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Bioactive Phenolic Derivatives from *Acaena splendens* Methanol Extract

N. Backhouse,[1]* C. Delporte,[1] R. Negrete,[1] S. A. San Feliciano[2] and J. L. López-Pérez[2]

[1]Pharmacognosy Laboratory, Faculty of Chemical and Pharmaceutical Sciences, University of Chile, P.O. Box 233, Santiago 1, Chile
[2]Department of Organic and Pharmaceutical Chemistry, Faculty of Pharmacy, University of Salamanca, 37007 Salamanca, Spain

***Acaena splendens* H. *et* A. has been used in Chilean folk medicine for the treatment of fever and inflammation. A description of the *in vivo* reduction of bacterial pyrogen-induced fever in rabbits and carrageenan-induced paw oedema in guinea pigs is presented. The methanol extract named ME-1, obtained after succesive extractions with petroleum ether and dichloromethane, showed a strong antipyretic action (45.7% of effect), though the antiinflammatory activity was only observed after submitting this extract to column fractionation, giving a crude mixture of flavonoids named C4 with both activities (55.7% and 98.9% of antiinflammatory and antipyretic effect respectively at a dose of 600 mg/kg). The bioassay-guided fractionation by column chromatography afforded the active fraction, which contained (-,-)-epicatechin, tiliroside, 7-O-acetyl-3-O-β-D-glucosyl-kaempferol and 7-β-D-glucosyloxy-5-hydroxy-chromone.**

Keywords: Acaena splendens; antiinflammatory; antipyretic activities; flavonoids; chromone.

INTRODUCTION

The genus *Acaena*, family Rosaceae, is represented by 150 species, of which 125 grow in America (Navas, 1976), and about 20 are native to Chile (Marticorena and Quezada, 1985). *Acaena splendens* H. *et* A., vernacular name in Spanish 'amores secos', grows in the lower Andes Mountains from Río Tora (IV Region-north) to Coihaique (XI Region-south) (Navas, 1976). Folk medicine uses infusions of the whole plant for the treatment of gout and rheumatism, fever and inflammation and as a diuretic in kidney complaints (Muñoz *et al.*, 1981; San Martìn, 1983).

Continuing with the chemical and pharmacological studies on *A. splendens*, initiated by Backhouse *et al.* (1997), and in the hope of discovering bioactive molecules, we attempted to isolate other bioactive molecules from the methanol extract (ME-1) obtained after successive extractions with petroleum ether and dichloromethane. The acute toxicity studies of the crude methanol extract (MEC) in mice, revealed no toxic effects at the doses tested (suspension of 9.2, 11.1 and 12.6 g/kg in saline arabic gum), and no side effects were recorded during the observation period (Backhouse *et al.*, 1997). The antipyretic and antiinflammatory properties of the infusion (INF), crude methanol (MEC), petroleum ether (PEE) and dichloromethane (DME) extracts of the whole plant had been already reported (Backhouse *et al.*, 1997), where the INF, the MEC and the DME extracts showed marked antipyretic and antiinflammatory activities. These previous results made us believe that we could find different types of metabolites responsible for the observed effects. As steroids and triterpenes had been already isolated from the non polar extracts, we attempted to isolate those possible polar active substances, which could explain the use of this plant in folk medicine.

* Correspondence to: Dr N. Backhouse, Pharmacognosy Laboratory, Faculty of Chemical and Pharmaceutical Sciences, University of Chile, PO Box 233, Santiago 1, Chile.
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MATERIALS AND METHODS

General experimental procedures. Melting points are uncorrected and were determined on a Kofler hot stage. IR spectra (KBr disc) were recorded using a Perkin-Elmer 1310 infrared spectrometer. NMR spectra in $CDCl_3$, DMSO-d_6 and CD_3OD with TMS as internal standard were recorded at 200/50, 300/75 and 400/100 MHz ($^1H/^{13}C$) on Bruker 200, 300 and 400 spectrometers, using commercially available pulse programmes for DEPT (Distortionless Enhanced Polarization Transfer) and 2D spectra. The monitoring of the fractions was done by TLC (silica gel GF_{254}, $CHCl_3$/MeOH = 9/1 v/v), spots were detected under UV (254 and 366 nm) and using NH_3 vapours together with heating the plates at 110 °C after spraying with Liebermann-Burchard reagent.

Plant material. *Acaena splendens* H. *et* A. was collected in Lagunillas at 2000 m altitude, Cajón del Maipo, SE of Santiago (34° S Lat.), Chile, in early summer (December). A voucher specimen is on deposit in the Herbarium of our Faculty (SQF: 16642).

Extraction and isolation. The air-dried ground plant material (whole plant) (4 kg) was extracted successively, at room temperature, with petroleum ether (60°–80 °C), CH_2Cl_2 and methanol, yielding respectively 85.9 g, 80.4 g and 360.0 g of crude extracts in respect of dry powdered material (termed PEE, DME and ME-1) after removing

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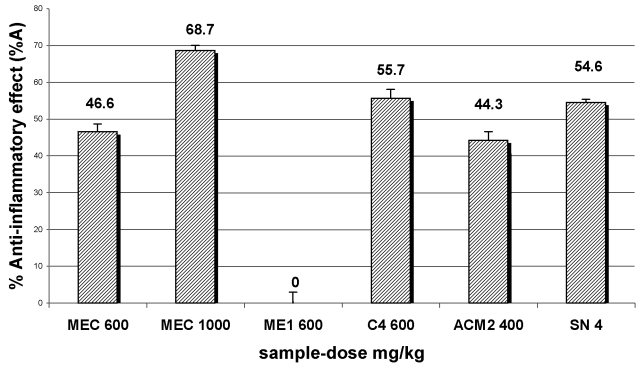


Figure 1. Comparative antiinflammatory effect (% A) of the methanol extracts MEC, ME1 fractions C4, ACM2 of Acaera splendens and sodium naproxen (SN)

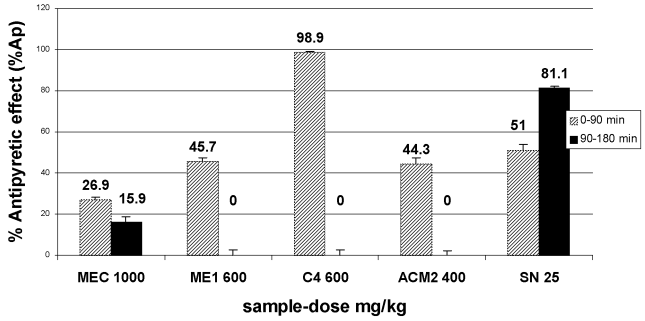


Figure 2. Comparative antipyretic effect (%A) of the methanol extracts MEC, ME1, fractions C4, ACM2 of *Acaena splendens* and sodium naproxen (SN), in two time intervals

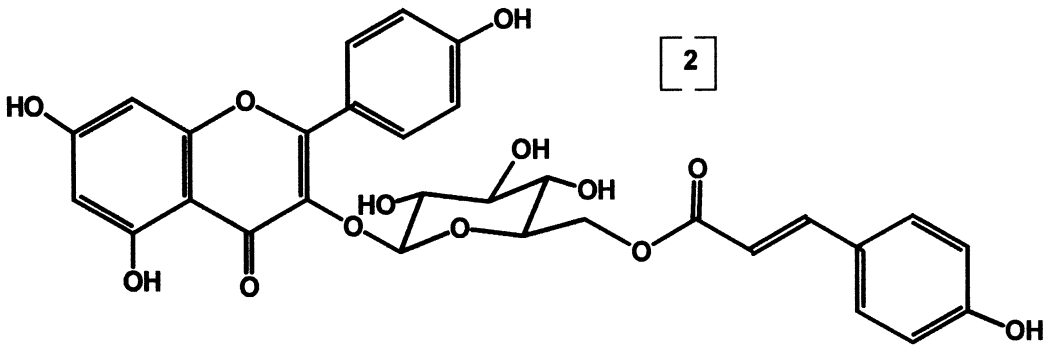
solvents *in vacuo*. The ME-1 was subjected to antiinflammatory and antipyretic assays to monitor potential activities, and flash-chromatographed on silica gel G, eluting with petroleum ether –CH_2Cl_2, CH_2Cl_2–MeOH mixtures of increasing polarity (100:0 up to 50:50, increasing in steps of 25), and the different fractions were tested for antiinflammatory activity. After TLC analysis of one of the active fractions named C4 (eluted with CH_2Cl_2–MeOH=75:25), 80.3 g was submitted to fractionation in column chromatography yielding a crude mixture of phenolic compounds termed ACM2 (38.8 g, corresponding to 48% of C4, eluted with CH_2Cl_2:MeOH=95:5 to 90:10) with antiinflammatory (Fig. 1) and antipyretic activities (Fig. 2), which was flash-chromatographed (9.0 g) on silica gel eluting with EtOAc–MeOH mixtures of increasing polarity (increasing in steps of 5) affording a purified mixture (3.5 g, eluted with EtOAc 100%) which was submitted to a new column chromatography and yielded: epicatechin (**1**) (50 mg eluted with EtOAc, corresponding to a 1.4% of C4), tiliroside (**2**) (100 mg eluted with EtOAc, 2.9% of C4), and after repeated column chromatography: 7-O-acetyl-3-O-β-D-glucosyl-kaempferol (**3**) (10 mg eluted with EtOAc, 0.3% of C4) and 7-β-D-glucosyloxy-5-hydroxy-chromone (**4**) (30.0 mg eluted with EtOAc, 0.9% of C4). The R_fs on TLC (silica gel, $CHCl_3$-MeOH 9:1) were 0.88, 0.75, 0.56 and 0.45 respectively.

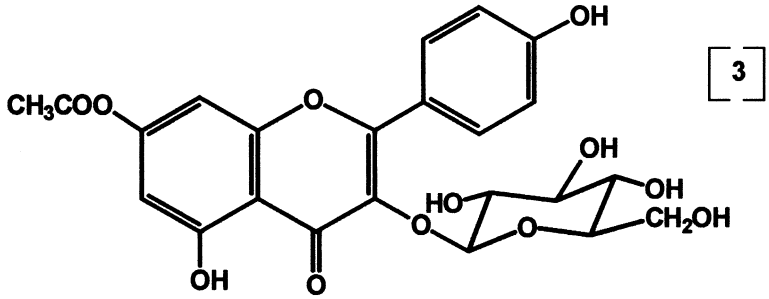
Compound **1** was identified through comparison of the

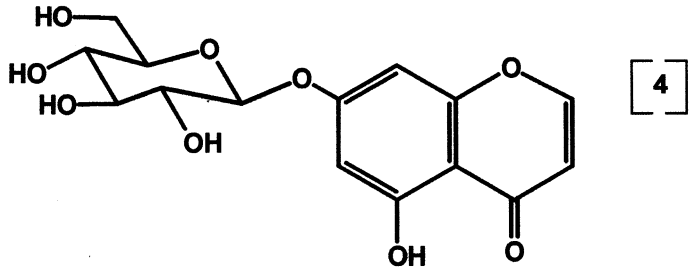
Table 1. NMR data (200/50 and 400/100 MHz) for tiliroside (obtained from fraction ACM2)

C	CHn	δ C ppm	δ H (multip.)	*J* Hz
2	C	156.2		
3	C	133.0		
4	C	177.2		
5	C	161.0	12.60 (OH. bs)	
6	CH	98.6	6.15 (d)	2.0
7	C	164.0	10.60 (OH, bs)	
8	CH	93.5	6.38 (d)	2.0
9	C	156.3		
10	C	103.7		
1′	C	120.7		
2′,6′	CH	130.5	7.98 (d)	8.8
3′,5′	CH	114.9	6.85 (d)	8.8
4″	C	159.8	10.60 (bs)	
1″	CH	101.1	5.45 (d)	7.4
2″	CH	74.0	5.49 (OH	bd 4.2)
3″	CH	76.2	3.2–3.5 (m)	
			5.21 (OH	bd 4.0)
4″	CH	69.9	5.26 (OH	bd 4.9)
5″	CH	74.1		
6″	CH_2	62.8	4.26 (d)	12.0
			4.02 (dd)	12.0;6.2
1‴	C	124.8		
2‴,6‴	CH	129.8	7.36 (d)	8.6
3‴,5‴	CH	115.6	6.78 (d)	8.6
4‴	C	159.5	10.6 (OH bs)	
7‴	CH	144.2	7.34 (d)	16.0
8‴	CH	113.6	6.08 (d)	16.0
9‴	C	165.9		

BRUKER, DMSO-d6. TMS as internal standard. Data from ^1H, ^{13}C (200/50 MHz), COSY , DEPT, HMQC, HMBC (400/100 Hz).







NMR data reported in the literature (Breitmaier, 1993; Harborne and Mabry, 1982).

Spectral data for compound **2** practically coincided with those published for tiliroside (Chari *et al.*, 1978) but, on the basis of direct and long-range homo/heteronuclear 2D-NMR correlations, previous assignments of several NMR signals have been corrected (Table 1). The MP 250°–256°C, differs from the α-glucoside tribuloside MP of 224°–226°C (Chari *et al.*, 1978, Kuroyanagi *et al.*, 1978).

Compound **3** was obtained impurified with a very small amount of triterpenoids, nevertheless the analysis of its NMR spectra permitted us to propose a structure for it. Comparison of absorptions in its ^{1}H and ^{13}C NMR spectra with those of tiliroside and kaempferol 3-O-β-glucoside (Harborne and Mabry, 1982), easily allowed us to propose its structure as an acetyl derivative of the latter. The location of the acetate group at position C-7 of the flavonoid was deduced from its phenolic nature (δ 2.25 ppm for the methyl signal of the acetate in the proton spectrum, δ 21.2 ppm in the ^{13}C spectrum) and from the shifting induced in the signals assigned to the methines C-6 and C-8 (+5.9 and +1.6 ppm respectively) in its ^{13}C spectrum, compared with those of the parent glucoside (Table 2).

Compound **4** was identified by comparison of its NMR spectral data with those reported as 7-β-D-glucosyloxy-5-hydroxy-chromone (Simon *et al.*, 1994). The assignments of the spectral signals have been revised on the light of hetero and homonuclear 2D-NMR correlations (Table 3).

Animals. Pirbright guinea-pigs (220–300 g) of both sexes were used for the antiinflammatory study and for antipyretic studies adult female New Zealand rabbits (2–3 kg) were used. The animals were kept under standard housing conditions at the Animal Maintenance Unit (UMA) of the Chilean Public Health Institute (ISP), and fasted overnight (12 h) before the day of the experiments.

Antipyretic activity assays. The antipyretic activity was assayed using three animals for each dose modified from USP XXII (1990) and repeating each experiment three times (nine results were obtained for each dose). The samples were administered as a suspension in saline arabic gum (5% w/v) at 600 mg/kg for ME-1 and C4 and at 400 mg/kg for the crude mixture of flavonoids (ACM2).

Pyrexia was produced by *E. coli* endotoxin at a dose of 13 ng/kg (prepared in sterile and apyrogenic 0.9% saline

Table 2. NMR data (300/75 MHz) for 7-O-acetyl-3-O-β-D-glucosyl-kaempferol (obtained from fraction ACM2)

C	CHn	δ C ppm	δ H (multip.)	J Hz
2	C	158.7		
3	C	135.8		
4	C	179.7		
5	C	161.8		
6	CH	104.6	6.45 (bs)	
7	C	163.2		
8	C	95.2	6.64 (bs)	
9	C	158.7		
10	C	106.0		
11	COO	166.2		
12	CH_3	21.2	2.25 (s)	
1′	CH	118.2		
2′,6′	C	132.7	8.30 (d)	8.5
3′,5′	CH	116.4	7.15 (d)	8.5
4′	C	159.4		
1″	CH	100.3		
2″	CH	76.7		
3″	CH	78.6[a]		
4″	CH	71.6		
5″	CH	78.3[a]		
6″	CH2	62.9		

[a] Interchangeable assignments.
BRUKER, CD_3OD. TMS as internal standard. Data from ^1H and ^{13}C.

solution) injected intravenously in the ear, 1 h after administering the sample.

Previously, temperature versus time curves were obtained for each animal. Temperature was recorded with an Ellab pyrogen tester, from the time of injection of the pyrogen up to 180 min afterwards. The observation period was divided into two time intervals: 0–90 and 90–180 min (Backhouse *et al.*, 1994).

The mean areas (for each animal) under the temperature/time curves obtained with pyrogen alone ($AREA_{pyr}$) and with pyrogen plus the sample ($AREA_{pyr+s}$) were compared, and then averaged over all the rabbits. The areas under the curves were calculated using the EXCEL program. The antipyretic effect (%Ap) was calculated according to: $1\text{-}(Area_{pyr+s}/Area_{pyr}) \times 100$. The significance of the effect was estimated using the ANOVA test (Kohout and Norwood, 1981), for $p \leq 0.05$.

Table 3. NMR data (300/75 MHz) for 7-β-D-glucosyloxy-5-hydroxy-chromone (obtained from fraction ACM2)

C	CHn	δ C ppm	δ H (multip.)	J Hz
2	CH	158.3	8.09 (d)	5.9
3	CH	111.0	6.17 (d)	5.9
4	C	181.8		
5	C	161.5	12.49 (OH bs)	
6	CH	99.9	6.27	1.9
7	C	163.3		
8	CH	95.1	6.50	1.9
9	C	157.7		
10	C	106.7		
1″	CH	100.1	4.85 (d)	7.0
2″	CH	73.3	ca 3.37 (m)	
3″	CH	77.4	ca 3.41 (m)	
4″	CH	69.8	ca 3.24 (m)	
5″	CH	76.6	ca 3.58 (m)	
6″	CH2	60.8	3.51 (d)	12.2
			3.25 (dd)	12.2;5.8

BRUKER, DMSO-d_6. TMS as internal standard. Data from ^1H, ^{13}C, DEPT, HMBC and HMQC.

Antiinflammatory activity assays. The antiinflammatory activity was evaluated in groups of 15 animals for each dose (except for ACM2), according to the carrageenan-induced paw oedema method described by Winter *et al.* (1963). Samples were administered 1 h before the injection of 0.1 mL of sterile saline λ-carrageenan (1% w/v) suspended in saline arabic gum (5% w/v) at the doses of 600 and 400 mg/kg for ME-1, C4 and ACM2, respectively.

Paw volume was measured with an Ugo Basile plethysmometer (model 7150), immediately (V_i) and 4 h after administration of the samples (V_f). Inflammation (%*I*) was calculated as the difference between final and initial paw volumes, divided by V_i. The results, reported as antiinflammatory effect, were calculated as the percent inhibition of inflammation ($\%A = \%Ic\text{-}\%I/\%Ic \times 100$) compared with a control group receiving only the vehicle 1 h before the injection of λ-carrageenan, which showed a $37.7\% \pm 1.26\%$, of inflammation (%*I*c) (Backhouse *et al.*, 1994). The significance of the drug-induced changes was estimated using Student's *t*-test (Spiegel, 1991), for $p \leq 0.05$.

Sodium naproxen at doses of 4 and 25 mg/kg, was used as a standard in the antiinflammatory and antipyretic evaluations respectively, showing 54.6% of effect for the first, and 51.1%.and 81.1% of antipyretic effect in the first and second time interval, respectively.

RESULTS AND DISCUSSION

Antiinflammatory and antipyretic activities

Although the antiinflammatory activity of the crude methanol extract (MEC) was found to be dose-related, reaching a maximum effect of 72.4% at 1200 mg/kg, higher than the maximum effect observed for sodium naproxen (54.6% at 4.0 mg/kg), the antipyretic activity of the ME was only observed at 1000 mg/kg (Backhouse *et al.*, 1997). Nevertheless, unlike the lack of antiinflammatory activity of ME-1 which was only observed in a purified fraction after submitting this extract to column chromatography (Fig. 1), it showed a strong antipyretic action (47.5%) at the first time interval (Fig. 2). Bioassay-guided fractionation of the residue of ME-1 allowed us to continue the study with the most active fraction C4 (55.7% of antiinflammatory effect and 98.9% of antipyretic effect at a dose of 600 mg/kg), affording an antipyretic and antiinflammatory active fraction named ACM2 with an equal percent of activities for both assays at 400 mg/kg, although lower than the maximum effect of sodium naproxen (54.6% of antiinflammatory effect and 51.0% and 81.1% of antipyretic effect in the first and second interval respectively) (Figs. 1, 2).

Chemical constituents of the active extract ACM2

Looking forward to explaining the activities mentioned above, the most abundant metabolites were separated,

purified and identified consisting mainly of epicatechin, tiliroside, 7-O-acetyl-3-O-β-D-glucosyl-kaempferol (in smaller amount) and 7-β-D-glucosyloxy-5-hydroxy-chromone.

For tiliroside previous ambiguous assignments of several NMR signals have been corrected: 4′ and 4‴, 2 and 9, 2′ and 6′/2‴and 6‴, 3′and 5′/3‴and 5‴ (Table 1). On the other hand, the *J* H-1″ 7.4 Hz confirms the presence of the β-glucoside derivative (Chari *et al.*, 1978).

In relation to 7-β-D-glucosyloxy-5-hydroxy-chromone previously described by Simon *et al.* (1994), the assignments of the spectral signals have been revised in the light of hetero and homonuclear 2D-NMR correlations allowing the ^{13}C reassignments for C-2 and C-9: δ158.3 and 157.7 ppm (Table 3), instead of 158.0 and 159.9 ppm, respectively.

In an exhaustive bibliography research, no references have been found for 7-O-acetyl-3-O-β-D-glucosyl-kaempferol.

Although most of the metabolites isolated have been previously described in other species, it is important to underline that many of the medicinal properties attributed to plants could be due to the presence of flavonoids. It is well known that these compounds have an influence in the metabolism of arachidonic acid, which could explain the mechanism of action in inflammation and fever (Alcaraz and Ferrándiz, 1987). Also, the major compound present in the active extract was tiliroside, which chemically is very similar to the flavonoids present in the *Ginkgo biloba* extract (*p*-coumaric esters of glucorhamnosides of quercetin and kaempferol) employed in the treatment of central and peripheral ischaemia, where radical scaveanger properties have been attributed to them (Fünfgeld, 1988), which interfere in arachidonic acid pathways. Tsuruga *et al.* (1991) reported the antiallergy effect of *Magnolia salcifolia* and found that tiliroside was able to inhibit induced histamine released in rat mast cells, an effect that could also explain its role in the inflammatory response. Recently, the *in vitro* anticomplementary activity of the methanol extract of *Magnolia fargesii* was reported, where tiliroside was isolated as an active constituent showing a very potent anticomplementary activity ($IC_{50} = 5.4 \times 10^{-5}$ M), on the classical pathway of the complement system (Jung *et al.*, 1998). As this is related to one of the complex mechanisms involved in inflammation, it could be considered as more evidence for the antiinflammatory effect of tiliroside, a major constituent of the purified fraction C4 of *Acaena splendens*, together with the antiinflammatory effect of (−)-epicatechin which was found to significantly reduce the rat paw oedema induced by carrageenin (ED_{50} 74 mg/kg, i.p.) (Swarnalakshmi *et al.*, 1981).

Finally, these results allowed us to explain the medicinal use attributed to this plant, employed in Chilean folk medicine for the treatment of fever and inflammatory conditions, as it in fact presents antiinflammatory and antipyretic effects *in vivo*, due to the presence of several active principles, probably flavonoids isolated in this study together with sterols and triterpenes previously reported (Backhouse *et al.*, 1997).

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