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Anticandidal pimaradiene diterpene from *Phlomis* essential oils

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Abstract

In the present study, it was aimed to investigate the phytochemical profile and antimicrobial effects of *Phlomis lunariifolia* Sm., *Phlomis amanica* Vierh., *Phlomis monocephala* P.H. Davis, *Phlomis sieheana* Rech. fil, *Phlomis armeniaca* Willd. essential oils collected from Turkey. The *Phlomis* essential oils were obtained from the aerial parts by hydrodistillation and were subsequently analyzed both by gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS). Chromatographic separations followed by structure identification of individual compounds of interest from *Phlomis* essential oils were conducted using 1D and 2D NMR, FT-IR, UV and HRMS techniques. In addition, antimicrobial studies using a microdilution assay and TLC bioautography were applied to the essential oils and the relevant components. The analysis of the essential oils led to the identification of 143 compounds, where an unknown volatile compound was detected as the major compound (22.8% and 12.7%) in the essential oils of *P. amanica* and *P. monocephala*, respectively. After chromatographic clean up, the isolation and characterization of this compound resulted in (−)-8(14),15-isopimaradien-11α-ol. The sesquiterpene germacrene-D was identified as the major constituent of *P. lunariifolia* (7.7%), *P. sieheana* (16.6%) and *P. armeniaca* (23.4%) oils. 4-Methoxycarbonyl-7-methyl cyclopenta[*c*]pyrane – a fulvoiridoid – was obtained by acid hydrolysis from iridoid ipolamiide which was shown to be present in the oils of *P. armeniaca* (1.4%) and *P. sieheana* (0.2%). Furthermore, *Phlomis* essential oils were investigated for their antifungal properties using a TLC bioautographic assay where the diterpene was shown as the active principle against *Candida albicans* and *Candida tropicalis* when compared with standard antifungal agents. Minimum inhibitory concentrations against various human pathogenic bacteria (from 125 to >1000 μg/ml), *C. albicans* and *C. tropicalis* (62.5–1000 μg/ml), were determined using a microdilution assay. The results obtained from this study suggest that essential oils and their individual compounds thereof may be potential resource and ingredients for pharmaceuticals or cosmetics with antimicrobial activity. ***To cite this article: B. Demirci et al., C. R. Chimie 12 (2009).***

Keywords: *Phlomis lunariifolia*; *Phlomis amanica*; *Phlomis monocephala*; *Phlomis sieheana*; *Phlomis armeniaca*; Lamiaceae; 8(14),15-Isopimaradien-11α-ol; Antimicrobial activity; Antifungal bioautography

1. Introduction

The genus *Phlomis* L. (Lamiaceae) is represented overall by 34 species and 52 taxa, in which 34 are

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endemic (including 12 natural hybrids) in Turkey [1–3]. It is documented that some *Phlomis* species are used as a tonic and stimulant in Anatolian folk medicine [4].

Previous phytochemical investigations on *Phlomis lunariifolia*, *Phlomis monocephala* and *Phlomis sieheana* were reported, where an aliphatic alcohol glycoside, a phenylethanoid glycoside, and a flavone glycoside were isolated from the aerial parts of *P. lunariifolia*, in addition to 15 known glycosides, by Calis and Kirmizibekmez [5]. Also from the overground parts of *P. monocephala*, two iridoid glycosides, three phenylethanoid glycosides, and one lignan glucoside were isolated by Yalcin and coworkers [6]. From the aerial parts of *P. sieheana*, an iridoid glucoside, six phenylethanoid glycosides, and a monoterpene glycoside were isolated and characterized by Ersoz et al. [7]. Very recently a pimarane-type diterpene was reported from *Phlomis amanica* [8].

As part of our ongoing research into essential oil of *Phlomis* species grown in Turkey, we report here the essential oil composition of five species, *P. lunariifolia* Sm., *P. amanica* Vierh., *P. monocephala* P.H. Davis, *P. sieheana* Rech. fil, and *Pholmis armeniaca* Willd., both by gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS). To the best of our knowledge, this is the first report on the essential oil chemistry of these five *Phlomis* species. Also the isolation of a diterpene and the identification of an iridoid within the essential oil are presented. In addition, antifungal *Candida* TLC bioautographic assay using *Phlomis* essential oils was applied initially to screen for bioactive constituents within the oils. Microbroth dilution assay was also used to determine the inhibitory activity against various human pathogenic Gram-positive and Gram-negative bacteria as well as against the yeast *Candida albicans* and *Candida tropicalis*.

2. Materials and methods

2.1. General

If not indicated otherwise, all chemicals, solvents, media and standards were purchased from Sigma/Aldrich at high purity (>99%).

Optical rotation was measured on a JASCO DIP-1000 polarimeter. IR spectra were measured using a Shimadzu FTIR-8400S. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Unity 600 system at 600 and 150 MHz, respectively. 1D and 2D NMR data were also obtained using the same system, and spectra were measured and reported in ppm. Tetramethylsilane (TMS) at 0.0 ppm or the solvent $CDCl_3$ was used as reference internal standard. HRMS analyses were obtained on a Jeol JMS-AX 500 system. GC and GC–MS analyses were carried out by Hewlett Packard 6890 and Hewlett–Packard GCD systems using a Innowax FSC column (60 m × 0.25 mm ∅, with 0.25 μm film thickness), respectively (see for analytical details as in Demirci et al. [9]).

2.2. Plant material

The aerial parts of the plants were collected from different regions and identified by one of us. Voucher specimens were deposited at the Herbarium of Erciyes University, Faculty of Science and Letters. Detailed information on the plant materials used is given in Table 1.

2.3. Isolation of the essential oils

The plant materials were dried in the shade at room temperature and were subjected to hydrodistillation for 3 h using a Clevenger-type apparatus [10] to produce essential oils. The percentage yields were calculated

Table 1
Information on the plant material and essential oils

Phlomis ssp.	Collection site	Altitude (m)	Date	Oil yield[a] (%)	MYD[b]
P. lunariifolia (PL)	Icel; Aydincik–Gulnar 13th km	50	03.07.2003	tr	1671
P. amanica (PAm)	Hatay; Arsuz, Haymaseki village	250–300	22.05.2004	0.14	1723
P. monocephala (PM)	Icel; Aydincik–Gulnar 13th km	50	03.07.2003	0.20	1669
P. sieheana (PS)	Kayseri; Gesi, Ildem Koop	1150	19.06.2003	0.04	1643
P. armeniaca(PAr)	Kayseri; Kayakevi–Develi road 13th km	1700	24.07.2002	0.10	1698

tr: Trace (<0.1%).
[a] Essential oil yields are given on moisture free basis.
[b] MYD: herbarium code of the collector.

on a dry weight basis as given in Table 2. The oils were obtained neat, dried over anhydrous sodium sulfate to remove the water and stored at +4 °C until analyzed and tested further.

2.4. Isolation of (−)-8(14),15-isopimaradiene-11α-ol (*1*)

Compound **1** was isolated initially from *P. monocephala* (PM) essential oil (65.8 mg) by column chromatography. Silica Gel 60 G (*ca.* 3 g, Merck 7734) was used as a packing material, and filled with wet *n*-hexane (column size: 10 × 500 mm). *n*-Hexane:-diethyl ether (100 → 0) was used as an eluant in a gradient system. The essential oil was applied and eluted with *n*-hexane:diethyl ether (90:10) to yield **1** (3.2 mg) as a colorless oily material. $[\alpha]_D$ −17.0° ($CHCl_3$; *c* 0.38), EI-MS *m/z* (rel. int.): 288 $[M]^+$ (5), 273(4), 270(3), 255(8), 137(100), 123(28), 95(31), 81(35), 69(28). HRMS: 288.2458 $[M]^+$ calcd for $C_{20}H_{32}O$, 220.2453. FT-IR (liquid film: $CHCl_3$) ν_{max} cm^{-1}: 3546 (−OH), 1635, 1602, 1458, 1436, 1367, 1020, 1004 (^1H, ^{13}C and 2D NMR data are given in Table 3).

2.5. Preparation of 4-methoxycarbonyl-7-methyl cyclopenta[c]pyrane (*2*)

Ipolamiide (5 mg) was treated with 0.1 N HCl at pH 5 and stirred vigorously at approx. 100 °C. After 1 h all ipolamiides were converted into its aglycone. The solution extracted with Et_2O and evaporated under nitrogen gave a red-orange residue (2 mg). The sample was analyzed by GC–MS. EI-MS *m/z* (rel. int.): 190$[M]^+$ (100), 189(69), 175(61), 131(15), 129(23), 103(10), 102(15), 77(21), 51(13). MS data were in agreement with those reported by Bianco et al. [11].

2.6. Bioassays

2.6.1. Microorganisms

All microorganisms were obtained from various culture collections (ATCC, NRRL) and were maintained in 15% glycerol as cryoprotectant at −85 °C. Prior to use in the assays, bacteria were inoculated in Mueller–Hinton agar (MHA, Merck, Germany), and fungi in Sabouraud dextrose agar (SDA, Acumedia, MD) in Petri dishes until sufficient growth under optimum conditions for purity check. Microorganisms were then transferred to Mueller–Hinton broth (MHB, Merck, Germany) and incubated at 37 °C for 24 h.

2.6.2. Antimicrobial bioassay

A microdilution broth susceptibility assay was used [12,13]. Stock solutions of the test samples were prepared in 25% (v/v) dimethylsulfoxide (DMSO, Carlo Erba). Serial dilutions of samples were prepared up to 1.95 μg/ml using sterile distilled water in a 96-well microtiter plate. Microbial suspensions previously grown as described above were standardized to 1×10^8 CFU/ml (McFarland No: 0.5) in double strength Mueller–Hinton broth (MHB, Merck, Germany). One hundred microliters of each microbial suspension was then added to the appropriate well. The last row, which contained only the serial dilutions of the essential oil without microorganism, was used as a negative control. 'Only DMSO' dilutions were considered as another control to eliminate solvent effects. After incubation at 37 °C for 24 h the first well without turbidity was determined as the minimum inhibition concentration (MIC, μg/ml). Chloramphenicol and Ampicillin were used as standard antibacterials, whereas Ketoconazole and Clotrimazole were used antifungal positive controls. All experiments were repeated in triplicate. Average MIC results are given in Table 4.

2.6.3. Bioautography method

For determining the active constituent a bioautography technique was used [12,14]. First the samples were subjected to TLC; following the separation, the inoculated medium was applied onto the developed TLC plate as described below to identify the active principle(s).

2.6.3.1. Thin layer chromatography. Precoated Silica Gel 60 GF 254 (0.2 mm) (Merck) plates, cut to appropriate size, on aluminum support were used. Essential oils (2 mg/ml; 2 × 1 μl) and Ketoconazole and Clotrimazole (2 mg/ml; conc., 2 μl) were applied using Drummond microcapillaries onto the TLC plates and developed (8:2, v:v, *n*-hexane:ethyl acetate). Separated compounds were visualized with UV light (365 and 254 nm) and developed with anisaldehyde/H_2SO_4 spray reagent followed by heating to 110 °C, for the duplicate which served as reference. The other plate was utilized for the assay after complete solvent evaporation.

2.6.3.2. Agar overlay bioautographic assay. Nutrient Agar (15 ml, Difco) was poured onto the Petri plate (12 cm diameter) for the formation of an agar base. One of the developed and untreated TLC plates was carefully placed on the agar base under aseptic conditions. *C. albicans* as well as *C. tropicalis* were previously grown and incubated as described above. MHB with an Agar (7.5%, Acumedia, MD) was used in molten form

Table 2
The essential oil composition of *Phlomis* species

RR_I	Compound	PL	PAm	PM	PS	PAr
1032	α-Pinene	tr	2.1	4.9	tr	tr
1035	α-Thujene	–	0.1	0.3	–	–
1118	β-Pinene	tr	0.1	0.3	0.1	tr
1146	δ-2-Carene	tr	–	tr	0.1	tr
1174	Myrcene	–	–	0.3	–	–
1203	Limonene	0.1	0.1	3.9	0.2	tr
1213	1,8-Cineole	tr	–	0.1	tr	0.1
1280	*p*-Cymene	0.1	–	0.5	tr	tr
1400	Nonanal	0.2	0.2	0.2	–	0.1
1400	Tetradecane	–	–	–	–	tr
1443	Dimethyl tetradecane[a]	0.1	–	–	0.5	0.6
1452	α,*p*-Dimethylstyrene	–	–	0.1	–	–
1452	1-Octen-3-ol	–	0.1	0.1	–	–
1466	α-Cubebene	2.0	0.1	0.7	–	0.1
1477	4,8-Epoxyterpinolene	–	–	0.1	–	–
1493	α-Ylangene	0.3	0.1	0.1	–	tr
1495	Bicycloelemene	0.1	–	0.2	0.4	0.4
1497	α-Copaene	1.5	0.5	0.7	0.1	0.5
1506	Decanal	0.2	tr	0.1	tr	0.1
1528	α-Bourbonene	0.1	0.1	0.1	0.1	0.1
1535	β-Bourbonene	0.2	0.7	0.6	1.5	1.1
1544	α-Gurjunene	0.5	–	tr	–	tr
1549	β-Cubebene	1.0	0.1	0.3	0.2	0.2
1553	Linalool	0.2	0.1	0.1	0.7	0.9
1577	α-Cedrene	1.0	–	–	–	–
1589	Isocaryophyllene	0.5	0.2	–	–	–
1589	β-Ylangene	0.4	0.2	–	0.7	0.8
1594	*trans*-β-Bergamotene	0.1	–	–	–	–
1597	β-Copaene	–	0.2	0.4	0.3	0.5
1600	β-Elemene	–	–	0.6	0.9	0.9
1604	2-Undecanone	–	–	–	0.2	–
1612	β-Caryophyllene	9.0	0.9	5.1	1.1	–
1628	Aromadendrene	0.1	0.2	0.1	–	0.1
1638	β-Cyclocitral	–	–	0.1	–	0.1
1650	γ-Elemene	–	–	0.2	1.4	0.5
1661	Alloaromadendrene	0.2	–	0.4	–	–
1668	(*Z*)-β-Farnesene	6.5	8.3	3.1	11.7	6.2
1659	γ-Gurjunene	–	–	0.1	0.2	tr
1669	Sesquisabinene	0.2	–	–	–	–
1674	Muurola-4,11-diene	0.3	–	–	–	–
1687	α-Humulene	0.9	–	0.4	0.3	0.2
1700	*p*-Mentha-1,8-dien-4-ol (=limonen-4-ol)	–	–	0.1	–	–
1704	γ-Muurolene	–	0.3	tr	–	0.5
1688	Selina-4,11-diene (=4,11-eudesmadiene)	2.0	0.5	2.9	0.7	–
1695	(*E*)-β-Farnesene	–	–	–	1.6	1.2
1700	Heptadecane	–	–	–	0.2	–
1708	Ledene	–	0.2	0.2	–	–
1726	Germacrene-D	7.7	14.7	6.0	16.6	23.4
1740	α-Muurolene	0.4	0.3	0.4	–	–
1742	β-Selinene	–	–	–	6.7	2.6
1744	α-Selinene	–	–	0.1	1.5	tr
1754	(*Z*)-γ-Bisabolene	0.5	–	–	–	–
1755	Bicyclogermacrene	2.6	10.7	1.5	1.6	2.3
1763	Naphthalene	–	–	0.1	–	–
1764	(*E*)-2-Undecenal	–	–	–	0.1	0.1
1765	(*E*)-γ-Bisabolene	0.1	–	–	–	–
1773	δ-Cadinene	1.7	0.8	0.4	0.6	1.0

(*continued on next page*)

Table 2 (*continued*)

RR_I	Compound	PL	PAm	PM	PS	PAr
1776	γ-Cadinene	0.2	0.3	0.3	0.2	0.3
1779	(*E*,*Z*)-2,4-Decadienal	–	–	–	0.5	–
1785	7-*epi*-α-Selinene	0.4	0.2	0.5	0.1	0.2
1786	*ar*-Curcumene	1.8	–	–	–	–
1798	Methyl salicylate	–	–	0.2	–	0.2
1800	Drimenene[a]	–	–	0.1	–	–
1808	Nerol	–	–		–	0.1
1827	(*E*,*E*)-2,4-Decadienal	0.1	0.1	0.1	0.4	0.2
1834	Ethyl salicylate	–	–	tr	–	–
1838	(*E*)-β-Damascenone	0.2	–	0.1	0.3	0.1
1849	Calamenene	0.3	0.1	0.3	–	–
1854	Germacrene-B	–	–	–	0.9	0.3
1857	Geraniol	–	–	–	–	0.4
1864	*p*-Cymen-8-ol	0.1	–	0.2	–	–
1868	(*E*)-Geranyl acetone	0.1	–	0.1	0.5	0.2
1884	1-Methyl naphthalene	–	0.1	–	–	–
1900	Nonadecane	–	–	–	–	0.1
1900	*epi*-Cubebol	0.3	–	0.2	0.1	0.1
1941	α-Calacorene	–	0.2	–	–	–
1945	1,5-Epoxy-salvial(4)14-ene	–	–	–	0.6	0.4
1953	Palustrol	–	0.1	–	–	–
1957	Cubebol	0.8	0.1	0.3	–	–
1958	(*E*)-β-Ionone	–	0.2	–	0.3	0.2
1969	*cis*-Jasmone	–	–	–	–	0.1
1972	1-Ethyl naphthalene	–	0.1	–	–	–
1984	γ-Calacorene	–	–	–	–	0.1
2001	Isocaryophyllene oxide	0.3	–	0.3	–	0.1
2008	Caryophyllene oxide	1.2	0.1	1.2	0.7	0.7
2033	*epi*-Globulol	–	0.2	–	–	–
2036	2-Pentadecanone	–	–	–	–	0.3
2037	Salvial-4(14)-en-1-one	–	–	0.1	0.5	0.2
2046	Norbourbonone	–	–		0.5	0.1
2050	(*E*)-Nerolidol	0.1	–	0.1	0.2	0.3
2057	Ledol	–	0.1	–	–	–
2069	Germacrene-D-4β-ol	–	–	–	–	0.1
2071	Humulene epoxide-II	–	–	0.2	–	–
2080	Cubenol	0.3	0.2		–	–
2084	Octanoic acid	–	–	–	–	0.3
2088	1-*epi*-Cubenol	0.6	0.3	0.4	–	0.1
2095	Hexyl benzoate	–	–	–	–	0.3
2098	Globulol	0.8	1.5	0.6	0.5	–
2104	Viridiflorol	0.4	1.1	0.5	–	0.3
2144	Rosifoliol		0.3	0.2	–	0.1
2131	Hexahydrofarnesyl acetone	1.0	0.7	0.5	1.9	2.3
2144	Spathulenol	3.9	6.3	3.8	3.0	1.9
2148	(*Z*)-3-Hexen-1-yl benzoate	0.2	–	–	0.4	0.2
2174	Fokienol	–	–	–	–	0.2
2179	3,4-Dimethyl-5-pentylidene-2(5*H*)-furanone	–	–	–	0.8	0.2
2179	Nor-Copaonone	–	–	–	0.4	–
2187	T-Cadinol	0.4	0.6	0.4	0.3	0.5
2192	Nonanoic acid	–	–	–	–	0.6
2200	Docosane	–	–	–	0.4	0.1
2209	T-Muurolol	0.9	0.7	0.5	0.5	0.6
2219	δ-Cadinol (=α-muurolol)	0.3	0.2	0.6	0.2	0.2
2239	Carvacrol	–	–	–	–	0.3
2240	1-Methyl ethyl hexadecanoate	–	–	–	–	0.2
2247	*trans*-α-Bergamotol	0.4	0.2	0.5	0.5	0.3
2255	α-Cadinol	1.2	1.4	1.2	1.4	1.2
2264	Intermedeol	0.4	–	0.3	–	–

Table 2 (*continued*)

RR_I	Compound	PL	PAm	PM	PS	PAr
2269	Guaia-6,10(14)-dien-4β-ol	0.4	–	0.4	–	–
2273	Selin-11-en-4α-ol	–	0.2	–	–	–
2278	Torilenol	–		–	0.5	–
2287	8,13-Epoxy-15,16-dinor-labd-12-ene	–	0.5	0.8	–	–
2298	Decanoic acid	–	–	–	0.5	1.0
2357	Sandracopimaradiene	–	0.4	1.1	–	–
2300	Tricosane	–	0.7	–	1.1	0.7
2357	Octadecanal	–	–	–	0.5	–
2369	Eudesma-4(15),7-dien-1β-ol	–	0.5	–	–	0.2
2376	Manoyl oxide	0.8	–	6.1	–	–
2380	8α,13-Oxy-14-en-epilabdane(=*epi*-manoyl oxide)	0.2	–	–	–	–
2384	Farnesyl acetone	–	–	–	0.7	–
2384	Hexadecanol	–	–	–	0.4	–
2392	Caryophylla-2(12),6-dien-5β-ol (=caryophyllenol II)	0.1	0.6	–	–	–
2396	γ-Dodecalactone	–	–	–	0.2	0.2
2400	Tetracosane	–	–	–	0.5	0.3
2485	4-Methoxycarbonyl-7-methyl cyclopenta[*c*]pyrane (**2**)	–	–	–	0.2	1.4
2500	Pentacosane	–	–	–	1.1	2.5
2503	Dodecanoic acid	–	0.7	–	1.8	1.4
2600	Hexacosane	–	–	–	0.2	0.1
2607	Octadecanol	–	–	–	0.7	0.6
2622	Phytol	–	–	–	0.5	0.4
2655	Benzyl benzoate	–	–	–	–	0.2
2670	Tetradecanoic acid	–	–	0.3	0.3	0.4
2700	Heptacosane	–	–	–	1.2	2.3
2900	Nonacosane	–	–	–	0.9	1.2
2917	8(14),15-Isopimaradien-11α-ol (**1**)	5.5	22.8	12.7	–	–
2931	Hexadecanoic acid	9.7	–	1.7	2.1	4.9
	Total	74.2	83.7	72.8	79.8	77.1
	Identified compounds	68	60	78	76	93

RR_I: relative retention indices calculated against *n*-alkanes; %: percentages calculated from FID data; tr: trace (<0.1%).

[a] Correct isomer not identified.

and kept at 45 °C after sterilization, where the pre-grown *Candida* suspensions were transferred and adjusted to McFarland No: 0.5 each. Immediately, the *Candida* inoculated media were poured onto the TLC plates to form a thin layer and incubated at 37 °C for 24–36 h, separately. After sufficient growth, the Petri plates were sprayed using a 1% (w/v, EtOH) Tetrazolium Violet (2,5-diphenly-3-[α-naphthyl] tetrazolium chloride, Sigma) reagent and further incubated at 37 °C for 1 h. Inhibition zones were visualized against the red colored background. Ketoconazole and Clotrimazole were used as standard antifungal agents for comparison.

3. Results and discussion

3.1. Determination of essential oil components

Except for *P. lunariifolia* all other investigated species are endemic plants of Turkey [1]. The essential oils were obtained by hydrodistillation from the aerial parts of *P. lunariifolia* Sm., *P. amanica* Vierh., *P. monocephala* P.H. Davis, *P. sieheana* Rech. fil, *P. armeniaca* Willd., respectively. The oils were subsequently analyzed using both GC and GC–MS systems with which the individual components were identified according to their relative retention indices (RR_I) and their relative percentages (see Table 2).

A sum of 68 individual compounds were identified in the oil of *P. lunariifolia* (PL), representing 74.2% of the total essential oil. This oil was characterized by a high content of hexadecanoic acid (9.7%), β-caryophyllene (9.0%), germacrene-D (7.7%), and (*Z*)-β-farnesene (6.5%). Overall, the oil was found to be rich in sesquiterpene hydrocarbons.

In the oil of *P. amanica* (PAm), 60 components were identified representing 83.7% of the total oil. This oil was characterized by germacrene-D (14.7%), bicyclogermacrene (10.7%), (*Z*)-β-farnesene (8.3%) and spathulenol (6.3%).

Table 3
^{1}H and ^{13}C NMR data for compound **1**

	^{1}H			^{13}C
No	Assignment	$^{1}H-^{13}C$ correlations	NOE correlations	Assignment
1	1.34 (ddd, 4,13,13) ax. 1.85 (br d, 13) eq.	C-20	H-5,9 H-11	40.2
2	1.51(m) ax. 1.56(m) eq.			19.1
3	1.21 (ddd, 4,13,13) ax. 1.42 (br d, 13) eq.	C-19		42.0
4	–			33.4
5	1.12 (dd, 3,13)	C-4,6,10,19,20	H-1_{ax},9,7_{ax},18	54.8
6	1.30 (dddd, 5,13,13,13) ax. 1.64 (m) eq.	C-5,7 C-8	H-19,20	23.0
7	2.05 (br ddd, 5,13,13) ax. 2.32 (ddd, 2,5,14) eq.	C-5,6,8,14 C-5,8,9,14	H-5 H-14	36.3
8	–			136.4
9	1.75 (br d, 5)	C-8,10,11,12,14,20	H-5,1_{ax}	59.9
10	–			39.1
11	4.03 (ddd, 5,7,7)	C-10,12,13	H-1_{eq},17, 20	66.2
12	1.65 (d, 7) 2H	C-9,11,13,14,15,17		43.4
13	–			37.5
14	5.30 (t,2)	C-7,9,12,13,15	H-7_{eq},17	127.4
15	5.88 (dd, 10,17)	C-12,13,17	H-17	149.1
16	5.01 (dd, 1,17) *trans* 4.92 (dd, 1,10) *cis*	C-13,15 C-13	H-17	110.6
17-CH_3	1.05	C-12,13,14,15	H-16_{trans},11,20,15,14	27.0
18-CH_3	0.89	C-3,4,5,19	H-4	33.8
19-CH_3	0.85	C-3,4,5,18	H-6_{ax}	22.1
20-CH_3	0.83	C-1,5,9,10	H-6_{ax},11,17	15.9

Measurements were performed with 600 MHz for ^{1}H NMR and 150 MHz for ^{13}C NMR in $CDCl_3$. Assignments were confirmed by HMQC spectrum. *J* values are shown in parenthesis.

Table 4
Minimum inhibitory concentrations (MICs, μg/ml) for *Phlomis* essential oils

Microorganisms	PL	PAm	PM	PS	PAr	St1	St4	St3	St4
Escherichia coli NRRL B-3008	500	500	500	500	500	7.8	7.8	nt	nt
Staphylococcus aureus ATCC 6538	1000	1000	1000	1000	>1000	1.9	1.9	nt	nt
Pseudomonas aeruginosa ATCC 27853	>1000	>1000	>1000	>1000	>1000	125	125	nt	nt
Enterobacter aerogenes NRRL 3567	1000	1000	1000	1000	1000	3.9	3.9	nt	nt
Proteus vulgaris NRRL B-123	500	500	500	500	500	15.6	15.6	nt	nt
Salmonella typhimurium ATCC 13311	>1000	1000	1000	>1000	500	3.9	3.9	nt	nt
Bacillus cereus NRRL B-3711	500	250	125	250	500	31.25	31.25	nt	nt
Candida tropicalis NRRL Y-12968	1000	500	500	0.25	500	nt	nt	250	15.6
Candida albicans NRRL 27077	nt	62.5	125	nt	nt	nt	nt	7.8	3.9

St1: Chloramphenicol; St2: Ampicillin; St3: Ketoconazole; St4: Clotrimazole; nt: not tested.

Analysis of *P. monocephala* (PM) oil revealed 78 components, representing 72.8% of the total oil. An unknown compound was detected as the major compound (22.8% and 12.7%) in both the essential oils of PAm and PM, respectively.

A total of 76 and 93 compounds were characterized in *P. sieheana* (PS) and *Phlomis armenica* (PAr) essential oils, representing 79.8% and 77.1% of the total oils, respectively. Germacrene-D (16.6% and 23.4%) and (*Z*)-β-farnesene (11.7% and 6.2%) were identified as the major constituents of PS and PAr essential oils, respectively.

Altogether a 143 volatile compounds were identified within the investigated *Phlomis* species in this study. To the best of our knowledge, this is the first report on the essential oil chemistry of these species. GC–MS analyses showed two interesting compounds to be present in some of the investigated *Phlomis* essential oils, a diterpene (1) and an iridoid derivative (2).

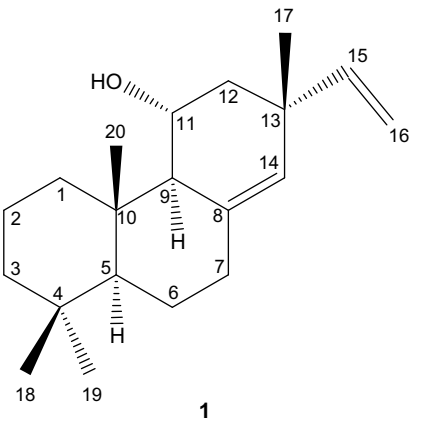
3.2. Structure elucidation

The unknown compound (**1**) was isolated from PM essential oil by column chromatography. The IR spectrum of **1** showed the absorption band characteristic for a hydroxyl group at 3546 cm^{-1}. Its EI-mass spectrum gave a molecular ion peak at *m/z* 288, and a fragment peak at *m/z* 273 [$M^+ - CH_3$] among others. The HR-EI-MS analysis of the molecular ion peak at *m/z* 288.2458 suggested a molecular formula of $C_{20}H_{32}O$, confirming 5° of unsaturation for **1**. The ^1H NMR spectrum (data given in Table 3) of **1** showed the presence of four tertiary methyl groups at δ 0.83, 0.85, 0.89 and 1.05, an oxygenated methine group at δ 4.03 (ddd, $J = 5$, 7, 7 Hz) and an sp^2 methine proton at δ 5.30 (br t, $J = 2$ Hz), and vinyl group protons, which were mutually spin coupled at δ 4.92 (dd, $J = 1$, 10 Hz), 5.01 (dd, $J = 1$, 17 Hz) and 5.88 (dd, $J = 10$, 17 Hz). The ^{13}C NMR spectrum of **1** indicated the presence of 20 carbons, including 4 sp^2 carbons and the oxygenated methine group at δ 66.2. Conclusively, the spectral data suggested that **1** was a tricyclic compound.

Analysis of the HMQC and HMBC spectra supported the structural assignment (details in Table 3). The long range $^1H-^{13}C$ correlation of two tertiary methyls at δ_H 0.85 and 0.89 (H_3-19, 18) with a methylene carbon at δ_C 42.0 (C-3), a quaternary carbon at δ 33.4 (C-4) and a methine carbon at δ 54.8 (C-5), and mutual $^1H-^{13}C$ correlation between the both methyl groups, supported that the two methyls were *gem*-dimethyl structure in compound **1**. Further correlation between the methyl proton signal at δ_H 0.83, the methine carbon at δ_C 54.8, and δ_C 59.9 was observed, respectively. The signal at δ_C 59.9 showed ^1J-direct coupling with a broad doublet signal at δ_H 1.75 in the HMQC spectrum. The methine proton at δ_H 1.75 showed spin–spin coupling with the oxygenated methine proton at δ_H 4.03 in the $^1H-^1H$ COSY spectrum of **1**. The remaining methyl group at δ_H 1.05 correlated with sp^2 carbon at δ_C 149.1 which was attached by the vinyl proton at δ_H 5.88, and methylene carbon at δ_C 43.4 whose proton showed spin–spin coupling with the oxygenated methine proton.

Consideration of these spectral data and further $^1H-^{13}C$ correlations (summarized in Table 2) led to the conclusion that the structure of **1** was 8(14),15-isopimaradiene-11α-ol.

Extensive NOESY (also summarized in Table 3) spectral analysis allowed us to define the stereochemistry of the hydroxyl group at C-11. Since NOE correlation (as shown in Fig. 2) between C-11 proton and H_3-17, 20 and H-1_{eq} protons was observed, the hydroxyl group at C-11 of compound **1** should have α-equatorial orientation. Further NOE correlation (summarized in Table 3) between H-5 proton and H-1_{ax} and H-9 protons was in particular important for the confirmation of the stereochemistry of the ring junction of **1** which was assigned in *trans* position. Accordingly, the structure of compound **1** was elucidated as 8(14),15-isopimaradiene-11α-ol (=11α-hydroxy-sandaracopimara-8(14),15-diene). To the best of our knowledge this is the first report on this compound isolated from essential oils. However, a very recent report coincides with the same structure; also isolated from a Turkish endemic *P. amanica*



1

Fig. 1. Assignment of (−)-8(14),15-isopimaradiene-11α-ol (**1**).

Fig. 2. NOE correlations for **1**.

where all the spectroscopic data are in agreement with amanicadol [8].

3.3. Determination of fulvoiridoid

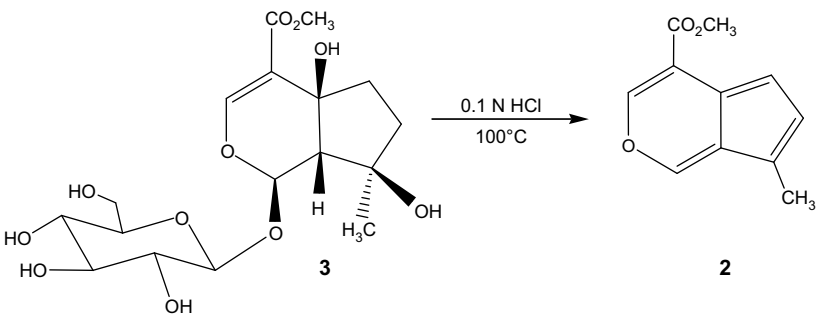
GC–MS analyses of the essential oils showed an unknown minor compound with a molecular weight of 190 corresponding to $C_{11}H_{10}O_3$ from the computer peak matching against the commercial Wiley GC–MS Library [15]. As the relative contents of **2** were in minor amounts within the oils, chemical derivatization aided in the structure elucidation. The suggested iridoid agylcone (**2**) was obtained from the iridoid (**3**) frequently occurring in *Phlomis* species [5–8] including the investigated species in this study.

For the identification of compound **2**, the iridoid ipolamiide (**3**) was subjected to acid hydrolysis. The resulting compound 4-methoxycarbonyl-7-methyl cyclopenta[*c*]pyrane (**2**) was characterized by co-injection of the *Phlomis* essential oils to confirm its presence in the oils of PAr (1.4%) and PS (0.2%). This compound was previously also reported as a transformation product of ipolamiide after acid hydrolysis, and was classified as a fulvoiridoid (or pseudoazulene) [11]. To the best of our knowledge, this is the first report of the rare compound **2** in an essential oil as a natural product. The presence of **3** in *Phlomis* species supports the hydrolytic formation of compound **2** from hydrodistillations possibly as an artifact as shown in Scheme 1.

3.4. Antimicrobial activity

Phlomis essential oils were investigated first for their antifungal properties using a TLC bioautographic assay where the diterpene (**1**) was shown as the active principle against *C. albicans* and *C. tropicalis* when compared to the standard antifungal agent. A strong inhibitory zone was observed in the bioautographic assay when compared with the antifungal standards, especially PAm essential oil was followed by PM and PL oils. Minimum inhibitory concentrations (MICs) against various human pathogenic bacteria and *C. albicans* were determined using a microdilution assay. As seen in Table 4, weak to moderate antibacterial activity of the tested essential oils was observed (from MICs 500 to >1000 μg/ml) with exception against the pathogen *Bacillus cereus* (125–500 μg/ml). PM oil showed relatively good inhibition against *B. cereus*. The activity observed in the bioautographic assay against *Candida* sp. was repeated with a strong inhibitory effect of the PAm essential oil with decreasing action of PM and PL, respectively (62.5–1000 μg/ml). The relative percentage amount of the diterpene 8(14),15-isopimaradiene-11α-ol (**1**) showed correlation with the anticandidal effect in a dose response fashion.

In conclusion, the essential oils of *Phlomis* species are a natural source for antimicrobial and in particular antifungal applications. It is worthwhile to investigate further parts and species from this widespread genus.



Scheme 1. Acid hydrolysis of ipolamiide (**3**).

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