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Lipophilicity and antibacterial activity of flavonols: Antibacterial activity of resinous exudates of *Haplopappus litoralis*, *H. chrysantemifolius* and *H. scrobiculatus*

[Lipofilia y actividad antibacteriana de flavonoles: Actividad antibacteriana de los exudados resinosos de *Haplopappus litoralis*, *H. chrysantemifolius* y *H. scrobiculatus***]**

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Abstract
The antibacterial properties of the resinous exudates from Haplopappus litoralis, H. chrysantemifolius and H. scrobiculatus from Central Chile were assessed against Gram negative and Gram-positive bacteria, and proved active against the latter. The results show that the antibacterial activities of the resinous exudates are independent from the flavonols isolated from each extract that proved to be inactive. The estimated lipophilicity of the flavonols isolated from the Haplopappus resinous exudates were compared with the lipophilicity of known antibacterial flavonols. This analysis showed that lipophilicity is an important variable to predict the antibacterial activity of flavonols.

Keywords: *Haplopappus*, Antibacterial resinous exudates, Flavonols, Lipophilicity.

Resumen
La actividad antibacteriana de los exudados resinosos de *Haplopappus litoralis*, *H. chrysantemifolius* y *H. scrobiculatus* de la Zona Central de Chile fueron evaluadas frente a bacterias Gram-negativas y Gram-positivas, y resultaron activos frente a estas últimas. Los resultados mostraron que la actividad antibacteriana de los exudados resinosos es independiente de los flavonoles aisladas de cada extracto que no mostraron actividad antibacteriana. La lipofilia estimada de los flavonoles aislados de los exudados resinosos de *Haplopappus* se compararó con la lipofilia de conocidos flavonoles antibacterianos. Este análisis mostró que la lipofilia es una variable importante para predecir la actividad antibacteriana de los flavonoles.

Palabras Clave: *Haplopappus*, Exudados resinosos antibacterianos, Flavonoles, Lipofilia.

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INTRODUCTION

Haplopappus Cass. (Asteraceae, Astereae) is a genus of 56 species categorized into four sections. The species are restricted to Chile and Argentina (Klingenberg, 2007) and, with a few exceptions, all of them show a characteristic production of resinous exudates in trichomes that populates twigs and leaves (Urzúa *et al*., 2004). According to Wilhelm de Mösbach (1992) who compiled the plant ethnopharmacological Mapuche tradition, *Haplopappus baylahuen* Remy ("Baylahuén", "Vaila-huen") contains a medicinal resin used in external applications for promoting wound-healing and ingested, as a digestive stimulant. He indicates in his remarkable study the existence of other *Haplopappus* species used for the same ethnopharmacological properties. Several publications mention other ethnopharmacological uses for around 20 *Haplopappus* species, among them: general antiseptic; to treat gastro- intestinal and urinary tract infections; for promoting wound-healing in animals; to stop complicated colds; for stomach, liver and kidney ailments; as hepatic and intestinal stimulants; and even as aphrodisiac (Ibañez and Paredes, 1937; Muñoz *et al*., 1980; Houghton *et al*., 1985; Montes and Wilkomirsky, 1987; Farga and Lastra, 1988; Hoffmann *et al.*, 1992; Mellado *et al*., 1996; Zin and Weiss, 1998; Vogel *et al*., 2005). Taking into account all the ethnopharmacological literature consulted the most important and spread medicinal property of "Baylahuén" is its use as antiseptic.

In a previous communication, the antimicrobial properties and preliminary information of the secondary metabolites present in the resinous exudates of nine *Haplopappus* species from Central Chile was informed (Urzúa *et al*., 1995). Years later, for *Haplopappus uncinatus* the antibacterial properties of the resinous exudate were correlated with the presence of the clerodane diterpenoid 18-acetoxy-*cis*-cleroda-3-en-15-oic acid (10β, 16ξ, 19β, 17β, 20α form). The flavonols 3, 5-dihydroxy-6, 7, 3', 4'-tetramethoxyflavone, also present in the extract, has shown to be inactive (Urzúa *et al*., 2006).

In this paper, the antibacterial properties of the resinous exudates of other three species of *Haplopappus* from Central Chile are presented. The results show that the antibacterial activity of the resinous exudates is independent from the flavonols isolated from each extract that proved to be inactive.

EXPERIMENTAL

Plant material and extraction of the resinous exudates

Aerial parts of *H. litoralis* Phil. and *H. chrysanthemifolius* (Lees.) DC. were collected during the flowering season in Los Molles Cape (V Region, Chile, 33º 19' S, 70º 14' W). Aerial parts of *H. scrobiculatus* (Lees.) DC. were collected during the flowering season in La Parva (Región Metropolitana, Chile, 33º 19' S, 70º 14' W). Voucher specimens were deposited in the Herbarium of the Museo Nacional de Historia Natural, Santiago, Chile.

The resinous exudates of *H. litoralis* (80 g), *H. chrysanthemifolius* (100 g) and *H. scrobiculatus* (650 g) were obtained by dipping fresh plant material in cold CH_2Cl_2 for 15-20 s. The extracts were concentrated to sticky residues.

Spectroscopy

IR-FT spectra were recorded on a Bruker IFS66V. Both ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker 400 Ultra Shield spectrometer and 2D spectra were obtained using standard Bruker Software.

Preliminary analysis of the resinous exudates

All the resinous exudates were comparatively studied by IR (film) and ^{1}H-NMR spectroscopy. TLC analysis was performed with Merck silica gel chromatoplates (0.2 mm) using CH_2Cl_2 and CH_2Cl_2-MeOH (97:3) as developing systems. Terpenoids were visualized by spraying the chromatoplates with *p*-anysaldehyde/H_2SO_4 and heating at 105 ºC. For flavonoids, natural product - polyethylene glycol reagent was used and visualized at UV-365 nm. (Wagner *et al*., 1984).

Separation of the resinous exudates

Part of the resinous exudates of *H. litoralis* (5 g), *H. chrysanthemifolius* (4 g) and *H. scrobiculatus* (5 g) were fractionated by CC (silica gel) using a pentane-CH_2Cl_2 step gradient, CH_2Cl_2 and a CH_2Cl_2- MeOH step gradient. The crude flavonols in each extract were obtained after having collected the individual fractions with the same composition of flavonols (TLC).

Antibacterial activity determination in solid medium

The antibacterial activity was evaluated against *Bacillus cereus* (NAS 569), *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 6538p), *Micrococcus luteus* (ATCC 9341), *Clavibacter*

michiganensis subsp. *michiganensis* (Cmm 623), *Escherichia coli* (ATCC 25922), *Salmonella paratyphi* B (ATTCC 2659), *Proteus vulgaris* (ATCC 6380), *Klebsiella pneumoniae* (ATCC 13883) and *Erwinia carotovora* (IC 2610).

The antibacterial activity was determined by the agar overlay method (Mayr-Harting *et al*., 1972). Bacteria grown overnight in LB-broth (Ausubel *et al*., 1995), were diluted to Mc Farland 0.5-1.0 (1.5 - 3 x 10^8 cells/ml) and 100 μl of this dilution were mixed with 3 ml of molten soft agar (0.7%) at 50 ºC. The soft agar was poured over Petri dishes containing 20 ml of 1.5% agar. Two-fold dilutions of the test samples (5 μl) in methanol were deposited over solidified agar, starting at 1000 μg/ml up to 2 μg/ml. After 18 h of incubation at 37 ºC, the diameter of the inhibition zone was determined. Control measurements were carried out with methanol. The minimum inhibitory concentration (MIC) corresponded to the minimum concentration that showed a transparent halo of growth inhibition. The MIC determination was carried out in five independent experiments.

RESULTS

The amount of resinous exudate from each species is shown in Table 1. ^{1}H-NMR spectroscopy and TLC analysis of each extract show the presence of a complex mixture of labdane or clerodane diterpenoids acids with minute amounts of lipophilic flavonols.

Table 1
Resinous exudates from *H. litoralis, H. chrysanthemifolius* and *H. scrobiculatus*

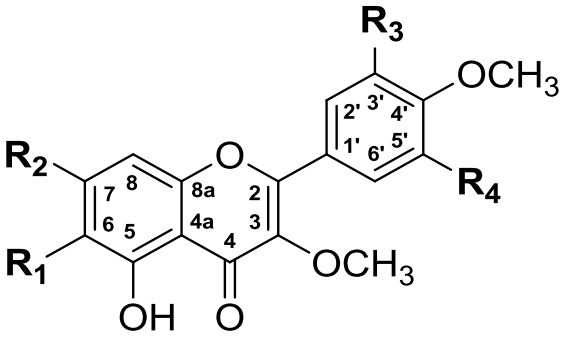
***Haplopappus* species**	**Resinous exudates (g) (%)**
Haplopappus litoralis	10 (12.5)
Haplopappus chrysanthemifolius	5 (4.5)
Haplopappus scrobiculatus	45 (6.9)

Part of the resinous exudates of *H. litoralis*, *H. chrysanthemifolius* and *H. scrobiculatus* were fractionated by CC. The crude flavonols fractions obtained from each extract were purified by extensive preparative TLC to yield the following compounds Table 2.

Table 2
Flavonols isolated from each resinous exudate

Resinous exudates from *Haplopappus*	**Flavonols (mg)**
H. litoralis	5-hydroxy-3,7,3',4'-tetramethoxyflavone [**1**] (50mg)
	5,3'-dihydroxy-3,7,4'-trimethoxyflavone [**2**] (100mg)
H. chrysanthemifolius	5,3'-dihydroxy-3,7,4'-trimethoxyflavone [**2**] (8mg)[1]
	5,3',5'-trihydroxy-3,7,4'-trimethoxyflavone [**3**] (10mg)
H. scrobiculatus	5,7-dihydroxy-3,6,4'-trimethoxyflavone[1] [**4**] (68mg)
	5,3'-dihydroxy-6,7,4'-trimethoxyflavone[1] [**5**] (25mg)

[1] previously isolated (Faini *et al*., 1999).



	R_1	R_2	R_3	R_4
[1]:	H	OCH_3	OCH_3	H
[2]:	H	OCH_3	OH	H
[3]:	H	OCH_3	OH	OH
[4]:	OCH_3	OH	H	H
[5]:	OCH_3	OCH_3	OH	H

Figure 1
Structures of compounds [1-5]

Antibacterial activity

The activities of the resinous exudates and of the flavonols isolated from each of them were assessed against Gram-positive and Gram negative bacteria, and proved inactive against the latter. Table 3 lists the activities of the resinous exudates, and flavonols **1-5**. In addition, the observed activities were compared with those of three commercial antibiotics (ampicillin, tetracycline and chloramphenicol).

Table 3
Antibacterial activity of the resinous exudates and flavonols isolated from each resin

Tested samples	MIC values in solid media (µg)[b]			
	A [c]	B [c]	C [c]	D [c]
	µg	µg	µg	µg
H. litoralis resinous exudate	0.625	0.32	2.5	1.25
H. chrysanthemifolius resinous exudate	0.625	0.32	2.5	2.5
H.scrobiculatus resinous exudate	1.25	1.25	1.25	2.5
5-hydroxy-3,7,3',4'-tetramethoxyflavone [**1**]	i	i	i	i
5,3'-dihydroxy-3,7,4'-trimethoxyflavone [**2**]	i	i	i	i
5,3',5'-trihydroxy-3,7,4'-trimethoxyflavone [**3**]	i	i	i	i
5,7-dihydroxy-3,6,4'-trimethoxyflavone[**4**]	i	i	i	i
5,3'-dihydroxy-6,7,4'-trimethoxyflavone [**5**]	i	i	i	i
Methanol(blank)	i	i	i	i
Chloramphenicol	1.25	2.5	1.25	1.25
Ampicillin	i	i	0.16	0.08
Tetracycline	2.5	2.5	0.16	0.04

([b]) µg deposited in 5 µl. ([c]) Tested microorganisms: A, *Bacillus subtilis* (ATCC 6633); B, *Bacillus cereus* (NAS 569); C, *Micrococcus luteus* (ATCC 9341); D, *Staphylococcus aureus* (ATCC 5638). i, inactive.

Discussion and Conclusion

The identity of the flavonols of each resinous exudate was established by NMR (400 MHz) experiments (^{1}H, ^{13}C, DEPT, COSY, HSQC and HMBC) and by comparison with spectroscopic data from the literature (Harborne, 1994; Andersen and Markham, 2006).

Previous work in *H. scrobiculatus* with plant material collected in the same area of the sample used in this communication, report the isolation of thirteen flavonoids. None of those flavonoids correspond to compounds [**4]** and [**5]**. In that work, the whole dry milled plant was used and the chloroform extracts were discarded (Ates *et al*., 1982). That extract is the one that should contain the less polar flavonoids.

As example of the structural determination of the flavonoids, the full assignation of the NMR spectra

and structural determination of 5, 7-hydroxy-3, 6, 4'-trimethoxyflavone [**4**], is shown below. Assignation of all the H and C resonances was done using DEPT-135 and HSQC experiments, Table 4. The position of the oxygenated substituents in the flavonoid structure, was obtained throw Heteronuclear Multiple Bond Correlations, Figure 2.

Table 4
NMR data of 5,7-hydroxy-3,6,4'-trimethoxyflavone [4]

Position	DEPT 135	^{13}C Chemical shift (ppm)	^{1}H Chemical shift (ppm)	Integration, multiplicity and coupling constant (Hz)
2	C	156,12	-	-
3	C	138,41	-	-
4	C	179,19	-	-
4ª	C	106,19	-	-
5	C	152,24	-	-
6	C	129,99	-	-
7	C	154,96	-	-
8	CH	93,07	6,54	1H; *s*
8ª	C	151,79	-	-
1'	C	122,77	-	-
2'	CH	130,16	8,06	1H; *d*; J = 8.97
3'	CH	114,07	7,01	1H; *d*; J = 8.78
4'	C	161,71	-	-
5'	CH	114,07	7,01	1H; *d*; J = 8.78
6'	CH	130,16	8,06	1H; *d*; J = 8.97
C5-OH	-	-	12,92	1H; *s*
C3-OMe	CH_3	60,13	3,85	3H; *s*
C6-OMe	CH_3	60,89	4,04	3H; *s*
C4'-OMe	CH_3	55,41	3,89	3H; *s*

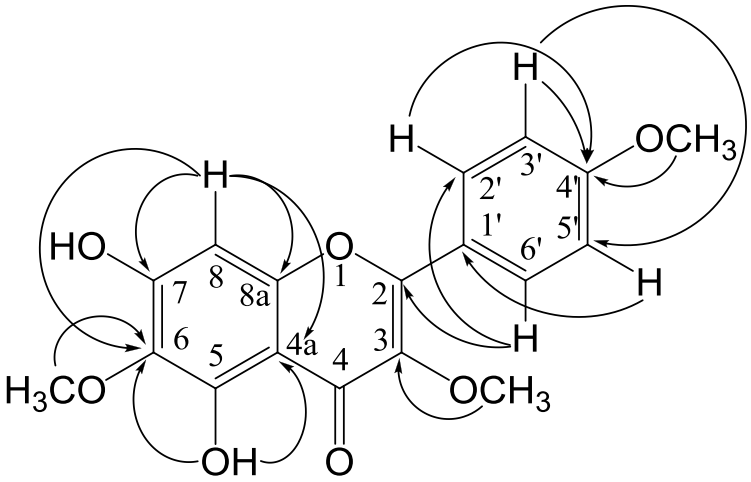


Figure 2
Structure of compound [4] showing major Heteronuclear Multiple Bond Correlations

Compound [**4]** has been previously isolated from the resinous exudates of *Haplopappus bustillosianus* (Urzúa *et al*., 2007).

This is the first report on the chemistry of the resinous exudates of *H. litoralis* from which two methylated derivatives of quercetin **[6]** were isolated: 5-hydroxy-3,7,3',4'-tetrametoxyflavone [**1**] and 5,3'-dihydroxy-3,7,4'-trimethoxyflavone [**2**]. Compound **[2]**, was previously reported from *Haplopappus chrysanthemifolius* (Faini *et al*., 1999), but in that work the method used, was not selective for extracting the plant surface compounds.

The flavonols isolated from *H. chrysanthemifolius*, in this work, were published previously and the purpose of re-study the plant species was to ensure that the flavonols are found in the resinous exudates and to have the material for antibacterial testing.

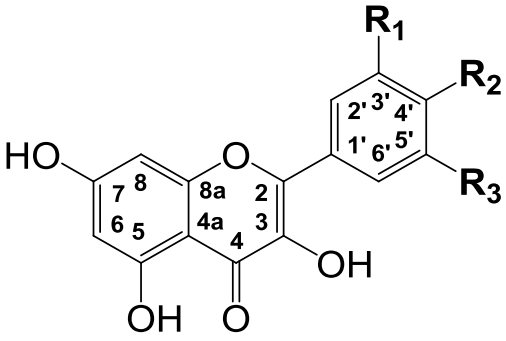
In general, the results of the resinous exudates composition of these three species of *Haplopappus* agree with literature data where the method of extraction was similar. Mixtures of diterpenic acid in combination with small amounts of flavonoids, in the resinous exudates of *Haplopappus*, have been very well documented (Tojo *et al*., 1999; Urzúa *et al*., 2004; Urzúa *et al*., 2006; Urzúa *et al*., 2007). Exceptions were observed to these generalizations. The resinous exudates of *Haplopappus taeda*, is rich in flavonoids, and the resinous exudates of *Haplopappus remyanus* that also is rich in flavonoids contains coumarins (Vogel *et al*., 2005).

Table 3 lists the activities of the resinous exudates, and flavonoids [**1-5]**. The resinous exudates shows activity against Gram-positive bacteria, but the flavonols, also present in the extracts show to be inactive. These results are in agreement with those obtained with the resinous exudates from *H. uncinatus* (Urzúa *et al*., 2006). The antibacterial properties of the resinous exudates was correlated with the presence of the clerodane diterpenoid 18-acetoxy-*cis*-cleroda-3-en-15-oic acid (10β, 16ξ, 19β, 17β, 20α form), but the flavonols 3,5-dihydroxy-6,7,3',4'-tetramethoxyflavone, also present in the extract, has shown to be inactive.

Almost without exception, flavonols that show antimicrobial activity have the characteristic presence of free hydroxyl groups. There are also glycosidic derivatives from these basic skeletons (Cushnie and Lamb, 2005).

Lipophilicity has proved to be an important variable in the antimicrobial activity of natural products. Table 5 shows the estimated lipophilicity of flavonols [**1-5**] isolated from *Haplopappus* resinous exudates that are not antibacterial as well as flavonols well known for its antibacterial properties [**6-9**] (Cushnie and Lamb, 2005). Flavonols with lipophilicity 2.79 and higher do not show antimicrobial activity but flavonols with lipophilicity 2.25 and lower are antibacterial. These results do not agree with the general statement that lipophilic flavonoids found in plant surface extracts are antibacterial (Wollenweber and Dietz, 1981).

This analysis shows that lipophilicity is an important variable to predict the antibacterial activity of flavonols. The correlation between lipophilicity and antibacterial activity of flavonols does not rule out the importance of the existence of other structural factors involved in the activity.



	R_1	R_2	R_3
[6]:	OH	OH	H
[7]:	H	H	H
[8]:	H	OH	H
[9]:	OH	OH	OH

Figure 3
Structures of compounds [6-9].

Table 5
Lipophilicity values of flavonols isolated from *Haplopappus litoralis, H. chrysantemifolius,* H. *scrobiculatus* and common antibacterial flavonols.

Flavonols	Lipophilicity[a]
3,5,7,3',4'-pentahydroxyflavone **[6]**	1.54
3,5,7- trihydroxyflavone **[7]**	2.25
3,5,7,4'-tetrahydroxyflavone **[8]**	1.90
3,5,7,3',4',5'-hexahydroxyflavone **[9]**	1.18
5-hydroxy-3,7,3',4'-tetramethoxyflavone **[1]**	3.47
5,3'-dihydroxy-3,7,4'-trimethoxyflavone **[2]**	3.14
5,3',5'-trihydroxy-3,7,4'-trimethoxyflavone **[3]**	2.79
5,7-dihydroxy-3,6,4'-trimethoxyflavone **[4]**	3.14
5,3'-dihydroxy-6,7,4'-trimethoxyflavone **[5]**	3.14

[a]: Lipophilicity estimated using XLOGP3 (Cheng *et al*., 2007).

Taking into consideration the small amounts of lipophilic flavoids found in the resinous exudates of *Haplopappus* and the fact that clerodane and labdane diterpenoids are the principal components they probably are the antimicrobial active compounds.

The structural features of Gram positive active antibacterial diterpenoids, included a substituted decalinic system (lipophilic region), and a hydrophilic fragment, in general a CO_2H group (Urzúa *et al*., 2008), these structural requirements fits with the structure of most of the diterpenoids present in the resinous exudates of *Haplopappus* (Tojo *et al*., 1999; Urzúa *et al*., 2004; Urzúa *et al*., 2006 and Urzúa *et al*., 2007).

Finally, considering the information presented we suggest that research efforts on the antibacterial capacity of the resinous exudates of *Haplopappus* should be focused on the diterpenes.

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