

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, and it was determined that sesamin and asarinin were extracted at relatively high levels from the roots (1.7–3.1 g/kg and 1.1–1.7 g/kg, respectively), but at lower levels from the 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 the 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

Article:

Introduction

A new arsenal of antibiotics is needed to address two problems involving the treatment of bacterial infections: the emergence of drug-resistance and the existence of bacteria that are 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 stems have been used medicinally by many Native American tribes [2–5]. Despite an 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 *Geotrichum 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 nonvolatile compounds present in *A. californica* roots and leaves. This is a significant gap in the literature, given that the 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 of 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 16000 cases in the United States of nontuberculous 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 a 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) [18] and its C-7 epimer, asarinin (2) (Fig. 1). Sesamin was a white solid with an 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 an 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 [19]. NMR data are included as

Supporting Information (Table 1S, Figs. 1S–4S, Supporting Information). 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 [20–22], while asarinin was first isolated from prickly ash bark [23]. Both sesamin and asarinin have been shown to act as insecticidal synergists with pyrethrins [21, 22]. Previous studies have shown that sesamin has moderate activity against *Staphylococcus aureus* and no activity against *Escherichia coli* [24]. In addition, asarinin was shown to be moderately active against *S. aureus* and *Bacillus subtilis*, and it has been suggested that it inhibits the NorA efflux pump system of *S. aureus* [25, 26].

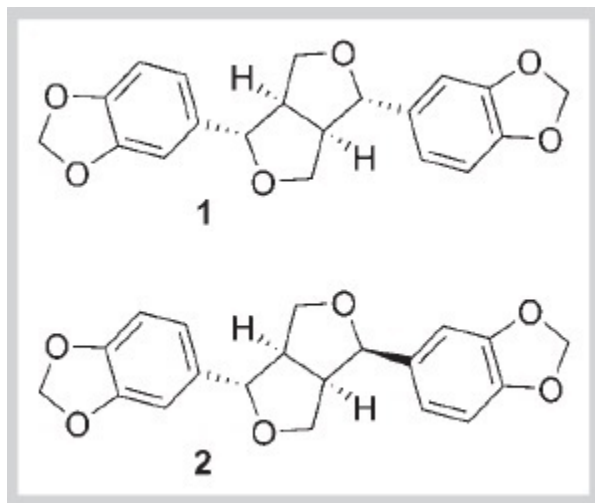


Figure 1. Structures of furofuran lignans sesamin (1) and asarinin (2) isolated from *A. californica*.

Sesamin and asarinin demonstrated a range of antimicrobial activities (8 to 140 $\mu\text{g/mL}$ or 22.6 μM to 395 μM) against the five different species of *Mycobacterium* that were evaluated (Table 1). This is the first report of activity of sesamin and asarinin against nontuberculous mycobacteria, although both sesamin and asarinin have been shown to lack activity against *Mycobacterium tuberculosis* [27]. These results are not surprising, given that members of the *M. tuberculosis* complex do not share the same susceptibilities to antimycobacterial 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 $\mu\text{g/mL}$, while sample 2 (aerial extract) demonstrated weak or no inhibition (MIC > 250 $\mu\text{g/mL}$). These differences in activity were likely due to the higher levels of sesamin and asarinin present in the roots as compared to the 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 $\mu\text{g/mL}$ against *Mycobacterium smegmatis* (Table 1). Sample 1 contained $8.8 \pm 1.0\%$ sesamin and $4.7 \pm 0.4\%$ asarinin (Table 2); thus, the MIC of 125 $\mu\text{g/mL}$ (expressed as mass of crude extract/volume media) is equivalent to 16.8 $\mu\text{g/mL}$ (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 $\mu\text{g/mL}$, respectively).

Table 1. Minimum inhibitory concentrations (MIC) of the crude extracts of *A. californica*, sesamin, and asarinin against *Mycobacterium* species.

Sample	MIC ($\mu\text{g/mL}$) against: <i>M. smegmatis</i>	<i>M. abscessus</i>	<i>M. chelonae</i>	<i>M. marinum</i>	<i>M. avium</i> 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. The negative control (vehicle, 2% DMSO) caused no significant growth inhibition

Table 2. Quantity of sesamin and asarinin in extracts prepared from the roots or leaves/stems of *A. californica*.

Sample name	Plant part	Sesamin concentration ^a yield ^b (ppt) \pm SD	% in extract ^c \pm SD	Asarinin concentration yield (ppt) \pm SD	% in extract \pm 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. The extract concentration was calculated based on linear regression analysis of 6-point calibration curves of the peak area versus the concentration with a 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 The yield is reported as parts per thousand or mass of pure compound (g) per mass of original plant material (kg).

^c The % in extract is reported as mass (g) of sesamin or asarinin per mass of solid extract (g) \times 100. The % in extract values are provided for the purpose of comparison with biological data

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 antimycobacterial 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 the roots than in the leaves suggest that the root extracts would be more effective than the leaf extracts for the treatment of mycobacterial infections.

Materials and Methods

Plant material

Cultivated *A. 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 dryweight, harvested from Apache Creek Ranch in November 2010). All plant material was air-dried prior to extraction.

Extraction and liquid chromatography-mass spectrometry

Three batches of *A. californica* plant material were cut and ground, and then 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 [28]. Briefly, the methanol extract was defatted by partitioning between a 1:1 ratio of hexane to methanol, and the latter fraction was then dried 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® Rf ISCO using 120 g RediSep Rf Gold® silica columns (20–40 µm particle size, Teledyne ISCO). 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 (360–450mL, 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 18mL/min) and pooled into 10 fractions. Fraction V (250–320mL, 420mg), the active fraction, was then subjected to further purification with two successive stages of isocratic separation with reversed-phase preparative HPLC on a C18 column (Phenomenex, Gemini-NX, 5 µm, 250 × 21.2 mm, flow rate 21mL/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, 75mg 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, 60mg, 98.3% purity) was eluted at 15 min and asarinin (2) (0.0096% yield, 60mg, 98.5% purity) was eluted at 16.5 min.

Test bacteria, chemicals, biochemicals, and minimal inhibitory concentration

Mycobacterium marinum (ATCC strain 927), *M. smegmatis* strain mc2 155 (ATCC strain 700084), *Mycobacterium abscessus* strain AAY-P-1, *Mycobacterium chelonae* strain EO-P-1, *Mycobacterium intracellulare* strain TMC 1406T (ATCC 13950), and *Mycobacterium avium* strain A5 [29,30] 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*). The minimal inhibitory concentration (MIC) of each fraction or compound was measured by broth microdilution with a starting inoculum of $0.5\text{--}1.0 \times 10^5$ CFU/mL [30, 31]. The plates were incubated for 4 days at 37°C or 30°C (*M. marinum* only) and the turbidity was measured (absorbance 580 nm). Extracts were tested over a concentration range of 0.12 to 250 µg/mL by twofold dilutions in the presence of a constant DMSO concentration (2%). The MIC was defined as the lowest concentration completely inhibiting bacterial growth. Rifampin (Sigma, purity $\geq 97\%$) served as the control.

Quantitative analysis

Sesamin and asarinin were identified in 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,) 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 highperformance liquid chromatograph (HPLC) (Agilent HP1200) with a pentafluorophenyl (PFP) column (5 µm, 150 × 4.6 mm; Phenomenex). An acetonitrile (1% formic acid):water (1% formic acid) gradient was employed at 1.0mL/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. The extracts were diluted so that sesamin and asarinin concentrations fell within the linear range of the calibration curve.

Supporting information

NMR spectroscopic data, ¹H-NMR spectra, and ¹³C-NMR spectra for sesamin and asarinin from the *A. californica* are available as Supporting Information.

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Conflict of Interest

The authors report no conflict of interest.

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