Chemical Analysis and Antimicrobial Activity of *Halimium voldii*

Costas Demetzos [a, *], Dimitrios Perdetzoglou[a] and Kit Tan[b]

[a] School of Pharmacy, Department of Pharmacognosy, Panepistimiopolis Zografou, Athens 15771, Greece. E-mail: demetzos@galenos.pharm.uoa.gr

[b] Botanical Institute, Botanical Laboratory, University of Copenhagen, Gothersgade 140, DK-1123, Copenhagen K, Denmark

* Author for correspondence and reprint request

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Volatile constituents and a hexane extract of the leaves of *Halimium voldii* Kit Tan, Perdetzoglou & Raus, sp. nova, were analyzed by GC and GC-MS. Thirty compounds were identified in the essential oil of *Halimium* representing 88.7% of the oil composition. The main components were nonanal (12.8%), dodecane (10.6%), *Z*-caryophyllene (8.2%), y-muurolene (10.9%), δ-cadidene (3.5%), caryophyllene oxide (5.1%), β-eudesmol (3.6%) and manoyl oxide (5.5%). Thymol was identified in the hexane extract as the main compound. A labdane diterpene *ent*-labd-7, 13 (E)-dien, 15-ol was detected by its mass spectra fragmentation pattern and its structure was determined by spectroscopic methods and its optical rotation. The essential oil and the hexane extract were assayed for their antimicrobial activity against Gram (+) and Gram (–) bacteria.

Introduction

In the Balkan Peninsula the genus *Halimium* is only represented by *H. voldii*. A sample, from Mt. Taygetos (Peloponnisos, Greece) has been recently described as a new species. *H. voldii* is an endemic small shrublet with laxly spreading lower branches procumbent at the base, growing in submontaneous region (940–1400 m), after *Pinus nigra* forest, in terra rossa and schistolithic rock. (Tan *et al.*, 2000). Recent studies on *Halimium viscosum* and *H. verticillatum* by Spanish group have been conducted and a variety of diterpenoid structures mostly with labdane skeleton have been isolated and identified (Urones *et al.*, 1989; Rodilla *et al.*, 1998) New chemotypes (the chemical fingerprint of a given locality of a taxon) of *H. viscosum* collected near the Spanish-Portuguese border have been confirmed and several natural products with different skeletal have been isolated (Markos *et al.*, 1996). This is the first phytochemical and antimicrobial study of the genus *Halimium* of the Balkan Peninsula. We continued our research on the chemical composition of the essential oils and of the extracts of the family *Cistaceae* studying particularly the compounds with a labdane skeleton (Anastasaki *et al.*, 1999; Angelopoulou *et al.*, 2001). This paper presents GC and GC-MS data of the volatiles and of the hexane extract as well as the isolation and structural characterization of labdanes from the hexane extract of the new species *H. voldii* Kit Tan, Perdetzoglou & Raus.

Experimental

Plant material

The aerial parts from *H. voldii* were collected on Mt. Taygetos (Peloponnisos, Greece) in April 1998. A voucher specimen has been deposited in the following herbaria: C (Copenhagen), G (Geneva), UPA (University of Patras, Greece) (personal herbarium of Dr. Kit Tan & Dr. G. Vold no. 20377).

Analysis of essential oil and of plant extract

The essential oil and the hexane extract were analyzed using a capillary GC-MS (Hewlett Packard HP 6890) spectrometry system operating in the EI mode. A HP-5 MS fused silica capillary column of a 30 m × 0.25 mm (0.25 μm film thickness) was used for the analysis. The column was temperature programmed as follows: 50 °C for 5 min and then the temperature was increased to 280 °C at a rate of 3 °C / min (split ratio 4:1). Mass unit conditions: ion source 230 °C ionization energy 70 eV electron current 1453 μA. Identification of the compounds was based on the comparison of their

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retention indices with those of the literature (Adams, 1995), and on the basis of their mass spectral fragmentation pattern using the Wiley 138.I/NBS, GC-MS spectrometry library. The essential oils were also analyzed by GC analysis (Perkin-Elmer 8500) (Table I) with a CP-WAX capillary column of a 60 m × 0.32 mm (0.25 μm film thickness). The column was temperature programmed as follows: 50 °C for 5 min and then the temperature was increased to 280 °C at a rate of 1.5 °C/min.

Extraction of plant material and isolation of **1**

Powdered leaves (90 g dry wt) were submitted to hydrodistillation for 3 h. The yield of the essential oil was 0.015% of dried material (w/w). The oil was dried over anhydrous Na_2SO_4 and kept in the refrigerator. The physical properties *i.e* the optical rotation ([α]) at different wavelengths and at concentration c = 1.2 g/100 ml and the density (d ^{25}) of the oil were: [α] $^{25}_{D}$: −0.83 (*c* 1.2 $CHCl_3$); [α] $^{25}_{436}$: −1.83 (*c* 1.2 $CHCl_3$); [α] $^{25}_{546}$: −0.91 (*c* 1.2 $CHCl_3$); d ^{25} : 0.67.

Powdered leaves (34 g dry wt) were extracted using hexane at room temperature to yield 2.05% (w/w) of extract. The extract (697 mg) then was purified by column chromatography (CC) over 20 g silica gel (230–400 mesh, Merck) using n-hexane: CH_2Cl_2 (1:1 v/v) (300 ml) to CH_2Cl_2 (100%) (100 ml), to remove chlorophylls. The purified extract was tested by TLC and then analyzed by GC-MS. Labdanes recently isolated by us (Anastasaki *et al.*, 1999) were used as standards to identify compounds into the extract having mass fragmentation pattern identical to that of authentic samples. Labd −7, 13 -dien, 15 -ol (**1**) (Fig. 1), was detected by GC-MS in the hexane extract. The purified dry residue of the hexane extract (580 mg) was subjected to column chromatography (CC) over 20 g silica gel (230–400 mesh Merck) using n-hexane:CH_2Cl_2 (400 ml) mixtures of increasing polarity, to yield fractions 1–4. Fraction 4 (100 ml) (22 mg) (CH_2Cl_2) was subjected to preparative TLC using silica gel plates (silica gel 60 F_{254} Merck) (n-hexane: CH_2Cl_2 1:1). Compound **1** was obtained in its pure state (1.5 mg) and its purity was tested by GC-MS. ^{1}H-NMR, MS were identical with those reported in the literature (Anastasaki *et al.*, 1999). The [α] $^{25}_{D}$ of **1** was negative ([α] $^{25}_{D}$: −7 (*c* 0.15 $CHCl_3$) and thus characterized as *ent*-labd-7,13 (E) dien 15-ol (**1**).

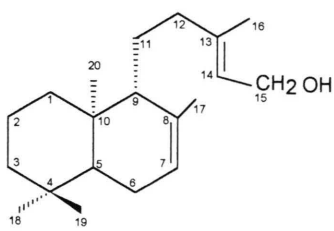

Fig. 1. Structure of *ent*-labd-7,13 (E) dien 15-ol **(1).**

Biological assay

The oil and the extract were assayed against Gram (+) microorganisms *i.e. Staphylococcus aureus*, and *S. epidermides* and against Gram (−) microorganisms *i.e. Escherichia coli* and *Klebsellia pneumonia* (Table II). The microbial strains were from the American Type Culture Collection. Carvacrol, a well-known natural antimicrobial compound, was used for comparative reasons, while streptomycin was used as standard in order to control the sensitivity of the microorganisms. The MICs were determined by microdilution assay as recommended by NCCLS (NCCLS 1995). MICs of carvacrol and of streptomycin were also determined in parallel experiments.

Results and Discussion

The volatiles from leaves of species of the genus *Halimium* have not been investigated before, and there are no literature data concerning the essential oil composition and the volatiles of the hexane extract. Both analyzed by GC-MS, which appears to be the best method for rapid analysis and identification of the volatiles constituents (Lienert *et al.*, 1998). Qualitative and quantitative analysis data of the essential oil of the new species *H. voldii* are presented in Table I. In total thirty compounds were identified accounting for 88.7% of the essential oil. The main components were nonanal (12.8%), dodecane (10.6%), *Z*-caryophyllene (8.2%), *γ*-muurolene (10.9%), δ-cadidene (3.5%), caryophyllene oxide (5.1%), β-eudesmol (3.6%) and manoyl oxide (5.5%). Manoyl oxide is a common volatile compound in the genus *Cistus* and the percentage content of its isomers was clarified. 13-*epi* manoyl oxide and manoyl oxide (5.5%) were detected and identified in the essential oil of *H. vordii* using their mass spectra fragmentation

Table I. Chemical and percentage (%) composition of the essential oil of *H. voldii*.

	Compounds	RI*	#	%
1	α-Pinene	926	1024	2.4
2	$C_{12}H_{16}$	983	–	0.9
3	Decane	1000	1000	0.4
4	Limonene	1022	1206	1.4
5	Benzeneacetaldehyde	1040	–	0.5
6	Undecane	1099	1100	0.3
7	Nonanal	1102	1410	12.8
8	(2 *E*,6 *Z*), Nonadienal	1146	–	0.3
9	(2 *E*), Nonenal	1153	1547	1.2
10	Naphthalene	1175	1766	1.6
11	Dodecane	1200	1200	10.6
12	Decanal	1202	1480	1.8
13	β-Cyclocitral	1213	–	0.6
14	Carvone	1235	1714	0.6
15	(2 *E*), Decanal	1254	1655	1.3
16	NÎ m/z: 204, 192, 177, 149, 136, 121, 107, 93, 77, 32.	1270	–	0.9
17	Undecanal	1303	–	1.1
18	(2 *E*, 4 *E*), Decadienal	1310	–	1.0
19	Tetradecane	1399	1400	0.6
20	*Z*-Caryophyllene	1407	1617	8.2
21	*E*-Geranylacetone	1450	1750	1.0
22	*y*-Muurolene	1475	1670	10.9
23	β-Ionone	1482	–	1.3
24	β-Himachalene	1497	1736	2.6
25	*y*-Cadinene	1510	1735	2.1
26	δ-Cadinene	1520	1754	3.5
27	Caryophyllene oxide	1583	1995	5.1
28	Viridiflorol	1590	2112	3.0
29	NI *m/z* 300, 287, 243, 173, 159, 143, 111, 83, 71, 43	1595	–	4.1
30	NI *m/z* 300, 204, 189, 161, 134, 119, 109, 95, 57, 43, 32.	1645	–	3.3
31	β-Eudesmol	1649	2248	3.6
32	NI *m/z* 212, 194, 110, 96, 82, 68, 57	1716	–	3.2
33	Manoyl oxide	1998	2331	5.5
34	13-*epi*-Manoyl oxide	2011	2351	2.5
	Total identified (%)			**88.7**

* RI, # RI: Retention indices on HP-5 and CP-WAX columns were calculated according to Van den Dool and Kratz (Van den Dool and Kratz, 1963) respectively.

NI : Not identified ; Compounds were listed according to their retention time (R_t) to HP-5 column.

pattern (Angelopoulou *et al.*, 2001). Since labdane type diterpenes were isolated from the genus *Halimium* (Urones *et al.*, 1989; Rodilla *et al.*, 1998; Markos *et al.*, 1996) they were not detected in the essential oil of *H. voldii* except for the manoyl oxide isomers. Thymol was identified in the hexane extract as the main compound, while a labdane diterpene *i.e.* labd-7, 13-dien, 15-ol was detected and identified by its mass spectra fragmentation pattern and with authentic sample. Its structure was determined using spectroscopic methods while its optical rotation was negative and thus characterized as *ent*-labd-7, 13 (E)-dien, 15-ol (**1**). To the best of our knowledge the occurrence of **1** as natural compound in its enantiomeric state has never been presented in the literature. The essential oil and the hexane extract were investigated for antimicrobial activity (Table II). Both showed moderate activity against Gram (+) bacteria while they had no effect against Gram (–) bacteria. The MICs of the essential oil and of the hexane extract of *H. voldii* against staphylococci were 500 μg/ml and 300 μg/ml respectively, while against Gram (–) bacteria the MICs were > 1000 μg/ml. The MIC of carvacrol was 300 μg/ml against all tested microorganisms.

Table II. Minimum inhibitory concentrations (μg/ml) of the essential oil of the hexane extract of *H. voldii* and of carvacrol. The values represent means of three independent experiments run in triplicate. SD never exceeded 12% of the mean value.

Microorganisms	Essential oil	Hexane extract	Carvacrol
S. auerus ATCC 6538	500	300	300
S. epidermidis ATCC 12228	500	300	300
E. coli ATCC 25922	>1000	>1000	300
K. pneumoniae ATCC 13883	>1000	>1000	300

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