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Antimycobacterial Furofuran Lignans from the Roots of *Anemopsis californica*

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Abstract

Topical preparations of *Anemopsis californica* have been used by Native American tribes in the southwestern United States and northern Mexico to treat inflammation and infections. We report results of bioassay-guided isolation conducted on a sample of *A. californica* roots. The furofuran lignans sesamin (**1**) and asarinin (**2**) were isolated and shown to have MIC values ranging from 23 to 395 μM against five different species of environmental nontuberculous mycobacteria. These findings are significant given that these bacteria can cause skin, pulmonary, and lymphatic infections. Crude *A. californica* extracts were analyzed by liquid chromatography - mass spectrometry (LC-MS), and it was determined that sesamin and asarinin were extracted at relatively high levels from roots (1.7–3.1g/kg and 1.1–1.7 g/kg, respectively), but lower levels from leaves (0.13 g/kg for both compounds). Our findings suggest that the majority of activity of crude *A. californica* root extracts against nontuberculous mycobacteria can be attributed to the presence of sesamin and asarinin. This paper is the first to report isolation of these compounds from a member of the Saururaceae family, and the first to describe their activity against nontuberculous mycobacteria.

Keywords

Anemopsis californica; Saururaceae; nontuberculous mycobacteria; antibacterial; botanical; sesamin; asarinin

Introduction

A new arsenal of antibiotics is needed to address two problems involving treatment of bacterial infections: the emergence of drug-resistance and the existence of bacteria that are

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Conflict of Interest

The authors report no conflict of interest.

innately resistant to most antibiotics. A promising source for new antibacterial compounds is the natural products produced by plants, bacteria, and fungi. It is estimated that 25 to 50 percent of anti-infective agents come from these natural sources [1].

This study focuses on the plant *Anemopsis californica* (Nutt.) Hook. & Arn. (Saururaceae) as a source of antimicrobial compounds. *A. californica*, commonly known as "yerba mansa," is native to the southwestern United States and northern Mexico, and its roots, leaves, and stem have been used medicinally by many Native American tribes [2–5]. Despite historical and modern precedent for the use of this plant to treat infection, only a few studies have focused on the chemicals responsible for its anti-infective properties [6–7]. The chemical compounds identified from *A. californica* thus far are exclusively from the volatile oils of the leaves and roots [8–9]. Volatile oil extracts from *A. californica* have been shown to inhibit the growth of endometrial, cervical, colon, and breast cancer cells *in vitro* [8–9], and demonstrated antimicrobial activity against *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Geotrichim candidum* [6]. Additionally, ethanol and ethyl acetate extracts of various parts of *A. californica* were shown to inhibit the growth of colon and breast cancer cells, and aqueous *A. californica* extracts inhibited cell migration and metastasis [10–11]. These studies did not indicate which chemical constituents were responsible for the observed effects.

There are currently no reports of the identities of non-volatile compounds present in *A. californica* roots and leaves. This is a significant gap in the literature, given that its traditional mode of application of *A. californica* is a whole plant poultice or decoction. For example, the Shoshoni tribe of Nevada applied boiled and mashed *A. californica* roots to areas of inflammation and infections [2]. The Pima tribe of Arizona and New Mexico and the Mahuna and Chumash tribes of California used a decoction of leaves and roots to treat wounds [3–4, 12], and the Nevada Paiute and the California Costanoan tribes used decoctions of *A. californica* roots or leaves to treat pain [2,5].

The ethnobotanical precedent for application of *A. californica* to treat infections encouraged us to screen extracts from this plant for activity against pathogenic microorganisms. Activity of crude *A. californica* extracts was noted against several species of nontuberculous mycobacteria. These findings were deemed significant, given the clinical relevance these organisms. Nontuberculous mycobacteria are commonly found in soils, natural waters, and engineered water systems, including household plumbing [13–14], and can cause pulmonary, skin, and lymph node infections [15]. The resultant chronic respiratory or soft tissue infections require long term antibiotic treatment that can have serious side effects [16]. Current estimates report a total number of over 16,000 cases in the United States of nontuberculosis mycobacterial disease per year with a total cost of over $425 million [17]. The objective of this research was to identify compounds from *A. californica* with potential for the treatment of nontuberculous mycobacterial infections. In addition, we sought to provide insight into the scientific basis for the ethnobotanical use of *A. californica* to treat bacterial infections.

Results and Discussion

Bioactivity-guided fractionation of *A. californica* resulted in the isolation of two compounds, sesamin (**1**) [22] and its C-7 epimer, asarinin (**2**). Sesamin was a white solid with a HRESIMS *m/z* of 355.1175 (calcd for $C_{20}H_{19}O_6$ $[M+H]^+$ *m/z* 355.1176, $[\alpha]_D^{25}=+104$, c = 0.0125 g/100 mL, methanol). Asarinin, also called episesamin or isosesamin, was a white solid with a HRESIMS *m/z* of 355.1167 (calcd for $C_{20}H_{19}O_6$ $[M+H]^+$ *m/z* 355.1176, $[\alpha]_D^{25}=+144$, c = 0.0125 g/100 mL, methanol). The ^1H and ^{13}C NMR of both sesamin and asarinin were in agreement with literature values [23]. NMR data are included as Supplemental Information (Table S1, Figures S1–S4).

Although sesamin and asarinin are both known compounds, this is the first report of their presence in a member of the Saururaceae plant family. Sesamin was first isolated from sesame seed oil [24–26], while asarinin was first isolated from prickly ash bark [27]. Both sesamin and asarinin have been shown to act as insecticidal synergists with pyrethrins [25–26]. Previous studies have shown that sesamin has moderate activity against *S. aureus* and no activity against *E. coli* [28]. In addition, asarinin was shown to be moderately active against *S. aureus* and *B. subtilis*, and it was suggested that it inhibits the NorA efflux pump system of *S. aureus* [29–30].

Sesamin and asarinin demonstrated a range of antimicrobial activities (8 to 140 μg/mL or 22.6 μM to 395 μM) against the five different species of *Mycobacterium* evaluated (Table 1). This is the first report of activity of sesamin and asarinin against nontuberculous mycobacteria, although both sesamin and asarinin were shown to lack activity against *M. tuberculosis* [31]. These results are not surprising, given that members of the *M. tuberculosis* complex do not share the same susceptibilities to anti-mycobacterial antibiotics with the nontuberculous mycobacteria [16].

Crude *A. californica* extracts were tested against representative *Mycobacterium* species (Table 1). Samples 1 and 3 (root extracts) demonstrated MIC values ranging from 125 to >250 μg/mL, while sample 2 (aerial extract) demonstrated weak or no inhibition (MIC >250 μg/mL). These differences in activity were likely due to the higher levels of sesamin and asarinin present in roots as compared to leaves (Table 2).

The root extracts (samples 1 and 3) were significantly less active compared to the pure compounds. However, these extracts are crude mixtures, and sesamin and asarinin constituted only a fraction of their content (Table 2). Accounting for this difference, it appeared that the antimycobacterial activity of the crude extracts could be largely attributed to the presence of sesamin and asarinin. For example, sample 1, a root extract of *A. californica*, demonstrated an MIC of 125 μg/mL against *M. smegmatis* (Table 1). Sample 1 contained 8.8 ± 1.0 % sesamin and 4.7 ± 0.4 % asarinin (Table 2); thus, the MIC of 125 μg/mL (expressed as mass of crude extract/volume media) is equivalent to 16.8 μg/mL (expressed as mass of sesamin and asarinin, combined, per volume of media). This value is within the range of the reported MICs for sesamin and asarinin alone against *M. smegmatis* (8 and 35 μg/mL, respectively).

In conclusion, the results of the quantitative analysis suggest that the majority of activity of *A. californica* root extracts against nontuberculous mycobacteria can be attributed to the presence of relatively high levels of sesamin and asarinin. Importantly, the presence of anti-mycobacterial compounds in *A. californica* roots supports the traditional use of this plant as a treatment for infection, although follow up studies would be necessary to evaluate the *in vivo* relevance of these findings. The higher levels of sesamin and asarinin in roots over leaves suggest that root extracts would be more effective than leaf extracts for treatment of mycobacterial infections.

Materials and Methods

Plant Material

Cultivated *Anemopsis californica* plant material was obtained from two sites, Horizon Herbs in Williams, OR (42°12' 17.21 "N, 123°19' 34.61"W; voucher number NCU592735, identified by Richard A. Cech) and Apache Creek Ranch in Santa Fe, NM (35°35' 56.40"N, 105°50' 27.22"W; voucher number NCU602027, identified by Amy Brown). Vouchers are retained at the University of North Carolina Herbarium. Harvested plant material was separated into three different portions, a root sample (sample #1, 9.8 g dry weight), a leaf/stem sample from the same plant (sample #2, 7.5 g dry weight, both harvested from Horizon Herbs in April, 2010), and a large batch of roots/rhizomes to facilitate isolation work (sample #3, 520 g dry weight) harvested from Apache Creek Ranch in November 2010. All plant material was air dried prior to extraction.

Extraction and LC-MS

Three batches of *A. californica* plant material were cut and ground, and then was macerated in methanol for 24 hours. The marc for each extract was subsequently soaked in methanol a total of three times, and the methanol was decanted and combined. The methanol extracts were evaporated to dryness with a rotary evaporator and subjected to liquid-liquid partitioning using published methods [18]. Briefly, the methanol extract was defatted by partitioning between a 1:1 ratio of hexane to methanol, and the latter fraction was then dried down and further partitioned between 4:1:5 of chloroform:methanol:water. The chloroform fraction was evaporated to dryness and its antimicrobial activity was tested using a broth microdilution assay. The yields from the chloroform fraction of samples 1, 2, and 3 were 343 mg, 104 mg, and 10 g, respectively.

For the isolation of the active compounds, the chloroform extract (10 g, sample #3, roots and rhizomes) was subjected to two stages of normal-phase chromatography on a CombiFlash®R_f ISCO using 120 g RediSep Rf Gold® Silica Columns (20 – 40 μm particle size, Teledyne ISCO, Lincoln, NE, USA). The first stage of normal-phase chromatography was performed with a hexane/chloroform/methanol gradient on silica gel (eluent chloroform and methanol, flow rate 18 mL/min) and the eluate was pooled into 12 fractions. Fraction VI (360–450 mL, 645 mg) was subjected to a second stage of separation with a hexane/acetone/methanol gradient on silica gel (eluent acetone and methanol, flow rate 18 ml/min) and pooled into 10 fractions. Fraction V (250–320 mL, 420 mg), the active fraction, was then subjected to further purification with two successive stages of isocratic separation with

reversed-phase preparative HPLC on a C_{18} column (Phenomenex, Gemini-NX, 5 μm, 250 × 21.2 mm, flow rate 21 mL/min). The first stage of reversed-phase separation employed a 50:50 acetonitrile:water isocratic mobile phase composition, and the eluate was pooled into 5 fractions. Fractions II and III from this separation (200–300 mL and 320–400 mL, 75 mg and 80 mg, respectively) were then combined and subjected to a second reversed-phase separation with an isocratic mobile phase composition of 75:25 methanol:water. Sesamin **(1)** (0.012 % yield, 60 mg, 98.3% purity) eluted at 15 min and asarinin **(2)** (0.0096% yield, 60 mg, 98.5% purity) eluted at 16.5 min.

Test Bacteria, Chemicals, Biochemicals, and MIC

Mycobacterium marinum (ATCC strain 927), *M. smegmatis* strain mc^2155 (ATCC strain 700084), *M. abscessus* strain AAy-P-1, *M. chelonae* strain EO-P-1, *M. intracellulare* strain TMC 1406^T (ATCC 13950), and *M. avium* strain A5 [19–20] were grown in Middlebrook 7H9 broth medium containing 0.5 % (v/v) glycerol and 10 % (v/v) oleic acid-albumin with aeration (120 rpm) for 7 days at 37°C or 30°C (only *M. marinum*). Minimal inhibitory concentration (MIC) of each fraction or compound was measured by broth microdilution with a starting inoculum of 0.5–1.0 $\times 10^5$ CFU/mL [20–21]. The plates were incubated for 4 days at 37°C or 30° C (only *M. marinum*) and the turbidity measured (absorbance 580 nm). Extracts were tested over a concentration range of 0.12 to 250 μg/mL by two-fold dilutions, in the presence of a constant DMSO concentration (2%). The MIC was defined as the lowest concentration completely inhibiting bacterial growth. Rifampin (Sigma, St. Louis, MO purity ≥ 97%) served as the control.

Quantitative Analysis

Sesamin and asarinin were identified in the crude *A. californica* extracts by matching retention time and fragmentation patterns with those of the isolated standard compounds. The concentrations of these compounds were then measured using selective reaction monitoring (SRM) on a triple quadrupole mass spectrometer (TSQ Access; Thermo Scientific, Waltham, MA, USA) with an electrospray ionization source in the positive ion mode. Transitions of 337.1 to 203.1 and 337.1 to 289.2 were employed for the isomeric compounds. The mass spectrometer was coupled to a reversed phase high performance liquid chromatograph (HPLC) (Agilent HP1200; Santa Clara, CA, USA) with a PFP column (5 μm, 150 × 4.6 mm; Phenomenex, Torrance, CA, USA). An acetonitrile (1% formic acid):water (1% formic acid) gradient was employed at 1.0 mL/min with HPLC grade solvents. A calibration curve (concentration range of 0.05 to 5.0 μg/mL) of concentration versus average peak area for triplicate injections was employed for quantitative analysis. Extracts were diluted so that sesamin and asarinin concentrations fell within the linear range of the calibration curve.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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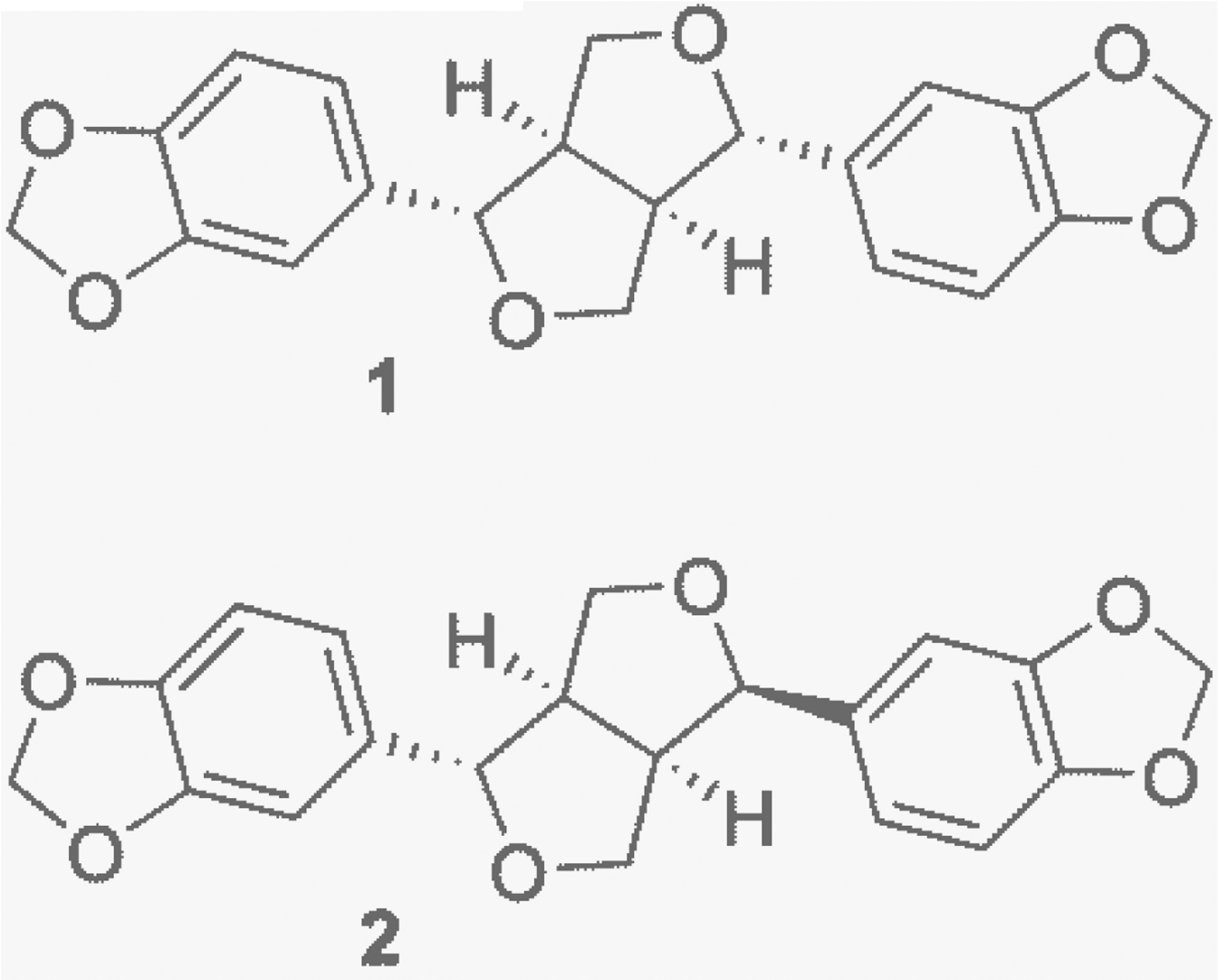

Fig 1.
Structures of furofuran lignans sesamin (**1**) and asarinin (**2**) isolated from *A. californica*.

Table 1

Minimum inhibitory concentrations (MIC) of crude extracts of *A. californica*, sesamin, and asarinin against *Mycobacterium* species

Sample	**MIC (μg/mL) Against:**				
	M. smegmatis	***M. abscessus***	***M. chelonae***	***M. marinum***	***M. avium* A5**
Sample 1[a]	125	>250	>250	250	125
Sample 2[a]	>250	>250	>250	>250	>250
Sample 3[b]	125	>250	250	>250	>250
Sesamin	8	>130	65	13	8
Asarinin	35	>140	140	35	35
Rifampin[c]	25	0.8	0.15	0.8	2

[a] Samples 1 and 2 are for root and aerial extracts-respectively from the same *A. californica* plant.

[b] Sample 3 represents a large batch root extract that was subjected to bioactivity-directed fractionation, resulting in the isolation of sesamin and asarinin.

[c] The antibiotic rifampin is included as a positive control. Negative control (vehicle, 2% DMSO) caused no significant growth inhibition.

Table 2

Quantity of sesamin and asarinin in extracts prepared from roots or leaves/stems of *A. californica*

Sample Name	Plant part	Sesamin Concentration[a]		Asarinin Concentration	
		yield[b] (ppt) ± SD	% in extract[c] ± SD	yield (ppt) ± SD	% in extract ± SD
Sample 1	Root	3.1 ± 0.3	8.8 ± 1.0	1.7 ± 0.1	4.7 ± 0.4
Sample 2	Leaf/stem	0.13 ± 0.02	0.95 ± 0.14	0.13 ± 0.02	0.9 ± 0.2
Sample 3	Root	1.7 ± 0.2	8.8 ± 0.9	1.1 ± 0.1	5.9 ± 0.2

[a]Concentrations were determined by LC - MS analysis of extracts prepared from the relevant plant parts. Standard deviations are for triplicate analyses of the same extract. Extract concentration was calculated based on linear regression analysis of 6 point calibration curves of peak area versus concentration with slope (m) = 587881 ± 4628, intercept (b) = −20512 ± 10554, and R^2 = 0.9998 for sesamin and a slope (m) = 647726 ± 16450, intercept (b) = −15594 ± 37516, and R^2 = 0.9974 for asarinin. Extracts were diluted so that the concentrations tested fell within the linear range of the calibration curve.

[b]Yield is reported as parts per thousand or mass of pure compound (g) per mass of original plant material (kg).

[c]% in extract is reported as mass (g) of sesamin or asarinin per mass of solid extract (g) × 100.

The % in extract values are provided for the purpose of comparison with biological data.