Journal of Ethnopharmacology 188 (2016) 200–203

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Ethnopharmacological communication

# Antimycobacterial triterpenes from the Canadian medicinal plant *Sarracenia purpurea*

Steven A. Morrison [a], Haoxin Li [a], Duncan Webster [b], John A. Johnson [a], Christopher A. Gray [a,c,*]

[a] Department of Biological Sciences, University of New Brunswick, Saint John, New Brunswick, Canada E2L 4L5
[b] Division of Infectious Diseases, Department of Medicine, Saint John Regional Hospital, Saint John, New Brunswick, Canada E2L 4L2
[c] Department of Chemistry, University of New Brunswick, Saint John, New Brunswick, Canada E2L 4L5

ARTICLE INFO

*Article history:*
Received 4 February 2016
Received in revised form
21 April 2016
Accepted 28 April 2016
Available online 9 May 2016

*Keywords:*
Antimycobacterial
Betulinaldehyde
β-Sitosterol
Betulinic acid
Ursolic acid
*Sarracenia purpurea*
*Mycobacterium tuberculosis*

ABSTRACT

*Ethnopharmacological relevance:* The purple pitcher plant, *Sarracenia purpurea*, is a medicinal plant used by the Canadian First Nations to treat a wide variety of illnesses. The Mi'kmaq and Wolastoqiyik (Maliseet) peoples of Eastern Canada have traditionally used infusions of *S. purpurea* for the treatment of tuberculosis-like symptoms. Previous investigations have shown methanolic extracts of *S. purpurea* to possess antimycobacterial activity.
*Aim of the study:* To isolate and identify antimycobacterial constituents from *S. purpurea*.
*Materials and Methods:* Methanolic extracts of *S. purpurea* were 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 triterpenes betulinaldehyde, β-sitosterol, betulinic acid, and ursolic acid were isolated from *S. purpurea*. Betulinaldehyde, betulinic acid, and ursolic acid exhibited MICs of 450, 950, and 450 μM and $IC_{50}$s of 98, 169, and 93 μM against *M. tuberculosis* H37Ra respectively whilst β-sitosterol was inactive (MIC and $IC_{50}$ of $>1000$ μM).
*Conclusions:* Betulinaldehyde, betulinic acid, and ursolic acid were identified as the principal constituents responsible for the antimycobacterial activity of *S. purpurea*. This work is consistent with the ethnopharmacological use of *S. purpurea* by Canadian First Nations as a treatment against infectious diseases.



## 1. Introduction

Tuberculosis is one of the leading causes of global morbidity and mortality associated with an infectious disease and it is estimated that approximately one third of the global population is currently infected with *Mycobacterium tuberculosis* (WHO, 2014). Due to the emergence of drug resistant strains of *M. tuberculosis*, there is a need for the development of new drugs with unique mechanisms of action (Koul et al., 2011). *Sarracenia purpurea* L. (Sarraceniaceae), or the purple pitcher plant, is a carnivorous herbaceous perennial that has been used by Canadian First Nations for the treatment of a wide variety of illnesses (Moerman, 1998). The Mi'kmaq and Wolastoqiyik (Maliseet) peoples of Eastern Canada have long used *S. purpurea* as a remedy for tuberculosis-like symptoms either by infusing the plant as a tea or through direct consumption of the herb (Moerman, 1998). Previous bioassays have shown that methanolic extracts of *S. purpurea* inhibited the growth of *M. tuberculosis* H37Ra (O'Neill et al., 2014) and these observations, in conjunction with the plants historical use by Canadian First Nations, have prompted our current research to identify and isolate the antimycobacterial constituents from *S. purpurea*.

*Abbreviations:* $[\alpha]_D$, specific rotation measured at 589 nm and the temperature indicated; 1D, one dimensional; 2D, two dimensional; ACS, American Chemical Society; c, concentration in g/100 mL; $CH_2Cl_2$, dichloromethane; EtOAc, ethyl acetate; HPLC, high performance liquid chromatography; HRESIMS, high resolution electrospray ionization mass spectrometry; $IC_{50}$, median inhibitory concentration; IR, infra-red; LC-MS, liquid chromatography – mass spectrometry; MeOH, methanol; MIC, minimum inhibitory concentration; MRA, microplate resazurin assay; MS, mass spectroscopy; nBuOH, 1-butanol; NMR, nuclear magnetic resonance; SD, standard deviation; TLC, thin layer chromatography

* Corresponding author at: Department of Biological Sciences, University of New Brunswick, Saint John, New Brunswick, Canada E2L 4L5.
*E-mail address:* cgray@unb.ca (C.A. Gray).

http://dx.doi.org/10.1016/j.jep.2016.04.052
0378-8741/

## 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 an Agilent 400-MR DD2 instrument at 400 MHz for $^{1}H$ 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 measured with a Rudolph Autopol III polarimeter. Flash chromatography was performed using a Biotage Flash+ chromatography system with KP-Sil 25+S and C18 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. 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 microlitre 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).

### 2.2. Plant material and extraction

*S. purpurea* was collected by hand in July 2011 from the woods of Prince of Wales, New Brunswick, Canada (45° 11.988′ N; 66° 13.814′ W). Plant tissue was cleaned by hand, rinsed with deionized water, freeze-dried 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-39665). The freeze dried plants (40.0 g) were ground in a domestic blender, exhaustively extracted in methanol (2 × 200 mL; 7 h per extraction) using a Soxhlet extractor and the resulting solution concentrated in vacuo to give a crude methanolic extract (12.9 g).

### 2.3. Mycobacterial strains and growth conditions

*M. tuberculosis* strain H37Ra (ATCC 25,177) was grown as described by O'Neill et al. (2014) and diluted to a turbidity equivalent to a 1.0 McFarland standard ($10^7$ CFU). Suspensions were cryogenically preserved for up to four weeks, thawed, and diluted prior to use.

### 2.4. Microplate resazurin assay (MRA)

The MRA was carried out as described by O'Neill et al. (2014) using rifampin (0.1 μg/mL) and 2% DMSO as the positive and negative controls, respectively. The percentage inhibition of mycobacterial growth was then defined as 1−(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 *M. tuberculosis* H37Ra.

### 2.5. Extraction, isolation and identification

The *S. purpurea* extract exhibited antimycobacterial activity against *M. tuberculosis* H37Ra in our screening bioassay (mean inhibition ± standard deviation =24.0 ± 0.1%; tested at 100 μg/mL) and fractionation of the extract was bioasay guided 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 (7.0 g) was dissolved in 9:1 MeOH/$H_2O$ (600 mL) and extracted with hexanes (3 × 200 mL), then diluted with $H_2O$ (300 mL) and extracted with $CH_2Cl_2$ (3 × 200 mL). The aqueous fraction was then concentrated, dissolved in $H_2O$ (600 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 (290 mg), $CH_2Cl_2$ (766 mg), EtOAc (366 mg), *n*-BuOH (1.31 g), and aqueous (3.08 g).

The hexanes fraction (290 mg) was subjected to silica gel flash chromatography using a stepwise gradient of hexanes to EtOAc (10% increments of EtOAc, 200 mL per eluent) to afford 11 fractions. Fraction 2 (220 mg) was further purified by silica gel flash chromatography using a stepwise gradient of 100% hexanes to 9:1 hexanes/EtOAc (2% increments of EtOAc, 130 mL per eluent) followed by washes of 17:3 hexanes/EtOAc and 4:1 hexanes/EtOAc. The eluents were combined according to their respective TLC profiles to yield nine fractions. Of these nine fractions, fraction 5 (18 mg) exhibited antimycobacterial activity and was further purified using normal phase HPLC (eluted with 9:1 hexanes/EtOAc) to give compounds **1** (2 mg) and **2** (1 mg). The third column fraction obtained from the hexanes partition (19 mg) also exhibited antimycobacterial activity and was further purified by normal phase HPLC directly (eluted using 17:3 hexanes/EtOAc) to give compound **3** (6 mg) and a mixture of fatty acid glycerides.

The $CH_2Cl_2$ liquid-liquid partition fraction (766 mg) was subjected to silica gel flash chromatography using a stepwise gradient of hexanes to EtOAc (10% increments of EtOAc, 200 mL per eluent) to afford 11 fractions. Fraction 4 (78 mg) exhibited antimycobacterial activity and was further purified by normal phase HPLC (4:1 hexanes/EtOAc) to give compound 4 (6 mg).

### 2.6. Spectroscopic and spectrometric data

Betulinaldehyde (1). White solid; $[\alpha]_D^{25} = -4°$ (c $6 \times 10^{-4}$, $CH_2Cl_2$); IR (thin film) $\upsilon_{max}$ 3438, 2936, 2868, 1723, 1456, 1373, 1247, 1038, 887 $cm^{-1}$; HRESIMS *m/z* 441.3728 [M + $H^+$] (calculated for $C_{30}H_{49}O_2$, 441.3727). $^{1}H$ and $^{13}C$ NMR data were consistent with literature values (Barthel et al., 2008).

β-Sitosterol (2). White solid; $[\alpha]_D^{25} = -29°$ (c $4 \times 10^{-3}$, $CH_2Cl_2$); IR (thin film) $\upsilon_{max}$ 3429, 2937, 2869, 1665, 1455, 1377, 1051, 958, 801 $cm^{-1}$; HRESIMS *m/z* 397.3807 [M – $H_2O$ + $H^+$] (calculated for $C_{29}H_{49}$, 397.3829). $^{1}H$ and $^{13}C$ NMR data were consistent with literature values (Chang et al., 2000).

Betulinic acid (3). White solid; $[\alpha]_D^{25} = -8°$ (c $3 \times 10^{-3}$, $CH_2Cl_2$); IR (thin film) $\upsilon_{max}$ 3448, 2938, 1686, 1455, 1373, 1232, 1034, 882, 803 $cm^{-1}$; HRESIMS *m/z* 457.3676 [M + $H^+$] (calculated for $C_{30}H_{49}O_3$, 457.3676). $^{1}H$ and $^{13}C$ NMR data were consistent with literature values (Peng et al., 1998).

Ursolic acid (4). White solid; $[\alpha]_D^{25} = 0°$ (c $6 \times 10^{-4}$, $CHCl_3$); IR (thin film) $\upsilon_{max}$ 3438, 2933, 2638, 1687, 1456, 1373, 1237, 1033, 881, 803 $cm^{-1}$; HRESIMS *m/z* 457.3677 [M + $H^+$] (calculated for $C_{30}H_{49}O_3$, 457.3676). $^{1}H$ and $^{13}C$ NMR data were consistent with literature values (Seebacher et al., 2003).

### 2.7. Determination of minimum inhibitory concentrations (MIC) and median inhibitory concentrations ($IC_{50}$)

MICs and $IC_{50}$ values against *M. tuberculosis* H37Ra were determined as previously described (O'Neill et al., 2014) on dilution series comprising 12 concentrations (400–0.20 μg/mL) in triplicate. The MIC of a compound was considered to be the lowest concentration at which it inhibited mycobacterial growth by more than a mean value of 90% (Collins and Franzblau, 1997), and the corresponding $IC_{50}$ was estimated by fitting a four parameter logistic curve (Sebaugh, 2011) to the mycobacterial growth data