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Further mulinane diterpenoids from *Azorella compacta*

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Keywords
Azorella compacta; diterpenoid; gastric ulcer; llareta; mulinane

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Abstract

Objectives The chemical study of a dichloromethane extract from *Azorella compacta* was directed to the isolation of characteristic mulinane and azorellane diterpenoids in order to determine their gastroprotective activity.

Methods Usual chromatographic techniques on the extract led to the isolation of 12 compounds, which were identified by their spectroscopic properties. The HCl/ethanol-induced gastric lesions model in mice was used to determine the gastroprotective activity.

Key findings The new diterpenoids, 13β-hydroxymulinane (**1**), mulin-11, 13-dien-20-ol (**2**), 13α-methoxyazorellanol (**3**) and mulin-11,13-dien-18-acetoxy-16,20-dioic acid (**12**) were isolated from *A. compacta*. The known diterpenoids mulin-11,13-dien-20-oic acid (**4**), 13α-hydroxyazorellane (**5**), 13β-hydroxyazorellane (**6**), mulinic acid (**7**), mulinolic acid (**8**) and azorellanol (**9**), and the aromatic compounds 5,7-dihydroxychromone (**10**) and isoflavonoid biochanin A (**11**), were also obtained from the extract. Compounds **6**, **9** and **12** at 20 mg/kg reduced gastric lesions by 69%, 71% and 73%, respectively, being statistically similar to lansoprazole at the same dose.

Conclusions The results corroborate the intraspecific chemical variations detected previously in specimens of *A. compacta* collected at different Chilean latitudes. A high concentration of azorellanol (**9**) could account in part for some of the therapeutic properties attributed to this species, in particular in ulcer treatment. Most of the mulinane and azorellane diterpenoids isolated in this study showed relevant gastroprotective activity at a low dose in the bioassay.

Introduction

Azorella compacta Phil. (Apiaceae, Umbelliferae), locally known as 'llareta', is a tiny evergreen flowering shrub distributed throughout the high Andean regions (3000–4500 m) of Peru, Bolivia, Chile and Argentina. Previous chemical studies on this species[1–8] suggest striking variations in the concentration of diterpenoids in extracts obtained from specimens growing at different latitudes in northern and central Chile. Thus, plants collected at Tatio (Atacama Desert, Chile) were reported to produce mainly mulin-11,13-dien-20-oic acid, mulinol, 11,12-epoxy-mulin-13-en-20-oic acid, azorellanol, mulinolic acid, mulinic acid and desacetylazorellanol.[1–6] On the other hand, specimens of *A. compacta* collected at Vallenar (III region, Chile) afforded 20-hydroxymulin-11,13-dienyl acetate and 13,14-dihydroxymulin-11-en-20-oic acid.[7] Finally, the extract of plants collected at Farellones (Santiago, Chile), led to the isolation of mulin-12,14-dien-11-on-20-oic acid and mulin-12-ene-11,14-dion-20-oic acid.[8]

The local reputation of llareta as an antidiabetic, antiulcer, anticholesterol, anti-inflammatory and antiseptic, among other curative properties attributed to the species, has led to several bioactivity studies that report interesting results with some of the unique diterpenoids found in the extract.[5–9] Mulinolic acid and azorellanol were reported to decrease glycaemia[6] in streptozotocin diabetic rats at a similar level as chlorpropamide. Azorellanol, found in high concentrations in most specimens of *A. compacta* studied, has been reported to display high tripanocidal,

anti-inflammatory and antituberculosis activity.[5] However, there are no reports in the literature on the potential antiulcer properties of llareta diterpenoids.

Continuing our ongoing research on bioactive diterpenoids from Chilean medicinal plants, we now report the results of a phytochemical study on extracts obtained from specimens of *A. compacta* collected at Copiapo (Chile) and the relative gastroprotective activity displayed by the diterpenes (**1**–**9** and **12**) isolated in this study, against HCl/ethanol-induced gastric lesions in mice.

Materials and Methods

Chemicals

Thin-layer chromatography (TLC) (Kieselgel 60 GF254; Merck KGaA, Darmstadt, Germany) was run in *n*-hexane/EtOAc mixtures (9 : 1; 7 : 3 and 1 : 1 v/v) and spots were revealed by spraying plates with H_2SO_4-MeOH (5 : 95, v/v) and heating at 120°C. Silica gel (Kieselgel 60, 0.063–0.200 mm; Merck) and Sephadex (LH-20; Merck) were used in column chromatography. Technical solvents used in bulk plant extraction were previously distilled and dried according to standard procedures.

Instrumentation

A Bruker Avance AM-400 spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany) equipped with 5-mm probes was used for all nuclear magnetic resonance (NMR) experiments. Compounds were dissolved in $CDCl_3$ with tetramethylsilane as internal standard. ^{1}H-NMR spectra were obtained at 400.13 MHz, acquisition time (AQ) = 3.958 s, relaxation delay (RD) = 1.0 s, 30° pulse width = 6.5 μs, spectral width (SW) = 8278.1 Hz, line broadening (LB) = 0.3 Hz, FT size = 32 K. For the ^{13}C-NMR spectra, SF = 100.61 MHz, AQ = 1.36 s, RD = 2.0 s, 30° pulse width = 14 μs, SW = 23 980.81 Hz, LB = 1.0 Hz, FT size = 32 K. For the HMBC spectra, AQ = 0.303 s, 0.0459 s, RD = 1.0 s, SW = 3378.38 Hz, 22 321.43 Hz, FT size = 1024 × 1024 K, and 7.7 Hz long-range coupling constants. For the HMQC spectra, parameters were very similar to those used in the HMBC experiments, except for the 145-Hz one-bond coupling constant. The NOESY spectrum was obtained with four scans, AQ = 0.303 s, 0.303 s, RD = 1.0 s, SW = 3378.38 Hz, 3378.38 Hz, FT size = 1024 × 1024 K and LB = 0.0 Hz. IR spectra were recorded on a Vector 22 FT-IR spectrometer (Bruker Biospin GmbH; samples were dissolved in $CHCl_3$ and solutions was placed on NaCl plates). Mass spectra were recorded on a MAT 95XP Thermo Finnigan model spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) with an accelerating voltage of 3 kV and ionization energy of 70 eV. The temperature of the ion source was maintained at 250°C. Optical rotations were obtained in $CHCl_3$ on a Polax-2 L ATAGO polarimeter (ATAGO Co. Ltd, Tokyo, Japan).

Plant material

Specimens were collected in 2011 at Cerro el Potro (3500 m Copiapo, III Region, Chile) and identified as *Azorella compacta* by Professor M. Eliana Ramirez (Laboratorio de algas, Museo Nacional de Historia Natural). A voucher specimen (no. 140111) was deposited at the Herbarium of the Museo Nacional de Historia Natural (Santiago, Chile).

Extraction and isolation

Dried and pulverized leaves of *A. compacta* (3 kg) were left to macerate (3 times, 8 l each time, 7 days/extraction) with dichloromethane (DCM) and then methanol (MeOH).

After filtration, the solutions were concentrated to dryness under reduced pressure to obtain the extracts (DCM 300 g and MeOH 100 g). The DCM extract (250 g) was submitted to flash chromatography on silica gel 60 H (300 g, column length 25 cm, i.d. 10 cm) and eluted with *n*-hexane/EtOAc mixtures (3 l each) of increasing polarity (9 : 1, 7 : 3, 1 : 1, 3 : 7, 0 : 1; v/v) and MeOH. Solutions were then concentrated under reduced pressure to produce fractions 1–6.

Further column chromatography (silica gel 700 g, particle size: 200–500 μm, column length 65 cm, i.d. 9 cm) on fraction 1 (20 g, *n*-hexane/EtOAc 9 : 1) eluted with EtOAc/*n*-hexane (0.2 : 9.8, v/v) afforded 50 subfractions (200 ml each). By TLC comparison they were joined into three main fractions (1A–1C) according to their similarity in composition. Column chromatography (silica gel, 150 g, particle size: 63–200 μm, column length 50 cm, i.d. 4.5 cm) on fraction 1A (1 g) eluted with *n*-hexane/EtOAc mixtures (0–5% EtOAc) led to the isolation of compounds **1** (40 mg), **2** (50 mg) and **3** (5 mg). Fraction 1B (6 g) was submitted to column chromatography using silica gel (400 g, particle size: 63–200 μm, column length 40 cm, i.d. 6.5 cm) and *n*-hexane/EtOAc mixtures of increasing polarity (0–10% EtOAc) afforded mulin-11,13-dien-20-oic acid (**4**) (3 g),[1] 13α-hydroxyazorellane (**5**) (550 mg)[9] and 13β-hydroxyazorellane (**6**) (90 mg).[9] An aliquot (600 mg) of the fraction 1C (5 g) was submitted to rotatory chromatography on a silica gel disk (4 mm layer thickness) using a chromatotron and *n*-hexane/EtOAc (1 : 99) as solvent system to give **2** (20 mg), **4** (200 mg), a mixture of **4** and **6** (200 mg), and **6** (20 mg).

Preliminary purification of fraction 2 (*n*-hexane/EtOAc 7 : 3; 45 g) using column chromatography (Sephadex LH-20, column length 46 cm, i.d. 7.5 cm) and MeOH (3 times, 5 l each time) allowed the separation of fatty acids, chlorophylls and pigments from the diterpenoids. Fractions of 50 ml were then collected and combined to give a

mixture containing the diterpenoids (10 g) as seen by TLC. Flash chromatography on the mixture (silica gel 200 g, particle size: 40–63 μm, column length 30 cm, i.d. 6.5 cm) using *n*-hexane/EtOAc mixtures (9 : 1, 7 : 3, 3 : 7 and 0 : 1; v/v) gave fractions 2A–2D (3 l each). Further column chromatography on fraction 2A (2 g) using silica gel column chromatography (200 g, particle size: 63–200 μm, column length 60 cm, i.d. 4 cm) afforded **4** (1 g) and **6** (200 mg).

Repeated column chromatography (silica gel 240 g, 63–200 μm, column length 60 cm, i.d. 6.5 cm) on fraction 2B (3 g) led to the isolation of mulinic acid (**7**) (400 mg),[10] mulinolic acid (**8**) (300 mg)[11] and a mixture of both (1.3 g).

Azorellanol (**9**) (3 g)[4] was isolated by column chromatography on fraction 2C (4 g) using silica gel and eluting with *n*-hexane/EtOAc (7 : 3). Column chromatography on fraction 2D (4 g) using silica gel (200 g, 63–200 μm, column length 60 cm, i.d. 4.5 cm), and *n*-hexane/EtOAc mixtures (7 : 3, 3 : 7 and 0 : 1; v/v) gave complex mixtures of compounds. NMR analysis of some of the fractions collected suggested that they were not diterpenoids.

Fraction 3 (*n*-hexane/EtOAc 1 : 1, 15 g) was submitted to a preliminary column chromatography separation of fats and pigments on a Sephadex LH-20 (column length 46 cm, i.d. 7.5 cm) using MeOH as mobile phase (3 times, 4 l each time). Fractions of 25 ml were then collected and combined according to TLC similarity to give a brown resin (2 g) that was submitted to column chromatography on silica gel (160 g, 63–200 μm, column length 40 cm, i.d. 5 cm). Elution with DCM/EtOAc mixtures (1 : 0, 7 : 3, 3 : 7 and 0 : 1; 3 l each time) afforded 5,7-dihydroxychromone (**10**) (20 mg),[12] biochanin A (**11**) (30 mg)[13] and compound (**12**) (25 mg).

The polar fractions 4–6 were submitted to column chromatography using Sephadex LH20 and MeOH but did not contain mulinane-type or azorellane-type diterpenoids as seen by TLC and proton NMR analysis of some fractions. NMR spectra of compound **1–3** and **12** (Figures S1–S14) are available as Supporting Information.

Gastroprotective activity

Animals

Swiss albino mice (30 ± 3 g) were purchased from the Instituto de Salud Pública de Chile (Santiago, Chile). Mice were fed a certified Champion diet with free access to water under standard conditions of 12-h dark/light cycle and 20°C room temperature. The protocols were followed according to the recommendations of the Canadian Council on Animal Care with the ethical guidelines for investigations in conscious animals,[14] and were approved by the Universidad de Chile Animal Use and Care Committee (certificate approved in July 2010 by Dr Nicolas Giuliani).

The gastroprotective activity of the diterpenes **1–9**, **12** was evaluated in the HCl/EtOH-induced lesion model as described previously.[15,16] Mice were randomly distributed into groups of seven animals each and fasted for 12 h with free access to water prior to the experiment. Diterpenoids and lansoprazole were suspended in Tween 80 (1% solution) and were administered intragastrically to mice at a dose of 20 mg/kg in a volume of 10 ml/kg. At 50 min after administration of the diterpenoids, lansoprazole or 1% Tween 80 (10 ml/kg), all groups were treated orally with 0.2 ml of a solution containing 0.3 M HCl/60% ethanol solution (HCl/EtOH) for gastric lesion induction. Animals were killed 1 h after the administration of HCl/EtOH and the stomachs were excised and inflated by saline injection (1 ml). The ulcerated stomachs were fixed in 5% formalin for 30 min and opened along the greater curvature. Gastric damage visible to the naked eye was observed in the gastric mucosa as elongated black/red lines, parallel to the long axis of the stomach similar to the HCl/EtOH-induced lesions in rats. The length (mm) of each lesion was measured and the lesion index was expressed as the sum of the length of all lesions.

Statistical analysis

Results are presented as the mean ± SD. In all experiments, statistical differences between treatments and their respective control were determined by one-way analysis of variance followed by Dunnet's pairwise test. The level of significance was set at $P < 0.01$. GraphPad Prism 4 for Windows (GraphPad Software, Inc., La Jolla, CA, USA) was used for all statistical tests.

Results

Repeated column chromatography on the dichloromethane extract led to the isolation of 12 compounds (Figure 1). The known diterpenoids **4–9**, biochanin A (**10**) and the chromone (**11**) were identified by their spectroscopic properties and in comparison with related data reported in the literature. Four other closely related diterpenoids were isolated in this study and structures **1–3** and **12** are proposed based on the spectroscopic evidence discussed below.

Compound **1** was obtained as an oil. Resonances in the ^{1}H-NMR spectrum at δ_H 5.48 (1H, *d*, $J = 12.4$, H-12), 5.39 (1H, *dd*, $J = 12.4$; 8.1, H-11), 2.58 (1H, *t*, $J = 14.4$; 13.4, H-15), along with signals arising from five methyl groups at δ_H 1.24 (3H, *s*, Me-16), 0.90 (3H, *s*, Me-17), 0.87 (3H, *d*, $J = 6.6$, Me-19), 0.78 (3H, *d*, $J = 6.6$, Me-18) and 0.63 (3H, *s*, Me-20), were consistent with a mulinane-type diterpenoid lacking the diene system. The molecular formula of **1** was deduced as $C_{20}H_{34}O$ (m/z = 290.2601) based on HREIMS and ^{13}C-NMR data. The DEPT 135° spectrum allowed the assignation of carbon multiplicities in the ^{13}C-NMR spectrum that were consistent with six methylene, six methine,

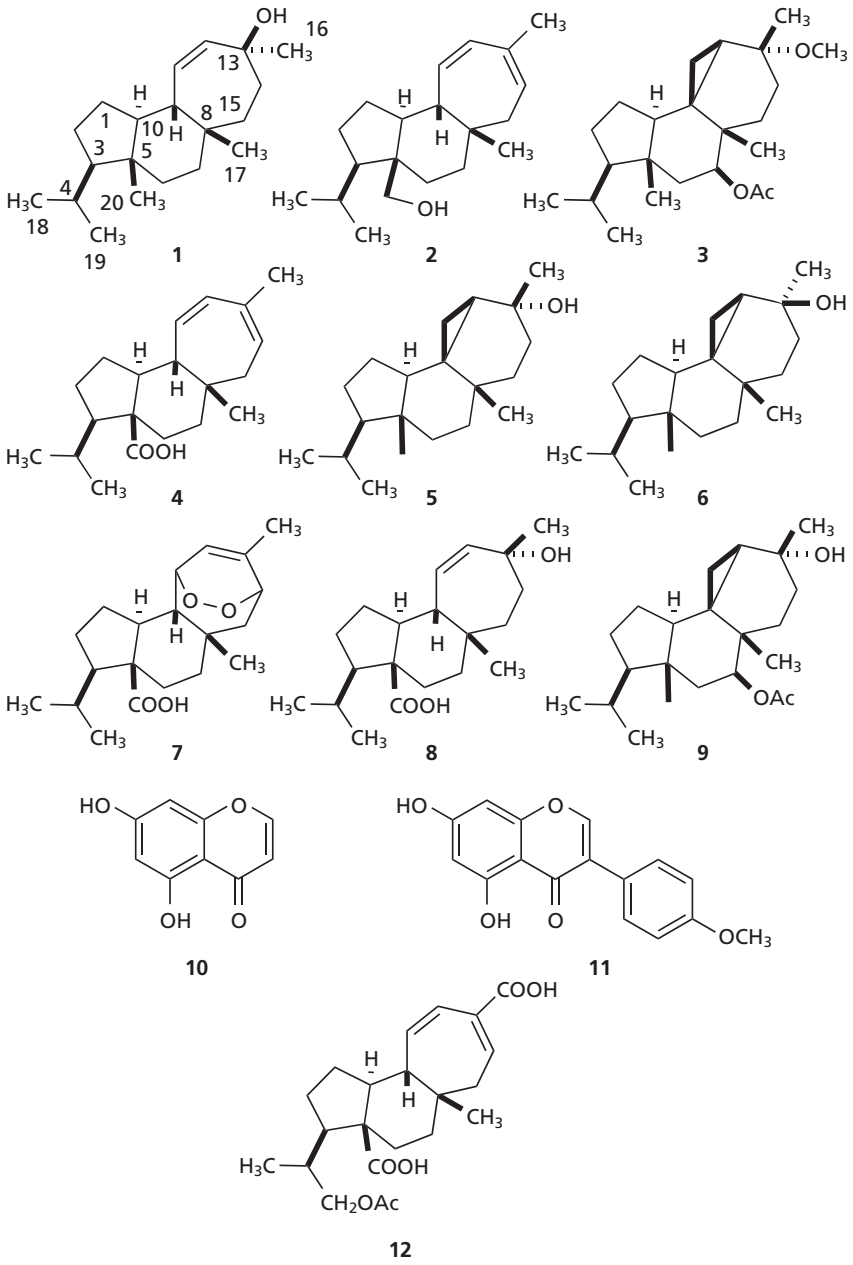

Figure 1 Chemical structures of compounds **1**–**12**.

and five methyl groups in the structure of **1**, along with three quaternary carbons at δ_C 70.9 (C-13), 43.5 (C-5) and 35.7 (C-8). An exception was observed in the ^{13}C-NMR spectrum: the presence of a methyl group at δ_C 11.3 instead of a carboxyl group. It was located at C-20 through the HMBC correlation between δ_H at 0.64 (Me-20) and the carbon signals at δ_C 57.5 (C-3), 51.1 (C-10), 43.5 (C-5) and 35.4 (C-6).

NOESY experiments suggested that **1** had the same relative configuration as 13-epimulinolic acid.[17] Thus, H-9 showed a dipolar correlation with H_3-17, both with β configuration, but not with H_3-16 protons. Besides, H_3-17 did not correlate with H_3-16, which confirms the β-orientation of the hydroxyl group at C-13. Other NOESY correlations were H_3-20/H-9, H_3-20/H-19 and H_3-20/H_3-18. Thus, compound **1** was identified as 13β-hydroxymulinane.

Compound **2** was obtained as a gum. The ^{1}H-NMR spectrum showed close similarity with that of 20-hydroxymulin-11,13-dienyl acetate[7] but it had resonances for a primary hydroxyl group signal at C-20 (δ_H 3.80, *d*, $J = 11.5$ and 3.58, *d*, $J = 11.5$; δ_C 60.1) instead of the acetate ester. This was further confirmed by comparison

with the NMR data reported for mulin-11,13-dien-20-oic acid.[1] Thus, compound **2** was identified as mulin-11,13-dien-20-ol.

Compound **3** was isolated as a gum. The resonances in the NMR spectra of compound **3** indicated a close similarity to azorellanol (**9**),[4] only differing in the presence of a methoxyl group at C-13 (δ_H 3.17; δ_C 49.1) instead of the hydroxyl group in **9**. Therefore, compound **3** was identified as 13α-methoxyazorellanol. Spectroscopic data reported for 13α-hydroxyazorellane,[9] 13β-hydroxyazorellane[9] and azorellanol[4] gave further support to this assignment.

Compound **12** was isolated as an oil. Its molecular formula, $C_{22}H_{30}O_6$, was established by HREIMS (m/z 390.2040, calcd. 390.2042). The ^{13}C-NMR spectrum showed well-resolved resonances for all 22 carbons. DEPT analysis indicated seven methine carbons at δ_C 143.6, 134.8, 120.7, 55.1, 52.6, 50.1 and 36.5, six methylene carbons at δ_C 68.9, 40.7, 37.3, 32.8, 28.4 and 24.5, three methyl carbons at δ_C 27.1, 20.9 and 17.0, and the quaternary carbons at δ_C 179.6, 172.5, 171.3, 128.4, 58.5 and 35.3. The almost identical ^{13}C chemical shifts of **12** and mulin-11,13-dien-20-oic acid (**4**) were indicative of the presence of a mulinane diterpenoid.[1,7] The ^{1}H-NMR spectra of **12** showed characteristic proton signals arising from a diene moiety at δ_H 7.21 (1H, *d*, *J* = 7.1, H-14), 6.42 (1H, *d*, *J* = 13.0, H-12) and 5.8 (1H, *dd*, *J* = 13.0; 6.5, H-11), along with three methyl groups at δ_H 2.06 s, 1.13 *d* (5.3) and 0.88 s, and a methylene group at δ_H 4.05 (1H, *d*, *J* = 11.0, H-18) and 3.85 (1H, *dd*, *J* = 11.0; 5.0, H-18). The HMQC spectra showed cross-peaks at δ_H 4.05, 3.85 and δ_C 68.9, and δ_H 1.13 and δ_C 17.0, while the proton resonances at δ_H 7.21, 6.42 and 5.80 were connected with carbon signals at δ_C 143.6, 120.7 and 134.8, respectively. These data confirmed the presence of an isopropyl group attached to an acetyl group and a diene system. HMBC correlation between H-12 (δ_H 6.42) and C-16 allowed the allocation of the carboxylic group. The same correlation between H-14 and C-16 confirmed this assignment. Similarly, HMBC correlations between H-3 and H-10 with C-20 were observed. In addition, the methyl signal at δ_H 1.13 *d* (H_3-19) correlated with δ_C 36.5, 52.6 and 68.9, indicating that the acetoxy group was attached to C-18. The main results from ROESY suggested that **12** had the same relative configuration shown, in agreement with the relative stereochemistry characteristic of mulinane diterpenoids.[1,7] This compound was identified as mulin-11,13-dien-18-acetoxy-16,20-dioic acid.

The spectroscopic and physical data for the new compounds **1**–**3** and **12** are presented.

13β-Hydroxymulinane

13β-Hydroxymulinane (**1**): white oil; $[\alpha]_D^{20} = -43.0$ (*c* 0.06, $CHCl_3$); FT-IR ν_{max}: 3110-2990, 1430, 1290, 1145/cm; HREIMS: calcd. for $C_{20}H_{34}O$ ($M^{+\cdot}$): 290.2610, found: 290.2601; EI-MS: m/z (rel. int. %): 290 [$M^{+\cdot}$] (4), 272 (25), 246 (24), 229 (100), 221 (17), 189 (8), 175 (43), 147 (57), 139 (33), 119 (30), 107 (37), 105 (42), 91 (48), 79 (43), 67 (33). ^{1}H NMR (400.13 MHz, $CDCl_3$): 1.41 m; 1.05 m (H-1); 1.75 m; 1.14 m (H-2); 1.02 m (H-3); 1.43 m (H-4); 1.61 m; 1.45 m (H-6); 1.50 m; 1.15 m (H-7); 1.90 m (H-9); 1.69 m (H-10); 5.39 dd (12.4; 8.1) (H-11); 5.48 d (12.4) (H-12); 1.78 m; 1.50 m (H-14); 2.58 t (14.4; 13.4); 0.88 m (H-15); 1.24 s (H-16); 0.90 s (H-17); 0.78 d (6.6) (H-18); 0.87 d (6.6) (H-19); 0.63 s (H-20). ^{13}C NMR (100.61 MHz, $CDCl_3$): 24.4 t (C-1); 28.3 t (C-2); 57.5 d (C-3); 30.9 d (C-4); 40.3 s (C-5); 35.4 t (C-6); 39.7 t (C-7); 35.7 s (C-8); 46.9 d (C-9); 51.1 d (C-10); 133.9 d (C-11); 136.0 d (C-12); 70.9 s (C-13); 36.0 t (C-14); 30.1 t (C-15); 33.2 q (C-16); 27.4 q (C-17); 22.3 q (C-18); 23.0 q (C-19); 11.3 q (C-20).

Mulin-11,13-dien-20-ol

Mulin-11,13-dien-20-ol (**2**): pale yellow oil; $[\alpha]_D^{20} = -185.9$ (*c* 0.05, $CHCl_3$); FT-IR ν_{max}: 3190, 2950, 1670, 1600, 1383, 1210, 1107/cm; HREIMS: calcd. for $C_{20}H_{32}O$ ($M^{+\cdot}$): 288.2453, found: 288.2445; EI-MS: m/z (rel. int. %): 288 [$M^{+\cdot}$] (16), 270 (29), 255 (17), 241 (33), 229 (13), 213 (21), 177 (23), 175 (10), 163 (25), 149 (18), 133 (35), 132 (17), 120 (52), 109 (36), 108 (35), 93 (100), 81 (27), 69 (39). ^{1}H NMR (400.13 MHz, $CDCl_3$): 1.50 m; 1.02 m (H-1); 1.63 m; 1.15 m (H-2); 0.85 m (H-3); 1.45 m (H-4); 2.06 m; 1.13 m (H-6); 1.61 m; 1.20 m (H-7); 2.17 dd (7.5; 6.4) (H-9); 1.43 m (H-10); 5.46 dd (12.6; 6.4) (H-11); 5.63 d (12.6) (H-12); 5.49 brd (5.3) (H-14); 2.73 brd (17.2); 1.48 m (H-15); 1.80 s (H-16); 0.88 s (H-17); 0.87 d (6.4) (H-18); 1.04 d (6.4) (H-19); 3.80 d (11.5); 3.58 d (11.5) (H-20). ^{13}C NMR (100.61 MHz, $CDCl_3$): 23.8 t (C-1); 28.3 t (C-2); 58.0 d (C-3); 31.6 d (C-4); 48.1 s (C-5); 30.0 t (C-6); 39.1 t (C-7); 34.9 s (C-8); 49.2 d (C-9); 54.0 d (C-10); 133.0 d (C-11); 127.8 d (C-12); 131.6 s (C-13); 125.6 d (C-14); 36.3 t (C-15); 25.6 q (C-16); 27.2 q (C-17); 23.3 q (C-18); 23.2 q (C-19); 60.1 t (C-20).

13α-Methoxyazorellanol

13α-Methoxyazorellanol (**3**): oil; $[\alpha]_D^{20} = -32.0$ (*c* 0.01, $CHCl_3$); FT-IR ν_{max}: 1740, 1457, 1383, 1250, 965/cm; HREIMS: calcd. for $C_{23}H_{38}O_3$ ($M^{+\cdot}$): 362.2821, found: 362.2815.

^{1}H NMR (400.13 MHz, $CDCl_3$): 1.20 m; 0.90 m (H-1); 1.80 m; 1.17 m (H-2); 1.25 m (H-3); 1.51 m (H-4); 2.21 dd (14.0;7.0); 1.50 m (H-6); 5.28 dd (11.5;7.0) (H-7); 2.41 dd (12.9;7.2) (H-10); 0.76 s; 0.15 brs (H-11); 0.76 s (H-12); 1.33 m; 1.21 m (H-14); 1.49 m; 1.15 m (H-15); 1.19 s (H-16); 1.03 s (H-17); 0.83 d (6.6) (H-18); 0.92 d (6.6) (H-19); 0.91 s (H-20); 3.17 s (OCH_3); 2.03 s (Ac). ^{13}C NMR

(100.61 MHz, $CDCl_3$): 20.7 t (C-1); 27.5 t (C-2); 58.9 d (C-3); 31.1 d (C-4); 42.1 s (C-5); 39.3 t (C-6); 78.4 d (C-7); 34.2 s (C-8); 26.2 s (C-9); 47.1 d (C-10); 10.9 t (C-11); 25.1 d (C-12); 73.6 s (C-13); 29.7 t (C-14); 31.5 t (C-15); 24.2 q (C-16); 18.8 q (C-17); 22.4 q (C-18); 22.3 q (C-19); 17.5 q (C-20); 49.1 q (OCH_3); 171.6 s; 21.3 q (Ac).

Mulin-11,13-dien-18-acetoxy-16,20-dioic acid

Mulin-11,13-dien-18-acetoxy-16,20-dioic acid (**12**): oil; FT-IR ν_{max}: 3300-2700 *br*, 1720, 1730, 1685, 1457, 1383, 1250/cm; HREIMS: calcd. for $C_{22}H_{30}O_6$ ($M^{+\cdot}$): 390.2042, found: 390.2039. ^{1}H NMR (400.13 MHz, $CDCl_3$): 2.06*; 1.68 m (H-1); 1.94 m; 1.55 m (H-2); 1.67 m (H-3); 1.68 m (H-4); 2.49 brd (12.1); 1.41 m (H-6); 1.63 m; 1.46 m (H-7); 2.33 dd (8.2; 6.5) (H-9); 1.68 m (H-10); 5.80 dd (13.0; 6.5) (H-11); 6.42 d (13.0) (H-12); 7.21 brd (7.1) (H-14); 2.87 brd (16.5); 2.03 m (H-15); 0.88 s (H-17); 4.05 d (11.0); 3.85 dd (11.0; 5.0) (H-18); 1.13 d (5.3) (H-19); 2.06 s (Ac). ^{13}C NMR (100.61 MHz, $CDCl_3$): 24.5 t (C-1); 28.4 t (C-2); 52.5 d (C-3); 36.5 d (C-4); 58.5 s (C-5); 32.7 t (C-6); 40.8 t (C-7); 35.3 s (C-8); 50.1 d (C-9); 55.1 d (C-10); 134.8 d (C-11); 120.7 d (C-12); 128.4 s (C-13); 143.6 d (C-14); 37.3 t (C-15); 172.5 s (C-16); 27.1 q (C-17); 68.9 t (C-18); 17.0 q (C-19); 179.6 s (C-20); 171.3 s; 20.9 q (Ac). *Hidden signal.

Gastroprotective activity

The results of the gastroprotective bioassays performed with compounds **1**, **2**, **4–6**, **8–9** and **12** are presented in Table 1. All diterpenoids tested showed gastroprotective activity in the HCl/EtOH-induced gastric lesions model in mice at a dose of 20 mg/kg (p.o.). The activity displayed by **6** (69%), **9** (71%) and **12** (73%) was similar to that observed with lansoprazole at the same dose, while **1**, **2**, **4**, **5**, **7** and **8** (59%, 26%, 39%, 56%, 12% and 55%, respectively) were less active in comparison. Compounds **3**, **10** and **11** were not tested for gastroprotective activity. Among mulinane diterpenes (**1**, **2**, **4**, **7–8** and **12**) **7** did not show any significant difference with the untreated control and the best gastroprotective activity was displayed by mulin-11,13-dien-18-acetoxy-16,20-dioic acid (**12**), with an extra acid group in its structure. Remarkable differences in gastroprotective activity were found among the azorellane diterpenoids, the most active being 13β-hydroxyazorellane (**6**) and azorellanol (**9**).

Table 1 Gastroprotective effect of compounds **1–9**, **12** and lansoprazole at 20 mg/kg on HCl/EtOH-induced gastric lesions in mice

Compound	*n*	Lesion index (mm)	Lesion reduction (%)	*P*
1	7	15.0 ± 2.3**	59*	<0.01
2	7	27.0 ± 2.2**	26*	<0.01
3[a]	–	–	–	–
4	7	22.4 ± 1.9**	39*	<0.01
5	7	16.1 ± 4.3**	56*	<0.01
6	7	11.4 ± 2.2	69*	<0.01
7	7	31.9 ± 3.8**	12	<0.01
8	7	16.4 ± 4.6**	55*	<0.01
9	7	10.4 ± 2.6	71*	<0.01
12	7	9.9 ± 3.6	73*	<0.01
Lansoprazole	7	8.1 ± 2.4	78*	<0.01
Control	7	36.4 ± 3.9	–	–

The results are expressed as mean ± SD (n = number of mice). *$P < 0.01$, significantly different compared with the control; **$P < 0.01$, significantly different compared with lansoprazole (analysis of variance followed by Dunnett's test). [a]Not evaluated.

Discussion

Azorellanol and mulin-11,13-dien-20-oic acid were the main diterpenes isolated from the dichloromethane extract of *A. compacta*. The new diterpenes reported in this study, 13β-hydroxymulinane (**1**), mulin-11,13-dien-20-ol (**2**), 13α-methoxyazorellanol (**3**) and mulin-11,13-dien-18-acetoxy-16,20-dioic acid (**12**) are present only in very small amounts. The aromatic compounds 5,7-dihydroxychromone (**10**) and biochanin A (**11**) are reported for the first time in this plant, although they are also minor components of the extract. The chemical composition of *A. compacta* found in this study confirms the existence of wide intraspecific chemical variations in this species, possibly derived from adaptive mechanisms to the environmental conditions of the natural habitat at different latitudes.

Balance impairment between aggressive (stress, chemical agents, drugs, gastric acid and pepsin secretion) and defensive factors (endogenous mechanisms) in the gastric mucosa may lead to gastric ulcers.[18] For instance, ethanol has a direct action on the gastric mucosa, inducing the formation of gastric ulcers, while the presence of HCl accelerates the process. Thus, the administration of the HCl/EtOH solution provokes an acute gastric ulcer in mice that allows the testing of new drugs that could protect the gastric mucosa. Medicinal plants that are traditionally used to treat ulcers are one of the most attractive sources of natural products as gastroprotective agents.[18–22]

The gastroprotective activity of terpenes of various skeletal types under different experimental conditions has been reported in the literature but showed significant activity only at a single oral dose of 100 mg/kg.[19–23] In a previous study, we found a gastroprotective effect of some of the ferruginol derivatives tested that was comparable with the *Azorella* diterpenoids reported here under the same experimental conditions (20 mg/kg).[24] However, in comparison with poligodial, carnosol, 6,7-royleanone, 7,20-epoxiroyleanone, royleanone and horminone, under the

same the conditions, the gastroprotective effect of *Azorella* diterpenes is lower.[25,26]

In compounds **2**, **4** and **12**, including the alcohol, acid and diacid, the greatest activity was related to the presence of an additional acid at C-16. In the series **5**, **6** and **9**, the gastroprotective effect of 13β-hydroxyazorellane was comparable with that of azorellanol, reducing the gastric lesions by 69% and 71%, respectively. There were statistically significant differences in the gastroprotective activity of 13α-hydroxyazorellanol and 13β-hydroxyazorellane, which could be attributed to the presence of the β-OH isomer. Compounds **1** and **8** presented similar gastroprotective activity. The gastroprotective effect of compound **7** collapsed, which could be associated with the presence of the peroxide function.

The results of this study confirm for the first time that the local use of *A. compacta* in the treatment of gastric ulcers could have its basis in the high content of azorellanol (**9**) in the leaves of this species. Other medicinal uses of the plant could also be attributed to this compound as deduced by several reports on the bioactivity of diterpenoids isolated from *A. compacta.* Azorellanol and mulin-11,13-dien-20-oic acid have trypanocidal activity against all stages of *Trypanosoma cruzi*.[5] Delporte *et al.*[27] reported the absence of toxicity or side-effects in mice of azorellanol, 13-hydroxy-7-oxoazorellanol and desacetylazorellanol as well as high topical anti-inflammatory activity. Azorellanol and desacetylazorellanol also showed potent anti-NF-κB activity.[28] The antitubercular activity of natural azorellane and mulinane diterpenoids has also been described.[29–32] Wächter *et al.* [8] reported the antibacterial activity of mulin-11,13-dien-20-oic acid and mulinolic acid. Finally, the azorellane diterpenes from *Azorella yareta*, including mulinane diterpenoids, displayed trichomonicidal activity over the range of 40–120 μM.[9]

Conclusions

The chemical composition of the extract obtained from specimens of *A. compacta* collected at Copiapo (Chile) showed qualitative differences in the composition of characteristic diterpenoids as compared with previous reports on specimens collected at other Chilean latitudes, thus corroborating intraspecific chemical differences. Minor components of the extract isolated in this study were the new diterpenoids 13β-hydroxymulinane, mulin-11,13-dien-20-ol, 13α-methoxyazorellanol and mulin-11,13-dien-18-acetoxy-16,20-dioic acid, the structures of which are proposed based on spectroscopic evidence. A high concentration of azorellanol (**9**) (1% of the extract) could account in part for some of the putative medicinal properties attributed to this species. Most of the mulinane and azorellane diterpenoids isolated in this study showed relevant gastroprotective activity at low doses in the bioassay. The most active compounds tested were **6**, **9** and **12**, showing an effect comparable with that of lansoprazole, thus confirming for the first time this type of activity in *Azorella* diterpenoids.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1 ^{1}H-NMR spectrum of compound **1** ($CDCl_3$).
Figure S2 ^{13}C-NMR spectrum of compound **1** ($CDCl_3$).
Figure S3 HMQC spectrum of compound **1** ($CDCl_3$).
Figure S4 HMBC spectrum of compound **1** ($CDCl_3$).
Figure S5 ^{1}H-NMR spectrum of compound **2** ($CDCl_3$).
Figure S6 ^{13}C-NMR spectrum of compound **2** ($CDCl_3$).
Figure S7 HMQC spectrum of compound **2** ($CDCl_3$).
Figure S8 HMBC spectrum of compound **2** ($CDCl_3$).
Figure S9 ^{1}H-NMR spectrum of compound **3** ($CDCl_3$).
Figure S10 ^{13}C-NMR spectrum of compound **3** ($CDCl_3$).
Figure S11 ^{1}H-NMR spectrum of compound **12** ($CDCl_3$).
Figure S12 ^{13}C-NMR spectrum of compound **12** ($CDCl_3$).
Figure S13 HMQC spectrum of compound **12** ($CDCl_3$).
Figure S14 HMBC spectrum of compound **12** ($CDCl_3$).