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Short communication

Composition of a volatile extract of *Eryngium duriaei* subsp. *juresianum* (M. Laínz) M. Laínz, signalised by the antifungal activity

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ABSTRACT

The composition of a volatile extract of *Eryngium duriaei* subsp. *juresianum*, signalised by the antifungal activity (MIC values = 0.16–0.32 μL mL^{-1}) against several dermatophyte species (*Trichophyton mentagrophytes*, *T. rubrum*, *Epidermophyton floccosum*; *T. verrucosum*, *T. mentagrophytes var interdigitale*, *Microsporum canis* and *M. gypseum*) was established following a combined methodology of GC, GC–MS and an exclusive ^{13}C NMR technique that does not require prior isolation of compounds. Twenty-five components were identified accounting 84.6% of the whole composition. Major compound was found to be α-neocallitropsene (26.0%) although the dominance of caryophyllane derived compounds, the most probable responsible for the antifungal activity, namely isocaryophyllen-14-al (16.2%), 14-hidroxy-β-caryophyllene (13.4%), caryophyllene oxide (7.6%) and *E*-β-caryophyllene (6.3%).

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1. Introduction

Exploring Iberian plants as resources of natural compounds available for the research of bioactive molecules and drug leads, several plant extracts have been prepared to be probed in activity *screening* programs hosted at the Drug Discovery Group, Center for Pharmaceutical Studies/Faculty of Pharmacy, University of Coimbra. The volatile extract isolated from the aerial parts of *Eryngium duriaei* Gay ex Boiss. subsp. *juresianum* (M. Laínz) M. Laínz was included in the screening for activity against dermatophyte fungi and yeasts species. In order to contribute for the knowledge of the composition of that volatile extract, aiming also the assignment of the activity to specific constituent(s), we undertook an exhaustive phytochemical analysis by combination of GC, GC–MS and ^{13}C NMR techniques. As our knowledge, this is the first report on the biological activity and on the chemistry of *Eryngium duriaei* Gay ex Boiss. subsp. *juresianum* (M.Laínz) M. Laínz.

The genus *Eryngium* L. is probably the most extensive and taxonomically complex genus of Apiaceae family, including about 250 species distributed all around the world, mainly in Eurasia, North of Africa and South of America. Although the medicinal usefulness of several species employed in traditional medicines for preparation of diuretic, appetite stimulant, laxative or anti-inflammatory remedies, only few of them were object of bioactivity surveys or chemical investigations. A noteworthy part of the chemical investigations and reports concerns to the composition of the volatile extracts [1–7], some of them revealing uncommon peculiar compositions dominated by methyl-derivatives of benzaldehyde, ex. *Eryngium corniculatum* [3], *E. amethystinum* [4] or *E. foetidum* [5] or rare sesquiterpenic compounds, ex.: muurol-9-en-15-al, cadina-9-en-15-al and cadina-9-en-15-ol from *Eryngium maritimum* [6] or eryng-9-en-15-al from *Eryngium creticum* [7]. Many of these plants are endangered or have a *sparse occurrence.* In Portugal a dozen of *taxa* grow wild, including the Iberian endemisms, *E. galioides*, *E. duriaei* subsp. *duriaei* and *E. duriaei* subsp. *juresianum*.

E. duriaei is an erect, spinosous and perennial herb, measuring 30–100 cm. The leaves show variable features: whereas plants from lower altitudes have the basal leaves more or less linear-spathulate and not undulate with a denticulate margin, those of higher altitudes have narrower basal leaves, linear-oblanceolate, undulate, pinnatifite, showing a regularly sinuate-dentate margin. According these different morphologies Laínz considered two different *taxa* [8–10], first at specific level [9], *E. juresianum* (M. Laínz) M. Laínz and *E. duriaei* J. Gay ex Boiss., latter defining two subspecies *E. duriaei* subsp. *juresianum* and *E. duriaei* subsp. *duriaei* [10].

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Table 1
Antifungal activity (MIC and MLC) of *Eryngium duriaei* subsp. *juresianum* volatile oil.

Strains	*Eryngium* oil		Fluconazole	
	MIC	MLC	MIC	MLC
Candida albicans ATCC 10231	>20	>20	1	>128
Candida tropicalis ATCC 13803	>20	>20	4	>128
Candida krusei H9	>20	>20	64	64–128
Candida guillermondii MAT23	2.5	2.5	8	8
Candida parapsilosis ATCC 90018	>20	>20	<1	<1
Cryptococcus neoformans CECT 1078	0.64	0.64–1.25	16	128
T. mentagrophytes FF7	0.16	0.32	16–32	32–64
Microsporum canis FF1	0.32	0.32	128	128
Trichophyton rubrum CECT 2794	0.16	0.32	16	64
M. gypseum CECT 2905	0.32	0.32–0.64	128	>128
Epidermophyton floccosum FF9	0.16	0.16	16	16
Trichophyton mentagrophytes var interdigitale CECT 2958	0.32	0.32	N.T	N.T
T. verrucosum CECT2992	0.16–0.32	0.32	N.T	N.T

N.T. = not tested. Results were obtained from 3 independent experiments performed in duplicate.
[a]MIC and MLC were determined by a macrodilution method and expressed in $\mu L\,mL^{-1}$ (V/V).
[b]MIC and MLC were determined by a macrodilution method and expressed in $\mu g/mL^{-1}$ (W/V).

2. Materials and methods

2.1. Plant material

Eryngium duriaei Gay ex Boiss. subsp. *juresianum* (M. Laínz) M. Laínz was collected in Serra-do-Açor, Portugal, near the village of Piodão. A voucher specimen was prepared for taxonomic determination and deposited in the Herbarium of the Botanic Institute of Coimbra University (COI).

The aerial parts of the plant were submitted to water distillation during 4 h using a Clevenger-type apparatus according the procedure described in the European Pharmacopoeia [11], rending a pale yellowish lighter water immiscible liquid with unpleasant woody odour. This oil was obtained at the yield of 0.15%.

2.2. Antifungal activity

The activity was evaluated against seven dermatophyte species – three isolated from nails and skin, *Microsporum canis* FF1, *Trichophyton mentagrophytes* FF7 and *Epidermophyton floccosum* FF9, four from Colección Española de Cultivos Tipo, *M. gypseum* CECT 2905, *T. rubrum* CECT 2794, *T. mentagrophytes var interdigitale* CECT 2958 and *T. verrucosum* CECT 2992; five *Candida* species, two clinical isolates from recurrent cases of vulvovaginal or oral candidosis, *C. krusei* (H9) and *C. guillermondii* MAT23 and three reference species from the American Type Culture Collection, *C. albicans* ATCC 10231, *C. tropicalis* ATCC 13803, and *C. parapsilosis* ATCC 90018; a strain of *Cryptococcus neoformans* CECT 1078. Clinical isolates were identified by standard methods.

A macrodilution broth method was used to determine the Minimal Inhibitory Concentrations (MIC) and Minimal Lethal Concentrations (MLC), according to NCCLS references M27-A2 [12] and M38A [13], for yeasts and filamentous fungi, respectively.

2.3. Extract analysis

Analysis of the volatile oil was carried out by combination of gas chromatography (GC), gas chromatography–mass spectroscopy (GC–MS) and by ^{13}C Nuclear Magnetic Resonance (^{13}C NMR) without previous isolation of compounds.

Analytical GC was carried out in a Hewlett-Packard 6890 (Agilent Technologies, Palo Alto, CA, USA) chromatograph with a HP GC ChemStation Rev. A.05.04 data handling system, equipped with a single injector and two flame ionization detectors (FID). A graphpak divider (Agilent Technologies, part no. 5021-7148) was used for simultaneous sampling to two Supelco (Supelco, Bellefonte, PA, USA) fused silica capillary columns with different stationary phases: SPB-1 (polydimethylsiloxane 30 m × 0.20 mm i.d., film thickness 0.20 μm), and SupelcoWax-10 (polyethyleneglycol 30 m × 0.20 mm i.d., film thickness 0.20 μm). Oven temperature program: 70–220 °C (3 °C min^{-1}), 220 °C (15 min); injector temperature: 250 °C; carrier gas: helium, adjusted to a linear velocity of 30 cm s^{-1}; splitting ratio 1:40; detectors temperature: 250 °C.

GC–MS was carried out in a Hewlett-Packard 6890 gas chromatograph fitted with a HP1 fused silica column (polydimethylsiloxane 30 m × 0.25 mm i.d., film thickness 0.25 μm), interfaced with an Hewlett-Packard mass selective detector 5973 (Agilent Technologies) operated by HP Enhanced ChemStation software, version A.03.00. GC parameters as described above; interface temperature: 250 °C; MS source temperature: 230 °C; MS quadrupole temperature: 150 °C; ionization energy: 70 eV; ionization current: 60 μA; scan range: 35–350 units; scans s^{-1}: 4.51.

^{13}C NMR spectrum of the mixture was recorded on a Bruker 400 Avance Fourier Transform spectrometer operating at 100.13 MHz for ^{13}C, equipped with a 5 mm probe, in deuteriochloroform ($CDCl_3$), with all shifts referred to internal tetramethylsilane (TMS). Spectrum was recorded with the following parameters: pulse width = 4 μs (flip angle 45°); acquisition time = 2.7 s for 128 K data table with a spectral width of 25,000 Hz (250 ppm); digital resolution = 0.183 Hz/pt. The number of accumulated scans was 3000 (50 mg of the oil in 0.5 mL $CDCl_3$).

Components of the volatile oil were identified by: (i) their retention indices on both SPB-1 and SupelcoWax-10 columns, calculated by linear interpolation relative to retention times of C_8–C_{24} of *n*-alkanes and compared with those of reference compounds included in CEF laboratory database or literature data [14]; (ii) their mass spectra by matching with reference spectra from the CEF laboratory own spectral database, Wiley/NIST database or literature data [14–16]; (iii) ^{13}C NMR spectroscopy (major compounds), following the methodology developed and computerized in UMR-CNRS 6134 laboratories, using a lab-made software and spectral data library [17,18]. Isocaryophyllen-14-al (β-betulenal) and 14-hydroxy-β-caryophyllene were identified by comparison of their spectral data with those reported in the literature [19,20]. Relative amounts of individual components were calculated based on GC raw data areas without FID response factor correction.

3. Results and discussion

Antifungal activity of the volatile extract of *E. duriaei* subsp. *juresianum* was signalised owing to data presented in Table 1. Activity

Table 2
Composition of the volatile oil of *Eryngium duriaei* subsp. *juresianum*.

RI[a]	RI[b]	Spectroscopy	Compounds	%
929	1030	MS; ^{13}C NMR	α-Pinene	0.7
978	1290	MS	*n*-Octanal	0.4[c]
978	1161	MS	Myrcene	*t*[c]
1019	1206	MS	Limonene	*t*
1024	1237	MS	*Z*-β-Ocimene	0.3
1034	1255	MS; ^{13}C NMR	*E*-β-Ocimene	0.9
1079	1393	MS	*n*-Nonanal	*t*
1181	1495	MS	*n*-Decanal	0.6
1235	n.d	MS	2-Decenal	0.2
1327	n.d.	MS	Bicycloelemene	*t*
1386	1585	MS	β-Elemene	0.7[c]
1386	n.d.	MS	*n*-Dodecanal	*t*[c]
1408	1595	MS; ^{13}C NMR	*E*-β-Caryophyllene	6.3
1440	1665	MS	α-Humulene	0.4
1444	1665	MS	*E*-β-Farnesene	0.4
1461	1685	MS; ^{13}C NMR	α-Neocallitropsene	26.0
1468	1676	MS	γ-Curcumene	0.8
1471	1711	MS; ^{13}C NMR	β-Selinene	3.0
1481	1727	MS; ^{13}C NMR	Bicyclogermacrene	3.8
1545	n.d.		Unknown 1	1.5
1556	1969	MS; ^{13}C NMR	Caryophyllene oxide	7.6[c]
1556	2112	MS; ^{13}C NMR	Spathulenol	1.4[c]
1561	2063	MS	Globulol	0.5
1586	2025	MS	Humulene epoxide II	1.0
1615	2145	MS; ^{13}C NMR	Isocaryophyllen-14-al (β-Betulenal)	16.2
1621	n.d.		Unknown 2	1.0
1637	2344	MS; ^{13}C NMR	14-Hydroxy-β-caryophyllene[d]	13.4
1655	n.d.		Unknown 3	1.2
			Monoterpene hydrocarbons	1.9
			Sesquiterpene hydrocarbons	41.4
			Oxygen-containing sesquiterpenes	40.1
			Aliphatic compounds	1.2
			Total identified	84.6

Compounds listed in order to their elution on the SPB-1 column; t: traces; n.d.: not determined.
Mass spectra of unknown compounds: *m*/*z* (relative intensity):
Unknown 1: 220 [M$^+$] (3), 43 (100), 91 (87), 119 (87), 123 (87), 132 (81), 41 (70), 105 (70), 109 (69), 107 (63).
Unknown 2: 218 [M$^+$] (2), 119 (100), 91 (48), 105 (41), 93 (40), 132 (36), 145 (32), 79 (29), 77 (29), 41 (28).
Unknown 3: 218 [M$^+$] (5), 107 (100), 150 (71), 135 (47), 41 (33), 79 (30), 91 (30), 53 (23), 77 (22), 108 (20).
[a] Retention indices on the SPB-1 column relative to C8–C24 *n*-alkanes.
[b] Retention indices on the SupelcoWax-10 column relative to C8 to C24 *n*-alkanes.
[c] Quantification based on peak areas from SupelcoWax-10 chromatogram.
[d] β·α- and β·β-conformers at identical proportions.

was revealed against dermatophyte species, with MIC values ranging from 0.16 to 0.32 μL mL^{-1}.

Twenty-five components (Table 2) were identified in the extract, accounting 84.6% of the whole composition. It is predominantly composed by sesquiterpenic hydrocarbons and oxygen-containing sesquiterpenes at identical proportions. α-Neocallitropsene (iso-α-acoradiene) (26.0%), and the caryophyllane derivatives, isocaryophyllen-14-al (β-betulenal) (16.2%), 14-hydroxy-β-caryophyllene (13.4%), caryophyllene oxide (7.6%) and *E*-β-caryophyllene (6.3%) were found to be the major constituents.

All other components are in concentrations under 4%. Three compounds, at non-negligible amounts (≥1.0%) could not be identified. Their mass spectra appear at the end of Table 2.

^{13}C NMR, used as complementary technique for major compounds identification, was particularly useful in the cases for which GC retention indices and mass spectra were not conclusive. Indeed, this methodology allowed to identify of β-betulenal (isocaryophyllen-14-al) instead its isomer caryophyllene-14-al (α-betulenal) [19]. Furthermore, ^{13}C NMR signals allowed to distinguishing α-neocallitropsene (*iso*-α-acoradiene) from its epimer, α-acoradiene. ^{13}C NMR also revealed carbon resonance signals of both, β·α- and β·β-, 14-hydroxy-β-caryophyllene conformers [20].

Considering previous reports on the biocidal activity of caryophyllane derived compounds [21,22], the antifungal activity of the volatile oil of *E. duriaei* subsp. *juresianum* can be related with the occurrence, at noteworthy concentrations, of β-betulenal (isocaryophyllen-14-al), 14-hydroxy-β-caryophyllene and caryophyllene oxide. In fact, Yang et al. [21] stated the effectiveness of caryophyllene oxide as antifungal agent in an *in vitro* experimental model of onychomycosis; Demirci et al. [22] revealed the activity of a saturated analogue of 14-hydroxy-β-caryophyllene, 14-hydroxy-4,5-dihydro-β-caryophyllene, against plant pathogenic fungi.

4. Conclusions

The composition of the volatile oil of *E. duriaei* subsp. *juresianum*, an endangered Iberian endemism, was, for the first time, established by means of a combination of GC, GC–MS and ^{13}C NMR analytical techniques. ^{13}C NMR was particularly useful in cases for which GC retention indices and mass spectra were could not support unequivocal identifications, such those of the major constituents, α-neocallitropsene, isocaryophyllen-14-al (β-betulenal) and 14-hydroxy-β-caryophyllene.

The specific activity of the volatile oil against pathogenic dermatophytes is very probably due to its caryophyllane derived components, namely isocaryophyllen-14-al, 14-hydroxy-β-caryophyllene and caryophyllene oxide.

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