Growth of microorganisms as affected by essential oil or pure compounds was determined relative to growth of control microorganisms. For the purposes of this study, inhibition is defined as ≤50% of control growth, as determined by optical density at 600 nm. Leaf oil bioassays were performed against several microorganisms, including gram-positive and gram-negative bacteria, yeast, and fungi. The growth of *Staph. aureus*, *Strep. pneumoniae*, and *G. candidum* was inhibited by *A. californica* leaf oil at 0.1%.

To determine whether the most abundant compounds in the oil were responsible for some of the antimicrobial effects observed, bioassays against *Staph. aureus* were repeated using single volatile oil compounds (α-pinene, sabinene, 1,8-cineole, piperitone, methyl eugenol, and elemicin) in place of the leaf oil. Pure α-pinene at 0.01% (v/v) resulted in a complete kill of the bacteria (**Figure 4**). None of the other constituents inhibited the growth of *Staph. aureus* (data not shown). It is probable that α-pinene in the leaf essential oil was only partially responsible for the inhibition of *Staph. aureus*. *A. californica* leaf essential oil at 0.1% (v/v) contains 0.002% (v/v) α-pinene. This concentration of leaf oil inhibited 73% of *Staph. aureus* growth. However, α-pinene at 0.005% (v/v) did not inhibit *Staph. aureus* growth by 70% (**Figure 4**). If α-pinene in the leaf oil was solely responsible for the antimicrobial effect, we would expect inhibition to occur at this concentration. Instead this dose (0.005%) of α-pinene only inhibited the growth of the bacteria by ∼10%. These results suggest that other compounds in the leaf essential oil are responsible for either direct inhibition of *Staph. aureus* or additive or synergistic enhancement of the inhibitive effect of α-pinene. No single microorganism has been specifically linked to any of the ailments historically treated with *A. californica*. Yet, the symptoms resulting from infection by the microorganisms tested in this study (*13*−*15*) are similar to symptoms traditionally treated with *A. californica* (*1*−*3*).

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