JPP
Journal of
Pharmacy and Pharmacology

JPP 2009, 61: 1689–1697

Received July 24, 2009
Accepted September 11, 2009
DOI 10.1211/jpp/61.12.0015
ISSN 0022-3573

Research Paper

Gastroprotective effect and cytotoxicity of abietane diterpenes from the Chilean Lamiaceae *Sphacele chamaedryoides* (Balbis) Briq.

Carlos Areche[a], Guillermo Schmeda-Hirschmann[a], Cristina Theoduloz[b] and Jaime A. Rodríguez[b]

[a]Laboratorio de Productos Naturales, Instituto de Química de Recursos Naturales and [b]Depto. de Ciencias Básicas Biomédicas, Facultad de Ciencias de la Salud, Universidad de Talca, Talca, Chile

Abstract

Objectives The aim of this report was to isolate, identify and assess the gastroprotective effect and cytotoxicity of abietane diterpenes from the Chilean medicinal plant *Sphacele chamaedryoides* (Balbis) Briq. (Lamiaceae).

Methods The isolated compounds were identified by spectroscopic means. The gastroprotective effect of the compounds was studied on the HCl/EtOH-induced gastric lesions model in mice. The cytotoxicity of the compounds was assessed on human normal lung fibroblasts (MRC-5) and gastric adenocarcinoma cells (AGS).

Key findings From the aerial parts of the plant, five phenolic and five *p*-quinone abietanes, the sesquiterpene spathulenol and two flavonoids were obtained. The main diterpene from the plant was carnosol (**7**). Lansoprazole at 20 mg/kg reduced gastric lesions by 64.7% ($P < 0.01$), being statistically similar to carnosol at doses of 10 and 20 mg/kg; the percent lesion reduction with **7** at 5 mg/kg was 49.3%. At a single oral dose of 5 mg/kg, the diterpenes bearing a *p*-quinone moiety – 6,7-dehydroroyleanone (**1**), royleanone (**2**), 7,20-epoxyroyleanone (**3**), taxoquinone (**5**) and horminone (**6**) – presented a gastroprotective effect of 54.4, 70.8, 65.0, 35.8 and 52.7%, respectively. Of the C-7 hydroxy derivatives, the activity was much lower for the 7β-OH isomer. The phenolic diterpenes **7** and 7-oxo-11,12,14-trihydroxy-8,11,13-abietatrien-20-al (**8**) inhibited gastric lesions by 49.3 and 53.0%, respectively. Royleanone (**2**), 7,20-epoxyroyleanone (**3**), horminone (**6**), **8** and spathulenol proved to be cytotoxic with IC50 values in the range of 11–67 μM. The selective cytotoxicity of compounds **1** (IC50: 61 and 366 μM) and **5** (IC50: 310 and 27 μM) against AGS cells and fibroblasts, respectively, merit additional studies.

Conclusions All the abietanes obtained from *S. chamaedryoides* present either one or two phenolic OH groups, a quinone system, or both. Several compounds present in the plant showed higher gastroprotective effect than lansoprazole. The cytotoxic effect of most compounds was found at fairly high concentrations and lacked cell specificity. Further studies are required using different tumour cell lines and viability/proliferation assays to assess the specificity of the isolated compounds. The selective cytotoxicity of compounds **1** and **5** against AGS cells and fibroblasts, respectively, merit additional studies.

Keywords abietane diterpenes; cytotoxicity; gastroprotective effect; Lamiaceae; *Sphacele chamaedryoides*

Introduction

Gastric and duodenal ulcers are digestive diseases that affect, with different intensity, 8–10% of the population living in the industrialized countries. In 2002, stomach cancer and peptic ulcer disease represented 1.5 and 0.5% of the total cause of deaths in the world, respectively.[1] Recently, it has been well established that chronic gastric ulcer might lead to the development of gastric and pancreatic cancer.[2,3] Gastric cancer is the fourth cause of mortality in individuals aged 45–64 years in Chile[4] with an annual rate of 4.9%. Chile has one of the highest incidences of gastric cancer, with a similar prevalence to Japan, Costa Rica and Singapore. The pharmacological therapeutics of gastric ulcer have evolved from the use of antacids, anticholinergic drugs and H_2-receptor blockers to the more recent antisecretory drugs that inhibit the gastric H^+-K^+ ATPase.[5] In the last few decades, much

Correspondence: Dr Guillermo Schmeda-Hirschmann, Laboratorio de Productos Naturales, Instituto de Química de Recursos Naturales, Universidad de Talca, Casilla 747, Talca, Chile.
E-mail: schmeda@utalca.cl

effort has been made to discover and develop new anti-ulcer drugs from natural sources, some plant-derived anti-ulcer drugs being carbenoxolone from *Glycyrrhiza glabra*, solon from sophoradin isolated from *Sophora subprostrata* and gefarnate from cabbage (*Brassica oleracea*) among others.[6]

Abietane diterpenes display several biological effects, including anti-ulcerogenic and antiproliferative activity in human tumour cell lines.[7,8] The gastroprotective effect and cytotoxicity of abietane diterpenes has been shown in studies carried out with natural products as well as semisynthetic derivatives. The work performed so far includes the classical investigations leading to ecabet sodium,[9–11] structure–activity trends in dehydroabietic acid derivatives,[12] ferruginol[13–15] and its semisynthetic products,[16] cytotoxic effect of *Salvia* diterpenes[17] and a dehydroabietylamine derivative.[18] The genus *Sphacele* is commonly found in central Chile and it constitutes are a good source of diterpenes. The isolation of antioxidant abietanes from *Sphacele salviae* was reported by Escuder *et al*.[19] According to these authors the compounds presented high free radical scavenging effect against the free radical diphenylpicrylhydrazyl (DPPH). Escuder *et al*.[19] described the isolation of phenolic diterpenes from the leaves of *S. salviae*, but quinone diterpenes were not obtained. The herb *Sphacele chamaedryoides* (Balbis) Briq. (Lamiaceae) (Syn. *Lepechinia chamaedryoides* (Balb.) Epl.) occurs in the coastal range of central Chile and is known under the common name 'algue-lahuen' or 'salvia macho'.[20,21] Some peasants use the leaf infusion as a digestive. The composition of *S. chamaedryoides* essential oil was described by Valenzuela *et al*.[22] The diterpene quinone horminone was identified from the leaf resin of *S. chamaedryoides*.[23] Abietane diterpenes have been described as constituents of *S. salviae* but *S. chamaedryoides* has not previously been investigated as a source of gastroprotective and cytotoxic constituents. Following our studies on bioactive terpenes from South American plants, we now report the isolation, gastroprotective effect and cytotoxicity of abietane diterpenes from 'algue-lahuen'.

Materials and Methods

Equipment

Melting points were determined on a Koffler hot stage apparatus (Electrothermal 9100) and were uncorrected. Optical rotations were obtained for solutions in $CHCl_3$ (concentrations expressed in g/100 ml) on a Jasco DIP 370 polarimeter. IR spectra were recorded on a Nicolet Nexus FT-IR instrument. ^1H NMR spectra were recorded at 400 MHz and ^{13}C NMR data were obtained at 100 MHz on a Bruker Avance 400 spectrometer (δ scale). Mass spectra are presented as m/z (rel. int. %). Si gel 60 (Merck, 63–200-μm particle size) was used for column chromatography, precoated Si gel plates (Merck, Kieselgel 60 F_{254}, 0.25 mm) were used for TLC analysis. TLC spots were visualized by spraying the chromatograms with *p*-anisaldehyde–ethanol–acetic acid–H_2SO_4 (2 : 170 : 20 : 10 v/v) and heating at 110°C for 3 min. High-speed counter-current chromatography (HSCCC) was carried out with a P.C. Inc. (Potomac, USA) equipment. The apparatus consisted of a multilayer coil of 1.68 mm i.d. poly(tetrafluoroethylene) tubing with a total capacity of 320 ml. The revolution radius or the distance between the holder axis and central axis of the centrifuge (R) was 10.5 cm, the β-value was 0.76 (β = r/R), where r is the distance from the coil to the holder shaft. The rotor speed varied from 0 to 1200 rev/min. The flow rate was controlled with a DC Analytic Gearmotor (Bodine Electric Company, Chicago, USA) with a P.C. Inc. injection module and with a 10-ml sample loop.[24]

Extraction and isolation of the compounds

S*phacele chamaedryoides* was collected in March 2005 in a forest near Salto de Agua, Chovellen (VII Region, Chile). A voucher specimen (No. 3006) was deposited in the herbarium of the Universidad de Talca. The plant was identified by Dr Patricio Peñailillo at the Universidad de Talca Herbarium.

Dried and powdered leaves (1 kg) were extracted under reflux with petroleum ether (PE) (3 × 30 min, 10 L PE each time) to obtain 45 g of a PE extract. The plant material was subsequently extracted by reflux with EtOAc (3 × 30 min, 10 L EtOAc each time) to obtain 25 g of an EtOAc extract.

The PE extract was permeated on Sephadex LH-20 (column length 50 cm, internal diameter 5 cm) with a 3 : 1 : 1 PE–DCM–MeOH (v/v/v) mixture as eluent. The procedure was repeated three times with 10 g of the crude extract. For every 10 g extract-permeation, some 25 fractions of 20 ml each were collected. According to the TLC pattern (silica gel, PE–EtOAc 7 : 3, v/v), the fractions were pooled into three fraction groups A–C. Fraction A consisted of chlorophyll and was discarded. Fraction B (13 g) was chromatographed on silica gel G 60 (120 g, 200–500 μm) in a 60 × 6 cm column using a PE–EtOAc 0–100% gradient to afford five subfractions named B-1 to B-5. The fractions B-1 to B-3 were further purified by HSCCC. Column chromatography (CC) of the fraction B-4 on silica gel (120 g, particle size 200–500 μm, column length 50 cm, internal diameter 3 cm) using a PE to EtOAc gradient yielded 183 fractions of 20 ml each. From the fractions 64–82, some 68 mg of 7-oxo-11,12,14-trihydroxy-8,11,13-abietatrien-20-al (**8**) was obtained. The fraction B-5 after CC on silica gel (100 g, 200–500 μm, 60 × 2.5 cm column) using DCM–MeOH (0–10%) afforded 151 fractions of 20 ml each. From fractions 1–30, compound **9** (2 mg) was obtained, while fractions 31–50 yielded compound **10** (4 mg) and fractions 73–99 afforded 250 mg carnosol (**7**). Column chromatography of fraction C afforded 60 mg horminone (**6**).

Some 25 g of the EtOAc extract was permeated in a Sephadex LH-20 column (50 cm length, 5 cm internal diameter) using MeOH as the mobile phase. Fractions were pooled according to the TLC patterns (silica gel, PE–EtOAc, 7 : 3, v/v). Chlorophyll-containing fractions were discarded and the mixtures were pooled into two subfractions named A and B. Further fractionation of fraction B by CC on silica gel (60 g, 200–500 μm, 50 × 5 cm column) resulted in 198 fractions of 20 ml each. Fractions 3–5 afforded 30 mg ferruginol (**4**), fractions 90–103 yielded after successive crystallizations in benzene 90 mg of carnosol (**7**), and fractions 115–198 after recrystallization in PE gave 22 mg of the flavonoid pinocembrin (Figure 1).

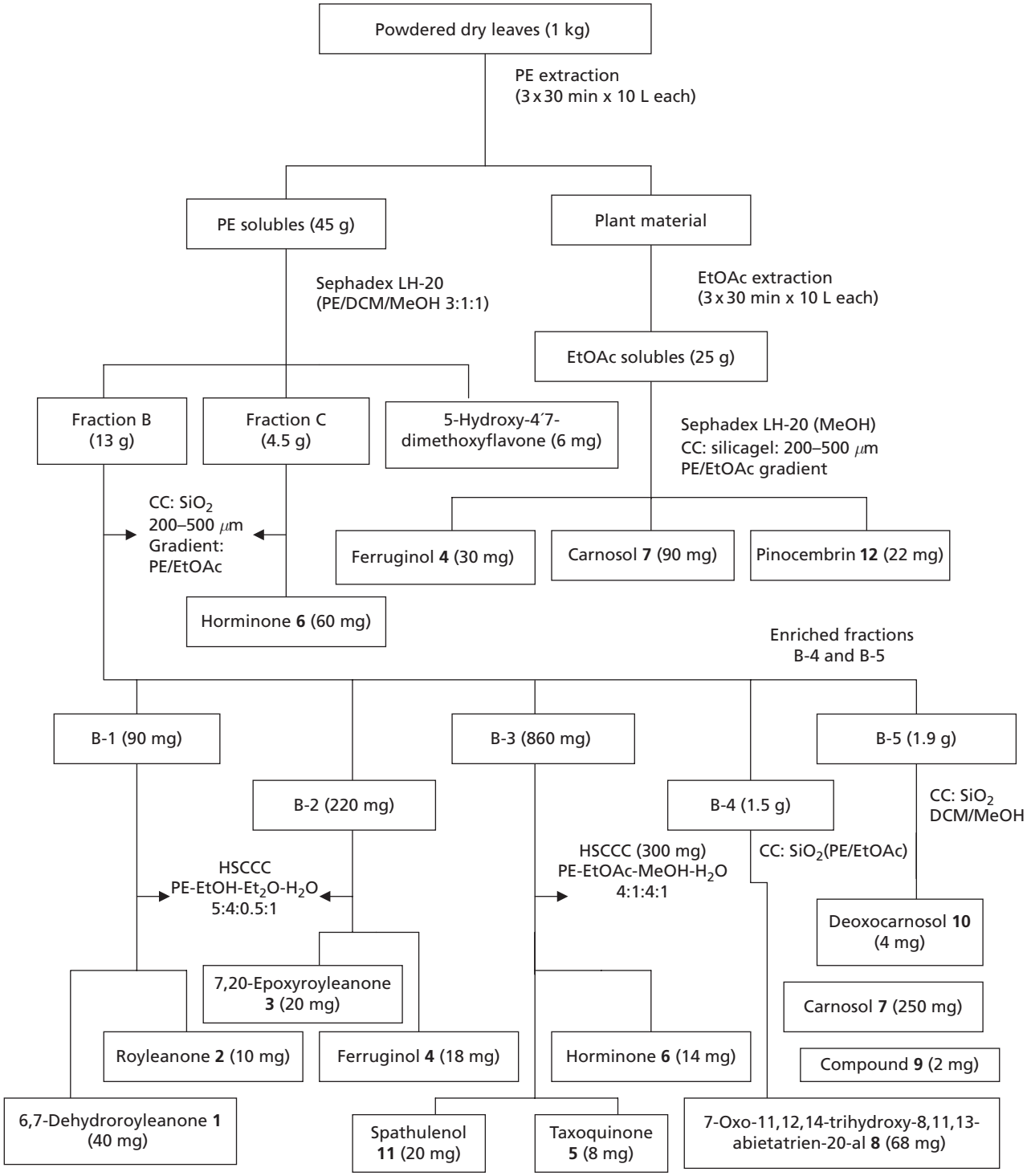

Figure 1 Isolation of the *Sphacele chamaedryoides* leaf constituents

Fractions B-1 to B-3 were separated with HSCCC equipment. The solvent mixtures: PE–EtOH–Et_2O–H_2O (5 : 4 : 0.5 : 1, v/v/v/v), PE–EtOAc–MeOH–H_2O (4 : 1 : 4 : 1, v/v/v/v) were used as the two-phase solvent system for HSCCC separation, which were selected by a previous experiment with the fractions in a series of solvent systems using TLC analysis and distribution of the fractions between a two-phase solvent system. Each mixture was thoroughly equilibrated overnight in a separation funnel at room temperature. Then, the upper phase and the lower phase were separated and degassed by sonication for 15 min before use.[25] The lower phase (aqueous phase) was used as the mobile phase while the organic phase (upper phase) was employed as stationary phase.

For the separation, first, the coiled column was filled with the upper phase (stationary phase) of the solvent system. Then, the HSCCC apparatus was rotated at 800 rev/min and at the same time, the lower phase (mobile phase) was pumped through the column in a tail to head (T to H) direction at a flow rate of 2.0, 2.0 and 2.5 ml/min and a pressure of 50 PSI for the subfractions 1, 2 and 3, respectively. After hydrodynamic equilibrium was reached (about an hour in all cases), the sample solution (subfractions were dissolved in 5 ml of upper phase and 5 ml lower phase) was injected through the injection valve. Fractions of 5 ml were collected and combined based on the TLC patterns on silica gel (PE–EtOAc 8 : 2, v/v). From the fraction B-1, 162 fractions of 5 ml were collected. From fractions 58–89, 40 mg of 6,7-dehydroroyleanone (**1**) and from fractions 101–110, 10 mg royleanone (**2**) were obtained. Fraction B-2 afforded 198 fractions of 5 ml each. After TLC comparison, fractions 18–25 yielded 20 mg 7,20-epoxyroyleanone (**3**) and fractions 83–97 afforded 18 mg ferruginol (**4**). From fraction B-3, some 180 fractions of 5 ml each were collected. Fractions 38–50 gave 14 mg horminone (**6**), fractions 56–59 contained 8 mg taxoquinone (**5**) and fractions 66–79 afforded 20 mg spathulenol (**11**). The isolation procedure is summarized in Figure 1.

HCl/EtOH-induced ulcer model in mice

Male Swiss albino mice, 30 ± 3 g were used. Mice were fed on certified Champion diet with free access to water under standard conditions of 12-h dark–light period, 50% relative humidity and 22°C room temperature. Mice were randomly distributed into groups of 8–10 mice each and fasted for 24 h with free access to water before the experiment. For carnosol, three different doses were used: 20, 10 and 5 mg/kg. For comparison purposes, the isolated compounds were assessed at a dose of 5 mg/kg. Fifty minutes after treatment, all groups received, orally, 0.2 ml of a solution containing 0.3 M HCl–60% EtOH (HCl/EtOH) for gastric lesion induction. The antisecretory drug lansoprazole was used as the reference compound. The mice were sacrificed by cervical dislocation 1 h after the administration of HCl/EtOH, and the stomachs were excised and inflated by injection of saline (1 ml). The ulcerated stomachs were fixed in 5% formalin for 30 min and opened along the greater curvature. The length (mm) of each lesion was measured, and the lesion index was expressed as the sum of the length of all lesions.[13] The protocols were approved by the Universidad de Talca Institutional Animal Care and Use Committee, which follows the recommendations of the Canadian Council on Animal Care.[26]

Cytotoxicity assay

Culture of both cell lines was carried out according to Areche *et al*.[13] Confluent cultures of human normal lung fibroblasts MRC-5 (ATCC CCL-171) or human gastric adenocarcinoma AGS cells (ATCC CRL-1739) were treated with medium containing the compounds at concentrations ranging from 0 up to 1000 μM. The substances were firstly dissolved in dimethyl sulfoxide (DMSO) and then in medium. The final concentration of DMSO in the test medium and controls was 1%. Cells were exposed for 24 h to test medium with or without (control) the compound. Each concentration was tested in quadruplicate together with the control and repeated three times in separate experiments. Cell viability was determined at the end of the incubation by means of the neutral red uptake (NRU) assay.[27] The results were transformed to percentage of controls and the IC50 values were graphically obtained from the dose–response curves.

Lipophilicity

The lipophilicity of the compounds was calculated using Chem Office 2002 version 8.0 software. The parameter is presented as log P.

Statistical analysis

Results were expressed as the mean ± SD. In all experiments, statistical differences between several treatments and their respective control were determined by one-way analysis of variance and when the F value was significant, post-hoc differences were determined by Dunnett's multiple comparison test. The level of significance was set at $P < 0.01$. All statistical analyses were performed using the software Statistica 5.1 (StatSoft, Inc.) and Statistical Package S-Plus 2000.

Results

From the aerial parts of *Sphacele chamaedryoides*, 10 abietane diterpenes, the sequiterpene spathulenol and two flavonoids were isolated and characterized by spectroscopic means. The isolation of the plant constituents is summarized in Figure 1 and the structure of compounds **1**–**10**, **12** and **13** is shown in Figure 2. While the diterpenes **1**–**7** and **10** were previously described from other plant sources, this is the first report on the occurrence of compounds **8** and **9** in nature.

The ^{1}H NMR spectrum of compound **8** showed characteristic signals for the isopropyl side chain (methyl d at δ 1.31 and 1.32 and m at δ 3.53 ppm), two methyl singlets at δ 1.05 and 0.95 and an aldehyde proton at 10.01 ppm, indicating that the C-20 methyl group was oxidized to an aldehyde function. The three coupling signals at δ 2.18, 2.76 and 2.95 ppm pointed to a carbonyl function at C-7 and this assumption was supported by the ^{13}C NMR as well as by the IR spectrum. The spectroscopic data of the product were very similar to those reported for the 12-methyl ether derivative isolated from *Salvia coulteri* roots.[28] The compound obtained from *S. chamaedryoides* differed only in the absence of the 12-methyl ether and it is reported for the first time.

The structure of compound **9** follows from the molecular formula $C_{20}H_{26}O_5$ that indicates eight degrees of unsaturation, consistent with a phenolic abietane bearing two carbonyl functions. The ^{1}H NMR spectrum of **9** presents the isopropyl side chain, two sp^3 methyl groups and an aldehyde function, in addition to an aromatic proton signal at δ 6.96. Instead of the three coupling signals for H-5 and H-6 observed in compound **8**, this product showed two singlets at δ 2.92 and 4.82, compatible with a 6-oxo-7-hydroxy function. The compound is related to 6-oxo-7-hydroxycarnosic acid but differs in the presence of an aldehyde function at C-20 instead of the carboxylic acid. The stereochemistry at C-7 was deduced by comparison with the NMR data of the α- and

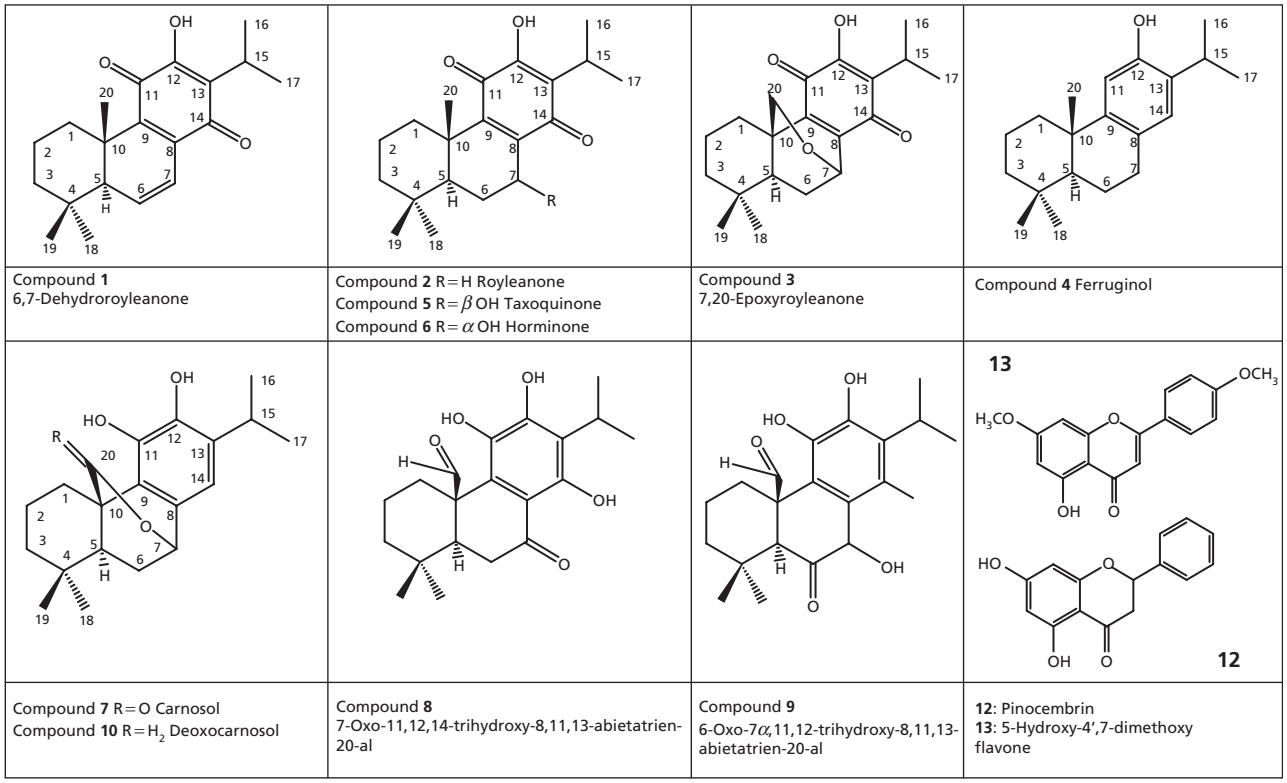

Figure 2 Structure of the main diterpenes from *Sphacele chamaedryoides*

β-derivatives of 6-oxo-7-hydroxycarnosic acid reported from the flowers of *Salvia canariensis*.[29] In this latter report, the H signal for H-7 resonates at δ 5.06 for the β- and at δ 4.89 for the 7-α derivative. Thus, the compound was identified as 6-oxo-7α,11,12-trihydroxy-8,11,13-abietatrien-20-al and is described for the first time in this report.

The spectroscopic data of diterpenes **1**–**7** and **10** are in agreement with those previously reported in the literature.[13,14,23,30–34] The NMR data of the compounds **11**–**13** are in concordance with those reported by Ulubelen *et al*.[35] and Agrawal.[36]

Compound **1**. 6,7-Dehydroroyleanone. Orange powder, mp 150-153°C. HRMS: calculated for $C_{20}H_{26}O_3$: 314.1884, found: 314.1882. EIMS: m/z (rel. int. %): 314 (100), 299 (28), 271 (18), 245 (60), 232 (78), 213 (20), 119 (16), 83 (28), 69 (37). IR ν_{max}: 3363, 2959, 2926, 2868, 1658, 1642, 1628, 1550, 1376, 1254, 1164, 913, 714 cm^{-1}. $[\alpha]_D^{20}$: 60 ($CHCl_3$; c = 0.02).

Compound **2**. Royleanone. Tan amorphous powder. HRMS: calculated for $C_{20}H_{28}O_3$: 316.2025, found: 316.2038. EIMS: m/z (rel. int. %): 316 (100), 301 (20), 249 (25), 231 (20), 219 (21), 205 (20), 161 (23), 149 (12), 119 (13), 91 (10), 83 (11), 69 (40). IR ν_{max}: 3360, 2958, 2927, 2869, 1641, 1631, 1603, 1461, 1377, 1360, 1252, 1162, 1102, 1029, 959, 902 cm^{-1}. $[\alpha]_D^{20}$: +140 ($CHCl_3$; c = 0.01).

Compound **3**. 7,20-Epoxyroyleanone. Tan amorphous powder. HRMS: calculated for $C_{20}H_{26}O_4$: 330.1831, found: 330.1841. EIMS: m/z (rel. int. %): 330 (13), 305 (5), 300 (100), 285 (11), 257 (11), 231 (35), 217 (11), 199 (6), 169 (6), 119 (10), 91 (6), 83 (11), 69 (27). IR ν_{max}: 3373, 2927, 1652, 1634, 1604, 1459, 1396, 1282, 1155, 1124 cm^{-1}. $[\alpha]_D^{20}$: −51 ($CHCl_3$; c = 0.01).

Compound **4**. 12-Hydroxyabieta-8(14),9(11),12-triene (ferruginol). Colourless powder, mp 50–53°C. EIMS: m/z (rel. int. %): 286 (66), 271 (88), 243 (6), 229 (22), 215 (21), 201 (58), 189 (100), 175 (91), 149 (42), 147 (35), 133 (24), 115 (24), 91 (18), 69 (96). IR ν_{max}: 3264, 2962, 1620, 1582, 1511, 1461, 861 cm^{-1}. $[\alpha]_D^{20}$: +38 ($CHCl_3$; c = 0.5).

Compound **5**. Taxoquinone. Orange powder, mp 200–202°C. HRMS: calculated for $C_{20}H_{28}O_4$: 332.1988, found: 332.1972. EIMS: m/z (rel. int. %): 332 (100), 314 (32), 299 (25), 281 (9), 261 (25), 247 (19), 219 (21), 195 (92), 118 (27), 109 (12), 91 (13), 69 (59). IR ν_{max}: 3350, 3362, 2929, 1651, 1626, 1602, 1459, 1394, 1253, 1155, 1061 cm^{-1}. $[\alpha]_D^{20}$: +210 ($CHCl_3$; c = 0.01).

Compound **6**. Horminone. Orange powder, mp 170–172°C. HRMS: calculated for $C_{20}H_{28}O_4$: 332.1988, found: 332.2003. EIMS: m/z (rel. int. %): 332 (100), 314 (67), 299 (41), 271 (11), 261 (27), 245 (19), 210 (18), 195 (70), 123 (28), 109 (7), 91 (9), 69 (10). IR ν_{max}: 3600, 3361, 2957, 2929, 2868, 1649, 1626, 1600, 1455, 1375, 1250, 1154, 1060 cm^{-1}. $[\alpha]_D^{20}$: −136 ($CHCl_3$; c = 0.014).

Compound **7**. Carnosol. Light green powder, mp 240–242°C. HRMS: calculated for $C_{20}H_{26}O_4$: 330.1831, found: 330.1841. IR ν_{max}: 3493, 3287, 2965, 2871, 1711, 1580, 1452, 1348, 1322, 1200, 918 cm^{-1}. $[\alpha]_D^{20}$: −61 (CH_3OH; c = 0.33).

Compound **8**. 7-Oxo-11,12,14-trihydroxy-8,11,13-abietatrien-20-al. Yellow solid, mp 172–175°C. HRMS: calculated

for $C_{20}H_{26}O_5$: 346.1780, found: 346.1782. MS (EI): m/z (rel. int. %): 346 (55), 317 (100), 275 (19), 249 (44), 247 (48), 235 (37), 221 (19). IR ν_{max}: 3410, 2964, 2932, 1688, 1621, 1425, 1290 cm^{-1}. $[\alpha]_D^{20}$: +180 ($CHCl_3$; c = 0.015). ^1H NMR (400 MHz, $CDCl_3$, δ-values, J in Hz): 3.31 brd (13.9) (H-1β); 2.18 dd (15.2; 2.7) (H-5α); 2.76 dd (16.6; 2.7) (H-6α); 2.95 dd (16.6; 15.2) (H-6β); 3.53 dq (7.1, 7.1) (H-15); 1.31 d (7.1) (H-16); 1.32 d (7.1) (H-17); 1.05 s (H-18); 0.95 s (H-19); 10.01 s (H-20); 6.82 s (12-OH); 13.41 s (14-OH). ^{13}C NMR (100 MHz, $CDCl_3$, δ-values, J in Hz): 30.16 t (C-1); 19.51 t (C-2); 40.93 t (C-3); 33.98 s (C-4); 49.35 d (C-5); 34.02 t (C-6); 201.16 s (C-7); 108.59 s (C-8); 119.16 s (C-9); 54.46 s (C-10); 136.31 s (C-11); 153.71 s (C-12); 121.84 s (C-13); 160.36 s (C-14); 24.28 d (C-15); 19.82 q (C-16); 19.92 q (C-17); 31.24 q (C-18); 21.20 q (C-19); 200.51 d (C-20).

Compound **9**. 6-Oxo-7α,11,12-trihydroxy-8,11,13-abietatrien-20-al. Yellow solid, mp 175–178°C. HRMS: calculated for $C_{20}H_{26}O_5$: 346.1789, found: 346.1778. MS (EI): m/z (rel. int. %): 346 (12), 317 (39), 300 (100), 285 (16), 257 (31), 230 (38). IR ν_{max}: 3532, 3386, 2959, 2929, 2858, 1709, 1660, 1634, 1619, 1368, 1256, 1218, 1054 cm^{-1}. $[\alpha]_D^{20}$: + 53 ($CHCl_3$; c = 0.01). ^1H NMR (400 MHz, $CDCl_3$, δ-values, J in Hz): 1.85–2.02 m (H-1α); 3.00 dt (13.5, 2.9) (H-1β); 1.35 m; 1.41 m (H-2); 1.35 m; 1.41 m (H-3); 2.92 br s (H-5α); 4.82 br s (H-7α); 6.96 s (H-14); 3.17 dq (6.9, 6.9) (H-15); 1.22 d (6.9) (H-16); 1.21 d (6.9) (H-17); 1.02 s (H-18); 0.77 s (H-19); 10.11 s (H-20).

Compound **10**. 20-Deoxocarnosol. Yellow solid, mp 180–183°C. HRMS: calculated for $C_{20}H_{28}O_3$: 316.2038, found: 316.2037. MS (EI): m/z (rel. int. %): 316 (52), 286 (100), 271 (16), 219 (26), 215 (32). IR ν_{max}: 3398, 2960, 2932, 1445, 1294 cm^{-1}. $[\alpha]_D^{20}$: 10 ($CHCl_3$; c = 0.01).

Compound **11**. Spathulenol. Light yellow oil. HRMS: calculated for $C_{15}H_{24}O$: 220.3505, found: 220.3505. IR ν_{max}: 3602, 3359, 2956, 1648, 1251 cm^{-1}. $[\alpha]_D^{20}$: +60 ($CHCl_3$; c = 0.01).

Compound **12**. (S)-5,7-dihydroxyflavanone (pinocembrin). Yellow-green powder, mp 195–198°C. ^1H RMN (400 MHz; $CDCl_3$ values of δ H and δ C in ppm, J in Hz): 12.04 s (OH-5), 7.42 brm (5H, 2′-6′); 6.01 brs (2H-6,8); 5.80 brs (OH-7); 5.43 dd (13.0; 2.9) (1H-2); 3.10 dd (17.1; 13.0) (1H-3); 2.84 dd (17.1; 2.9) (1H-3). $[\alpha]_D^{20}$: –31.3 (c = 0,016, MeOH).

Compound **13**. 5-Hydroxy-4′,7-dimethoxyflavone. Yellow powder, mp 167–170°C. ^1H RMN (400 MHz; $CDCl_3$ values of δ H and δ C in ppm, J in Hz): 12.81 s (OH-5), 7.85 d (8.8) (2H-2′,6′); 7.02 d (8.8) (2H-3′,5′); 6.58 s (1H-3); 6.49 d (2.1) (1H-6); 6.37 d (2.1) (1H-8); 3.90 s (3H); 3.89 s (3H).

The effect of the isolated compounds was assessed by oral administration using the HCl/EtOH-induced gastric lesion model in mice. When carnosol was administered at 5, 10 and 20 mg/kg, the reduction of gastric lesions at 5 mg/kg was 49.3% with a protective activity of 63.0 and 69.9% at 10 and 20 mg/kg, respectively. Under the same experimental conditions, the reference compound lansoprazole at 20 mg/kg reduced gastric lesions by 64.5%. The effect was statistically similar to carnosol at the doses of 10 and 20 mg/kg. In our study, using the HCl/EtOH induced gastric ulcer model in mice, the ED50 was close to 5 mg/kg. A comparison of the gastroprotective activity of the eight compounds obtained in amounts > 3 mg was undertaken at 5 mg/kg. The results are presented in Table 1. Of the group of compounds **1**–**3**, **5** and **6**, presenting a *p*-quinone moiety, the best gastroprotective effect was elicited by royleanone (**2**) (71.0%), lacking functionality at C-7. The activity of 6,7-dehydroroyleanone (**1**), 7,20-epoxyroyleanone (**3**) and horminone (**6**) was similar, preventing gastric lesions in the range of 52.7–65.0%. Of the C-7 hydroxy derivatives, the activity was much lower for the 7β-OH isomer (**5**). At 5 mg/kg the phenolic diterpenes **7** and **8** inhibited gastric lesions by 49.3 and 53.0%, respectively, being more active than lansoprazole. The most active plant constituents identified in this work were royleanone (**2**) and its 7,20-epoxide (**3**), which reduced gastric lesions by 70.8 and 65.0%, respectively (Table 1).

The compounds isolated from *S. chamaedryoides* were assessed for cytotoxicity against human normal lung fibroblasts (MRC-5) and gastric adenocarcinoma (AGS) cells. The results are presented in Table 2. Among the studied compounds, the flavonoids **13** (IC50 = 503 and 720 μM) and **12** (IC50 = 215 and 369 μM) as well as carnosol (**7**) (IC50 = 186 and 309 μM) were the least toxic products against AGS cells and fibroblasts, respectively. Compounds **1** (IC50 = 61 and 366 μM) and **5** (IC50 = 310 and 27 μM) showed low and selective effect against AGS cells and fibroblasts, respectively. However, compounds **2**, **3**, **6**, **8** and spathulenol proved to be quite cytotoxic with IC50 values in the range of 11–67 μM. According to our results, most of the *S. chamaedryoides* diterpenes were more cytotoxic than carnosol and lansoprazole. The reference compound presented an IC50 value of 306 μM for fibroblasts and 162 μM for AGS cells, respectively.

Table 1 Dose–response effect of the diterpene carnosol (**7**) and gastroprotective activity of compounds **1**–**3**, **5**, **6** and **8** in HCl/EtOH-induced gastric ulcers in mice

Treatment	*n*	Lesion index (mm)	Gastroprotective effect (%)
Carnosol 20 mg/kg	9	10.5 ± 1.7*	69.9
Carnosol 10 mg/kg	9	12.9 ± 1.3*	63.0
Carnosol 5 mg/kg	9	17.7 ± 1.4*	49.3
6,7-Dehydroroyleanone (**1**)	9	15.9 ± 1.7*	54.4
Royleanone (**2**)	8	10.2 ± 1.0*	70.8
7,20-Epoxyroyleanone (**3**)	9	12.2 ± 1.3*	65.0
Taxoquinone (**5**)	9	22.4 ± 1.9*,†	35.8
Horminone (**6**)	9	16.5 ± 1.8*	52.7
7-Oxo-11,12,14-trihydroxy-8,11,13-abietatrien-20-al (**8**)	9	16.4 ± 1.3*	53.0
Lansoprazole	9	12.4 ± 1.1*	64.5
Control	8	34.9 ± 1.8	n/a

Compounds **1**–**3**, **5**, **6** and **8** were administered at a dose of 5 mg/kg and lansoprazole at 20 mg/kg. *n*, animal number; n/a, not applicable. Results are presented as mean values ± SEM. *$P < 0.01$ compared with control group and †$P > 0.05$ compared with lansoprazole (analysis of variance followed by Dunnett's test).

Table 2 Lipophilicity and cytotoxicity of compounds **1**–**3**, **5**–**8**, **12**, **13** and spathulenol towards AGS cells and human lung fibroblasts

Compound	Lipophilicity (log P)	Cytotoxicity IC50 (μM)	
		AGS	Fibroblasts
6,7-Dehydroroyleanone (**1**)	2.51	61 ± 3	366 ± 26
Royleanone (**2**)	2.83	59 ± 4	18 ± 1
7,20-Epoxyroyleanone (**3**)	1.31	57 ± 5	67 ± 4
Taxoquinone (**5**)	1.74	310 ± 21	27 ± 1
Horminone (**6**)	1.74	12 ± 1	11 ± 1
Carnosol (**7**)	4.58	186 ± 13	309 ± 19
7-Oxo-11,12,14-trihydroxy-8,11,13-abietatrien-20-al (**8**)	3.13	43 ± 2	45 ± 3
Spathulenol (**11**)	3.01	23 ± 2	32 ± 3
Pinocembrin (**12**)	2.02	215 ± 13	369 ± 26
5-Hydroxy-4′,7-dimethoxyflavone (**13**)	2.43	503 ± 25	720 ± 43
Lansoprazole	n.d.	162 ± 10	306 ± 21
Etoposide	n.d.	0.36 ± 0.01	3.9 ± 0.1

Confluent cultures of human normal lung fibroblasts MRC-5 or human gastric adenocarcinoma AGS cells were treated with medium containing the compounds at concentrations ranging from 0 up to 1000 μM. n.d., not determined. Data are expressed as the mean of three different experiments in quadruplicate ± SD.

Discussion

Carnosol was the main diterpene constituent isolated from the aerial parts of *S. chamaedryoides*. The abietane diterpenes present in the plant are structurally related to the main product. Wenkert *et al.*[37] suggested the cascade oxidation of carnosic acid to *ortho*-benzoquinones, semiquinones and carnosol. Masuda *et al.*[38] reported the oxidation of carnosic acid to two *o*- and *p*-quinone derivatives. The closely related 20-deoxocarnosol (**10**) was also present in the plant as well as the abietane derivatives 7-oxo-11,12,14-trihydroxy-8,11,13-abietatrien-20-al (**8**) and 6-oxo-7α,11,12-trihydroxy-8,11,13-abietatrien-20-al (**9**), both described for the first time. The quinone diterpenes isolated comprise 6,7-dehydroroyleanone (**1**), royleanone (**2**), 7,20-epoxyroyleanone (**3**), taxoquinone (**5**) and horminone (**6**). Ferruginol (**4**) was also present in the diterpene fraction of the plant. The sesquiterpene spathulenol (**11**) and the known flavonoids pinocembrin (**12**) and 5-hydroxy-4′,7-dimethoxyflavone (**13**) were also isolated from the plant. Both flavonoids are widespread in the Lamiaceae.[39]

The single oral dose of 5 mg/kg at which compounds **1**–**8** were assessed should be regarded as a very low dose compared with other studies on the gastroprotective effect of natural products and semisynthetic derivatives. Only the diterpene polygodial presented higher activity in the animal models of gastric lesions in rodents.[12,15,40–45]

Several biological actions have been reported for the diterpene carnosol, including anti-inflammatory,[46] antitumour,[17] antiproliferative,[47] antioxidant,[48] antimicrobial[49] and antiplatelet activity,[50] and it is reported to be an activator of the peroxisome proliferator-activated gamma receptor[51] and have a hepatoprotective effect.[52] Miyazaki *et al.*[53] reported the gastroprotective effect of carnosol, with an ED50 of 9.6 mg/kg in the gastric lesion model of absolute ethanol in rats. A recent report on the gastroprotective effect of the hydroalcoholic extract of sage (*Salvia officinalis* L.) pointed to carnosol as a possible active constituent of the plant. The authors suggested that the gastroprotective effect of the sage extract could be related to the antioxidant activity displayed by both the extract and carnosol.[54] The quinone-type diterpenes did not present antioxidant activity because they are considered to be termination oxidative products from phenolic diterpenoids on the basis of their stability against radicals. This fact suggests a mechanism other than the antioxidant effect for the gastroprotective effect of the obtained quinone compounds.[19]

These results relate well with those reported for *p*-quinonic diterpenes of the royleanone type, which proved to be highly cytotoxic against the nasopharynx carcinoma KB and P-388 leukaemia lines in previous reports.[31,55] Interestingly, compounds **1** and carnosol (**7**) were more cytotoxic against gastric tumour cells AGS than normal fibroblasts. However, the variation seen may be due to the different tissue types rather than because one cell line is from a tumour while the other is normal. No clear relationship between cytotoxicity and lipophilicity could be observed.

The diterpenes royleanone, horminone and acetyl horminone isolated from *Salvia officinalis* were assessed for cytotoxic effect and DNA damage in human liver and colon cancer cells *in vitro*.[56] Horminone has been reported to inhibit protein synthesis in bacteria,[57] as well as to be hepatotoxic in rats.[58] The antiproliferative activity of abietane diterpenes isolated from *Plectranthus grandidentatus* (Lamiaceae) has been described.[59] The abietanes from *P. grandidentatus*, including royleanone derivatives, presented different effects in the selected cancer cell lines.[60]

Conclusions

Our results confirm the trend in the diterpene chemistry of the genus *Sphacele*, since the main abietanes isolated from the aerial parts of *S. salviae* were carnosol, rosmanol, carnosic acid and 20-deoxocarnosol. In our study, mainly carnosol and *p*-quinone derivatives were isolated but not carnosic acid, suggesting that the isolated products can be derived from the metabolism of carnosic acid. Two diterpenes (**8** and **9**) are described for the first time. All the abietanes obtained from *S. chamaedryoides* present either one or two phenolic OH groups, a quinone system, or both. Several compounds present in the plant showed higher gastroprotective effect than lansoprazole. The cytotoxic effect of most compounds was found at fairly high concentrations and lacked cell specificity. Further studies are required using different tumour cell lines and viability/proliferation assays to assess the specificity of the isolated compounds. Additional work should be undertaken to look for other constituents present in different populations of *S. chamaedryoides* growing under different environmental/stress conditions and in different phenological stages.

Declarations

Conflict of interest

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

Acknowledgements and funding

This work was supported by FONDECYT No. 1060841 and Programa de Investigación en Productos Bioactivos, Universidad de Talca. C. Areche thanks the Universidad de Talca for a Doctoral grant.

References

1. WHO. *The world health report: shaping the future*. Switzerland, 2003: 154–159.
2. Luo J *et al*. The risk of pancreatic cancer in patients with gastric or duodenal ulcer disease. *Int J Cancer* 2007; 120: 368–372.
3. Bahmanyar S *et al*. Long-term risk of gastric cancer by subsite in operated and unoperated patients hospitalized for peptic ulcer. *Am J Gastroenterol* 2007; 102: 1185–1191.
4. Departamento de Estadísticas e Información de Salud, Ministerio de Salud, Gobierno de Chile. 2006; www.minsal.cl (accessed September 2009). http://deis.minsal.cl/deis/salidas06/causas06.asp?temp=TODAS_EDADES.htm
5. Brunton L. Agents for control of gastric acidity and treatment of peptic ulcers. In: Hardman J *et al.,* eds. *The Pharmacological Basis of Therapeutics.* New York: McGraw Hill, 1996: 901–916.
6. Lewis DA, Hanson D. Anti-ulcer drugs of plant origin. In: Ellis GP, West GB, eds. *Progress in Medicinal Chemistry*. Amsterdam: Elsevier Science, 1991: 201–231.
7. San Feliciano A *et al*. Abietane acids: sources, biological activities, and therapeutic uses. *Planta Med* 1993; 59: 485–490.
8. Gaspar C *et al*. Effect of abietane diterpenes from *Plectranthus grandidentatus* on the growth of human cancer cell lines. *Planta Med* 2002; 68: 839–840.
9. Kinoshita M *et al*. Effect of a combination of ecabet sodium and cimetidine on experimentally induced gastric lesions and gastric mucosal resistance to ulcerogenic agents in rats. *Biol Pharm Bull* 1995; 18: 223–226.
10. Kinoshita M *et al*. Roles of prostaglandins, nitric oxide and the capsaicin sensitive sensory nerves in gastroprotection produced by ecabet sodium. *J Pharmacol Exp Ther* 1995; 275: 494–501.
11. Ichikawa T *et al*. Effects of ecabet sodium, a novel gastroprotective agent, on mucin metabolism in rat gastric mucosa. *Dig Dis Sci* 2000; 45: 606–613.
12. Sepúlveda B *et al*. Gastroprotective and cytotoxic effect of dehydroabietic acid derivatives. *Pharmacol Res* 2005; 2: 429–437.
13. Areche C *et al*. Gastroprotective and cytotoxic effect of semisynthetic ferruginol derivatives. *J Pharm Pharmacol* 2007; 59: 289–300.
14. Areche C *et al*. Gastroprotective activity of ferruginol in mice and rats: effects on gastric secretion, endogenous prostaglandins and non-protein sulfhydryls. *J Pharm Pharmacol* 2008; 60: 245–251.
15. Rodríguez JA *et al*. Gastroprotective and ulcer healing effect of ferruginol in mice and rats: assessment of its mechanism of action using in vitro models. *Life Sci* 2006; 78: 2503–2509.
16. Espinoza M *et al*. New gastroprotective ferruginol derivatives with selective cytotoxicity against gastric cancer cells. *Planta Med* 2008; 74: 802–808.
17. Guerrero IC *et al*. Abietane diterpenoids from *Salvia pachyphylla* and *S. clevelandii* with cytotoxic activity against human cancer cell lines. *J Nat Prod* 2006; 69: 1803–1805.
18. Li F *et al*. Cytotoxic effects and pro-apoptotic mechanism of TBIDOM, a novel dehydroabietylamine derivative, on human hepatocellular carcinoma SMMC-7721 cells. *J Pharm Pharmacol* 2008; 60: 205–211.
19. Escuder B *et al*. Antioxidant capacity of abietanes from *Sphacele salviae*. *Nat Prod Lett* 2002; 16: 277–281.
20. Hoffmann A. *Flora Silvestre de Chile, Zona Central*, 4th edn. Santiago de Chile: Fundación Claudio Gay, 1998.
21. Riedemann P, Aldunate G. *Flora nativa de valor ornamental; identificación y propagación. Chile, Zona Centro,* 2nd edn. Santiago de Chile: Editorial Andrés Bello, 2004.
22. Valenzuela L *et al*. The essential oil of *Sphacele chamaedryoides*. *Planta Med* 1992; 58: 273–274.
23. Pastene E *et al*. Application of HPTLC to the analysis of horminone in *Sphacele chamaedryoides* (Balbis) Briq. *J Planar Chromatogr* 2005; 18: 221–223.
24. Ito Y. Golden rules and pitfalls in selecting optimum conditions for high-speed counter-current chromatography. *J Chromatogr A* 2005; 1065: 145–168.
25. Hostettmann K *et al*. *Preparative chromatography techniques. Applications in natural product isolation.* Berlin, Heidelberg: Springer-Verlag, 1998.
26. Olfert ED *et al. Guide to the care and use of experimental animals*. Ottawa, Ontario: Canadian Council on Animal Care, 1993.
27. Rodríguez JA, Haun M. Cytotoxicity of *trans*-dehydrocrotonin from *Croton cajucara* (Euphorbiaceae) on V79 cells and hepatocytes. *Planta Med* 1999; 65: 522–526.
28. Frontana B *et al*. Diterpenoids from *Salvia coulteri*. *Phytochemistry* 1994; 36: 739–741.
29. González AG *et al*. Diterpenes from the flowers of *Salvia canariensis*. *Phytochemistry* 1987; 26: 1471–1474.
30. Rodríguez B. Spectral assignments and reference data. ^{1}H and ^{13}C NMR spectral assignments of some natural abietane diterpenoids. *Magn Reson Chem* 2003; 41: 741–746.
31. Jonathan L *et al*. 7-O-Methylhorminone and other cytotoxic diterpene quinones from *Lepechinia bullata*. *J Nat Prod* 1989; 52: 571–575.
32. Ulubelen *et al*. Abietane diterpenes from *Salvia napifolia*. *Phytochemistry* 1995; 40: 861–864.
33. Amaro-Luis JM. Abietane diterpenoids from *Salvia rubescens* ssp. *truxillensis*. *Pharm Acta Helv* 1997; 72: 233–238.
34. Kelekom A. An abietane diterpene from the Labiate *Coleus barbatus*. *Phytochemistry* 1984; 23: 1677–1679.
35. Ulubelen *et al*. Terpenoids from *Salvia sclarea*. *Phytochemistry* 1994; 36: 971–974.
36. Agrawal PK. *Carbon-13 NMR of Flavonoids.* Studies in Organic Chemistry, 39. Elsevier, Amsterdam, 1989.
37. Wenkert E *et al*. Chemical artifacts from the family Labiatae. *J Org Chem* 1965; 30: 2931–2934.
38. Masuda T *et al*. Recovery mechanism of the antioxidant activity from carnosic acid quinone, an oxidized sage and rosemary antioxidant. *J Agric Food Chem* 2002; 50: 5863–5869.
39. *Dictionary of Natural Products*. CD-ROM. London: Chapman & Hall/CRC, 2009.
40. Albino de Almeida AB *et al*. Antiulcerogenic effect and cytotoxic activity of semi-synthetic crotonin obtained from *Croton cajucara* Benth. *Eur J Pharmacol* 2003; 472: 205–212.
41. Arrieta J *et al*. Purification of gastroprotective triterpenoids from the stem bark of *Amphipterygyum adstringens*; role of prostaglandins, sulfhydryls, nitric oxide and capsaicin-sensitive neurons. *Planta Med* 2003; 69: 905–909.

42. Hiruma-Lima CA *et al*. Antiulcerogenic mechanisms of dehydrocrotonin, a diterpene lactone obtained from *Croton cajucara*. *Planta Med* 1999; 65: 325–330.
43. Matsuda H *et al*. Protective effects of polygodial and related compounds on ethanol-induced gastric mucosal lesions in rat: structural requirements and mode of action. *Bioorg Med Chem Lett* 2002; 12: 477–482.
44. Schmeda-Hirschmann G *et al*. Gastroprotective effect and cytotoxicity of natural and semisynthetic labdane diterpenes from *Araucaria araucana* resin. *Z Naturforsch C* 2005; 60: 511–522.
45. Silva Melo P *et al*. Comparation of the gastroprotective effect of a diterpene lactone isolated from *Croton cajucara* with its synthetic derivatives. *J Ethnopharmacol* 2003; 87: 169–174.
46. Poeckel D *et al*. Carnosic acid and carnosol potently inhibit human 5-lipoxygenase and suppress pro-inflammatory responses of stimulated human polymorphonuclear leukocytes. *Biochem Pharmacol* 2008; 76: 91–97.
47. Visanji JM *et al*. Induction of G2/M phase cell cycle arrest by carnosol and carnosic acid is associated with alteration of cyclin A and cyclin B1 levels. *Cancer Lett* 2006; 237: 130–136.
48. Wijeratne SS, Cuppett SL. Potential of rosemary (*Rosmarinus officinalis* L.) diterpenes in preventing lipid hydroperoxide-mediated oxidative stress in Caco-2 cells. *J Agric Food Chem* 2007; 55: 1193–1199.
49. Horiuchi K *et al*. Potentiation of antimicrobial activity of aminoglycosides by carnosol from *Salvia officinalis*. *Biol Pharm Bull* 2007; 30: 287–290.
50. Lee JJ *et al*. Antiplatelet activity of carnosol is mediated by the inhibition of TXA2 receptor and cytosolic calcium mobilization. *Vascul Pharmacol* 2006; 45: 148–153.
51. Rau O *et al*. Carnosic acid and carnosol, phenolic diterpene compounds of the labiate herbs rosemary and sage, are activators of the human peroxisome proliferator-activated receptor gamma. *Planta Med* 2006; 72: 881–887.
52. Sotelo-Félix JI *et al*. Protective effect of carnosol on CCl_4-induced acute liver damage in rats. *Eur J Gastroenterol Hepatol* 2002; 14: 1001–1006.
53. Miyazaki T *et al*. Method of treating ulcers. United States Patent assigned to Nagase & Company, Ltd., Osaka (JP). Patent N° US 6,638,523 B1. Priority: JP 11-305383. Application: 09/696,183. 2003.
54. Mayer B *et al*. Gastroprotective constituents of *Salvia officinalis* L. *Fitoterapia* 2009; 80: 421–426.
55. Kupchan SM *et al*. Taxodione and taxodone, two novel diterpenoid quinone methide tumor inhibitors from *Taxodium distichum*. *J Am Chem Soc* 1968; 9: 5923–5924.
56. Slamenová D *et al*. Cytotoxic and DNA-damaging effects of diterpenoid quinones from the roots of *Salvia officinalis* L. on colonic and hepatic human cells cultured in vitro. *Pharmacol Toxicol* 2004; 94: 282–290.
57. Nicolás I, Castro M. Theoretical study of the complexes of horminone with Mg^{2+} and Ca^{2+} ions and their relation with the bacteriostatic activity. *J Phys Chem A* 2006; 110: 4564–4573.
58. Ferreira R *et al*. Effects of horminone on liver mixed function of mono-oxygenases and glutathione enzyme activities of Wistar rat. *J Ethnopharmacol* 1998; 58: 21–30.
59. Cerqueira F *et al*. Effect of abietane diterpenes from *Plectranthus grandidentatus* on T-and B-lymphocyte proliferation. *Bioorg Med Chem* 2004; 12: 217–223.
60. Marques CG *et al*. Effect of abietane diterpenes from *Plectranthus grandidentatus* on the growth of human cancer cell lines. *Planta Med* 2002; 68: 839–840.