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A novel pentacyclic triterpene from *Leontodon filii*

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Abstract

A novel oleanene triterpenetetrol was isolated from the chloroform extract of the aerial parts of *Leontodon filii*. Its structure was shown to be 2β,3β,15α,21β-olean-12-ene-2,3,15,21-tetrol by chemical and spectroscopic methods. The fungicidal efficacy of the chloroform and methanol extracts of the plant was also evaluated, a protective effect being found against *Plasmopara viticola*, *Botrytis cinerea*, particularly powerful against *Pyricularia oryzae*.
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1. Introduction

Leontodon filii (Hochst. ex Seub.) Paiva et Orm [1] is one of some 50 species of the *Leontodon* spp., endemic to the Azores Archipelago and growing wild at 400–800 m above sea-level in S. Miguel, Terceira, S. Jorge, Pico and Flores Islands. This plant is used as a herbal remedy in the Azores, mainly due to its diuretic, choleretic and cholagogue effects.

The natural products described in the literature as constituents of *Leontodon* spp. are mainly sesquiterpene lactones, phenolic compounds, sterols and pentacyclic triterpenes. The occurrence of guaiane type compounds has been reported from two species of the *Leontodon* spp., namely *L. autumnalis* [2,3] and *L. hispidus* [4,5] of which the hypocretenolides were found to possess cytotoxic [5] and antiinflammatory activities [6]. The sesquiterpene lactone glucoside glucozaluzanin C [7] and a germacranolide glucoside [8] were found in the methanol extract of the aerial parts of *L. cichoraceus*. Phenolic compounds were detected in the aerial parts of *L. taraxacoides*. The major compounds were the flavonoids apigenin, luteolin and their glucosides, as well as caffeic acid, and chlorogenic and isochlorogenic acids [9]. HPLC-UV and HPLC-MS analyses of the subgenus Oporinia [10] and of *L. helveticus*, *L. autumnalis* and *L. hispidus* [11] led to the identification of flavonoids, phenolic acids and sesquiterpene lactones.

The pharmacological activities reported for plant sterols and triterpenes include antitumor and cytotoxic, antihypercholesteremic, antiinflammatory, anticonvulsant, antibacterial, analgesic, antitussive and expectorant [12]. The aerial parts of *L. autumnalis* contain campesterol, but β-sitosterol, β-amyrin and α-amyrin, which are minor constituents common in Compositae, were not found in the extract studied [12].

We now wish to present the evidence which led to the establishment of the structure of the major constituents of the chloroform extract of *L. filii*, which include the new triterpenetetrol (1) (Fig. 1), in addition to β-sitosterol, β-amyrin, α-amyrin and its acetate.

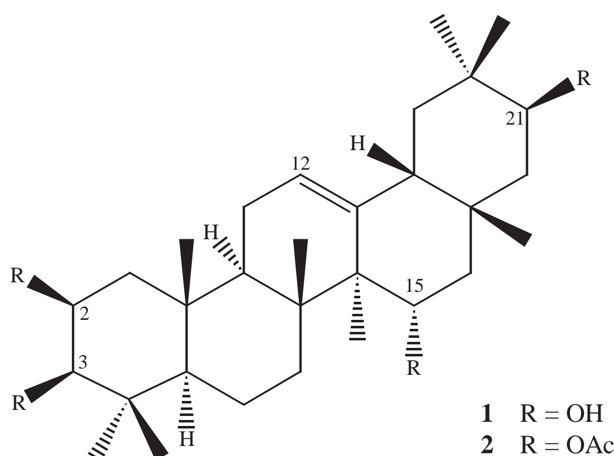

Fig. 1. Structure of Compounds **1** and **2**.

Moreover we assayed the effectiveness of the chloroform and methanol extracts on some plant pathogen fungi, namely *Plasmopara viticola*, *Pyricularia oryzae* and *Botrytis cinerea* with a view to the possible use of the plant extract as a natural pesticide.

2. Experimental

2.1. Methods

Melting points were determined with a digital melting point apparatus (electrothermal) and are uncorrected. The optical rotation was measured with a Perkin–Elmer 343 polarimeter. ^{1}H NMR (400.1 MHz) and ^{13}C NMR (100.6 MHz) spectra, DEPT, HMQC, HMBC, COSY and NOESY spectra were acquired on a Bruker ARX 400, in $CDCl_3$ using TMS as internal standard. The 1D and ^{1}H and ^{13}C were acquired under standard conditions. The 2D inverse hydrogen detected hetereonuclear shift correlation spectrum was acquired with the gradient selected HMQC pulse sequence [^{1}J(C,H)] with 256 time increments collected for each dataset and zero filling to 512, and sixteen transients were collected for each time increment. The 2D inverse hydrogen detected hetereonuclear long-range shift correlation was carried out with the gradient selected HMBC pulse sequence [^{n}J(C,H) optimized for 7 Hz] with 256 time increments collected for each dataset and zero filling to 512, thirty-two transients being collected for each time increment. For 2D ^{1}H,^{1}H NOESY the gradient selected pulse sequence was used with 1.5 s as the mixing time, ten transients were collected for each of the 256 time increments and zero filled to 512 [13].

IR spectra (cm^{-1}) were obtained on a Mattson Satellite FTIR spectrophotometer in KBr pellets. Low-resolution EIMS were taken at 70 eV using a Hewlett-Packard 5930 spectrometer, and HRMS was carried out with a Micromass VG ZAB-2F spectrometer. Analytical and PTLC were carried out on Merck 60 GF_{254} silica gel plates (absorbent thickness: 0.25 and 0.75 mm, respectively). CC was performed using silica gel 60G (0.040–0.063 mm, E. Merck) and eluted under low pressure. The standard compounds α-amyrin, β-amyrin and β-sitosterol were supplied by Extrasynthese, France.

2.2. Plant material

L. filii aerial parts, collected in July, in the Azores were dried in the shade. A voucher specimen nr. INOVA-129 is deposited in the Museum "Carlos Machado" Herbarium (Azores).

2.3. Extraction and isolation

The air-dried and finely powdered aerial parts of *L. filii* (266 g) were extracted with $CHCl_3$ at room temperature. Removal of the solvent under reduced pressure gave an extract (6.5 g), which was subjected to CC eluted with EtOAc/hexane mixtures of increasing polarity. The two eluates (A—1.5 g and B—430 mg) containing the major compounds of the extract were refractionated with benzene (A) to give α-amyrin acetate

(17 mg), β-amyrin (126 mg), α-amyrin (130 mg) and β-sitosterol (200 mg), and (B) with EtOAc/toluene (1:3) affording the new triterpenetetrol 1 (90 mg).

2β,3β,15α,21β-Olean-12-ene-2,3,15,21-tetrol (**1**). Colourless needles, mp 261–263 °C; $[\alpha]_{D}^{20}$+40.0° (c 0.1, MeOH); IR bands (KBr) 3442 (OH), 1602 (>C=CH–) cm^{-1}; EIMS *m/z* (rel. int.%): 474 $[M]^+$(4.3), 456 $[M–H_2O]^+$(6.9), 441 $[M–H_2O–Me]^+$(4.2), 438 $[M^+–2H_2O]^+$(5.5), 423 $[M–2H_2O–Me]^+$(4.4), 250 $[C_{16}H_{24}(OH)_2]^+$(89), 232 $[C_{16}H_{24}O]^+$(61), 203 $[C_{15}H_{23}]^+$(100); HRMS (EI) *m/z*: 474.37090, (Calc. for $C_{30}H_{50}O_4$: 474.37091), 456.36486 $[M–H_2O]^+$, 441.33453 $[M–H_2O–Me]^+$, 438.35305 $[M^+–2H_2O]^+$, 423.32594 $[M–2H_2O–Me]^+$, 250.19363 $[C_{16}H_{24}(OH)_2]^+$, 232.18433 $[C_{16}H_{24}O]^+$, 203.17957 $[C_{15}H_{23}]^+$. ^{1}H NMR and ^{13}C NMR see Table 1.

2β,3β,15α,21β-Olean-12-ene-2,3,15,21-tetroltetraacetate (**2**). Acetic anhydride (45 ml) was added to a solution of **1** (30 mg) in pyridine (1 ml) and the reaction mixture was stirred at r.t. for 24 h. Co-evaporation with toluene gave 2β,3β,15α,21β-olean-12-ene-2,3,15,21-tetroltetraacetate (**2**) (35 mg, 88%) as a syrup. IR bands (KBr): 1740, 1725

Table 1
^{1}H and ^{13}C NMR data of compound **1**[a]

C	^{13}C	^{1}H	HMBC
1	44.18	1.12 *m* (H-1α), 2.11 *m* (H-1β)	Me-25, H-9, H-5
2	71.05	4.09 *br dd* ($J_{2\alpha,3\alpha}$ =3.8)	–
3	78.39	3.22 *d*	Me-24, Me-23, H-1α, H-1β
4	40.10	–	Me-25, H-9, H-7α, H-7β
5	55.11	0.81–0.87 *m*	Me-25, Me-24, Me-23, H-7α, H-1α, H-1β
6	18.08	0.81–0.87 *m* (H-6α), 1.55 *m* (H-6β)	Me-26, Me-24, Me-23, H-3, H-1α, H-1β
7	31.97	1.36 *m* (H-7α), 1.52 *m* (H-7β)	Me-27, Me-26, H-9, H-5
8	41.08	–	Me-27, Me-25, H-1β
9	47.24	1.48 *m*	Me-27, Me-26, Me-25, H-7α
10	38.10	–	Me-26, Me-24, Me-23
11	23.63	1.88 *m* (H-11α), 1.97 *m* (H-11β)	Me-26, H-18β, H-9, H-1α
12	123.12	5.29 *br s*	H-18
13	142.06	–	Me-27, H-19α, H-19β, H-11α
14	42.80	–	Me-26, H-19α, H-19β, H-18, H-16β, H-9, H-7α
15	66.99	4.56 *dd* ($J_{15\beta,16\alpha}$ 11.2, $J_{15\beta,16\beta}$ 5.0)	M-28, Me-27, H-22β, H-21
16	35.63	1.26 *m* (H-16α), 1.63–1.68 *m* (H-16β)	Me-27
17	36.50	–	H-19α, H-19β, H-15
18	45.87	1.78 *m*	Me-30, Me-29, Me-27, H-16β
19	49.25	2.08 *m* (H-19β), 2.13 *m* (H-19α)	Me-30, Me-29, Me-28, H-16α
20	32.50	–	H-22β, H-18
21	78.84	3.74 *dd* ($J_{21\alpha,22\alpha}$ 4.0, $J_{21\alpha,22\beta}$ 12.4)	Me-30, Me-29, Me-28, H-19α, H-19β, H-16β
22	43.31	1.59 *m* (H-22α), 1.63–1.68 *m* (H-22β)	Me-30, Me-29, H-16α, H-16β
23	29.69	1.02 *s*	H-5, H-3
24	17.29	1.02 *s*	H-5, H-3
25	16.44	1.26 *s*	H-9, H-1α, H-1β
26	16.75	0.95 *s*	H-9, H-7α, H-7β
27	27.48	1.23 *s*	H-15β
28	18.67	1.09 *s*	H-22α, H-22β, H-16
29	33.31	0.95 *s*	H-22β, H-18
30	24.94	1.02 *s*	H-22β, H-18

[a] δ in ppm, *J* in Hz.

(C=O, Ac) cm^{-1}; ^1H NMR: δ 0.92, 0.97, 0.99, 1.03, 1.07, 1.23, 1.27, 1.61 (each s, 3H, Me-23, Me-24, Me-25, Me-26, Me-27, Me-28, Me-29, Me-30), 2.02, 2.04, 2.05, 2.06 (each *s*, 3H, OAc), 4.63 (1H, *d*, $J_{2\alpha,3\alpha}$ 4.0 Hz , H-3α,), 4.89 (1H, *dd*, $J_{21\alpha,22\alpha}$ 4.0 Hz and $J_{21\alpha,22\beta}$ 12.0 Hz, H-21α), 5.25–5.31 (2 H, *m*, H-2α, H-12), 5.79 (1 H, *dd,* $J_{15\beta,16\alpha}$ 11.9 Hz and $J_{15\beta,16\beta}$ 5.5 Hz, H-15β); EIMS *m*/*z* (rel. int.%): 582 $[M–HOAc]^+$(1.3), 522 $[M–2HOAc]^+$(8.8), 462 $[M–3HOAc]^+$(3.4), 402 $[M–4HOAc]^+$(32), 387 $[M–4HOAc–Me]^+$(15.7), 274 $[M–C_{18}H_{28}O_4–HOAc]^+$(88.6), 214 $[M–C_{18}H_{28}O_4–2HOAc]^+$(90.8), 201 (61), 199 $[M–C_{18}H_{28}O_4–2HOAc–Me]^+$(89.8), 60 $[HOAc]^+$(100).

2.4. Biological assays

Detection of fungicidal efficacy of the crude methanol and chloroform extract solutions against *Botrytis cinerea*, *Erysiphe graminis*, *Fusarium culmorum*, *Plasmopara vitícola*, and *Pyricularia oryzae* was performed in greenhouse using the plants *Capsicum annum* (pepper) (*B. cinerea)*, *Triticum aestivum* (wheat) (*E. graminis*, *F. culmorum*), *Vitis vinifera* (grapevine) (*P. viticola*) and *Oryza sativa* (rice) (*P. oryzae*). The crude extract (200 mg) was dissolved in acetone/water 9:1 (v/v) (200 ml). Each tested plant was sprayed with the extract solution (50 ml). One day (*C. annum*, *T. aestivum*, *E. graminis*, *F. culmorum*, *O. sativa*) or 1 week (*V. vinifera*) after spraying, the plant was inoculated by spraying with the spore suspension (10^6 spores/ml) of the pathogenic fungus. The results of the protective effect of the extracts were evaluated after 1 week of incubation using a scale from 0 to 8 (0—total infection; 8—complete control of the infection). The screening includes a control test, in which the plant is inoculated without being previously sprayed with the crude extract, and also a standard test with the commercial specific fungicides for the tested fungi with well-known efficacy.

3. Results and discussion

3.1. Structure elucidation

The chloroform extract of the aerial parts of *L. filii* was subjected to column chromatography to give the new compound **1**, together with β-sitosterol, β-amyrin, α-amyrin and α-amyrin acetate. Compound **1** gave a positive Liebermann–Burchard reaction and had a molecular formula of $C_{30}H_{50}O_4$ (HRMS *m*/*z* 474.37090).

The IR absorption band of **1** at 3442 cm^{-1} indicates the presence of the hydroxyl groups. Acetylation of compound **1** gave the tetraacetate **2** whose ^1H NMR spectrum exhibited the signals of the four acetoxymethyl groups detected at δ 2.02, 2.04, 2.05 and 2.06. The EIMS spectrum of this compound showed peaks characteristic of the fragmentation of one to four molecules of acetic acid at *m*/*z* 582 (1.3%), *m*/*z* 522 (8.8%), *m*/*z* 462 (3.4%) and *m*/*z* 402 (32%). The retro Diels–Alder fragmentation with loss of one or two molecules of acetic acid gave the fragment ions at *m*/*z* 274 and 214.

^1H NMR and ^{13}C NMR data of the oleanene tetrol **1** are given in Table 1. Two-dimensional correlation experiments (COSY, HMQC, HMBC and NOESY) were carried out in order to deduce that four hydroxyl groups are present at positions 2, 3 on ring A,

position 15 on ring D and position 21 on ring E. The olefinic proton at δ 5.29 (H-12) is characteristic of the oleanene skeleton type, together with the presence of eight methyl groups, appearing as singlets at δ 0.95 (2 Me), δ 1.02 (3 Me), δ 1.09, 1.23 and δ 1.26. The resonance of H-2 appears at δ 4.09 as *br dd*, coupled with H-3 at δ 3.22 with a coupling constant of $J_{2,3}$ 3.6 Hz, consistent with an equatorial/axial coupling of two vicinal *cis*-protons. The NOESY spectrum presented the correlation of H-3 with the multiplet at δ 0.81–0.87, which includes H-5 and H-6α,β, from which the orientation of OH-2 and OH-3, both in position β, can be confirmed (Fig. 2). H-15 appears as a *dd* at δ 4.54, the signal corresponding to C-15 being detected at δ 67.04 by HMQC. The coupling constants $J_{15\beta,16\alpha}$ 11.2 Hz and $J_{15\beta,16\beta}$ 5.0 Hz are in agreement with OH-15 in α-position, which is also confirmed by NOE experiments, due to the observed interactions of H-15 with H-18, with Me-28, and with the multiplet at δ 1.63–1.68, which includes H-16α and H-16β. The resonance of H-21 at δ 3.74 is a *dd* due to the coupling with H-22α and H-22β presenting a $J_{21\alpha,22\alpha}$ 4.0 Hz and $J_{21\alpha,22\beta}$ 12.4 Hz suggesting that H-21 is in α-orientation, which is confirmed by its NOESY correlations with H-19α, H-22α, Me-29 and Me-30.

The ^{13}C NMR chemical shifts of the detected signals for the methyl groups of **1** are in the same order of magnitude as the corresponding methyl groups of the β-amyrin skeleton [14] with the exception of Me-28 which appears at δ 18.7, due to the presence of OH-15 and OH-21. Their assignments and those of C-4, C-8, C-10, C-14, C-17 and C-20 were confirmed by HMBC (Table 1).

The HRMS pattern also supports the proposed structure. The loss of a water molecule from the molecular ion resulted in the peak corresponding to the fragment at *m*/*z* 456. The other three peaks are due to the loss of water molecules and methyl groups respectively at *m*/*z* 441 $[M–H_2O–Me]^+$, at *m*/*z* 438 $[M^+–2H_2O]^+$and *m*/*z* 423 $[M^+–2H_2O–Me]^+$. The *retro* Diels–Alder fragmentation results in the fragment ion at *m*/*z* 250, which is characteristic of Δ^{12}-unsaturated pentacyclic triterpenes and contains the D and E rings corresponding to $[C_{16}H_{26}O_2]^+$, confirming the presence of two hydroxyl groups in these rings. The loss of water gave the fragment $[C_{16}H_{24}O]^+$. The ion peak at *m*/*z* 203 corresponds to the fragment ion $[C_{15}H_{23}]^+$.

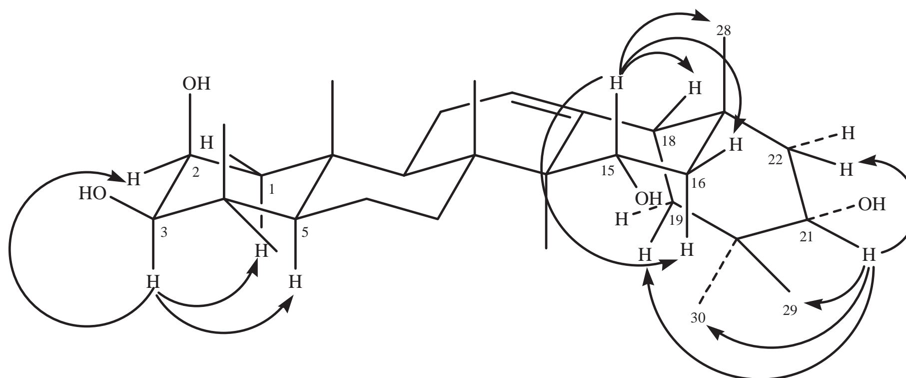

Fig. 2. NOESY correlations of **1** for the assignment of the 2, 3, 15, 21 chiral centres stereochemistry.

Table 2
Fungicidal activity of the *L. filii* aerial parts crude extracts[a]

Fungi	Plant tested	Crude extract (1000 ppm)		Control[b]	Standard[c]
		Chloroform	Methanol		
Botrytis cinerea	*Capsicum annum* (pepper)	5	3	2	7
Erysiphe graminis	*Triticum aestivum* (wheat)	2	2	2	8
Fusarium culmorum	*Triticum aestivum* (wheat)	2	2	2	8
Plasmopara vitícola	*Vitis vinifera* (grapevine)	4	4	2	8
Pyricularia oryzae	*Oryza sativa* (rice)	7	7	4	8

[a] 0—No effect (total infection), 2—hardly any effect (heavy infection), 3—moderate/heavy infection, 4—slight efficacy (moderate infection), 5—moderate efficacy (light/ moderate infection), 6—good efficacy (light infection), 7—intermittent infection, 8—very good efficacy (no infection).

[b] Assay without crude extract.

[c] Standard fungicides: ROVRAL (*Botrytis cinerea*), CORBEL (*Erysiphe graminis*), BENOMYL (*Fusarium culmorum*), POLYRAM COMBI (*Plasmopara vitícola*), BEAM (*Pyricularia oryzae*).

3.2. Fungicidal activity

A total of five microorganisms were used, namely *E. graminis*, *F. culmorum*, *P. vitícola*, *P. oryzae* and *B. cinerea*. These fungi are often responsible for the decay of foodstuff, and damage of economically important crops such as wheat, wine, rice and pepper. The plants tested (Table 2) were previously sprayed with methanol or chloroform extract solutions in acetone/water 1:9 (v/v) and then inoculated with the tested fungi spore suspension (10^6 spores/ml, distilled water). Control tests were prepared using the same procedure in the absence of the extract solution. The protective effect of the extracts was assessed based on comparison with the results obtained using standard fungicides under the same experimental conditions (Table 2). These preliminary tests showed that the chloroform extract is moderately efficient against *B. cinerea*, while both extracts are considered good rice protective agents, exhibiting a high level of inhibition of *P. oryzae*, which produces only intermittent infection in the tested plant. These extracts also demonstrated slight efficacy against *P. viticola*.

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References

[1] Amaral-Franco J. Nova flora de Portugal (Continente e Açores). Vol II. Lisboa: Sociedade Astória Ltd.; 1984. p. 505. [Translation of flora Europeae II and IV. Tutin TG, Heywood VW, Burges NA, Moore DM, Valentine DH, Walters SM, Webb DA, editors. Cambridge: Univ. Press.].
[2] Pyrek St. J. Phytochemistry 1985;24:186.
[3] Zidorn C, Ellmerer-Müller EP, Ongania K-H, Sturm S, Stuppner H. J Nat Prod 2000;63:812.
[4] Zidorn C, Ellmerer-Müller EP, Stuppner H. Phytochemistry 1998;49:797.

[5] Zidorn C, Stuppner H, Tiefenthaler M, Konwalinka G. J Nat Prod 1999;62:984.
[6] Zidorn C, Dirsch VM, Rungeler P, Sosa S, Della Loggia R, Merfort I, et al. Planta Med 1999;65:704.
[7] Zidorn C, Ellmerer-Müller EP, Stuppner H. Biochem Syst Ecol 2001;29:545.
[8] Zidorn C, Ellmerer-Müller EP, Dobner MJ, Stuppner H. Z Naturforsch, C 2001;56:904.
[9] Giner RM, Recio MC, Cuellar MJ, Máñez S, Peris JB, Stübing G, et al. Biochem Syst Ecol 1993;21:613.
[10] Zidorn C, Stuppner H. Biochem Syst Ecol 2001;29:827.
[11] Zidorn C, Stuppner H. Taxon 2001;50:115.
[12] Hooper SN, Chandler RF. J Ethnopharmacol 1984;10:181.
[13] Braun S, Kalinowski H-O, Berger S. 150 and more basic NMR experiments: a practical course. 2ed. Weinheim: Wiley-VCH; 1998. pp. 485, 489, 517.
[14] Heupel RC. Phytochemistry 1985;24:2929.