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Antimycobacterial agents from selected Mexican medicinal plants

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Abstract

As part of the ICBG program *Bioactive Agents from Dryland Biodiversity of Latin America*, the present investigation was undertaken to explore the possible antimycobacterial potential of compounds derived from selected Mexican medicinal plants. Bioassay-guided fractionation of the crude extracts of *Rumex hymenosepalus* (Polygonaceae), *Larrea divaricata* (Zygophyllaceae), *Phoradendron robinsonii* (Loranthaceae) and *Amphipteryngium adstringens* (Julianiaceae) led to the isolation of several antimycobacterial compounds. Four stilbenoids, two flavan-3-ols and three anthraquinones were isolated from *R. hymenosepalus*. Two flavonols and nordihydroguaiaretic acid were obtained from *L. divaricata*. Sakuranetin was the antimycobacterial agent isolated from *P. robinsonii*. Two known triterpenoids and the novel natural product 3-dodecyl-1,8-dihydroxy-2-naphthoic acid were obtained from *A. adstringens*. In general, the isolates were identified by spectral means. The antimycobacterial activity of the secondary compounds isolated from the analysed species, as well as that of nine pure compounds previously isolated in our laboratories, was investigated; the MIC values ranged from 16 to 128 μg mL^{-1}. Among the tested compounds, the glycolipids, sesquiterpenoids and triterpenoids showed the best antimycobacterial activity. The antimycobacterial property of the glycolipids is reported for the first time. Although the tested compounds showed moderate antimycobacterial activity, their presence in the analysed species provides the rationale for their traditional use in the treatment of tuberculosis.

Introduction

Tuberculosis (TB) is a deadly disease that kills almost 3 million people per year worldwide. Because of a combination of economic decline, the breakdown of health systems, insufficient application of TB control measures, the spread of HIV/AIDS and the emergence of multidrug-resistant (MDR) strains, TB is an increasing health problem in many developing countries. Despite the availability of inexpensive treatments, a variety of plant preparations used in folk medicine worldwide have played an important role in the treatment of patients with TB. Although these species might contain new leads for the development of novel anti-TB drugs, the medicinal value and active principles of these species are totally unknown. The emergence of MDR-TB urges research in the area of pure plant compounds, many of which have shown important activity against *Mycobacterium tuberculosis* (Newton et al 2000; Copp 2003; Okunade et al 2004).

The main goal of the present investigation was to isolate the potential anti-*M. tuberculosis* compounds from selected Mexican medicinal plants as part of the ICBG program *Bioactive Agents from Dryland Biodiversity of Latin America* (Timmermann et al 1999; Mata et al 2002, 2003; Rojas et al 2003 inter alia). In addition, the evaluation of some previously purified medicinal plant compounds was jointly performed.

The species selected for this study included *Rumex hymenosepalus* Torr. (Polygonaceae), *Larrea divaricata* Cov. (Zygophyllaceae), *Phoradendron robinsonii*

Urban (Loranthaceae) and *Amphipteryngium adstringens* Schiede ex Schlecht (Julianiaceae). The four vegetable plant materials selected are employed in traditional medicine for the treatment of TB, cough and other lung infections. *R. hymenosepalus* is a perennial leafy herb with 1-foot-long leaves and tiny greenish flowers that are replaced by showy pink seed pods. It grows in open dry habitats from the Western USA to Northern Mexico. The leaves are also used as a food and to alleviate fevers and gastrointestinal disturbances; in addition, a decoction of the roots is drunk to purify the blood and for treating wounds and skin irritations. Previous chemical investigation of this plant yielded emodin, physcion and chrysophanol (Rada & Brazdova 1972). *L. divaricata* is a perennial bush with dark green stems and yellowish-green leaves. Its leaves and stems are also used as an antiseptic, a blood purifier, a diuretic and an anti-inflammatory agent. Previous phytochemical analysis afforded several flavonoids and lignans (Sakakibara et al 1976; Ayres & Loike 1990). *P. robinsonii* is a hemiparasitic and dioecious plant with inconspicuous flowers. The female plants produce numerous spherical, translucent, white, pink or red berries that are eaten by birds. The plant mainly parasitizes *Acacia, Olneya, Parkinsonia* and *Prosopis* species. To our knowledge *P. robinsonii* has not been chemically investigated, however *P. coryae* and *P. tomentosu* have yielded several flavonoids (Dosaji et al 1983). *A. adstringens* is a tree that grows along the Pacific coast, reaching up to about 25 feet in height with an astringent bark. In addition to its reputed use for the treatment of TB, this species is widely appreciated in traditional medicine for the treatment of cancer, malaria and gastric ulcers (Navarrete et al 1998). *A. adstringens* has been shown to biosynthesize triterpenoids with anti-inflammatory, hypocholesterolaemic and antiulcer activities (Navarrete et al 1990, 1998; Mata et al 1991; Domínguez et al 1993; Olivera et al 1999; Arrieta et al 2003; Oviedo-Chavez et al 2004), as well as anacardic acids and aldehydes (Navarrete et al 1989; Mata et al 1991).

Materials and Methods

Plant materials

R. hymenosepalus roots (voucher no.: Bye 26598) were collected in the state of Sonora, Mexico, in November 1999. *P. robinsonii* (whole plant; voucher no.: Bye 20473) and *L. divaricata* (leaves; voucher no.: Bye 20321) were collected in San Luis Potosí, Mexico, in August 1995. The stem-bark of *A. adstringens* (voucher no.: Bye 22564) was collected on March 1998 in Oaxaca, Mexico. Voucher specimens were deposited at the Ethnobotanical Collection of the National Herbarium (MEXU), Instituto de Biología, UNAM.

Phytochemistry: general procedures

All solvents, with the exception of the ones used for HPLC, were of laboratory grade. Column chromatography was performed on silica gel 60 (Merck) or Sephadex LH-20 (Pharmacia). Silica gel 60 F_{254} (Merck) plates were used for TLC. HPLC was carried out with a Waters HPLC instrument equipped with a Waters UV photodiode array detector (900) set at 229–285 nm, using a μ-Porasil or Nova-Pak HR C_{18} column (19 mm i.d. $\times$ 300 mm). Control of the equipment, data acquisition, processing and management of chromatographic information were performed by the Millennium 2000 software program (Waters). IR spectra were obtained on a Perkin-Elmer 599B spectrophotometer. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on a Varian VXR-300S spectrometer in $CDCl_3$ using tetramethylsilane as an internal standard. EIMS were obtained on a JEOL JMS-AX505HA mass spectrometer. FABMS were recorded on a JEOL DX300 mass spectrometer in the positive mode using NBA as the matrix.

Isolation of the active principles from plant material

Rumex hymenosepalus

The air-dried and pulverized roots of *R. hymenosepalus* (3 kg) were macerated with CH_2Cl_2/MeOH (1:1; 10 L). The extract was evaporated under reduced pressure to yield 695 g of a dark residue (growth index (GI) = 67%), which was consecutively subjected to solvent partition between CH_2Cl_2, EtOAc and 10% H_2O in MeOH. The dichloromethane extract (4 g; GI = 66%) was column chromatographed over silica gel (175 g), eluting with a gradient of n-hexane/CH_2Cl_2 (0:10 $\rightarrow$ 10:0) and CH_2Cl_2/MeOH (0:10 $\rightarrow$ 10:0) to afford seven fractions (I–VII). Preparative TLC on silica gel (CH_2Cl_2/MeOH, 95:5) of the active fraction V (GI = 75%), eluted with hexane/CH_2Cl_2 (3:2), led to the isolation of **1** (61 mg, Rf = 0.7) and **9** (24 mg, Rf = 0.5). Fraction II, eluted with hexane/CH_2Cl_2 (9:1), was chromatographed over silica gel (60 g), eluting with hexane/CH_2Cl_2 (9:1), to afford **7** (80 mg) and **8** (9 mg). An inactive solid residue (45 g) was precipitated from the EtOAc extract (GI = 70%). The active mother liquors (185 g) were chromatographed on a silica gel (3 kg) column, eluting with n-hexane/EtOAc (7:3 $\rightarrow$ 3:7) and EtOAc/MeOH (10:0 $\rightarrow$ 1:1) to yield 10 subfractions. **1** (270 mg) spontaneously crystallized from subfraction II, eluted with n-hexane/EtOAc (3:2). Eluates from n-hexane/EtOAc (2:3) (subfraction IV) yielded 322 mg of **2**. Subfraction V (1.2 g), eluted with n-hexane/EtOAc (3:7), was column chromatographed over silica gel (60 g) using a gradient n-hexane/EtOAc (1:1 $\rightarrow$ 1:9) to afford 15 mg of **5** and 905 mg of **6**. Finally, 2 g of an inactive precipitate was subjected to flash column chromatography over silica gel (80 g) eluted isocratically with EtOAc/MeOH (9:1) to yield 100 mg of **3** and 20 mg of **4**.

Phoradendron robinsonii

Dried and powdered whole plant (2.1 kg) was exhaustively macerated with CH_2Cl_2/MeOH (1:1) at room temperature, affording 172 g of crude extract. The active extract of *P. robinsonii* (GI = 100%) was column

chromatographed over silica gel (1.75 kg), eluting with gradients of n-hexane/CH_2Cl_2 (0:10 → 10:0) and CH_2Cl_2/MeOH (0:10 → 10:0) to afford 12 primary fractions (I–XII). Antimycobacterial assay indicated that fractions VII (eluted with CH_2Cl_2; GI = 99%) and VIII (CH_2Cl_2/MeOH, 9:1; GI = 100%) were active. The active fractions were pooled based on their TLC profiles and were further chromatographed on a silica gel column (43 g) using a gradient of CH_2Cl_2/MeOH (0:10 → 10:0) to yield 11 subfractions. The antimycobacterial activity was concentrated on subfraction IX (GI = 99%). Preparative TLC (CH_2Cl_2/MeOH, 9:1) of this subfraction allowed the isolation of **10** (Rf = 0.5, 1.5 g).

Larrea divaricata

The air-dried aerial parts (250 g) were ground into powder and extracted by maceration with CH_2Cl_2/MeOH (1:1) at room temperature. After filtration, the extract was evaporated under reduced pressure to yield 60 g of a green residue (GI = 99%). The crude active extract was fractionated on a silica gel column (600 g), eluting with n-hexane/CH_2Cl_2 (0:10 → 10:0) to yield 11 primary fractions (I–XI). According to biological testing, fractions VIII (eluted with CH_2Cl_2; GI = 100%) and IX (CH_2Cl_2/MeOH, 9:1; GI = 100%) were the most active against *M. tuberculosis*. Extensive preparative TLC (n-hexane/CH_2Cl_2/MeOH, 1:94:5) of fraction VIII led to the isolation of compounds **11** (345 mg, Rf = 0.45, mp 236–238°C) and **12** (345 mg, Rf = 0.65, mp 166–268°C). Finally, preparative TLC on silica gel (CH_2Cl_2/MeOH, 95:5) of fraction IX allowed the isolation of 685 mg of **13** (Rf = 0.62, mp 179–180°C).

Amphipteryngium adstringens

The stem-bark (2.6 kg) of *A. adstringens* was macerated with CH_2Cl_2/MeOH (1:1) at room temperature to yield 865 g of an active crude extract (GI = 99%). The extract was primarily fractionated by column chromatography over silica gel (600 g), eluting with a gradient of n-hexane/CH_2Cl_2 (0:10 → 10:0) and CH_2Cl_2/MeOH (0:10 → 10:0) to produce eight primary fractions (I–VIII). Antimycobacterial testing revealed two active fractions: IV and V, eluted with n-hexane/CH_2Cl_2 (1:1) and CH_2Cl_2, respectively (GI = 99% in both cases). Compounds **24** (2.2 g; mp 176–178°C) and **25** (mp 142–145°C) spontaneously crystallized from active fractions IV (7 g) and V (90 g), respectively. Finally, from inactive fraction III (7 g), eluted with n-hexane/CH_2Cl_2 (7:3), precipitated a waxy white powder (17 mg), which was purified by HPLC (8.3 mL min^{-1}; MeOH/CH_3CN/H_2O, 50:45:5) to yield the novel naphthalene derivative **23** (3 mg, Rt = 25 min).

Physical and spectroscopic properties of compound 23

White solid mp 62–64°C. IR ν_{max} (film) cm^{-1}: 3500, 3005, 2915, 1681, 1637, 1571, 1471, 1459, 1226, 715. UV λ_{max} MeOH (log ε) nm: 341 (2408), 258 (4598), 234 (10280), 228 (10355), 222 (9168), 206 (11831). ^{1}H NMR $CDCl_3$ (300 MHz): δ_H 11.02 (brs OH); 7.56 (dd, J = 8.8 Hz, H-6); 6.92 (dd, J = 8.4 and 1.0 Hz, H-7); 6.81 (dd, J = 8.1 and 1.0 Hz, H-5); 6.26 (sa, H-4); 2.51(t, J = 7.5 Hz, H-1′); 1.65 (m, H-2′); 1.33–1.26 (m, H-3′–H-11′); 0.99 (t, J = 7.5 Hz, H-12′). ^{13}C NMR $CDCl_3$ (75 MHz): δ_C 166.9 (C-9); 161.6 (C-8); 157.8 (C-1); 157.7 (C-3); 139.2 (C-4a); 137.3 (C-6); 115.3 (C-5); 115.3 (C-2); 114.6 (C-7); 106.1 (C-8a); 104.6 (C-4); 33.3 (C-1′); 29.6–28.9 (C-3′–C-11′); 26.8 (C-2′); 14.5 (C-12′). EIMS: m/z 372 (M^+) (100), 189 (18), 176 (25), 134 (20), 105 (8). HREIMS m/z 372.4978 (calcd for $C_{23}H_{32}O_4$, 372.4980).

Preparation of the acetyl derivatives 1a and 23a

A solution of 2 mg of each compound in pyridine (0.1 mL) and Ac_2O (0.1 mL) was kept at room temperature for 48 h, diluted with CH_2Cl_2 (10 mL), washed with 1 N HCl (3 × 20 mL) and saturated $NaHCO_3$ solution (3 × 20 mL), dried over anhydrous Na_2SO_4 and evaporated to dryness, affording the triacetate **1a** (1.2 mg) and the diacetate **23a** (1.4 mg), respectively.

The spectral and physical properties of compound **1a** were identical to those previously described (Oleszek et al 2001). Compound **23a** was obtained as an amorphous solid; IR ν_{max} (film) cm^{-1}: 2924, 2853, 1760, 1693, 1599, 1460, 1400, 1160. ^{1}H NMR $CDCl_3$ (300 MHz): δ_H 7.63 (t, J = 8.8 Hz, H-6); 7.23 (dd, J = 8.4 and 1.0 Hz, H-7); 7.10 (dd, J = 8.1 and 1.0 Hz, H-5); 6.65 (sa, H-4); 2.51(t, J = 7.5 Hz, H-1′); 2.40 (s, CH_3CO-); 2.20 (s, CH_3CO-); 1.60 (m, H-2′); 1.33-1.26 (m, H-3′-H-11′); 0.99 (t, J = 7.5 Hz, H-12′). FABMS (positive, NBA): 457 ($M^+ + H$).

Pure compounds

Known pure compounds were isolated in our laboratories: the sesquiterpene lactones **14**, **15** and **26** were obtained from *Cosmos pringlei* Rob. & Fern. (Asteraceae) (Mata et al 2002); sesquiterpene **16** from *Iostephane heterophylla* (Cav.) Hemsl. (Asteraceae) (Mata et al 2001); the biflavonoid **17** from *Celaenodendron mexicanum* Standl. (Euphorbiaceae) (Castañeda et al 1992); and the glycolipids **18**–**22** from *Ipomoea tricolor* Cav. (Convolvulaceae) (Bah & Pereda-Miranda 1996).

Bioassay

The activity of the crude extracts, fractions and isolated compounds was determined against *M. tuberculosis* $H_{37}Rv$ (ATCC 27294) in the BACTEC 460 assay as previously described (Cantrell et al 1996; Collins & Franzblau 1997). Briefly, stock solutions of test compounds were solubilized at 80 mg mL^{-1} in DMSO, sterilized by passage through 0.22 μm PFTE filters (Millex-FG, Millipore, Bedford, MA, USA) and stored at −80°C until used. A 1:10 dilution was performed in DMSO; stocks and dilutions (50 μL) were added to 4 mL of 7H12 broth (BACTEC 12B; Becton Dickinson Diagnostic Instrument Systems, Sparks, MD, USA) to achieve the desired final

concentrations (ranging from 1 to 128 $\mu g\,mL^{-1}$) to determine the MICs. Controls received 50 μL of DMSO (MIC > 128 $\mu g\,mL^{-1}$). Rifampin (Sigma Chemical, Co., St Louis, MO, USA) and compound **26** were included as a positive drug controls.

M. tuberculosis was cultured in BACTEC 12B broth. Bacterial growth was monitored in a confined atmosphere using the Bactec 460-TB apparatus (Becton Dickinson, Sparks, MD, USA); this determines the ability of bacteria to catabolize [^{14}C]-palmitic acid in 7H12 broth by measuring the $^{14}CO_2$ released. The growth of the bacteria is represented as a numerical value called the growth index (GI), which ranges from 400 to 999 in 10 days; 0.1 mL of the diluted inoculum was used to inoculate 4 mL of fresh BACTEC 12B broth containing the test compounds. Cultures were incubated at 37°C and the GI determined daily (starting on the third day of incubation) until (solvent) control cultures achieved a GI of 999. The percentage of inhibition was defined as 1 – (GI of test sample/GI of control) × 100. The MIC was defined as the lowest concentration for which the ΔGI was less than the ΔGI of the 1:100 control. Activity criteria: MIC values higher than 128 $\mu g\,mL^{-1}$ indicate no activity; MIC values between 100 and 128 $\mu g\,mL^{-1}$ correspond to marginal activity; MIC values between 99 and 32 $\mu g\,mL^{-1}$ mean moderate activity; values lower than 32 $\mu g\,mL^{-1}$ correspond to good activity.

Statistical analysis

For each concentration of crude extracts and fractions tested the inhibition percentage is the average of the three different experiments. The MIC values for the isolated compounds were determined by the average of three different experiments repeated three times.

Results and Discussion

Phytochemical analysis

R. hymenosepalus, *L. divaricata*, *P. robinsonii* and *A. adstringens* were initially selected as a source of anti-TB compounds following the ethnomedical rationality since these species are used in popular medicine for the treatment of TB and other diseases. Thereafter, a preliminary test against *M. tuberculosis* (Table 1) using the BACTEC 460 assay (Cantrell et al 1996; Collins & Franzblau 1997) confirmed the ethnomedical hypothesis. Accordingly the four species were selected for activity-guided fractionation.

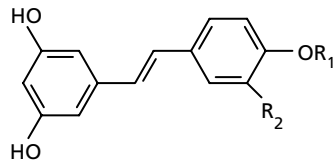
Bioassay-guided fractionation of the active extract (Table 1) prepared from the roots of *R. hymenosepalus* yielded compounds **1**–**9** (Figures 1–3) identified as 5-[(*E*)-2-(4-hydroxyphenyl)ethenyl]-1,3-benzenediol (**1**), 4-[(*E*)-2-(3,5-dihydroxyphenyl)ethenyl]-1,2-benzenediol (**2**) (Oleszek et al 2001), 4-[(*E*)-2-(3,5-dihydroxyphenyl)ethenyl]phenylhexopyranoside (**3**), 4-[(*E*)-2-(3,5-dihydroxyphenyl)ethenyl]-2-hydroxyphenyl hexopyranoside (**4**) (Nyemba et al 1995), (2*R*,3*R*)-2-(3,4-dihydroxyphenyl)3,4-dihydro-2*H*-chromene-3,5,7-triol (**5**), (2*R*,3*R*)-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3,4-dihydro-2*H*-chromen-3-yl-3,4,5-trihydroxybenzoate (**6**) (Chen et al 1993), 1,8-dihydroxy-3-methylanthra-9,10-quinone (**7**), 1,8-dihydroxy-3-methoxy-6-methylanthra-9,10-quinone (**8**) and 1,3,8-trihydroxy-6-methylanthra-9,10-quinone (**9**) (Dominguez et al 1991). This analysis represents the first report of the presence of stilbenoids and catechins in this important herbal drug.

From the active crude extract of *P. robinsonii* (Table 1), the known flavanone 5-hydroxy-2-(4′-hydroxyphenyl)-7-

Table 1 Inhibition of the growth of *M. tuberculosis* induced by the extracts CH_2Cl_2/MeOH (1:1) at a concentration of 50 $\mu g\,mL^{-1}$

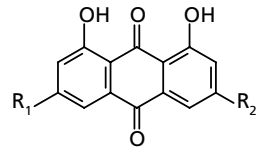
Substance	Inhibition (%)
R. hymenosepalus extract	67
P. robinsonii extract	99
L. divaricata extract	100
A. adstringens extract	95
Rifampin at 0.25 $\mu g\,mL^{-1}$*	100

*Positive control



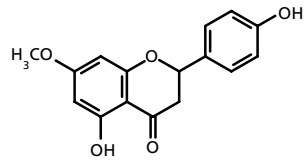
COMPOUND	R_1	R_1
5-[(*E*)-2-(4-hydroxyphenyl)ethenyl]-1,3-benzenediol **(1)**	H	H
4-[(*E*)-2-(3,5-dihydroxyphenyl)ethenyl]-1,2-benzenediol **(2)**	H	OH
4-[(*E*)-2-(3,5-dihydroxyphenyl)ethenyl]phenyl hexopyranoside **(3)**	β-D-Gluc	H
4-[(*E*)-2-(3,5-dihydroxyphenyl)ethenyl]-2-hydroxyphenyl hexopyranoside **(4)**	β-D-Gluc	OH

Figure 1 Structures of stilbenoids tested.

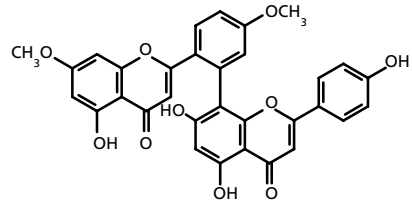


COMPOUND	R_1	R_2
1,8-dihydroxy-3-methylanthra-9,10-quinone **(7)**	H	CH_3
1,8-dihydroxy-3-methoxy-6-methylanthra-9,10-quinone **(8)**	CH_3	OCH_3
1,3,8-trihydroxy-6-methylanthra-9,10-quinone **(9)**	CH_3	OH

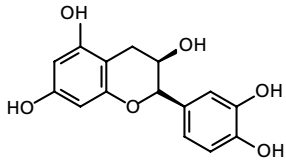
Figure 2 Structures of anthraquinones tested.



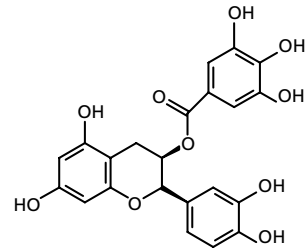
5-hydroxy-2-(4'-hydroxyphenyl)-7-methoxy-
2,3-dihydro-4*H*-chromen-4-one **(10)**



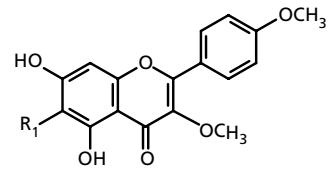
5-hydroxy-2-[2'-(5,7-dihydroxy-2-(hydroxyphenyl))-4*H*-chromen-4-one-4'-methoxyphenyl]
-7-methoxy-4*H*-chromen-4-one **(17)**



(2*R*,3*R*)-2-(3,4-dihydroxyphenyl)3,4-dihydro
-2*H*-chromene-3,5,7-triol **(5)**

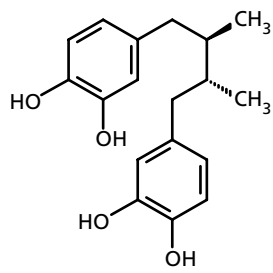


(2*R*,3*R*)-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3,4-dihydro
-2*H*-chromen-3-yl-3,4,5-trihydroxybenzoate **(6)**



COMPOUND	R_1
5,7-dihydroxy-3-methoxy-2-(4-methoxyphenyl)-4*H*-chromen-4-one **(11)**	H
5,6,7-trihydroxy-3-methoxy-2-(4-methoxyphenyl)-4*H*-chromen-4-one **(12)**	OH

Figure 3 Structures of flavonoids tested.



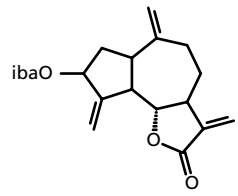
β,γ-dimethyl-,α,δ-bis(3,4-dihydroxyphenyl)butane **(13)**

Figure 4 Structure of lignan tested.

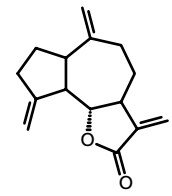
methoxy-2,3-dihydro-4*H*-chromen-4-one (**10**; Figure 3) (Dosaji et al 1983) was isolated.

L. divaricata yielded three active compounds identified as 5,7-dihydroxy-3-methoxy-2-(4-methoxyphenyl)-4*H*-chromen-4-one (**11**), 5,6,7-trihydroxy-3-methoxy-2-(4-methoxyphenyl)-4*H*-chromen-4-one (**12**) and β,γ-dimethyl-α,δ-bis(3,4-dihydroxyphenyl)butane (**13**) (Figures 3 and 4) (Sakakibara et al 1975, 1976, 1977). Compounds **11** and **12** are reported for the first time as constituents of this species.

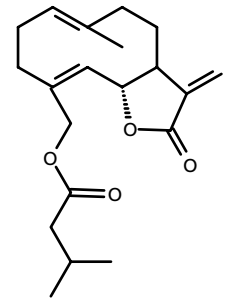
Bioassay-guided fractionation of the active extract (Table 1) prepared from the stem-bark of *A. adstringens* yielded (14b,24*E*)-3-oxolanosta-7,24-dien-26-oic acid (**24**) and (14b,24*E*)-3-hydroxylanosta-7,24-dien-26-oic acid (**25**) (Navarrete et al 1989) as the only activecompounds (Figure 8). In addition, a new naphthalene derivative was obtained from an inactive fraction and characterized as 3-dodecyl-1,8-dihydroxy-2-naphthoic acid (**23**) (Figure 7). This compound (mp 62–64°C) was isolated as a white solid. Its molecular formula was established as $C_{23}H_{32}O_4$ from HREIMS. The IR spectrum was consistent with the presence of hydroxyl (3500 cm^{-1}), aromatic (3005, 1571 and 1471 cm^{-1}) and a conjugated carboxyl (1681 cm^{-1}) functionalities. The UV spectrum had maxima absorptions at 228, 258 and 341 nm. On treatment with Ac_2O/pyridine, **23** afforded the diacetyl derivative **23a**, chemically confirming the presence of the two phenolic groups in the molecule. The NMR spectra indicated that **23** possessed a naphthalene moiety:



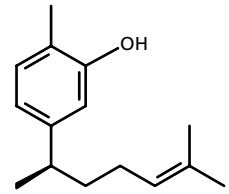
(3a*S*,6a*R*,8*S*,9a*R*,9b*S*)-3,6,9-trimethylene-2-oxododecahydroazulen[4,5-b]furan-8-yl-2-methylpropanoate **(14)**



(3a*S*,6a*R*,9a*R*,9b*S*)-3,6,9-trimethylenedecahydroazulen[4,5-b]furan-2(3*H*)-one **(26)**

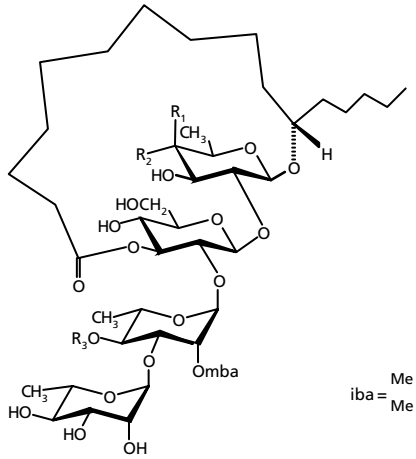


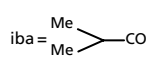
[(12a*R*)-7-methyl-3-methylene-2-oxo-3,3a,4,5,6,9,10,12a-octahydro-2*H*-cycloundeca[b]furan-11-yl]methyl-3-methylbutanoate **(15)**

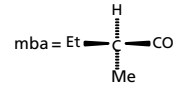


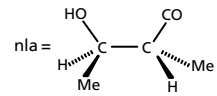
5-[(1*R*)-1,5-dimethyl-4-hexenyl]-2-methylphenol **(16)**

Figure 5 Structures of sesquiterpenes tested.

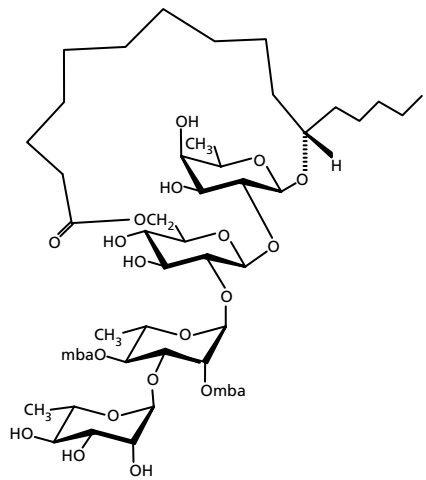








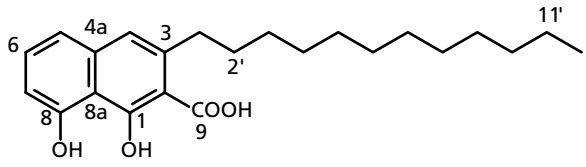
COMPOUND	R_1	R_2	R_3
(11*S*)-hydroxyhexadecanoic acid 11-*O*-α-L-rhamnopyranosyl-(1→3)-*O*-α-L-[2-*O*-(2*S*-methylbutyryl)-4-*O*-(2*S*-methylbutyryl)]-rhamnopyranosyl-(1→2)-*O*-β-D-glucopyranosyl-(1→2)-β-D-fucopyranoside-(1,3"-lactone) **(18)**	OH	H	mba
(11*S*)-hydroxyhexadecanoic acid 11-*O*-α-L-rhamnopyranosyl-(1→ 3)-O-α-L-[2-*O*-(2*S*-methylbutyryl)-4-*O*-(2-methylpropyl)]-rhamnopyranosyl-(1→2)-*O*-β-D-glucopyranosyl-(1→2)-β-D-fucopyranoside-(1,3"-lactone) **(19)**	OH	H	iba
(11*S*)-hydroxyhexadecanoic acid 11-*O*-α-L-rhamnopyranosyl-(1→3)-*O*-α-L-[2-*O*-(2*S*-methylbutyryl)-4-*O*-(3R-hydroxy-2*S*-methylbutyryl)]-rhamnopyranosyl-(1→2)-*O*-β-D-glucopyranosyl-(1→2)-β-D-fucopyranoside-(1,3"-lactone) **(20)**	OH	H	nla
(11*S*)-hydroxyhexadecanoic acid 11-*O*-α-L-rhamnopyranosyl-(′→ 3)-*O*-α-L-[2-*O*-(2*S*-methylbutyryl)-4-*O*-(2*S*-methylbutyryl)]-rhamnopyranosyl-(1→2)-*O*-β-D-glucopyranosyl-(1→2)-β-D-quinovopyranoside-(1,3"-lactone) **(22)**	OH	OH	mba



(11*S*)-hydroxyhexadecanoic acid 11-*O*-α-L-rhamnopyranosyl-(1→3)-*O*-α-L-[2-*O*-(2*S*-methylbutyryl)-4-*O*-(2*S*-methylbutyryl)]-rhamnopyranosyl-(1→2)-*O*-β-D-glucopyranosyl-(1→ 2)-β-D-fucopyranoside-(1,6"-lactone) **(21)**

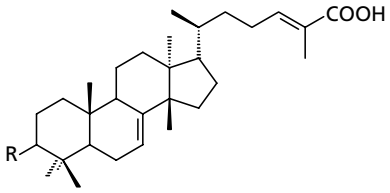
Figure 6 Structures of glycolipids tested.

δ_H/δ_C 7.56 (dd, J = 8.8 Hz, H-6)/137.3; 6.92 (dd, J = 8.4 and 1.0 Hz, H-7)/114.6; 6.81 (dd, J = 8.1 and 1.0 Hz, H-5)/115.3; 6.26 (sa, H-4)/104.6; two phenolics (δ_H 11.02 brs); one carboxyl group (δ_C 166.9) and one aliphatic side chain: δ_H/δ_C 2.51(t, J = 7.5 Hz, H-1′)/33.3; 1.33–1.26 (m, H-3′-H-11′)/29.6–28.9; 0.99 (t, J = 7.5 Hz, H-12′)/14.5. The HMBC correlations H-4/C-2, H-4, H-5/C-8a, H-5, OH-8/C-7 and H-1′/C-4 suggested the position of the alkyl chain at C-3 and the phenolics groups at C-1 and C-8. The carboxyl group was placed at C-2 on the basis of the HMBC correlations between H-4 (δ_H 6.26) and C-2 (δ_C 115.3). Finally, the strong NOESY correlations between H-4 (δ_H 6.26) and H-5 (δ_H 6.81)/H-1′ (δ_H 2.51) are in agreement with this



3-dodecyl-1,8-dihydroxy-2-naphthoic acid **(23)**

Figure 7 Structure of naphthalene tested.



COMPOUND	R_1
(14b,24*E*)-3-oxolanosta-7,24-dien-26-oic acid **(24)**	CO
(14b,24*E*)-3-hydroxylanosta-7,24-dien-26-oic acid **(25)**	OH

Figure 8 Structures of triterpenes tested.

proposal. On the basis of the above data, the novel natural product was characterized as 3-dodecyl-1,8-dihydroxy-2-naphthoic acid (**23**).

Antimycobacterial testing

The antimycobacterial activity of the constituents **1**–**13** and **23**–**25** (Figures 1–4, 7 and 8, respectively) isolated in this investigation as well as that of the nine pure compounds **14**–**22** (Figures 5 and 6), previously isolated in our laboratories from other medicinal plants, was examined using the BACTEC 460 assay. The tested compounds (Table 2) belong to different types of secondary metabolites, including sesquiterpenoids, triterpenoids, glycolipids, flavonoids, anthraquinones, stilbenoids and a lignan. (3a*S*,6a*R*,9a*R*,9b*S*)-3,6,9-trimethylenedeca-hydroazulen[4,5-b]furan-2(3H)-one (**26**; MIC 8 $\mu g\,mL^{-1}$), which represents an active principle of plant origin (Cantrell et al 1998), and rifampin (MIC 0.125 $\mu g\,mL^{-1}$) were used as the positive controls.

According to the data summarized in Table 2, the MIC values for the active compounds ranged between 16 and 128 $\mu g\,mL^{-1}$; the most active metabolites were the sesquiterpenes **15** and **16** (Figure 5) as well as the glycolipids **18**, **19**, **21** and **22** (Figure 6), which showed the same level of activity. The antimycobacterial activity of **15** could be related to both its alkylating properties and its lipophilic nature as reported for other sesquiterpene lactones (Cantrell et al 2001); its potency was comparable to lactone **26**, which was used in this study as one of the positive controls. Stilbenoids **1** and **2** (Figure 1) showed marginal activity while the acetyl derivative **1a** was four times more active than the parent compound, indicating that the lipophilicity favours the interaction with target cell membrane as previously described for other compounds (Cantrell et al 2001); the other natural stilbenoids tested, namely glycosides **3** and **4** (Figure 1), were inactive with MIC values higher than 128 $\mu g\,mL^{-1}$. Among the anthraquinones (**7**–**9**; Figure 2) tested, only **9** showed marginal activity with a MIC of 128 $\mu g\,mL^{-1}$. In the flavonoids series the activity was rather moderate, except in the subgroup of the flavan-3-ols (**5** and **6**; Figure 3), which were inactive; the MIC values of the active flavonoids ranged between 50 and 128 $\mu g\,mL^{-1}$. Few flavonoids have demonstrated good activity against *M. tuberculosis*. The most active are those isolated from the medicinal plants *Glycyrrhiza glabra* and *Erythrina gibbosa*, with MIC values in the range of 8–25 $\mu g\,mL^{-1}$ (Copp 2003). The only lignan tested was compound **13** (MIC = 50 $\mu g\,mL^{-1}$; Figure 4), which exhibited a similar effect to the active flavonoids. Finally, as other tetracyclic triterpenes (Cantrell et al 2001; Copp 2003), the tirucallanes **24** and **25** (Figure 8) from *A. adstringens* were active with MIC values of 64 and 32 $\mu g\,mL^{-1}$, respectively.

Conclusions

The species *R. hymenosepalus*, *L. divaricata*, *P. robinsonii* and *A. adstringens*, selected for this investigation following the ethnomedical criterion, yielded some antimycobacterial compounds belonging to different types of secondary metabolites. Although most of these substances showed modest activity, the original plant extracts had significant effects on the mycobacteria, thus providing the rationale for the traditional use of the plants in the treatment of TB.

Among the tested compounds the glycolipids, sesquiterpenoids and triterpenoids showed the best antimycobacterial activity. The antimycobacterial property of the glycolipids is reported for the first time; however, numerous triterpenoids and sesquiterpenoids have already been tested. According to the relative potency found in this study for such compounds, their level of activity is similar to that previously described for similar compounds tested in the same type of assay.

The presence of stilbenoids **1** and **2** in *R. hymenosepalus* might explain its use as a hypolipidaemic agent in Mexican folk medicine (Arichi et al 1982). On the other hand, the presence of **5** with demonstrated antiulceric and anti-inflammatory actions may explain the value of this species for the treatment of gastrointestinal disturbances (Calzada 2000; Lin et al 2001).

A. adstringens biosynthesizes triterpenoids and anacardic acid derivatives as well as naphthalene-type compounds such as **23**, discovered during the present investigation. Biogenetically, **23** could be generated from tridecanoic acid (acting as a starter moiety), which condenses with five units of malonyl-CoA to generate a suitable intermediate polyketide; in turn, the latter intermediate, on appropriate tailoring reactions, yields the alkyl naphthalene derivative **23**.

Table 2 Antimycobacterial activity of compounds **1**–**25**

Compound	MIC ($\mu g\,mL^{-1}$)
5-[(*E*)-2-(4-hydroxyphenyl)ethenyl]-1,3-benzenediol (**1**)	128
5-[(*E*)-2-(4-acetoxyphenyl)ethenyl]-1,3-benzenediol (**1a**)	32
4-[(*E*)-2-(3,5-dihydroxyphenyl)ethenyl]-1,2-benzenediol (**2**)	128
4-[(*E*)-2-(3,5-dihydroxyphenyl)ethenyl]phenyl hexopyranoside (**3**)	> 128
4-[(*E*)-2-(3,5-dihydroxyphenyl)ethenyl]-2-hydroxyphenyl hexopyranoside (**4**)	> 128
(2*R*,3*R*)-2-(3,4-dihydroxyphenyl)3,4-dihydro-2*H*-chromene-3,5,7-triol (**5**)	> 128
(2*R*,3*R*)-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3,4-dihydro-2*H*-chromen-3-yl-3,4,5-trihydroxybenzoate (**6**)	> 128
1,8-dihydroxy-3-methylanthra-9,10-quinone (**7**)	> 128
1,8-dihydroxy-3-methoxy-6-methylanthra-9,10-quinone (**8**)	> 128
1,3,8-trihydroxy-6-methylanthra-9,10-quinone (**9**)	128
5-hydroxy-2-(4′-hydroxyphenyl)-7-methoxy-2,3-dihydro-4*H*-chromen-4-one (**10**)	128
5,7-dihydroxy-3-methoxy-2-(4-methoxyphenyl)-4*H*-chromen-4-one (**11**)	50
5,6,7-trihydroxy-3-methoxy-2-(4-methoxyphenyl)-4*H*-chromen-4-one (**12**)	50
β,γ-dimethyl-α,δ-bis(3,4-dihydroxyphenyl)butane (**13**)	50
(3a*S*,6a*R*,8*S*,9a*R*,9b*S*)-3,6,9-trimethylene-2-oxododecahydroazulen[4,5-b]furan-8-yl-2-methylpropanoate (**14**)	32
[(12a*R*)-7-methyl-3-methylene-2-oxo-3,3a,4,5,6,9,10,12a-octahydro-2*H*-cycloundeca[b]furan-11-yl]methyl-3-methylbutanoate **(15)**	16
5-[(1*R*)-1,5-dimethyl-4-hexenyl]-2-methylphenol (**16**)	16
5-hydroxy-2-[2′-(5,7-dihydroxy-2-(hydroxyphenyl))-4*H*-chromen-4-one-4′-methoxyphenyl]-7-methoxy-4*H*-chromen-4-one (**17**)	64
(11*S*)-hydroxyhexadecanoic acid 11-*O*-α-L-rhamnopyranosyl-(1 → 3)-*O*-α-L-[2-*O*-(2*S*-methylbutyryl)-4-*O*-(2*S*-methylbutyryl)]-rhamnopyranosyl-(1 → 2)-*O*-β-D-glucopyranosyl-(1 → 2)-β-D-fucopyranoside-(1,3″-lactone) (**18**)	16
(11*S*)-hydroxyhexadecanoic acid 11-*O*-α-L-rhamnopyranosyl-(1 → 3)-*O*-α-L-[2-*O*-(2*S*-methylbutyryl)-4-*O*-(2-methylpropyl)]-rhamnopyranosyl-(1 → 2)-*O*-β-D-glucopyranosyl-(1 → 2)-β-D-fucopyranoside-(1,3″-lactone) (**19**)	16
(11*S*)-hydroxyhexadecanoic acid 11-*O*-α-L-rhamnopyranosyl-(1 → 3)-*O*-α-L-[2-*O*-(2*S*-methylbutyryl)-4-*O*-(3*R*-hydroxy-2*S*-methylbutyryl)]-rhamnopyranosyl-(1 → 2)-*O*-β-D-glucopyranosyl-(1 → 2)-β-D-fucopyranoside-(1,3″-lactone) (**20**)	32
(11*S*)-hydroxyhexadecanoic acid 11-*O*-α-L-rhamnopyranosyl-(1 → 3)-*O*-α-L-[2-*O*-(2*S*-methylbutyryl)-4-*O*-(2*S*-methylbutyryl)]-rhamnopyranosyl-(1 → 2)-*O*-β-D-glucopyranosyl-(1 → 2)-β-D-fucopyranoside-(1,6″-lactone) (**21**)	16
(11*S*)-hydroxyhexadecanoic acid 11-*O*-α-L-rhamnopyranosyl-(1 → 3)-*O*-α-L-[2-*O*-(2*S*-methylbutyryl)-4-*O*-(2*S*-methylbutyryl)]-rhamnopyranosyl-(1 → 2)-*O*-β-D-glucopyranosyl-(1 → 2)-β-D-quinovopyranoside-(1,3″-lactone) (**22**)	16
3-dodecyl-1,8-dihydroxy-2-naphthoic acid (**23**)	> 128
(14b,24*E*)-3-oxolanosta-7,24-dien-26-oic acid (**24**)	64
(14b,24*E*)-3-hydroxylanosta-7,24-dien-26-oic acid (**25**)	32
(3a*S*,6a*R*,9a*R*,9b*S*)-3,6,9-trimethylenedeca-hydroazulen[4,5-b]furan-2(3*H*)-one (**26**)*	8
Rifampin*	0.25

*Positive controls.

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