Constituents of *Quinchamalium majus* with Potential Antitubercular Activity

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Antitubercular bioassay-guided fractionation of the dichloromethane extracts of the above-ground biomass and roots of *Quinchamalium majus* led to the identification of six known constituents, betulinic acid (**1**), daucosterol (**2**), 5,7-dihydroxyflavone (**3**), oleanolic acid (**4**), (–)-2*S*-pinocembrin (**5**), and ursolic acid (**6**), for the first time in this species. Their chemical structures were determined on the basis of spectroscopic evidence and chemical transformation methods. All of these compounds along with additional 11 analogues were evaluated for their antitubercular potential against *Mycobacterium tuberculosis* in a microplate alamar blue assay, and the primary structure-activity relationships (SARs) for **4** and **6** were discussed. In addition, all the isolates were tested for cytotoxicity against African green monkey Vero cells in order to evaluate for their selectivity potential.

Key words: Quinchamalium majus, Antitubercular Activity, Structure-Activity Relationship

Introduction

Quinchamalium majus Brong. (Santalaceae) is a short and twiggy perennial herb with a tuberous ligneous white root, fleshy linear and succulent leaves, and head inflorescences with yellow, orange or red flowers (Hoffmann *et al.*, 1998). This species is indigenous to Chile where it grows on the sunny hillsides from the coast to high altitudes in the Andes Mountains. It also grows in Argentina, Bolivia, and Peru. Infusions of its aerial parts and roots are traditionally used in Chile for treating liver and stomach inflammations, cold, and fever (Montenegro, personal communication). Up to the present, very limited phytochemical and biological studies have been reported for this plant. The infusion of its aerial parts was found to exhibit the acetylcholine-like effect on isolated intestines, atria, and auricles of a guinea pig and/or a rabbit (Tampier de Jong, 1963) and only rutin was reported in 1973 by Horhammer *et al.* As part of a collaborative search for novel antitubercular principles of plants and microbial organisms from dryland biodiversity of Latin America (Timmermann *et al.*, 1999), the dichloromethane-soluble extract of the above-ground biomass and roots of *Q. majus* exhibited an inhibitory effect on the growth of *Mycobacterium tuberculosis* H37Rv in a microplate alamar blue assay (MABA) system with a minimum inhibitory concentration (MIC) of 50 μg/ml. Fractionation of the extract led to the identification of six previously known compounds (Fig. 1). All of these constituents along with 11 additional analogues were evaluated for their antitubercular and cytotoxic activities as pure compounds, and the results are described herein.

Material and Methods

General experimental procedures

Melting points were determined using a Fisher-Scientific melting point apparatus and are uncorrected. Optical rotations were measured with a Jasco P-1010 polarimeter. CD measurements were performed using a Jasco J-810 CD spectropolarimeter, which was calibrated with a positive standard, ammonium d-10-camphorsulfonate. IR (as a film on a diamond cell) was obtained on a Thermo Nicolet Avatar 360 FT-IR spectrometer. EI-MS data were obtained with a Varian Saturn 2100T GC-MS Workstation including data system software (Version 5.2) interfaced to a 3900-GC, a

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2000-MS detector, and a 1079-Injector. NMR spectra were recorded at room temperature on a Bruker Avance 300 NMR spectrometer in 5-mm NMR tubes with TMS as the internal standard. Standard pulse sequences were employed for the measurement of 2D NMR spectra ($^1H-^1H$ COSY, HSQC, and HMBC). Column chromatography (CC) was conducted on silica gel (32–63 μm or 63–200 μm; Scientific Adsorbents Incorporated, Atlanta, Georgia, USA) and Sephadex LH-20 (Pharmacia Biotech, Uppsala, Sweden). Semi-preparative reversed-phase HPLC was performed on a Varian LC system equipped with two 215 Solvent Delivery Modules and a 320 UV-VIS Detector, using a Dynamax-100 Å C_{18} column (21.4 × 250 mm, 10 μm) (Varian, Lake Forest, California, USA). Semi-preparative normal-phase HPLC was carried out on a Varian LC system equipped with a 9012 Solvent Delivery System and a 9065 Polychrom detector, using a 10 × 250 mm, 10 μm Econosphere CN column (Alltech, Deerfield, Illinois, USA). Analytical TLC was performed on Whatman Diamond K6F silica gel 60A (250 μm) and Merck RP-18 WF_{254S} (200 μm) plates. Compounds were visualized on TLC plates by dipping in phosphomolybdic acid (Sigma-Aldrich, Milwaukee, Wisconsin, USA) or vanillin/sulfuric acid reagents followed by charring at 110 °C for 5–10 min.

Plant material

Aerial parts and roots of *Quinchamalium majus* were collected in February 2002 in Termas De Chillan, VIII Región, Chile (36° 54′ S; 71° 25′ W) by one of us (G. M.). A voucher specimen (No. 1097) has been deposited in the herbarium at the Pontificia Universidad Católica de Chile, Santiago, Chile. Intellectual Property Rights Agreements for plant collections and collaborative research have been fully executed between the University of Arizona and Pontificia Universidad Católica de Chile.

Extraction and isolation

The milled dried plant material (500 g) was extracted by maceration with MeOH (3 × 1.8 l). After filtration and evaporation of the solvent *in vacuo*, the resultant extract was diluted with H_2O to afford a 90% aqueous MeOH solution (0.6 l) and then partitioned with *n*-hexane (3 × 0.6 l) and CH_2Cl_2/H_2O (1:1 v/v, 2 × 1 l), sequentially, to afford dried *n*-hexane-soluble (8.5 g) and CH_2Cl_2-soluble (3.5 g) residues. The CH_2Cl_2 extract was found to inhibit the growth of *M. tuberculosis* at a MIC value of 50 μg/ml while the *n*-hexane extract was inactive. Therefore, the CH_2Cl_2 extract (3.5 g) was subjected to silica gel CC (200 g, 63–200 μm) by elution with a step gradient of CH_2Cl_2/MeOH (100:0, 75:1, 40:1, 20:1, 10:1, 0:100 v/v, each 1 l) to give six fractions (1–6), respectively. Fractions 2 and 3 were combined on the basis of their similar TLC profiles and the pooled fraction (520 mg) was applied to CC (SiO_2, 52 g, 63–200 μm) by elution of *n*-hexane/EtOAc (8:1, 7:1, 6.5:1, 6:1, 1:1 v/v, each 1 l) to afford five further fractions 2–1 to 2–5. (–)-2*S*-Pinocembrin (**5**, 3 mg) was obtained from fr. 2–2 (45 mg) by CC over Sephadex LH-20/MeOH. Purification of fr. 2–3 (60 mg) by HPLC (CN column, *n*-hexane/isopropanol/methanol 97.5:1.5:1.5 v/v, 4 ml/min, 200 nm) afforded betulinic acid (**1**, 15 mg; t_R = 13.9 min). Fractionation of fr. 2–5 (0.9 g) over CC (SiO_2, 100 g, 63–200 μm) gave a further fraction (95 mg, CH_2Cl_2/MeOH/H_2O 15:1:0.1 v/v, 2 l), which was purified over Sephadex LH-20/MeOH to afford daucosterol (**2**, 60 mg). In turn, fractionation of fr. 2–4 (70 mg) by CC over Sephadex LH-20/MeOH afforded 5,7-dihydroxyflavone (**3**, 6 mg) and a further mixture fraction 2–4–1 (34 mg), which could not be separated by HPLC using Econosphere CN, NH_2, SiO_2, and C_{18} columns (Alltech, Deerfield, Illinois, USA). Therefore, this mixture was treated as described below to achieve separation and identification of its components.

^1H and ^{13}C NMR analysis of fraction 2–4–1 indicated that it was a mixture of triterpenoids containing hydroxyl group(s). After treatment with pyridine (0.8 ml) and acetic anhydride (0.3 ml) overnight, the resultant mixture (36 mg) still could not be separated with the above-mentioned HPLC methods. Next, the dried acetylated mixture was further treated with an excess of diazomethane in an ethyl ether solution prepared using the Diazald® Kit diazomethane generator (Sigma-Aldrich) for 10 min to obtain a product, which was purified by HPLC (Dynamax-100 Å C_{18} column, 100% CH_3CN, 10 ml/min, 200 nm) to afford **4b** [12 mg; t_R = 20.1 min; [m.p. 210–212 °C; $[\alpha]_D^{22}$ + 69° (*c* 0.1, $CHCl_3$)] (O'Neil *et al.*, 2001) and **6b** [22 mg; t_R = 21.0 min; m.p. 238–241 °C; $[\alpha]_D^{22}$ + 68° (*c* 0.1, $CHCl_3$)] (Hota and Bapuji, 1993).

A portion of **6b** (12 mg) was sequentially treated with 3 ml of 2% NaOH in MeOH overnight, 3 ml of 2 *N* HCl for neutralization, and partitioned with

CH_2Cl_2/H_2O 1:1 (3 × 10 ml) to give **6c** [7.5 mg; m.p. 112–114 °C; $[\alpha]_D^{22}$ + 58° (*c* 0.1, $CHCl_3$)] (Seo *et al.*, 1981), which was identical to the product generated from commercially purchased ursolic acid (**6**) after treatment with an excess of diazomethane. In addition, **6a** [8 mg; m.p. 287–289 °C; $[\alpha]_D^{22}$ + 71° (*c* 0.1, $CHCl_3$)] (O'Neil *et al.*, 2001) was prepared from **6** (8.2 mg) by routine treatment with C_5H_5N/Ac_2O for comparison of activity. In turn, commercially purchased oleanolic acid (**4**, 10 mg) was treated with C_5H_5N/Ac_2O to give **4a** [10 mg; m.p. 215–217 °C; $[\alpha]_D^{22}$ +75° (*c* 0.1, $CHCl_3$)] (O'Neil *et al.*, 2001), a portion of which was further treated with an excess of diazomethane to give a product identical to **4b**. In addition, **4c** [m.p. 201–203 °C; $[\alpha]_D^{22}$ + 68° (*c* 0.1, $CHCl_3$)] (O'Neil *et al.*, 2001) was prepared from **4** by direct treatment with CH_2N_2 for the biological testing.

Identification

Betulinic acid (**1**): White amorphous powder. – M.p. 293–295 °C. – $[\alpha]_D^{22}$ + 9.4° (*c* 0.1, $CHCl_3$/MeOH 1:1). – IR, ^{1}H NMR, and ^{13}C NMR data were identical to those of the authentic standard (Sigma-Aldrich) and the reported values (Siddiqui *et al.*, 1988).

Daucosterol (**2**): White amorphous powder. – M.p. 294–296 °C (dec.). – $[\alpha]_D^{22}$ – 41° (*c* 0.1, pyridine). – IR, ^{1}H NMR and ^{13}C NMR data were identical to the reported values (Faizi *et al.*, 2001).

5,7-Dihydroxyflavone (**3**): Pale yellow powder. – M.p. 288–290 °C. EI-MS: *m/z* = 254 (100) $[M]^+$, 226 (38), 152 (15), 124 (18), 96 (8). – ^{1}H NMR and ^{13}C NMR data were in agreement with reported values (Wagner *et al.*, 1976).

(–)-2S-Pinocembrin (**5**): Amorphous powder. – M.p. 195–197 °C. – $[\alpha]_D^{22}$ – 56° (*c* 0.1, $CHCl_3$/MeOH 10:1). – CD (*c* 0.001, $CHCl_3$/MeOH 10:1): $\Delta\varepsilon_{289}$ – 4.77 nm (Gaffield, 1970). – EI-MS *m/z*: 256 (100) $[M]^+$, 238 (21), 179 (68), 152 (50), 124 (57), 103 (17). – ^{1}H NMR and ^{13}C NMR data were identical to those of the authentic standard (Sigma-Aldrich) and the reported values (Tanaka *et al.*, 1985).

Microplate alamar blue assay

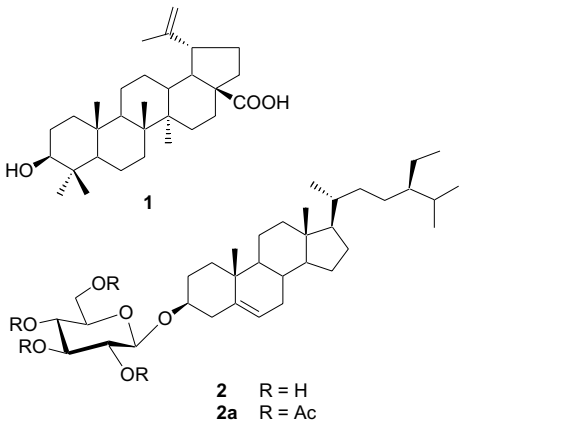
Antimycobacterial activity was determined against *Mycobacterium tuberculosis* H37Rv (ATCC 27294) in a microplate alamar blue assay system as described previously by Collins and Franzblau (1997). The minimum inhibitory concentration (MIC) value of < 128 μg/ml was considered as active. The antitubercular drug rifampin was used as a positive control.

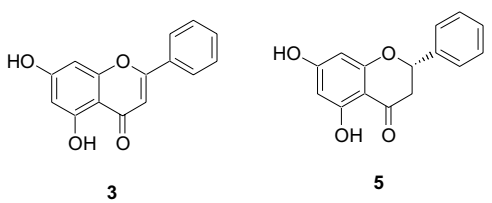
Cytotoxicity assay

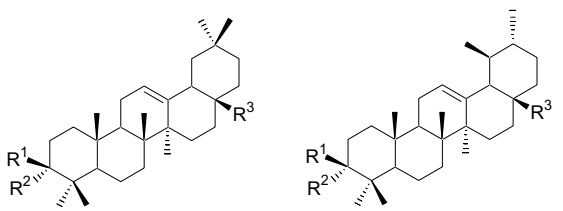
Evaluation of cytotoxicity of the CH_2Cl_2-soluble extract and pure compounds was performed as a modification of an established protocol (Cantrell *et al.*, 1996). In brief, test compounds were dissolved at 10–40 mg/ml in DMSO. Geometric three-fold dilutions were performed in growth medium M199 (Gibco, Grand Island, NY, USA) to which 5% fetal bovine serum was added (HyClone, Logan, UT, USA), 25 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (HEPES; Gibco), 0.2% $NaHCO_3$ (Gibco) and 2 mM glutamine (Irvine Scientific, Santa Ana, CA, USA) to obtain final concentrations ranging from 0.42 to 102.4 μg/ml. Final DMSO concentrations did not exceed 1% v/v. Samples were distributed in duplicate in 96-well tissue culture plates (Becton Dickinson Labware, Lincoln Park, NJ, USA) at a volume of 50 μl/well. An equal volume containing 5 × 10^3 log phase Vero cells (CCL-81; American Type Culture Collection, Rockville, MD, USA) were added to each well, and the cultures were incubated at 37 °C in an atmosphere of 5% CO_2 in air. After 72 h, cell viability was measured using the CellTiter 96 aqueous nonradioactive cell proliferation assay (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions. Absorbance at 490 nm was read in a BioRad Model 3550 microplate reader (Hercules, CA, USA). The IC_{50} value is defined as the reciprocal dilution resulting in 50% inhibition of the Vero cells. In addition, selective index (SI = IC_{50}/MIC) values were determined.

Results and Discussion

Six compounds of previously known structures were identified as betulinic acid (**1**), daucosterol (**2**), 5,7-dihydroxyflavone (**3**), oleanolic acid (**4**), (–)-2*S*-pinocembrin (**5**), and ursolic acid (**6**) (Fig. 1) for the first time from the dichloromethane-soluble extract of the above-ground biomass and roots of *Q. majus*, as described in Material and Methods. Among these constituents, the presence of **4** and **6** was confirmed by observation of their respective acetyl and/or methyl derivatives (**4a**, **4b**, **6b**, **6c**), which were identified by comparison of their physical and ^{1}H NMR data with reported values. Six related triterpenoids, α-amyrenone (**10**), α-amyrin (**8**), β-amyrenone (**9**), β-amyrin (**7**), oleanolic acid







	R^1	R^2	R^3		R^1	R^2	R^3
4	OH	H	COOH	**6**	OH	H	COOH
4a	OAc	H	COOH	**6a**	OAc	H	COOH
4b	OAc	H	$COOCH_3$	**6b**	OAc	H	$COOCH_3$
4c	OH	H	$COOCH_3$	**6c**	OH	H	$COOCH_3$
7	OH	H	CH_3	**8**	OH	H	CH_3
9	O		CH_3	**10**	O		CH_3

Fig. 1. Chemical structures of constituents of *Q. majus* and their related analogues.

(**4**), and ursolic acid (**6**) were purchased from Sigma-Aldrich to allow for comparison of antitubercular activities.

All of the isolates obtained from *Q. majus* along with 11 analogues were evaluated for their potential to inhibit the growth of *M. tuberculosis* and African green monkey Vero cells, respectively, according to established protocols (Collins and Franzblau, 1997; Cantrell *et al.*, 1996). The results (Table I) showed that three common triterpenes, betulinic acid (**1**), oleanolic acid (**4**) and ursolic acid (**6**), along with the flavanone **5** exhibited significant inhibitory activity in a microplate alamar blue assay with MIC values of 62, 30, 31, and 90 μg/ml, respectively, while the other two isolates **2** and **3** were inactive (MIC > 128 μg/ml).

It was observed that a reduction of the carboxylic acid group in compounds **4** and **6** to the corresponding methyl group resulted in the complete loss of activity even though the 3β-hydroxy group was not modified as in **7** and **8** or was oxidized to a ketone group as in **9** and **10**. In addition, the complete loss of activity as in **4b** and **6b** was also observed for both **4** and **6** if the 3β-hydroxy and carboxylic acid groups were simultaneously changed to the corresponding acetate and methyl ester, respectively. However, modification of only the carboxylic acid of **4** to its corresponding methyl ester (**4c**) retained the antitubercular activity in the same order of magnitude while the cytotoxicity against Vero cells was enhanced ca. threefold. Replacement of only the 3β-hydroxy group of **4** to its corresponding acetate (**4a**) resulted in a remarkable reduction of the antitubercular activity. In contrast to the observations with compound **4**, methyl ursolate (**6c**) decreased the antitubercular activity twofold and enhanced the cytotoxicity somewhat when compared to **6**. More interestingly, modification of the 3β-hydroxy group of **6** to the corresponding acetate (**6a**) reduced the cytotoxicity about three times while still keeping the same antitubercular activity.

The results of this study support our previous observations of the structure-activity relationships for pentacyclic triterpenoids (Wachter *et al.*, 1999), in that the presence of a hydroxy group in the A ring combined with the presence of a carboxylic acid or its methyl ester group in the E ring of triterpenes **4** and **6** is associated with the observed antitubercular activity. In addition, as demonstrated for **6** and **6a**, semi-synthesis of lead compounds of interest could provide more interesting analogues with improved selectivity indices, which could also exhibit lower cytotoxicity while conserving the same activity. Furthermore, it is worthy to point out that after reviewing the phytochemical and biological data from our laboratory, fractionation of several initially active plant-derived extracts often led to the isolation of common triterpenoids with only a moderate activity such as betulinic acid, oleanolic acid, ursolic acid, and their *epi*-isomers (Wachter *et al.*, 1999; Caldwell *et al.*, 2000; Cantrell *et al.*, 2001; Gu *et al.*, 2004; Woldemichael *et al.*, 2004). Therefore, it has become necessary to develop a methodology

Table I. Growth inhibition of *M. tuberculosis* and green monkey Vero cells by constituents of *Q. majus* and related analogues.

Compound	MIC [μg/ml]	IC_{50} [μg/ml]	IC_{50} [μM]	SI[a]
Rifampin[b]	0.03	98	119	3277
Betulinic acid (**1**)[c]	62	79	173	1.3
Daucosterol (**2**)	> 128	45	78	n.c.[d]
Daucosterol tetraacetate (**2a**)	> 128	> 102	> 137	n.c.[d]
5,7-Dihydroxyflavone (**3**)	> 128	84	331	n.c.[d]
Oleanolic acid (**4**)[c]	30	83	182	2.8
3-*O*-Acetyloleanolic acid (**4a**)	113	> 102	> 205	n.c.[d]
Methyl-3β-acetoxyolean-12-en-28-oate (**4b**)	> 128	> 102	> 199	n.c.[d]
Methyl oleanolate (**4c**)	31	32	68	1.0
(–)-2*S*-Pinocembrin (**5**)	90	30	117	0.3
Ursolic acid (**6**)[c]	31	37	81	1.2
3-*O*-Acetylursolic acid (**6a**)	31	> 102	> 205	> 3.3
Methyl-3β-acetoxyurs-12-en-28-oate (**6b**)	> 128	> 102	> 199	n.c.[d]
Methyl ursolate (**6c**)	63	25	53	0.4
β-Amyrin (**7**)	> 128	> 102	> 239	n.c.[d]
α-Amyrin (**8**)	> 128	> 102	> 239	n.c.[d]
β-Amyrenone (**9**)	> 128	> 102	> 241	n.c.[d]
α-Amyrenone (**10**)	> 128	> 102	> 241	n.c.[d]

[a] Selectivity index (SI = IC_{50}/MIC, in which both IC_{50} and MIC values are expressed in μg/ml).
[b] Positive control.
[c] Retested and included in the table for comparison of activities.
[d] Not calculated.

for dereplication of the most commonly found triterpenoids in initially active plant extracts in order to facilitate the search for novel antitubercular agents.

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