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Free radical scavengers, anti-inflammatory and analgesic activity of *Acaena magellanica*

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Abstract

Extracts of the whole plant *Acaena magellanica* (Rosaceae) were assessed for anti-inflammatory, antipyretic and analgesic activity in animal models. At 600 mg kg^{-1}, the global ethanolic extract (GEE), dichloromethane (DCM) and defatted methanol (MeOH) fractions showed a mild anti-inflammatory effect in the carrageenan-induced guinea-pig paw oedema. The GEE, DCM and defatted MeOH fractions significantly reduced inflammation by 43.2, 40.5 and 42.1 %, respectively. The GEE did not showed any significant antipyretic activity in doses up to 600 mg kg^{-1}. A 20 % w/v infusion administered orally at 16 mL kg^{-1} presented analgesic effect in the acetic acid-induced abdominal constriction test in mice. The GEE and MeOH extract of *A. magellanica* showed free radical scavenging activity in the diphenylpicrylhydrazyl decolouration assay. Assay-guided isolation led to quercetin, Q-3-*O*-β-D-glucoside, Q-3-*O*-β-D-galactoside, ellagic acid and catechin as the free radical scavengers. The saponins tormentic acid 28-*O*-β-D-galactopyranoside and 28-*O*-β-D-glucopyranoside were isolated from the polar extract. The structures were determined by spectroscopic methods.

Introduction

The Province of San Juan in Argentina has an important tradition in folk medicine. The flora comprises a large number of species distributed in different ecosystems, characterized by particular edaphic and climatic conditions. Plants of the high Cordillera de Los Andes have been used as medicines since pre-hispanic times and are still used for their reputed therapeutic properties. *Acaena magellanica* (Lam.) Vahl (Rosaceae), known as ''cadillo'', is widely distributed in Argentina and Chile from sea level to 4000 m above sea level (Grondona 1964; Kiesling 1994). The infusion of the whole plant is taken orally as a cholagogue, as a sedative and for wound healing (Ratera & Ratera 1980; Bustos et al 1996).

The analgesic and anti-inflammatory activities of an infusion of *A. magellanica* are reported here. The global ethanolic extract (GEE), hexane, dichloromethane (DCM) and methanol (MeOH) extracts were assessed for anti-inflammatory effects. The GEE was also evaluated for antipyretic activity. The GEE and defatted MeOH extract of *A. magellanica* displayed a strong free radical scavenging activity in the diphenylpicrylhydrazyl (DPPH) radical decolouration assay. We report the fractionation of extracts of *A. magellanica* and an investigation into their relative ability to scavenge the free radical DPPH.

Material and Methods

Plant material

A. magellanica was collected during the blossoming period in the Bauchazeta district, San Juan Province, Argentina, in December 1999. The plant was identified by Dr L. Ariza Espinar, INVIB-CONICET, Universidad Nacional de Córdoba, Argentina. Voucher specimens are deposited at INVIB-CONICET (BT 17) and at the herbarium of the Escuela de Química y Farmacia, Universidad de Chile, Santiago de Chile (SQF 21010).

Extracts

Extracts were prepared from the air-dried ground aerial parts. The GEE was prepared by extracting, under reflux, 300 g of the plant with ethanol (3 × 1500 mL, for 30 min each time). After concentration under reduced pressure, the GEE (28.6 g, 9.5% w/w yield in terms of dry starting material) was used for toxicity, anti-inflammatory, antipyretic and free radical scavenging studies.

A second plant sample from the same batch (1800 g) was successively extracted at room temperature (3 × 96 h) with hexane, DCM and MeOH. After filtration, each extract was evaporated to dryness to give semi-solid residues that constituted the hexane (22.68 g), DCM (12.78 g) and defatted MeOH (150 g) extracts. The w/w yields in terms of dry starting material were: 1.26, 0.71 and 8.3% for the hexane, DCM and defatted MeOH extracts, respectively. A 20% (w/v) infusion was prepared by adding boiling water to a sample. The infusion was used for the anti-inflammatory and analgesic activity assays.

A third plant sample (1000 g) was successively extracted at room temperature with MeOH:H_2O 9:1 (2.5 L, 24 h) and MeOH:H_2O 1:1 (2 L, 24 h). The methanol was removed under reduced pressure and the aqueous phase was successively extracted with diethyl ether (Et_2O; 5 × 100 mL) and ethyl acetate (EtOAc; 7 × 100 mL) affording 4.6 g of EtOAc solubles.

Isolation

As the most active extracts in the DPPH decolouration assay were the defatted MeOH and GEE, the MeOH extract was worked-up to isolate the free radical scavengers. The MeOH extract (150 g) was resuspended in water and partitioned with Et_2O and EtOAc to yield an Et_2O (18 g) and an EtOAc (4.17 g) extract. The remaining aqueous phase was lyophilized (127 g). The Et_2O extract (18 g) was treated with MeOH to afford a MeOH-soluble (16 g) fraction and a MeOH-insoluble yellow precipitate (0.23 g). Preparative thin-layer chromatography (TLC) of the MeOH-insoluble fraction (70 mg) (acetic acid:H_2O, 1:1; cellulose) afforded isorhamnetin **1** (15 mg) and quercetin **2** (33 mg), identified by NMR and UV spectra, and TLC comparison with standards. The EtOAc extract was treated with MeOH to afford a MeOH-soluble and a MeOH-insoluble fraction. The MeOH-soluble fraction from the EtOAc extract (569.3 mg) was applied onto a Sephadex LH-20 column (length 35.5 cm, 2.5 cm i.d.; equilibrated to MeOH). Some 45 fractions (10 mL each) were obtained. After TLC comparison (silica gel; EtOAc:formic acid:glacial acetic acid:water, 10:1:1:3, as mobile phase; detection under UV light and after spraying with diphenyl boric acid, ethanolamine complex in methanol) according to Wagner et al (1984), fractions with similar TLC patterns were combined affording the saponin **7** (36 mg; fractions 6–8), quercetin 3-*O*-*β*-D-galactoside **3** (9 mg, fraction 22) and quercetin 3-*O*-*β*-D-glucoside **4** (28 mg, fraction 23). From the MeOH-insoluble part of the EtOAc extract, 378 mg of ellagic acid **5** was obtained as colourless needles and identified by micro-melting point, NMR, UV, MS and co-chromatography with a standard sample. Some 2.5 g from the 4.6 g EtOAc-solubles from the third plant sample were applied onto a Sephadex LH-20 column (length 40 cm, 4 cm i.d., MeOH). Some 17 fractions (75 mL each) were collected. Fraction 2 contained a nearly pure saponin mixture (800 mg) of compounds **7** and **8**. Fraction 11 yielded 680 mg catechin **6**. The spectroscopic data of the compounds **1–6** are in agreement with those reported by Mabry et al (1970), Agrawal (1989) and The Merck Index (1989). ^{13}C NMR data of compounds **1–4** and **6** are presented in Table 1. The saponins **7** and **8** were identified by NMR spectroscopy and comparison with literature data (Jia et al 1993; Ahmad & Rahman 1994). After acid hydrolysis, both compounds yielded the triterpene tormentic acid and a sugar, identified by co-chromatography with a standard sample as galactose and glucose, respectively. The ^{13}C NMR data for both compounds confirmed the identity of the sugars and is in agreement with the published data (Jia et al 1993). ^{1}H and ^{13}C NMR data of compound **7** and ^{13}C NMR data of compound **8** are presented in Table 2. The structures of the isolated compounds are presented in Figure 1.

Isorhamnetin ***1***
Yellow solid; UV (MeOH): 250, 270 sh, 305 sh, 370. ^{1}H NMR (200 MHz, DMSO-d_6, δ ppm): 12.6 br s (Ph-OH); 7.92 br s (H-2′); 7.60 brd (8) (H-6′); 7.00 d (8) (H-5′); 6.35 d (1.9) (H-8); 6.50 d (1.9) (H-6); 3.91 s (OMe).

Quercetin ***2***
Yellow solid; UV (MeOH): 255, 270 sh, 300 sh, 372. ^{1}H

Table 1 ^{13}C NMR data of compounds **1–4** and **6** (50 MHz, MeOH-d_4, δ ppm).

C	Compound 1	Compound 2	Compound 3	Compound 4	Compound 6
2	156.28 s	156.66 s	156.48 s	156.56 s	80.89 d
3	132.99 s	133.53 s	133.77 s	133.81 s	66.27 d
4	177.18 s	177.51 s	177.67 s	177.65 s	25.38 t
5	161.07 s	161.39 s	161.44 s	161.42 s	144.69 s
6	98.79 d	99.01 d	99.02 d	98.86 d	114.23 d
7	164.64 s	164.76 s	164.70 s	164.21 s	144.00 s
8	93.93 d	93.86 d	93.80 d	93.82 d	107.93 d
9	156.42 s	156.74 s	156.59 s	156.56 s	144.76 s
10	103.93 s	104.01 s	104.06 s	104.20 s	111.85 s
1′	120.97 s	121.79 s	122.15 s	121.47 s	130.67 s
2′	113.31 d	115.48 d	115.44 d	115.43 d	117.97 d
3′	149.40 s	145.00 s	145.06 s	144.83 s	142.88 s
4′	146.85 s	148.71 s	148.74 s	148.76 s	132.68 s
5′	115.19 d	116.47 d	116.23 d	116.53 d	115.01 d
6′	122.22 d	121.34 d	121.33 d	121.44 d	118.43 d
OMe	55.64 q	–	–	–	–
Sugar			Galactose	Glucose	
1	–	–	102.23 d	101.42 d	–
2	–	–	71.48 d	74.33 d	–
3	–	–	73.48 d	76.78 d	–
4	–	–	68.18 d	70.32 d	–
5	–	–	76.04 d	77.49 d	–
6	–	–	60.38 t	61.32 d	–

Compounds: **1**, isorhamnetin; **2**, quercetin; **3**, quercetin 3-*O*-β-D-galactoside; **4**, quercetin 3-*O*-β-D-glucoside; **6**, catechin.

NMR (200 MHz, DMSO-d_6, δ ppm): 12.60 br s (Ph-OH); 7.52 br s (H-2′); 7.54 br d (8.9) (H-6′); 6.84 d (8.9) (H-5′); 6.38 d (1.9) (H-8); 6.18 d (1.9) (H-6).

Quercetin-3-O-β-D-*galactoside* ***3***

Yellow solid. UV spectra: MeOH: 257, 298 sh, 357; MeOH/$AlCl_3$: 272, 300 sh, 438; $AlCl_3$/HCl: 266, 300 sh, 401. MeOH/NaOMe: 267, 325, 405; MeOH/NaOAc: 232 sh, 271, 320, 393; NaOAc/H_3BO_3: 262, 300, 380. Acid hydrolysis yielded quercetin and galactose, identified by co-chromatography with a standard sample. ^1H NMR (200 MHz, DMSO-d_6, δ ppm): 7.53 d (2.1) (H-2′); 7.44 dd (8.4; 2.1) (H-6′); 6.84 d (8.4) (H-5′); 6.35 d (1.9) (H-8); 6.20 d (1.9) (H-6). Sugar: 4.91 d (7.7); 3.81 d (2.8); 3.78 dd (7.9); 3.60 m; 3.45–3.55 m; 3.38 t (5.9).

Quercetin-3-O-β-D-*glucoside* ***4***

Yellow solid. UV spectra: MeOH: 258, 300 sh, 358; MeOH/$AlCl_3$: 275, 300 sh, 438; $AlCl_3$/HCl: 267, 300 sh, 401. MeOH/NaOMe: 267, 327, 407; MeOH/NaOAc: 232 sh, 272, 322, 390; NaOAc/H_3BO_3: 262, 300, 382. Acid hydrolysis yielded quercetin and glucose, identified by co-chromatography with a standard sample. ^1H NMR (200 MHz, DMSO-d_6, δ ppm): 7.53 d (2.1) (H-2′); 7.45 dd (8.4; 2.1) (H-6′); 6.83 d (8.4) (H-5′); 6.36 d (1.9) (H-8); 6.22 d (1.9) (H-6). Sugar: 4.95 d (7.6); 3.80 m; 3.80 dd (7.9); 3.60 m; 3.45–3.55 m; 3.40 t (6).

Ellagic acid ***5***

Colourless crystals, mp: 368°C (dec); EI-MS: 302.19 (calc. for $C_{14}H_6O_8$: 302.19). UV spectra: MeOH: 368, 308 sh, 255, 207; MeOH/$AlCl_3$: 389, 323 sh, 270, 250, 209; $AlCl_3$/HCl: 368, 304 sh, 255, 205. EtOH: 366, 257; EtOH/$AlCl_3$: 388, 319 sh, 272, 249; $AlCl_3$/HCl: 369, 258. IR (KBr, cm^{-1}): 3094 (OH), 1699 (C═O) (s), 1619, 1583, 1511, 1448, 1397, 1338, 1195, 1112 (s), 1056 (s), 923, 883, 813, 759. ^1H NMR (200 MHz, DMSO-d_6): 7.57 s. Treatment with acetic anhydride/pyridine afforded the corresponding acetate. Acetate: ^1H NMR (200 MHz, $CDCl_3$): 8.08 s, 2.47 s (3H), 2.40 s (3H).

Catechin (3′,4′,5,7-tetrahydroxyflavan-3-ol) ***6***

$[\alpha]_D^{20°}$: −18.58 (c = 0.99; MeOH) ^1H NMR (200 MHz, MeOH-d_4, δ ppm): 4.74 d (H-2); 3.98 m (H-3); 2.81 dd (H-4); 2.66 dd (H-4a); 6.38 s br (2H; H-6 and H-8); 6.82 d (H-2′); 6.78 d (H-5′); 6.68 dd (H-6′). J (Hz: 2,3 = 6.6; 4,4a = 15.6; 4,3 = 4.8; 4a,3 = 8.5; 2′, 6′ = 1.7; 5′, 6′ = 8.1.

Table 2 ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) data of compound **7** (in MeOH-d_4) and ^{13}C NMR data of compound **8** (pyridine-d_5).

H	Compound 7	C	Compound 7	Compound 8
1	1.94 dd (12.4; 4.4); 0.88 m	1	46.72 t	48.02 t
2	3.62 m	2	68.04 d	68.66 d
3	2.88 d (9.6)	3	83.04 d	84.03 d
4	–	4	38.99 s	38.73 s
5	0.84 m	5	55.20 d	56.05 d
6	1.43 m; 1.50 m	6	18.19 t	19.12 t
7	1.34 m; 1.54 m	7	32.57 t	33.66 t
8	–	8	39.78 s	40.68 s
9		9	47.88 d	48.74 d
10	–	10	37.69 s	39.60 s
11	1.98 dd (10.4;3)	11	23.28 t	24.30 t
12	5.28 br s	12	128.02 d	128.29 d
13		13	138.18 s	139.48 s
14	–	14	41.19 s	42.65 s
15		15	28.13 t	29.66 t
16		16	25.71 t	26.82 t
17	–	17	47.98 s	48.66 s
18	2.49 s	18	53.45 d	53.98 d
19	–	19	72.14 s	73.00 s
20	1.35 m	20	41.43 d	42.23 d
21	2.60 ddd (12;12;6.7)	21	25.01 t	27.30 t
22	1.62 dd (12.7; 3.8); 1.82 m	22	36.80 t	37.87 t
23*	0.80 s	23	16.14 q	29.26 q
24*	1.00 s*	24	15.96 q	17.52 q
25	0.77 s	25	15.65 q	17.70 q
26	1.00 s*	26	27.81 q	17.07 q
27	1.32 s	27	23.18 q	24.66 q
28	–	28	177.03 s	177.02 s
29	1.19 s	29	25.57 q	27.01 q
30	0.93 d (6.7)	30	15.10 q	16.84 q
Sugar			Galactopyranose	Glucopyranose
1	5.29 d (8.1)	1	94.27 d	95.92 d
2	3.3–3.4 m	2	72.36 d	74.10 d
3		3	76.80 d	79.00 d
4		4	69.63 d	71.26 d
5		5	77.07 d	79.33 d
6	3.77 dd (12;2.2) 3.65 dd (12;4.7)	6	60.94 t	62.66 t

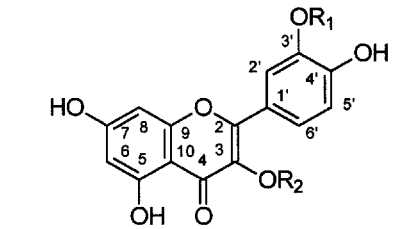
*May be interchangeable.

*2α,3β,19α-Trihydroxyurs-12-en-28-*O*-β-D-galactopyranoside* **7** *(tormentic acid 28-*O*-β-D-galactopyranoside): white amorphous powder*
Acid hydrolysis afforded tormentic acid and galactose, identified by co-chromatography with a standard sample. The ^{1}H and ^{13}C NMR data are in agreement with the literature (Jia et al 1993; Ahmad & Rahman 1994).

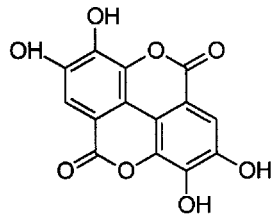
2α,3β,19α-trihydroxyurs-12-en-28-*O*-β-D-glucopyranoside **8** (tormentic acid 28-*O*-β-D-glucopyranoside): white amorphous powder. Acid hydrolysis afforded tormentic acid and glucose, identified by co-chromatography with a standard sample. The ^{1}H and ^{13}C NMR data are in agreement with the literature (Jia et al 1993; Ahmad & Rahman 1994).

β-Sitosterol ***9***
EI-MS: $C_{29}H_{50}O$ m/z 414.3792 (M^+) calc. for $C_{29}H_{50}O$: 414.3792 (52), 396 (35) (M-H_2O), 381 (21), 329 (22), 303 (32), 273 (20), 255 (39), 218 (100), 203 (40), 189 (22), 81 (72), 69 (61), 55 (80).

Oleanolic acid (3β-hydroxyolean-12-en-28-oic acid) ***10***
Colourless crystals, mp 196–198°C; $[\alpha]^{20}$: 35.8 (c = 0.33, $CHCl_3$).

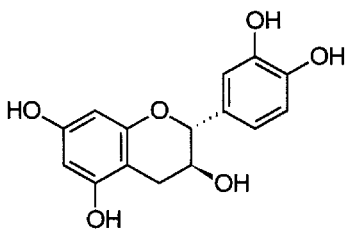


Compound	R_1	R_2
1	CH_3	H
2	H	H
3	H	β-D-galactose
4	H	β-D-glucose



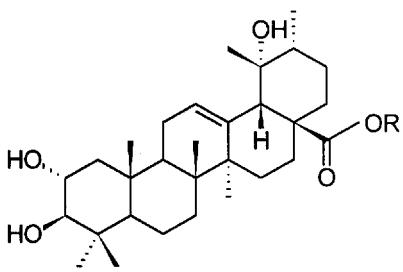
5

Ellagic acid

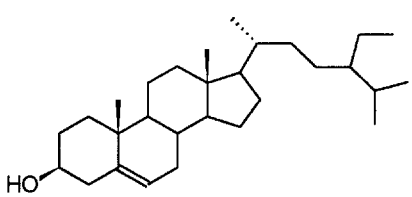


6

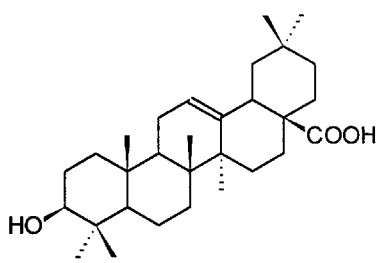
Catechin



	Compound	R
	Tormentic acid (TA)	H
7	TA β-D-galactopyranoside	β-D-galactose
8	TA β-D-glucoside	β-D-glucose



9 β-Sitosterol



10 Oleanolic acid

Figure 1 Structure of phenolics, steroids, triterpenes and saponins from *Acaena magellanica*.

Bioassays

Animals

Pirbright guinea-pigs (220–300 g) of both sexes were used for the anti-inflammatory assay. Adult female New Zealand rabbits were used for the antipyretic study and CF-1 mice of both sexes were selected for analgesic and acute toxicity studies. The animals were kept under standard housing conditions at the Animal Maintenance Unit of the Instituto de Salud Pública, Santiago de Chile (ISP) and fasted overnight before experiments. Experiments were carried out in accordance with internationally accepted principles and the protocols were approved by the ISP. The number of animals used was determined on the basis of statistical power calculations as reported in the methodology by Backhouse et al (1994), Davis et al (1997) and Delporte et al (1998). The dose level was set at 600 mg kg^{-1} according to previous work on Chilean medicinal plants (Backhouse et al 1996).

Acute toxicity

Male and female CF-1 mice (21–24 g; n = 10), kept under standard laboratory conditions and allowed free access to water, received a single oral dose of 8 g kg^{-1} GEE suspended in propylene glycol by gavage. Animals

were observed and weighed daily for a week. The 50% lethal dose (LD50) was calculated using the Morgan scoring method (Morgan 1992).

For the anti-inflammatory, antipyretic and analgesic assays, the GEE, hexane, DCM, MeOH extracts were suspended in propylene glycol or arabic gum and administered orally 1 h before the carrageenan, endotoxin injection or acetic acid by means of an intragastric catheter. Sodium naproxen (Laboratorios Saval, Chile) was used as a reference drug and was suspended in the same vehicle. λ-Carrageenan was purchased from Sigma Chemical Company, MO. *Escherichia coli* endotoxin was obtained from the Instituto de Salud Pública, Santiago de Chile.

Anti-inflammatory assay

The assay was carried out using groups of 13 animals according to the carrageenan-induced paw oedema method reported by Winter et al (1963). Extracts were suspended in propylene glycol and administered orally by gavage at a dose of 600 mg kg^{-1}, 1 h before the λ-carrageenan injection (0.1 mL sterile saline λ-carrageenan (1%) suspension). Paw volume was measured with an Ugo Basile plethysmometer (model 7150) immediately and 3 h after injecting λ-carrageenan suspension. Sodium naproxen (4 mg kg^{-1}) suspended in the same vehicle was used as a positive control (Backhouse et al 1994). Percentage anti-inflammatory activity was evaluated by the following formula:

$$\%\ \text{Anti-inflammatory activity} = ((\%\ \text{Ic} - \%\ \text{Id})/\%\ \text{Ic}) \times 100$$

where % Ic is the mean inflammation in control guinea-pigs (37.7±1.3% paw oedema increase for a group of 96 animals) and % Id is the average inflammation in drug-treated animals. The significance of the drug-induced changes was estimated using the Wilcoxon test for independent data (Hollander & Wolfe 1973). The effect was considered significant at a value of $P < 0.05$.

Antipyretic activity

The antipyretic activity of GEE at 600 mg kg^{-1} was determined using three animals and repeating each experiment three times to obtain nine results. GEE suspended in propylene glycol was orally administered 1 h before injecting the pyrogen. Pyrexia was produced by intravenous injection of *E. coli* endotoxin at 13 ng kg^{-1} into the ear 1 h after administering the samples. Temperature vs time curves were obtained previously for each animal. Rectal temperature was recorded with an Ellab pyrogen tester from the time of injection of the pyrogen to 180 min afterwards. The observation period was divided into two time intervals (0–90 min and 90–180 min), since active principles with fast absorption and elimination could be present in the sample under study, showing an effect only in the first 90 min of the assay, however compounds could have a slow absorption and the effect would only be seen after 90–180 min. Sodium naproxen (25 mg kg^{-1}) suspended in the same vehicle was used as a positive control (Delporte et al 1998). The mean area under the temperature–time curves for each animal was obtained with pyrogen alone (AUC_{pyr}) and with pyrogen plus the samples ($AUC_{pyr+extract}$). Areas were compared and averaged for all rabbits. The areas under the curves were calculated by means of a specially designed computer program. The significance of the effect was calculated using the Wilcoxon test. The antipyretic effect was calculated according to the following equation:

$$\%\ \text{Antipyretic effect} = (1 - AUC_{pyr+extract}/AUC_{pyr}) \times 100$$

Analgesic activity

The analgesic activity was evaluated by a single oral dose of the infusion at 16 mL kg^{-1}. Infusion-treated animals (n = 8), animals treated with sodium naproxen (n = 8) and control animals (n = 14) received an intraperitoneal injection of 0.5 mL 0.6% acetic acid. The analgesic effect was calculated as the percentage inhibition of abdominal constrictions compared with a control group receiving only the vehicle (Davis et al 1997). Sodium naproxen (12.5 mg kg^{-1}) was used as a reference drug according to previous work (Delporte et al 2002). The mean pain (D) was calculated according to the following equation:

$$\%\ D = (C_{sample}/C_{control}) \times 100$$

where C_{sample} is the mean number of abdominal constrictions in sample-treated animals and $C_{control}$ is the mean number of abdominal constrictions in control animals that received only the vehicle. The analgesic effect was calculated according to the following equation:

$$\text{Analgesic effect}\ (\%) = 100 - \%\ D$$

DPPH decolouration assay

The quenching of free radicals by extracts and compounds was evaluated spectrophotometrically at 517 nm against the absorbance of the DPPH radical (Aldrich). The free radical scavenging effect of crude extracts and compounds was assessed by the decolouration of a methanolic solution of DPPH according to Astudillo et al (2000) and Viturro et al (1999). A freshly prepared

DPPH solution (20 mg L^{-1}) was used for the assays. Samples were dissolved in methanol and the methanolic solution of DPPH served as a control. The degree of decolouration indicates the free radical scavenging efficiency of the substances. Catechin was used as a reference free radical scavenger. The percentage of DPPH decolouration was calculated as follows:

Decolouration (%) = 1 − ((Absorbance with compound − absorbance of blank sample)/Absorbance of control) × 100

Extracts and isolated compounds were assessed at 100, 50, 10 and 1 μg mL^{-1}. Values are presented as mean ± s.d. of three determinations.

HPLC analysis of phenolics

HPLC analysis was performed using Merck-Hitachi equipment consisting of a L-6200 pump, L-4000 UV detector and D-2500 chromato-integrator. Screening of flavonoids and phenolic acids in the sample was performed by the methodology of Häkkinen et al (1998) using a Lichrospher RP 18 column (250 mm × 4 mm). The solvent system used to assess the presence of gallic acid, catechin, ellagic acid and quercetin was as follows: solvent A: 50 mM ammonium dihydrogen phosphate, pH 2.6; solvent B: 0.20 mM *o*-phosphoric acid, pH 2.0; solvent C: 20% solvent A in 80% acetonitrile. The gradient elution programme was as described by Häkkinen et al (1998). Under these conditions, gallic acid, catechin, chlorogenic acid, ellagic acid, benzoic acid, quercetin, rutin and kaempferol eluted with retention times of 15.43; 42.72; 44.64; 55.50; 56.43; 62.08; 54.80 and 63.52 min, respectively.

The total phenolic content of the plant was determined by the Folin-Ciocalteau technique using a calibration curve with tannic acid (Scalbert et al 1989; Heldrich 1990). The total flavonoid content was estimated by the aluminium chloride method reported by Bahorun et al (1994). Ellagic acid, quercetin and its 3-*O*-galactoside content were determined by HPLC using a calibration curve with reference compounds.

Results and Discussion

Extracts of *A. magellanica* were assessed for anti-inflammatory, antipyretic and analgesic activity in animal models. The GEE showed no toxic effects up to 8 g kg^{-1}. No mortality or side-effects were observed.

At 600 mg kg^{-1}, the GEE showed a mild anti-inflammatory effect in the carrageenan-induced guinea-pig paw oedema, reducing inflammation by 43.2% (P = 0.0013). Serial extraction of the crude drug showed an increase in the bioactivity ranging from 18.8% (P = 0.1190) in the hexane extract to 40.5% (P = 0.0037) in the DCM extract and 42.1% (P = 0.0014) in the MeOH extract. Under the same experimental conditions, the anti-inflammatory effect of sodium naproxen was 54.6% at the dose of 4 mg kg^{-1}.

The GEE did not showed any significant antipyretic activity at 600 mg kg^{-1}. The infusion presented an analgesic effect in the acetic acid-induced abdominal constriction test in mice. The mean number of abdominal constrictions in drug-treated groups was significantly lower ($P < 0.0004$) than the respective control groups. The results are summarized in Table 3.

The GEE showed a strong free radical scavenging effect in the DPPH decolouration assay. Serial extraction of the crude drug clearly indicates that the activity is present mainly in the MeOH fraction. The defatted MeOH extract was fractionated with Et_2O and EtOAc to obtain the MeOH-soluble and MeOH-insoluble portions of the Et_2O and EtOAc solubles. The results of the assay are presented in Table 4. A preliminary phytochemical screening of the GEE, according to the methodology reported by Wagner et al (1984), indicated the occurrence of steroids, triterpenoids and flavonoids. Assay-guided isolation of the DPPH-active extracts led to the identification of flavonoids and phenolics as active components of the *A. magellanica* extracts. Compounds were isolated by permeation on Sephadex LH-20, column chromatography on silica gel and preparative TLC. The compounds were identified by NMR, UV, IR, micro-melting point and co-chromatography with reference samples.

On a dry weight basis, the total phenolic content of the crude drug *A. magellanica* was 5.30%, with 3.91% tannins and 0.057% flavonoids. The MeOH extract and fractions were also analysed by HPLC to determine the pattern of the phenolic compounds (Häkkinen et al 1998). The ellagic acid, quercetin and quercetin-3-*O*-galactoside contents in the MeOH extract as determined by HPLC were 0.18, 0.19 and 0.03% in terms of dry starting material. The main phenolic constituent of the MeOH extract was ellagic acid. The flavonoids and catechin occurring in the crude drug can be related with the free radical scavenging effect observed in the extracts.

After column chromatography of the hexane extract (10 g) on silica gel with a hexane: EtOAc gradient, 150 mg of β-sitosterol **9** was obtained. The DCM extract (10 g), under similar chromatographic conditions, afforded 1.2 g oleanolic acid **10**. Both compounds were identified by ^{1}H and ^{13}C NMR, MS and co-chromatography with reference samples.

Table 3 Percentage anti-inflammatory and analgesic activity of extracts and an infusion of *Acaena magellanica*, and sodium naproxen.

Sample	Dose (mg kg^{-1})	Anti-inflammatory activity (%)	*P*	Analgesic activity (%)	*P*
Global ethanolic extract	600	43.2±3.3	0.0013	NA	
Hexane extract	600	18.8±4.6	0.1190 (NS)	NA	
Dichloromethane extract	600	40.5±5.0	0.0037	NA	
MeOH extract	600	42.1±5.2	0.0014	NA	
20% Infusion	16 mL kg^{-1}	5.0±3.6	0.352	47.9±2.4	0.0004
Sodium naproxen	4	54.6±3.4		NA	
Sodium naproxen	12.5	NA		70.0±4.3	0.0069

Results are expressed as the mean±s.e.m., n = 13 for the anti-inflammatory assays and n = 8 for the analgesic assays. NA, not assayed; NS, not significant.

Table 4 Free radical scavenging activity of *Acaena magellanica* extracts and phenolics.

Sample	% Yield[a]	DPPH decolouration (μg mL^{-1})			
		100	50	10	1
Global ethanolic extract	9.5	98±2	48±2	4±2	–
Hexane extract	1.26	14±4	0	0	0
Dichloromethane extract	0.71	17±4	0	0	0
MeOH extract	8.33	93±4	89±4	49±4	ND
Et_2O, MeOH-soluble	0.89	85±5	73±5	76±5	76±5
Et_2O, MeOH-insoluble	0.09	92±3	92±3	88±3	76±3
EtOAc	0.23	92±4	90±4	72±4	70±4
EtOAc, MeOH-soluble	0.21	87±4	90±4	86±4	52±4
EtOAc, MeOH-insoluble	0.01	91±4	91±3	82±3	78±4
Aqueous	7.1	75±5	89±4	36±4	45±4
Quercetin	0.19	100±5	ND	94±3	18±3
Quercetin-3-*O*-glucoside	0.03	45±3	ND	30±4	0
Quercetin-3-*O*-galactoside	0.01	78±3	72±3	67±3	59±3
Ellagic acid	0.18	91±3	91±3	82±4	78±3
Catechin	0.10	79±3	ND	67±3	52±4

DPPH, diphenylpicrylhydrazyl; Et_2O, diethyl ether extract; EtOAc, ethyl acetate extract. [a]In terms of dry starting material. ND, not determined.

The infusion of *A. magellanica* is taken orally as a cholagogue, sedative and to heal wounds. The compounds isolated from the unpolar hexane and DCM extracts are triterpenes and steroids with known anti-inflammatory effects. The pharmacology of oleanolic acid has been revised by Liu (1995). Oleanolic acid is effective against chemically induced liver injury and has anti-inflammatory and antihyperlipidaemic properties in laboratory animals and antitumour-promotion effects. Oleanolic acid and its glycosides display hepatoprotective action. Kinjo et al (1999) studied the protective effects of oleanolic acid saponins and derivatives on in-vitro immunological liver injury of primary cultured rat hepatocytes. Oleanolic acid showed both hepatoprotective action and weak hepatotoxicity, while the effect of the saponins represented a balance between hepatoprotective action and hepatotoxicity. The protective effects of oleanolic acid against carbon tetrachloride-induced hepatotoxicity may, at least in part, be owing to its ability to block bioactivation of carbon tetrachloride mainly by the inhibition of expression and activities of P_{450} 2E1 (Jeong 1999). Oleanolic acid displays anti-inflammatory, antipyretic and analgesic activity, but does not have an ulcerogenic effect, suggesting that prostaglandins are not involved in its mechanism of action (Singh et al 1992). β-Sitosterol is also active as an anti-inflammatory and antipyretic (Gupta et al 1980).

The saponins **7** and **8** were isolated as white amorphous solids. On the basis of the spectroscopic information, the main saponin from *A. magellanica* is 2α,3β,19α-trihydroxyurs-12-en-28-*O*-β-D-galactopyranoside **7** (tormentic acid 28-*O*-β-D-galactopyranoside), while **8** is the corresponding 28-*O*-β-D-glucopyranoside (Shiojima et al 1992; Jia et al 1993; Ahmad & Rahman 1994).

Some of the biological activities displayed by extracts of *A. magellanica* can be related to constituents isolated in the present study. The DPPH radical scavenging activity of *A. magellanica* crude extracts, fractions and active compounds is presented in Table 4.

Catechin is also an inhibitor of cyclooxygenase 1 and 2 catalysed prostaglandin biosynthesis (Noreen et al 1997). Prostaglandins are important mediators in the inflammatory process, and the inhibition of cyclooxygenase is involved in the mechanism of action of many anti-inflammatory drugs, although the in-vitro effects may not have a correlation in-vivo. In this assay, the IC50 of catechin was 80 μM.

Barch et al (1996) studied the structure–function relationships of ellagic acid as a dietary anticarcinogen and found a variety of activities against known cancer promoters. The inhibition of arylamine *N*-acetyltransferase activity in *Helicobacter pylori* by ellagic acid has been reported by Chung (1998), suggesting its potential as a possible chemopreventive drug. Ellagic acid exhibits both antimutagenic and anticarcinogenic activity in a wide range of assays in-vitro and in-vivo. Loarca-Pina et al (1998) showed that ellagic acid inhibits aflatoxin B1 direct-acting mutagenicity. Further research on the antimutagenic properties of ellagic acid was reported by Kaur et al (1997) working on *Terminalia arjuna*. Ellagic acid was an effective inhibitor of oesophageal tumourigenesis in F344 rat oesophagus, inhibiting cytochrome P_{450}-mediated activation of NMBA (Stoner & Morse 1997). Other activities of ellagic acid include inhibition of carbon tetrachloride-induced toxicity and liver fibrosis in rats (Thresiamma & Kuttan 1996). Catechins and quercetin are peroxyl and hydroxyl free radical scavengers and present protective effect against lipid peroxidation (Zhang et al 1997).

The genus *Acaena* is placed in the section Sanguisorbeae (Poterieae) of the Rosoideae. According to Hegnauer (1990), hydrolysable gallic and ellagitannins seem to be common in the Rosoideae with biologically active tannins reported from *Agrimonia japonica* and *Potentilla kleiniana*. Triterpenes and saponins have been reported in the Rosaceae, including tormentoside, the 28-*O*-β-D-glucopyranoside of tormentic acid. Tormentoside seems to be the most common pseudosaponin in the Rosaceae (Hegnauer 1990). Tormentic acid 28-*O*-β-D-galactopyranoside has been previously reported as a constituent of the Rosaceae *Sanguisorba alpina* (Jia et al 1993). The triterpene tormentic acid has been previously reported from the Rosaceae *Rosa roxburghii* Tratt, *Geum japonicum* Thunberg, *Rosa multiflora* and *Potentilla tormentilla* Neck (Ahmad & Rahman 1994). Triterpenes, including tormentic acid are constituents of *Acaena pinnatifida* (Valcic et al 1997) and *Geum japonicum* (Xu et al 1996).

Tormentic acid, the aglycone of the saponins **7** and **8** showed a strong hypoglycaemic effect (Villar et al 1986; Ivorra et al 1988). According to Numata et al (1989), tormentic acid itself showed no cytotoxic activity, but its derivative 3-*O*-*cis*-*p*-coumaroyltormentic acid was one of the active constituents of the Chinese medicine Goreishi. Tannins and polyphenols can also reduce the absorption of glucose in the gastrointestinal tract (Miwa et al 1986).

The flavonoid quercetin, its 3-*O*-glycosides, ellagic acid and catechin were isolated and identified as the main free radical scavengers of *A. magellanica* methanolic extract. HPLC analysis showed ellagic acid as the main phenolic constituent in all DPPH active extracts and fractions. Ellagic acid and catechin are widespread in the plant kingdom and their phenolic nature make them powerful antioxidants.

The mashed aerial parts of *Acaena* species are used in poultices to heal wounds and in decoction to treat ulcers. The biological activity observed in the present study and the occurrence of bioactive compounds in *A. magellanica* support its use as a medicinal plant in San Juan folk medicine. Furthermore, the occurrence of tormentic acid derivatives and phenolics suggests potential as a hypoglycaemia crude drug. The closely related *Acaena splendens* has been shown to display anti-inflammatory and antipyretic activity (Backhouse et al 1997). Glycosidic steroids and triterpenes have been reported from *A. splendens*.

References

Ahmad, V. U., Rahman, A. U. (1994) *Handbook of natural products data*. Vol. 2. *Pentacyclic triterpenoids*. Elsevier, Amsterdam

Agrawal, P. K. (1989) *Carbon-13 NMR of flavonoids. Studies in organic chemistry 39*. Elsevier, Amsterdam

Astudillo, L., Schmeda-Hirschmann, G., Herrera, J. P., Cortés, M. (2000) Proximate composition and biological activity of Chilean *Prosopis* species. *J. Sci. Food Agric.* **80**: 567–573

Backhouse, N., Delporte, C., Negrete, R., Muñoz, O., Ruiz, R. (1994) Antiinflamatory and antipyretic activities of *Maytenus boaria*. *Int. J. Pharmacogn.* **32**: 239–244

Backhouse, N., Delporte, C., Negrete, R., Salinas, P., Pinto, A., Aravena, S., Cassels, B. K. (1996) Antiinflammatory and antipyretic

activities of *Cuscuta chilensis*, *Cestrum parqui* and *Psoralea glandulosa*. *Int. J. Pharmacogn.* **34**: 53–57

Backhouse, N., Delporte, C., Negrete, R., Suárez, S., Cassels, B. K., Breitmaier, E., Schneider, C. (1997) Antiinflammatory and antipyretic metabolites of *Acaena splendens*. *Int. J. Pharmacogn.* **35**: 1–6

Bahorun, T., Trotin, F., Pommery, J., Vasseur, J., Pinkas, M. (1994) Antioxidant activities of *Crataegus monogyna* extracts. *Planta Med.* **60**: 323–328

Barch, D. H., Rundhaugen, L. M., Stoner, G. D., Pillay, N. S., Rosche, W. A. (1996) Structure–function relationships of the dietary anticarcinogen ellagic acid. *Carcinogenesis* **17**: 265–269

Bustos, D., Tapia, A., Feresin, G., Ariza-E., L. (1996) Ethnopharmacobotanical survey of Bauchazeta district, San Juan Province, Argentina. *Fitoterapia* **67**: 411–415

Chung, J. G. (1998) Inhibitory actions of ellagic acid on growth and arylamine N-acetyltransferase activity in strains of *Helicobacter pylori* from peptic ulcer patients. *Microbios* **93**: 115–127

Davis, N. M., Roseth, A. G., Appleyard, C. B., Mcknight, W., del Soldado, P., Calignano, A., Cirino, G., Wallace, J. L. (1997) Nonaproxen vs. naproxen: ulcerogenic, analgesic and antiinflammatory effects. *Aliment. Pharm. Ther.* **11**: 69–79

Delporte, C., Backhouse, N., Negrete, R., Salinas, P., Cassels, B. K., San Feliciano, A. (1998) Antipyretic, hypothermic and antiinflammatory activities and metabolites from *Solanum ligustrinum* Lood. *Phytother. Res.* **12**: 1–5

Delporte, C., Muñoz, O., Rojas, J., Ferrándiz, M., Payá, M., Erazo, S., Negrete, R., Maldonado, S., San Feliciano, A., Backhouse, N. (2002) Pharmaco-toxicological study of *Kageneckia oblonga* (Rosaceae). *Z. Naturforsch.* (In press)

Grondona, E. (1964) Las especies Argentinas del género *Acaena*. *Darwiniana* **13**: 208–342

Gupta, M. B., Nath, R., Srivastava, N., Shanker, K., Kishor, K., Bhargava, K. P. (1980) Anti-inflammatory and antipyretic activities of β-sitosterol. *Planta Med.* **39**: 157–163

Häkkinen, S. H., Kärenlampi, S. O., Heinonen, I. M., Mykkänen, H. M., Törrönen, A. R. (1998) HPLC method for screening of flavonoids and phenolic acids in berries. *J. Sci. Food Agric.* **77**: 543–551

Hegnauer, R. (1990) *Chemotaxonomie der Pflanzen*. Band 9. Birkhäuser Verlag, Basel, pp 369–405

Heldrich, K. (ed.) (1990) *Official methods of analysis of the association of official analytical chemists*. VA 50th edn, Vol. 2. Association of Official Chemists, Arlington, p. 703

Hollander, M., Wolfe, D. A. (1973) *Nonparametric statistical methods*. John Wiley and Sons, New York

Ivorra, M. D., Paya, M., Villar, A. (1988) Hypoglycemic and insulin release effects of tormentic acid: a new hypoglycemic natural product. *Planta Med.* **54**: 282–285

Jeong, H. G. (1999) Inhibition of cytochrome P450 2E1 expression by oleanolic acid: hepatoprotective effects against carbon tetrachloride-induced hepatic injury. *Toxicol. Lett.* **105**: 215–222

Jia, Z.-J., Liu, X.-Q., Liu, Z.-M. (1993) Triterpenoids from *Sanguisorba alpina*. *Phytochemistry* **32**: 155–159

Kaur, S., Grover, I. S., Kumar, S. (1997) Antimutagenic potential of ellagic acid isolated from *Terminalia arjuna*. *Indian J. Exp. Biol.* **35**: 478–482

Kiesling, R. (1994) In: Mazzini, V. (ed.) *Flora de San Juan, Republica Argentina*. Vol. I. Buenos Aires

Kinjo, J., Okawa, M., Udayama, M., Sohno, Y., Hirakawa, T., Shii, Y., Nohara, T. (1999) Hepatoprotective and hepatotoxic actions of oleanolic acid-type triterpenoidal glucuronides on rat primary hepatocyte cultures. *Chem. Pharm. Bull.* (*Tokyo*) **47**: 290–292

Liu, J. (1995) Pharmacology of oleanolic acid and ursolic acid. *J. Ethnopharmacol.* **49**: 57–68

Loarca-Pina, G., Kuzmicky, P. A., de Mejia, E. G., Kado, N. Y. (1998) Inhibitory effects of ellagic acid on the direct-acting mutagenicity of aflatoxin B1 in the *Salmonella* microsuspension assay. *Mutat. Res.* **398**: 183–187

Mabry, T. J., Markham, K. R., Thomas, M. B. (1970) *The systematic identification of flavonoids*. Springer Verlag, New York

Miwa, I., Horie, T., Nakayama, M. (1986) Inhibition of intestinal α-glucosidases and sugar absorption by flavones. *Chem. Pharm. Bull.* (*Tokyo*) **34**: 838–844

Morgan, B. (1992) *Analysis of quantal response data*. Chapman and Hall, London, pp 46–50

Noreen, Y., Ringbom, T., Perera, P., Bohlin, L. (1997) Further studies of the inhibitory activities on the COX-1 and COX-2 catalyzed prostaglandin biosynthesis by (+) catechin. *J. Nat. Prod.* **60**: 2–7

Numata, A., Yang, P., Takahashi, C., Fujiki, R., Nabae, M., Fujita, E. (1989) Cytotoxic triterpenes from a Chinese medicine, Goreishi. *Chem. Pharm. Bull.* (*Tokyo*) **37**: 648–651

Ratera, E., Ratera., N. (1980) *Plantas de la flora Argentina empleadas en medicina popular*. Hemisferio Sur Argentina

Scalbert, A., Monties, B., Janin, G. (1989) Tannins in wood: comparison of different estimation methods. *J. Agric. Food Chem.* **37**: 1324–1329

Shiojima, K., Arai, Y., Masuda, K., Takase, Y., Ageta, T., Ageta, H. (1992) Mass spectra of pentacyclic triterpenoids. *Chem. Pharm. Bull.* (*Tokyo*) **40**: 1683–1690

Singh, G. B., Singh, S., Bani, S., Gupta, B. D., Banerjee, S. K. (1992) Anti-inflammatory activity of oleanolic acid in rats and mice. *J. Pharm. Pharmacol.* **44**: 456–458

Stoner, G. D., Morse, M. A. (1997) Isothiocyanates and plant polyphenols as inhibitors of lung and esophageal cancer. *Cancer Lett.* **19**: 113–119

The Merck Index (1989) 11th edn. Merck & Co., NJ, p 555

Thresiamma, K. C., Kuttan, R. (1996) Inhibition of liver fibrosis by ellagic acid. *Indian J. Physiol. Pharmacol.* **40**: 363–366

Valcic, S., Wachter, G. A., Montenegro, G., Timmermann, B. N. (1997) Triterpenoids from *Acaena pinnatifida* R. et P. *Z. Naturforsch. [C]* **52**: 264–266

Villar, A., Paya, M., Hortiguela, M. D., Cortes, D. (1986) Tormentic acid, a new hypoglycemic agent from *Poterium ancistroides*. *Planta Med.* **52**: 43–45

Viturro, C., Molina, A., Schmeda-Hirschmann, G. (1999) Free radical scavengers from *Mutisia friesiana* (Asteraceae) and *Sanicula graveolens* (Apiaceae). *Phytother. Res.* **13**: 422–424

Wagner, H., Bladt, S., Zgainski, E. (1984) *Plant Drug Analysis*. Springer Verlag, Berlin

Winter, C. A., Risley, E. A., Nuss, G. W. (1963) Anti-inflammatory and antipyretic activities of indomethacin, 1-(*p*-chlorobenzoyl)-5-methoxy-2-methyl-indole-3-acetic acid. *J. Pharmacol. Exp. Ther.* **141**: 369–373

Xu, H. X., Zeng, F. Q., Wan, M., Sim, K. Y. (1996) Anti-HIV triterpene acids from *Geum japonicum*. *J. Nat. Prod.* **59**: 643–645

Zhang, Z., Inserra, P. F., Watson, R. R. (1997) Antioxidants and AIDS. In: Garewal, H. S. (ed.) *Antioxidants and disease prevention*. CRC Press, pp 31–43