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The Canadian medicinal plant *Heracleum maximum* contains antimycobacterial diynes and furanocoumarins

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ABSTRACT

Ethnopharmacological relevance: *Heracleum maximum* is amongst the most commonly used plants by the indigenous peoples of North America. The First Nations of the eastern Canada use infusions of *Heracleum maximum* roots for the treatment of respiratory ailments including tuberculosis. Previous investigations of extracts derived from the roots of *Heracleum maximum have* shown it to possess antimycobacterial activity. Aim of the study: To isolate and identify antimycobacterial constituents from the roots of *Heracleum maximum*.

Materials and methods: A methanolic extract of *Heracleum maximum* roots was subjected to bioassay guided fractionation using the microplate resazurin assay (MRA) to assess inhibitory activity against *Mycobacterium tuberculosis* strain H37Ra. The antimycobacterial constituents were identified by NMR, MS and polarimetry.

Results: The polyacetylene (3*R*,8*S*)-falcarindiol and the furanocoumarins bergapten, isobergapten, angelicin, sphondin, pimpinellin, isopimpinellin and 6-isopentenyloxyisobergapten were isolated from the *Heracleum maximum* root extract. (3*R*,8*S*)-Falcarindiol and 6-isopentenyloxyisobergapten exhibited MICs of 24 μM and 167 μM and IC_{50}s of 6 μM and 27 μM against *Mycobacterium tuberculosis* H37Ra respectively. The remaining furanocoumarins bergapten, isobergapten, angelicin, sphondin, pimpinellin, and isopimpinellin were less active, with MICs of 925, 1850, 2149, 1859, 812 and 1625 μM and IC_{50}s of 125, 344, 350, 351, 389 and 406 μM.

Conclusions: (3*R*,8*S*)-Falcarindiol, bergapten, isobergapten, angelicin, sphondin, pimpinellin, isopimpinellin and 6-isopentenyloxyisobergapten were identified as the principal constituents responsible for the antimycobacterial activity of the roots of *Heracleum maximum*. This work supports the ethnopharmacological use of *Heracleum maximum* by Canadian First Nations and Native American communities as a treatment for infectious diseases, specifically tuberculosis.

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1. Introduction

The global prevalence of tuberculosis infection is currently estimated to be one-third of the population, causing close to one and a half million deaths and infecting as many as nine million people every year (WHO, 2012). Due to the highly infectious nature of tuberculosis, the limited number of therapeutic agents currently available, and the growing number of drug-resistant strains, there is an urgent need for the development of new anti-tuberculosis therapies (Koul et al., 2011). The medicinal ethnobotanical knowledge of the Canadian First Nations peoples has been shown to be a valid resource for identifying bioactive plants with studies demonstrating a high degree of correlation between traditional medicinal uses and observed biological activity (Carpenter et al., 2012; Jones et al., 2000; Li et al., 2012; McCutcheon et al., 1992, 1994, 1995, 1997). Canadian plants that have documented applications as treatments for tuberculosis therefore represent an important resource in the search for antimycobacterial natural products.

Heracleum maximum, or cow parsnip, is a member of the plant family Apiaceae (previously known as Umbelliferae) and is ranked as the sixth most commonly used plant by the indigenous peoples

Abbreviations: $[\alpha]_D$, specific rotation measured at 589 nm and the temperature indicated; 1D, one dimensional; 2D, two dimensional; ACS, American Chemical Society; bs, broad singlet; *c*, concentration in g/100 mL; CH_2Cl_2, dichloromethane; d, doublet; DMSO, dimethyl sulfoxide; EtOAc, ethyl acetate; HPLC, high performance liquid chromatography; HRESIMS, high resolution electrospray ionization mass spectrometry; IC_{50}, median inhibitory concentration; IR, infrared; LC–MS, liquid chromatography–mass spectrometry; m, multiplet; MeOH, methanol; MIC, minimum inhibitory concentration; MRA, microplate resazurin assay; MS, mass spectroscopy; NaCl, sodium chloride; *n*BuOH, 1-butanol; NMR, nuclear magnetic resonance; q, quartet; s, singlet; SD, standard deviation; t, triplet; TLC, thin layer chromatography.

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of North America (Moerman, 1998) where it has been used for numerous purposes including medicine, food, fiber, and the preparation of dyes. Eastern Canadian First Nations communities use *Heracleum maximum* as a traditional medicine to treat various infectious diseases (Mechling, 1959) and respiratory ailments (Hinds, 2000; Walker, 2008) that include tuberculosis (Lacey, 1993). Most commonly, *Heracleum maximum* is prepared as a tea by steeping the roots in hot water (Webster et al., 2006), and aqueous extracts of the root have previously been shown to possess significant antimycobacterial activity (Webster et al., 2010), stimulate the immune system through the production of interleukin-6 (Webster et al., 2006) and are not toxic to a variety of primary human cell lines (Webster et al., 2010). Methanolic extracts of *Heracleum maximum* have also demonstrated antimycobacterial, antibacterial and antifungal activity (McCutcheon et al., 1992, 1994, 1997).

The previous reports of antimycobacterial activity being observed for both aqueous and methanolic extracts of *Heracleum maximum* roots (McCutcheon et al., 1997; Webster et al., 2010) prompted us to investigate the antimycobacterial constituents of this plant through bioassay guided fractionation.

2. Materials and methods

2.1. General experimental procedures

All solvents for extraction and isolation were ACS certified or HPLC grade. NMR spectra were recorded on Varian Unity 300 and 400 instruments at 400 MHz for ^{1}H and 75 and 100 MHz for ^{13}C using standard 1D and 2D pulse programs. HRESIMS data were recorded on Thermo LTQ Exactive Orbitrap LC-MS. Optical rotations were determined on an Optical Activity Ltd AA-10 polarimeter. Flash chromatography was performed using a Biotage Flash+ chromatography system and KP-Sil 25+S silica cartridges (40–63 μm, 60 Å). Normal phase semi-preparative HPLC was performed using a Waters 510 pump, a Phenomenex silica column (10 μm, 100 Å, 250 × 10 mm) and a Waters R401 refractive index detector at a flow rate of 4 mL/min. Infrared spectra were recorded on a Nicolet NEXUS 470 Fourier transform infrared spectrometer with compounds as films on NaCl discs. Optical rotations were measured on an Optical Activity Ltd. AA-10 polarimeter at the sodium-D line (589 nm). Antimycobacterial testing was performed using the culture broth supplied in Mycobacteria Growth Indicator Tubes (BBL™ MGIT™) in non-tissue culture treated, low-binding, black 96-well microtitre plates sealed with polyester films (50 μm). Fluorometric readings (in relative fluorescence units, RFU) were recorded using a Molecular Devices Gemini EM dual-scanning microplate spectrofluorometer (530 nm excitation filter and a 590 nm emission filter operating in top-scan mode) for the antimycobacterial assays.

2.2. Plant material and extraction

Roots of *Heracleum maximum* (wet weight: 594 g) were collected by hand in November 2010 from the shore of the Kennebecasis River, Kingston Peninsula, New Brunswick, Canada (45°30.794′ N; 65° 53.956′ W). The roots were cleaned by rinsing with deionized water, freeze dried (dry weight: 128 g) and stored at −20 °C. Plants were identified by Dr. Stephen Clayden of the New Brunswick Museum and a voucher specimen has been deposited in the New Brunswick Museum Herbarium (Number: NBM VP-37491). The freeze dried roots (100.0 g) were ground in a domestic blender, exhaustively extracted in methanol (600 mL) for 8 hours using a Soxhlet extractor and the resulting solution concentrated *in vacuo* to give a crude methanolic extract (18.36 g).

2.3. Mycobacterial strains and growth conditions

Mycobacterium tuberculosis strain H37Ra (ATCC 25177) was grown in the growth medium supplied within the Mycobacteria Growth Indicator Tubes (MGIT™) [casein peptone (8.8 mg) and modified Middlebrook 7H9 broth base (41.3 mg) in deionized water (7 mL)] with the addition of BBL™ MGIT™ PANTA™ (polymyxin B, amphotericin B, nalidixic acid, trimetho-prim and azlocillin) antibiotic mixture (280 μg) in BBL™ MGIT™ oleic acid–albumin–dextrose–catalase enrichment (800 μL) and Tween 80 (4 μL). Cultures were incubated (37 °C; 5% CO_2) in a humid environment for one week before being diluted to a turbidity equivalent to a McFarland 1.0 standard (10^7 CFU) using 0.05% Tween 80 in MGIT growth medium. The resulting mycobacterial suspensions (1.5 mL) were cryogenically preserved for up to four weeks (−20 °C), thawed and diluted to a concentration of 2.0×10^6 cells/mL with MGIT growth medium (1:5) immediately prior to use in bioassays.

2.4. Microplate resazurin assay (MRA)

Stock solutions of rifampin (10 μg/mL) and test fractions (5 mg/mL) were prepared with sterile-filtered DMSO and stored at 4 °C. Antibiotic solutions were used within one month of preparation and fraction solutions were used within one week. Immediately prior to use, stock solutions (20 μL) were diluted with MGIT growth medium (480 μL) and the resulting test solutions (100 μL) transferred to non-peripheral wells of a 96-well microtitre plate and inoculated with suspensions of *Mycobacterium tuberculosis* H37Ra (100 μL). To reduce evaporation from the plates, sterile water (200 μL) was added to perimeter wells. In addition to the rifampin positive controls, negative controls [4% DMSO in MGIT growth medium (100 μL) inoculated with suspensions of *Mycobacterium tuberculosis* (100 μL)] and blanks [2% DMSO in MGIT growth medium (200 μL), and test solutions (100 μL) with MGIT growth medium (100 μL)] were included in each plate. All controls and fractions were tested in triplicate. Plates were incubated (37 °C; 5% CO_2) for 72 h in a humid environment before a 1:1 mixture of an aqueous solution of resazurin (125 μg/mL) and 10% Tween 80 (50 μL) was added to all wells. Plates were then incubated for a further 24 h, sealed with an adhesive polyester film, and mycobacterial growth was assessed fluorometrically at 37 °C. Fluorescence values were corrected for any background fluorescence of the media and test fractions by subtracting the fluorescence readings of the appropriate blanks from the mean fluorescence readings of the control and test wells. The percentage inhibition of mycobacterial growth was then defined as 1 −(mean test or positive control well fluorescence/mean negative control well fluorescence) × 100 (Collins and Franzblau, 1997). Fractions that caused more than 50% inhibition were considered to have significant activity against *Mycobacterium tuberculosis* H37Ra.

2.5. Bioassay guided fractionation

The fractionation of the *Heracleum maximum* extract was guided by inhibitory activity against *Mycobacterium tuberculosis* H37Ra using the MRA. Initially, the crude extract was fractionated by a modified Kupchan solvent–solvent partition protocol to give five fractions as follows: the organic extract (18.32 g) was dissolved in 9:1 MeOH/H_2O (1.0 L) and extracted with hexanes (3 × 300 mL), then diluted with H_2O (500 mL) and extracted with CH_2Cl_2 (3 × 300 mL). The aqueous fraction was then concentrated, dissolved in H_2O (400 mL) and extracted with EtOAc (3 × 200 mL) and *n*BuOH (3 × 200 mL). The five partition fractions were concentrated *in vacuo* to give the following fractions: hexanes (1.66 g), CH_2Cl_2 (1.85 g), EtOAc (328 mg), *n*BuOH (1.90 g), and aqueous (11.90 g). Based on the observed antimycobacterial activities and similarities in TLC profiles, the hexanes (1.66 g), CH_2Cl_2 (1.85 g)

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