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Biological activity of a furanoeremophilane isolated from *Senecio filaginoides* var. *filaginoides*

[Actividad biológica de un furanoeremofilano aislado de *Senecio filaginoides* var. *filaginoides*]

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Abstract
Compound, 10αH-9-oxofuranoeremophilane was isolated from *Senecio filaginoides* var. *filaginoides*, and its structure established on the basis of spectral analysis and and comparison with literature data. Antimicrobial activity was demonstrated against *Candida spp* but not against bacteria (*Staphylococcus aureus*, *Escherichia coli* and *Pseudomona aeruginosa*). Besides, the compound showed antifungal activity and effect on mycelial growth of the phytopathogenic fungus *Botrytis cinerea*. and comparison with literature data.

Keywords: ***S****enecio*, furanoeremophilane, antimicrobial activity, antifungal activity, *Botrytis cinerea*..

Resumen
Un compuesto, 10αH-9-oxofuranoeremofilano fue aislado de *Senecio filaginoides* var. *filaginoides*, esta estructura fue establecida sobre la base del análisis espectral y comparación con datos bibliográficos. La actividad antimicrobiana fue demostrada contra *Candida spp* pero no contra las bacterias *(S.aureus, E.coli* y *P. aeruginosa).* Además el compuesto mostró actividad antifúngica y efectos sobre el crecimiento micelial del hongo fitopatógeno *Botrytis cinerea.*

Palabras Clave: *Senecio*, furanoeremofilano, actividad antibacteriana, actividad antifúngica, *Botrytis cinerea*

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LIST OF ABBREVIATIONS: HRP (Herbario Regional Patagónico); ASTM (American Society for Testing and Materials); ATCC (American type culture collection); NIM (Número Instituto Malbrán); MIC (Minimal Inhibition Concentration); ANLIS (Administración Nacional de Laboratorios e Institutos de Salud); DMSO (dimetil sulfóxido).

INTRODUCTION

The genus *Senecio* (Asteraceae) is widely distributed all over the word (except Antarctica and Amazonas) and includes more than 2000 species. *Senecio filaginoides* var. *filaginoides* is one of the 300 species which occur in Argentina and grows from Jujuy to Santa Cruz province (Cabrera, 1971). Many studies of this genus have been focused on essential oil composition, terpenes, pyrrolizidine alkaloids and furanoeremophilanes (Bohlmann *et al*., 1986; Romo de Vivar *et al*., 2007; Mericli *et al*., 1987).The latter have been previously reported on *Senecio filaginoides* (Salmeron *et al*., 1983). It is well known that *Senecio* species are used in folk medicine; their properties are attributed to its essential oils and secondary metabolites such as terpenoids (Peris *et al*., 1996; Alonso *et al*., 2006). In this paper we report the isolation of 9-oxofuranoeremophilane from *Senecio filaginoides*, previously reported from *Petasites hybridus* and *Senecio aureus* (Bohlmann *et al*., 1976; Zalkow *et al*., 1979), but not in *S. filaginoides*.

This compound was analyzed for antimicrobial as well as for the antifungal properties against the phytopathogenic fungus *Botrytis cinerea*. Antimicrobial activity against bacteria and yeast of extracts (Cos *et al.*, 2002), essential oils (Pérez *et al*., 1999; Arancibia *et al.*, 2010; El-Shazly *et al.*, 2002), furanoeremophilanes (Gu *et al* ., 2004), eremophilanes (Mao *et al.*, 2002; Mao *et al.*, 2003) and sesquiterpenoids (Medeiros da Silva *et al.*, 2010) has been previously reported. On the other hand, most secondary compounds play an important defensive role against herbivores, other plants and phytopathogenic fungi; this fact could be useful as a strategy for novel pesticides with less environmental effects (Rimando and Duke, 2006). *Botrytis cinerea* is a phytopathogenic fungus that causes many losses in grape crops. The sesquiterpenoid botrydial and related metabolites produced by the fungus are responsible for the phytotoxic activity, producing necrotic lesions in the host plant (Collado *et al.*, 2007). An important strategy for the control of *B. cinerea* is based on the inhibition of the biosynthesis of these metabolites (Aleu *et al.*, 1999). Eremophilanes and furanoeremophilanes have been previously reported for their inhibitory effect on mycelial growth of *B. cinerea* (Collado *et al.*, 2004; Arias-Casará *et al.*, 2010).

MATERIALS AND METHODS

Plant material

Senecio filaginoides aerial parts were collected in Saavedra, Comodoro Rivadavia city (province of Chubut, Argentina) in May 2004. The plant was extracted and processed in august of the same year. A voucher specimen was authenticated and deposited in the Patagonia Regional Herbarium of Universidad Nacional de la Patagonia San Juan Bosco (UNPSJB) under the following herbarium number: HRP 6159-6170.

Extraction and isolation

Air-dried aerial plant parts (1.1 kg) were powdered, ground and extracted with 95° ethanol at room temperature. The extract was filtered and concentrated under vacuum (40° C) to give a residue (166 g). The whole extract was fractionated by vacuum column chromatography (Silica gel 60, Merck 230-400 mesh ASTM) and eluted with n-hexane:ethyl acetate step gradient (Reina *et al.*, 2001). The fractions were analyzed by thin layer chromatography (Silica gel 60 F254, Merck 0.2 mm thickness) with *n*-hexane: ethyl acetate (90:10) and then revealed by spraying a 25% H_2SO_4 solution followed by heating. Fractions obtained from mixtures with 10% ethyl acetate in hexane, gave compound (**1**) as yellow crystals (7.6 g).

General experimental procedures

All 1D and 2D NMR spectra (COSY, HSQC and HMBC) were recorded on a Bruker Avance 400 MHz spectrometer operating at 400.13 MHz for ^{1}H and 100.61 MHz for ^{13}C nucleus, and equipped with a 5 mm broadband inverse probehead incorporating a z-gradient coil. The spectra were recorded at 300 K from $CDCl_3$ solution. Chemical shifts (in ppm), were calibrated from residual solvent signal and reported relative to Me_4Si.

IR spectra were measured on a Bruker IFS-66 V FTIR spectrophotometer in solid state (KBr cell, 0.2 mm length).

Antimicrobial and antifungal activity

The compound was assayed against eight microorganisms including Gram positive and Gram

negative bacteria and yeast: *Staphylococcus aureus* (ATCC 29213), *Pseudomona aeruginosa* (ATCC 27853), *Escherechia coli* (ATCC 25299), *Candida albicans* (NIM 982879/982891), *Candida tropicalis* (ATCC 2000956), *Candida parapsilopsis* (ATCC 22019), *Candida krusei* (ATCC 6258), donated by Administración Nacional de Laboratorios e Institutos de Salud (ANLIS) Malbrán Institute (Buenos Aires, Argentina). The antimicrobial activity was determined in solid phase by Agar Dilution Method (Arancibia *et al.*, 2010), using sterile nutritive agar for bacteria and 4% glucose-sabouraud as culture medium for yeast. An aliquot from a 2500 µg/ml stock solution of compound (**1**) in dimethyl sulfoxide (DMSO) was inoculated into the liquid warm medium in order to obtain different final concentrations (250, 100 and 50 µg/ml), then stirred for a minute in a vortex at 3000 rpm and allowed to solidify. An inoculum of 10^6 CFU/ml was applied on the medium surface, and then incubated for 24 hours at 37º C (bacteria) and 48 hours at 28º C (yeasts). The minimal inhibitory concentration (MIC) endpoint was determined visually by recording the lowest concentration of the compound that prevented the appearance of visible growth.

Botrytis cinerea: isolation and culture conditions

The strain U29 of *B. cinerea* was isolated from infected grapes (Province of Mendoza, Argentina). The fungus was grown and maintained in Petri dishes on malt-yeast extract agar (1.5% agar, 2% malt extract and 0.2 % yeast extract). Cultures were incubated at 22°C (Wright *et al.*, 1983).

Fungitoxicity assay: effect on mycelial growth of B. cinerea

Antifungal activity of compound (**1**) was assessed using a radial growth inhibition test on malt-yeast extract agar (1.5% agar, 2% malt extract and 0.2 % yeast extract). Compound (**1**) was dissolved in DMSO to obtain different concentrations (0.5, 1, 2, 4, 6, 8 and 10 mg/ml) and 100 µl of this solutions were added to 5 ml of medium (0.6% agar, 2% malt extract and 0.2% yeast extract). The final DMSO concentration was the same in controls and treatment assays. The treated or control culture medium was poured into 9 cm diameter Petri dishes, which were left open in a laminar-flow hood for 30 min to remove the solvent. Cultures were incubated at 22° C for seven days and mycelial growth diameters were measured daily. Each experiment was carried out six times. The percentage of mycelial growth inhibition was calculated as I% = (C-T) 100/C where C is the diameter (cm) of the control and T is the diameter (cm) of the treatment with compound (**1**). Results were analyzed by ANOVA and mean values were compared with the LSD Fisher test ($P \leq 0.05$) (Cotoras *et al.*, 2001).

RESULTS AND DISCUSSION

Structure elucidation

Compound (**1**), 10α,H-9-oxofuranoeremophilane was crystallized from fraction 2 (0.48% of the plant) as yellow crystals. Its molecular formula, $C_{15}H_{20}O_2$ was deduced by comparison with the NIST mass spectral library and spectral data published in the literature (Salmeron *et al.*, 1983). The IR absorption bands at 1708 cm^{-1} (carbonyl group), 1670 cm^{-1} (furan ring), 15 carbon resonances in ^{13}C NMR and 20 hydrogen resonances in ^{1}H NMR suggested this structure (Figure 1). COSY, HSQC and HMBC spectra allowed the assignment of all the protons and carbons (Table 1).

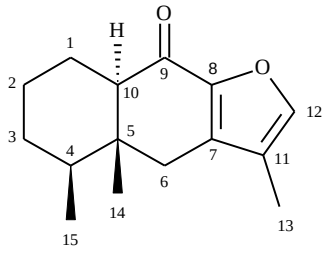


Figure 1: Structure of compound (**1**).

The ^{13}C NMR spectrum shows a keto carbonyl group (C-9, 209.8 ppm), and four further signals in the sp^2 chemical shift range (C-7, C-8, C-11 and C-12) which belong to the furan ring. The structure information accounted for six degrees of insaturation in accordance to its molecular formula and explains the junctions between two cyclohexanes and the furan ring, as well as the presence of a keto carbonyl group. Also four methylenes, two methines and a quaternary carbon were assigned with HMBC and COSY spectroscopy. Besides three methyl groups, characteristic of furanoeremophilanes, were detected in the ^{1}HNMR spectrum: 1.04 (3H, d, J=6.7 Hz), 0.64 (3H, s) and 1.90 (3H, s); the latter is bound to the furan ring and was deduced from the COSY spectrum. The methyl group, C-13 is joined to the furan ring (C-11) due to the correlations between C-12 and H-13 observed in HMBC spectroscopy.

The correlation between H-10 and C-14, as well as between H-14 and C-5, C-6, and C-10, suggested the junction between C-5 and C-10 and

allowed constructing a cyclohexane ring. In addition, the decalin structure was deduced by HMBC correlations between C-9 and H-10, C-6 and H-4 and H-14, and finally, between C-4 and H-6.

The relative configuration of (**1**) was deduced by the ^{1}H-^{1}H coupling constant between H-1 and H-10 (*J*=11.2 Hz), suggesting a trans-fused A/B ring system. This could also be explained by the chemical shift of protons H-14 at 0.64 ppm. This trans-diaxial coupling constant of H-10 is in accordance with other compounds previously reported (Oh, 2005; Gu *et al.*, 2004).

Table 1
NMR spectral data for compound 1: δ values, $CDCl_3$, ^{13}C NMR at 100 MHz, ^{1}H NMR at 400 MHz and HMBC correlations.

C	$\delta^{13}C$	Type of C	$\delta^{1}H(J)$	HMBC of C
1	19.1	CH_2	2.60(1H,ddd,1.51,5.3,17.2) 2.75(1H,m)	H-10
2	33.8	CH_2	2.27(1H,da,15.6) 2.36(1H,dd,1.52,15.6)	H-3, H-10
3	29.9	CH_2	1.68(1H,m) 1.85(1H,m)	H-2, H-4, H-15
4	41.0	CH	1.91 (1H,m)	H-2, H-3, H-6, H-10
5	53.2	C	---	H-1, H-2, H-14
6	40.3	CH_2	2.43(2H,d,4.8)	H-4, H-10, H-14
7	118.5	C	---	H-12,H-13
8	147.1	C	---	H-12
9	209.8	C	---	H-6, H-10
10	53.2	CH	2.53(1H,dd,5.3,11.2)	H-1, H-14
11	114.2	C	---	H-12,H-13
12	136.2	CH	7.05(1H,s)	H-13
13	6.87	CH_3	1.90(3H,s)	---
14	10.7	CH_3	0.64(3H,s)	H-10
15	13.6	CH_3	1.04 (3H,d,6.7)	---

δ is expressed in ppm, multiplicity, number of H, multiplicity and *J* in Hz are shown in parentheses.

Table 2
Antimicrobial activity expressed as minimal inhibitory concentration (MIC)

Microorganism	MIC (µg/ml)
Candida albicans (NIM 982879)	250
Candida albicans (NIM 982891)	250
Candida tropicalis (ATCC 200956)	50
Candida krusei (ATCC 6258)	250
Candida parapsilopsis (ATCC 22019)	250
Staphylococcus aureus (ATCC 29213)	(-)[a]
Escherichia coli (ATCC 25299)	(-)[a]
Pseudomona aeruginosa (ATCC 278531)	(-)[a]

[a]No antimicrobial activity observed in the tested concentrations.

The results on the antimicrobial activity against Gram negative and Grampositive bacteria and yeast are shown (Table 2).

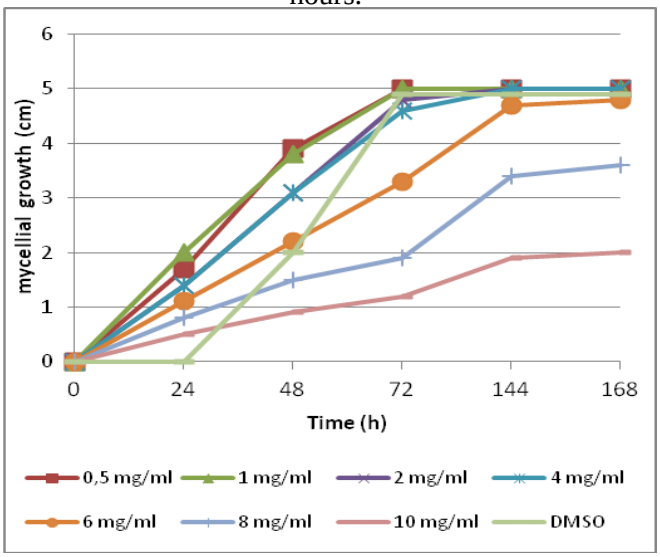
Compound (**1**) was no active against the bacteria tested. However, compound (**1**) showed antifungal properties against some *Candida* species. The most sensitive yeast was *Candida tropicalis* (ATCC 200956), with a minimal inhibitory concentration (MIC) of 50 µg/ml, whereas the other *Candida* strains, exhibited a MIC of 250 µg/ml.

Compound (**1**) reduced the fungal growth of *B. cinerea* on solid culture medium; the most important effect of antifungal activity was observed 72 hours after incubation (Figure 2), at the highest concentrations (Table 3).

Table 3
Antifungal activity against *B.cinerea* expressed as percentage of inhibition of mycelial growth (I%) after 72 hours of incubation

Concentration(**1**) (mg/ml)	Percentage of inhibition of mycelial growth
0.5	0 ± 0
1	0 ± 0
2	5.00 ± 2.05
4	8.00 ± 3.39
6	34.67 ± 6.34
8	61.67 ± 4.54
10	77.00 ± 3.57

Figure 2
Antifungal activity of (**1**) against *B. cinerea* expressed as mycelial growth (cm). (a) Mycelial growth (cm) recorded for different concentrations of (**1**) for 168 hours.



After 96 hours of incubation, the inhibition of radial growth becomes lower for the highest concentrations (8 and 10 mg/ml), whereas, for the remaining concentrations (from 0.5 to 6 mg/ml) the diameter of mycelial growth is not statistically different from those of the control.

CONCLUSIONS

The compound that is reported in this work has been previously isolated (Zalkow *et al.*, 1979; Bohlmann and Zdero, 1986) and synthesized (Bohlmann *et al.*, 1976). The 9-oxofuranoeremophilane, as a pure compound, has not previously been analyzed for its biological activity. Extracts from *Petasites hybridus*, with a minor content of 9-oxofuranoeremophilane, were reported for its anti-inflammatory activity (Fiebich *et al*., 2005).

Finally, the furanoeremophilane isolated from *Senecio filaginoides* shows antifungal activity against *Botritys cinerea.* In addition, the furanoeremophilane was also found to be active against *Candida spp*, leading us to further investigation about the applications of this kind of compounds.

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