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Antioxidant activity of coumarins and flavonols from the resinous exudate of *Haplopappus multifolius*

René Torres [a,*], Francesca Faini [b], Brenda Modak [a], Francisco Urbina [a], Cecilia Labbé [b], Juan Guerrero [c]

[a] *Departamento de Ciencias del Ambiente, Fascultad de Química y Biología, Universidad de Santiago de Chile, Avda. Bernardo O'Higgins 3363, Santiago, Chile*
[b] *Departamento de Química, Facultad de Ciencias, Universidad de Chile, Las Palmeras 3425, Santiago, Chile*
[c] *Departamento de Ciencias de los Materiales, Facultad de Química y Biología, Universidad de Santiago de Chile, Avda. Bernardo O'Higgins 3363, Santiago, Chile*

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Abstract

The antioxidant activity of eight coumarins and two flavonols isolated from *Haplopappus multifolius* was studied with the DPPH radical method. Results show that a high concentration of phenolic coumarins and the presence of quercetin and rhamnetin in the exudates could account for the protection of the plant against oxidative stress. Structures for the coumarins 6-hydroxy-7-[(*E*,*E*)-3′,7′-dimethyl-2′,4′,7′-octatrienyloxy] coumarin and 7-[(*E*)-3′-methyl-4′-hydroxy-2′-butenyloxy] coumarin are proposed on the basis of spectroscopic evidence.

Keywords: *Haplopappus multifolius*; Asteraceae; Resinous exudates; Coumarins; Flavonoids; Antioxidants; DPPH

1. Introduction

In order to elucidate the role of secondary metabolites in the defence of plants against oxidative stress, we have been studying resinous exudates of native species growing in arid and semi-arid zones of Chile exposed to extreme conditions of the environment. In particular, we have tested their antioxidant activities against DPPH radical (Lissi et al., 1999; Torres et al., 2003).

The large American genus *Haplopappus* is well represented in Chile with ca. 60 species (Marticorena and Quezada, 1985), with many of them being resinous and frequently used in folk medicine for liver ailments. *Haplopappus multifolius* Reiche is a small shrub growing at the mountain skirts near Santiago (Chile, Central Valley). Previous chemical studies on this species reported that it mainly accumulates coumarins in the resin that covers leaves and stems (Torres et al., 2004).

Very few systematic studies have been reported on structure-antioxidant activity correlations in coumarins. In a study of superoxide scavenging capacity, performed with 16 synthetic or plant derived coumarins with different substitution patterns, Paya found that only 7,8-dihydroxylated coumarins were active (Paya et al., 1994). Similarly, it has been recently reported that DPPH radical scavenging activity in furanocoumarins correlates with the number of phenolic hydroxyl groups present in their structures (Kogure et al., 2004; Piao et al., 2004).

We now report the results of an assay-guided chemical study of the resinous exudate of *H. multifolius* that afforded the new coumarins **1** and **2** along with six known coumarins **3**–**8**, and two flavonoids, quercetin and isorhamnetin.

* Corresponding author. Tel.: +56 2 6812575; fax: +56 2 6812108.
E-mail address: rtorres@lauca.usach.cl (R. Torres).

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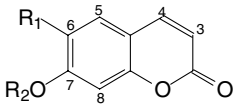
Scavenging activity towards the DPPH radical was measured for all compounds isolated.



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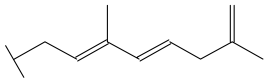
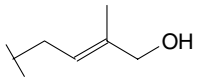
1	OH	
2	H	
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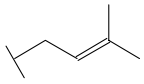
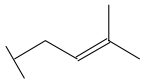
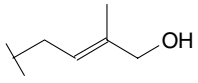
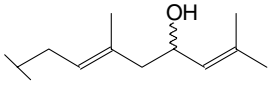
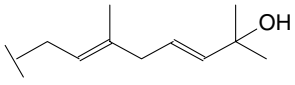
4	H	
5	OH	
6	OH	
7	OH	
8	OH	

2. Results and discussion

2.1. Isolation and identification of compounds

Repeated column chromatography of the extract monitored by TLC bioautography using DPPH solution, led to the separation of the active compounds. The known coumarins: hernianin **3**, *O*-prenyl-umbelliferone **4**, prenyletin **5**, haplopinol **6**, 6-hydroxy-7-[(*E*)-3′,7′-dimethyl-5′-hydroxy-2′,6′-octadienyloxy] coumarin **7** and 6-hydroxy-7-[(*E*,*E*)-3′,7′-dimethyl-7′-hydroxy-2′,5′-octadienyloxy] coumarin **8**, and the flavonols quercetin and isorhamnetin were identified by NMR analysis and TLC comparison with authentic samples. Two new coumarins were also isolated in this study and their structures **1** and **2** are proposed on the evidence discussed below.

Compound **1** was obtained as white needles. The HREIMS gave a molecular peak at *m*/*z* 312.1382 corresponding to the molecular formula $C_{19}H_{20}O_4$. The ^{1}H NMR spectrum (Table 1) had resonances for protons in the coumarin nucleus corresponding to H-3, H-4, H-5 and H-8. Additional signals in the spectrum were consistent with five olefinic protons [δ 4.92 (2H), 5.52 (1H), 5.63 (1H) and 6.19 (1H)], one oxymethylene group (δ 4.69), two methylene protons (δ 2.83) and two olefinic methyls (δ 1.72 and 1.85) in the structure of **1**.

The ^{13}C NMR spectrum (Table 1) showed in addition to the resonances of carbons belonging to the coumarin nucleus, 10 other signals arising from two methyls; one oxymethylene, one sp^3 methylene and six olefinic carbons that could only be located at the side-chain. Joint analysis of HSQC spectra and HMBC correlations (Fig. 1) confirmed the presence of an oxygeranyl group attached to the coumarin skeleton. A correlation observed between H-8 and the oxymethylene group at C′1 in the NOESY spectrum defined the position of the geranyloxy group at C-7. NOESY correlations (Fig. 2) observed between H-1′/CH_3-10′ and H-2′/H-4′ indicate that the configuration of the double bond between C-2′ and C-3′ must be *E*. The same *E* configuration was deduced for the other side chain double bond (C-4′ = C-5′) on the basis of the large coupling ($J = 15.6$ Hz) between H-4′ and H-5′. This evidence along with the UV and IR spectra are consistent with the new structure **1** (6-hydroxy-7-[(*E*,*E*)-3′,7′-dimethyl-2′,4′,7′-octatrienyloxy] coumarin).

Compound **2** was obtained as a colourless oil. The HREIMS gave a molecular peak at *m*/*z* 246.0849 corresponding to the molecular formula $C_{14}H_{14}O_4$. IR and UV spectra suggested that it was a coumarin substituted

Table 1
^{1}H and ^{13}C NMR spectroscopic data for compounds **1** and **2**, *J* (Hz) in parentheses

1			**2**	
C	^{13}C	^{1}H	^{13}C	^{1}H
C-2	161.6	–	162.1	–
C-3	114.5	6.28 *d*, (9.5)	113.4	6.28*d*, (9.5)
C-4	143.4	7.59 *d*, (9.5)	143.6	7.66 *d*, (9.5)
C-5	112.2	6.97 *s*	128.9	7.37 *d*, (8.4)
C-6	149.5	–	112.9.	6.85 *dd*, (8.4, 2.1)
C-7	143.1	–	161.4	–
C-8	100.5	6.83 *s*	101.9	6.82 *d*, (2.1)
C-9	149.4	–	156.1	–
C-10	112.4	–	111.6	–
C-1′	66.5	4.69*d*, (6,7)	65.2	4.67*d*, (6.4)
C-2′	118.8	5.52 *dt*, (6.7, 1.2)	118.9	5.77 *dt*, (6.4, 1.2)
C-3′	142.4	–	121.8	–
C-4′	135.5	6.19 *d*, (15.6)	67.7	4.11 *s*
C-5′	127.0	5.63 *dt*, (15.6, 7.1)	14.3	1.79 *s*
C-6′	42.9	2.83 *d*, (7.1)		
C-7′	142.0	–		
C-8′	115.6	4.92 *bs*		
C-9′	18.9	1.85 *s*		
C-10′	17.2	1.72 *s*		

Connectivities C–H through HSQC and HMBC experiments.

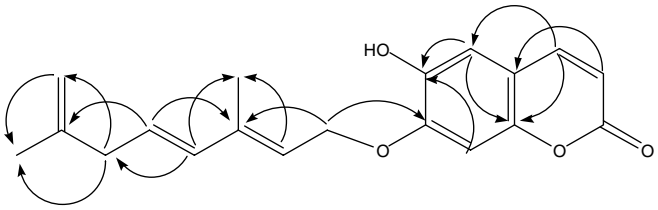


Fig. 1. Selected HMBC correlations of **1**.

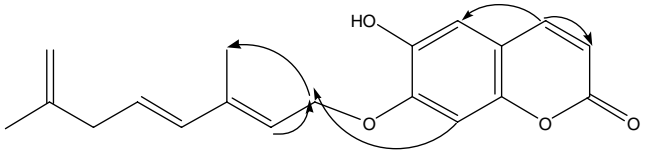


Fig. 2. Key NOESY correlations of **1**.

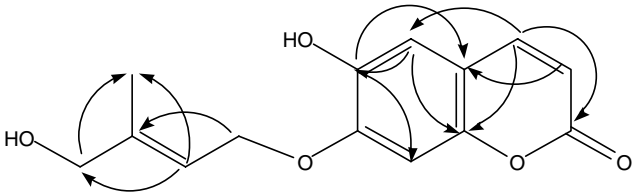


Fig. 3. Selected HMBC correlations of **2**.

at C-7. The ^{1}H NMR spectrum (Table 1) was very similar to that of haplopinol **6** (Chiang et al., 1982), lacking the hydroxyl group at C-6. The ^{13}C NMR spectrum (Table 1) has the resonances for the coumarin nucleus and five additional signals arising from carbons at the side-chain that accounted for: a methyl group, two oxymethylene groups and two olefinic carbons. Attachment of the side chain to C-7 followed from the correlation between H-1′ and C-7 in the HMBC spectrum of **2** (Fig. 3). Further confirmation for the proposed structure was obtained from HSQC, HMBC and NOESY experiments. The *E* configuration of the double bond was assigned on the basis of NOESY correlations observed between H-1′/H-5′ and H-4′/H-2′. Structure **2** (7-[(*E*)-3′-methyl-4′-hydroxy-2′-butenyloxy] coumarin) is proposed for this new compound which corresponds to 6-deoxyhaplopinol.

2.2. Antioxidant activity of resinous exudate and pure compounds

Results of assays with the 1,2-diphenyl-2-picrylhydrazyl radical (DPPH) are summarized in Table 2. The radical scavenging activity displayed by the extract (IC_{50} 45.5 mg/mL) in this assay would thus confirm the protective role of the resin towards ROS present in the environment. As expected, *O*-substituted phenolic coumarins (**2**, **3** and **4**) were almost inactive ($IC_{50} > 400$ μM) while phenolic coumarins **1**, **5**, **6**, **7** and **8** displayed a moderate antioxidant activity (IC_{50} 247.9, 109.3 125.9, 250.1 and 251.5 μM, respectively). A structure-activity correlation

Table 2
Antioxidant activity of phenolic coumarins and flavonoids

Compounds	DPPH radical $IC_{50} \pm$ SEM (μM)
1	247.9 ± 4.2
5	109.3 ± 8.1
6	125.9 ± 6.1
7	250.1 ± 10.3
8	251.5 ± 11.7
Quercetin	9.8 ± 0.2
Isorhamnetin	12.7 ± 0.9
Trolox (standard)	59.0 ± 2.4

within this series of phenolic coumarins is not straightforward in terms of type of prenyl units or the effect of hydroxyl groups at the side chain. Although the experiments confirmed the well known free radical scavenging activity of quercetin and isorhamnetin (IC_{50} 9.7 and 12.8 μM, respectively), the small amounts isolated from the resin extract (0.5% w/w) would indicate that their effective concentration is smaller than needed to account for the scavenging activity of the extract. Coumarins, although less active, must therefore be the main responsible for this activity due to their high concentration in the resinous exudate.

On the other hand, prenyletin **5**, the best radical scavenger phenolic coumarin of the extract, has been reported to protect human cells from oxidative stress induced by peroxides, probably through a mechanism that induces the production of protective genes (Wang et al., 2005)). In this way, the high concentration of prenyletin found in *H. multifolius* resin must be directly related to a mechanism of protection of the plant cells against oxidative damage and peroxidation of lipids in the resin.

3. Experimental

3.1. General experimental procedures

Melting points were determined on a Kofler micromelting apparatus and are uncorrected. UV spectra were recorded on a Varian Cary 500 UV/vis spectrophotometer. IR spectra were obtained on a Bruker IFS 66v instrument. ^{1}H NMR (400 MHz), ^{13}C (100 MHz) and 2D NMR spectra were recorded in $CDCl_3$ on a Bruker Avance DRX400 spectrometer with TMS as internal standard. HREIMS were recorded on a VG Platform (Fisons) Spectrometer using chemical ionization. Known compounds were identified by comparison of their spectroscopic data with those in the literature and by co-chromatography with authentic samples. Silica gel 60 (70–230 mesh ASTM; 63–200 μm) for open CC and GF_{254} for analytical TLC were purchased from Merck Ltd. (Darmstadt, Germany). DPPH was purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). All solvents and chemicals used were of analytical grade.

3.2. Plant material

Leaves and stems of *H. multifolius* were collected at Farellones (Región Metropolitana, Chile, 32°21′S, 70°21′W, 1770 m height, skirts of Andes Range) in summer 1999 and 2004. Voucher specimens (AJIM 2488) are deposited at the Instituto de Biología Vegetal y Biotecnología, Universidad de Talca and were authenticated by Dr. José San Martin.

3.3. Extraction and isolation

Fresh leaves and stems (1.5 kg) were extracted by dipping into CH_2Cl_2 at room temperature for 30 s. The solution was filtered and concentrated under reduced pressure at 30 °C to give an oily residue (105 g, 7% of resin/fresh plant). About 15 g of the extract was submitted to column chromatography (silica gel) and eluted with EtOAc–hexane mixtures of increasing polarity. Twelve fractions were collected and tested for antioxidant activity using DPPH as reactant. Further purification of fractions F-3, F-5 and F-7 afforded coumarins **3** (1.3 g), **4** (1.8 g), **5** (5.5 g) and **2** (0.5 g). Compounds **6** (0.5 g), **7** (1.2 g) and **8** (0.9 g) were obtained from F-9 while F-10 afforded **1** (0.8 g). A mixture of flavonoids from F-11 gave quercetin (0.05 g) and isorhamnetin (0.02 g).

3.4. 6-Hydroxy-7-[(E,E)-3′,7′-dimethyl-2′,4′,7′-octatrienyloxy] coumarin (*1*)

Colourless needles (m.p. 86 °C) (EtOH); UV (MeOH) λ_{max} nm: 352 ($\log\varepsilon$ 4.2), 305 ($\log\varepsilon$ 3.8); IR (KBr) ν_{max} cm^{-1}: 3430, 1730, 1615; for ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) spectra, see Table 1; HREIMS: *m/z* 312.1382 (M^+, calcd. for $C_{19}H_{20}O_4$, 312.3646).

3.5. 7-[(E)-3′-methyl-4′-hydroxy-2′-butenyloxy] coumarin (6-deoxyhaplopinol) (*2*)

Colourless oil. UV (MeOH) λ_{max} nm: 322 ($\log\varepsilon$ 4.1); IR (KBr) ν_{max} cm^{-1}: 1730, 1615; for ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) spectra, see Table 1; HREIMS: *m/z* 246.0849 (M^+, calcd. for $C_{14}H_{14}O_4$, 246.2622).

3.6. DPPH assays

3.6.1. TLC autographic assay

After developing and drying the TLC plates (samples ranging from 0.1 to 100 μg) were sprayed with 0.2% solution of DPPH in methanol. Active compounds appeared as yellow spots against a purple background after 90 min of spraying (Chacha et al., 2005).

3.6.2. Antioxidant capacity

One ml of 500 μM solution of DPPH in EtOH was thoroughly mixed with an equal volume of a solution of test compounds and resin at various concentrations and kept in the dark for 30 min. UV absorbances of the solutions were measured at 517 nm. The concentration in μM at which the absorbance at 517 nm decreases to 50% of its initial value was used as the IC_{50} value for each test solution. Experiments were done in triplicate and the mean value was recorded.

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