

Chemical Composition of the Essential Oil of *Phlomis linearis* Boiss. & Bal., and Biological Effects on the CAM-Assay: A Safety Evaluation

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Phlomis linearis Boiss. & Bal. of the Lamiaceae family growing in central, east and south-east Anatolia is an endemic species for Turkey. The essential oil obtained from the aerial parts by hydro distillation was subsequently analyzed by GC/MS. The main components of the oil were found as β -caryophyllene (24.2%), germacrene D (22.3%) and caryophyllene oxide (9.2%), among 49 identified compounds, representing 94.5% of the total essential oil. The overall biological activity of the essential oil (100 μ g/pellet) was tested on the chorioallantoic membrane (CAM) of the fertilized hen's egg in order to examine the anti-angiogenic and anti-inflammatory activity. None of the tests showed pronounced activity, toxicity or irritation at the tested concentration.

Key words: *Phlomis linearis*, Essential Oil, CAM-Assay

Introduction

The genus *Phlomis* as perennial herbs or shrubs of Lamiaceae are represented by thirty four species of which twenty one are endemic for Turkey (Huber-Morath, 1982). The leaves and flowers of *Phlomis* species are used as aromatic amara, carminative, stimulant and are locally known as “Ballıkotu, Calba, Çalba, Şalba” in Turkish traditional medicine (Baytop, 1997, 1999). The endemic species *Phlomis linearis* Boiss. & Bal. grows in steps, rocky igneous slopes, volcanic rubble at 1400 to 2400 m and has a distribution area from central to east and southeast Anatolia. This species is distinguished by a wide tubular calyx with a leave size 4 to 8 times longer than the width. The calyx and bracteoles also contain intense long hairs, the corolla is two lipped, generally in yellow and the upper lip is occasionally brownish (Dadandı, 2002)

Chemical constituents of *Phlomis linearis* have been investigated by Çalıř and co-workers (1990, 1991); new glycosides, iridoids, neolignans, and their derivatives have been isolated. Some *Phlomis* species have also been investigated for both

their biological activities and essential oil compositions by different groups (Mirza and Nik, 2003; Couladis *et al.*, 2000; Sokovic *et al.*, 2002a,b; Ristic *et al.*, 2000)

The *in vivo* application on the chorioallantoic membrane of the fertilized egg (CAM-assay) is a useful and well established method to determine anti-angiogenic and anti-inflammatory activity of individual compounds or complex plant extracts. It can be used for testing natural compounds in small amounts for revealing various modes of action and the complex mechanisms related to angiogenesis and inflammation. Furthermore, possible side effects such as membrane irritation, toxic and anticoagulant properties of the investigated material in question can be revealed (Paper, 1998; D'Arcy and Howard, 1967; Bürgermeister *et al.*, 2002; and references cited herein).

In this study, we have isolated the volatiles by water distillation from the aerial parts of *P. linearis*. The essential oil was then analyzed by gas chromatography/mass spectroscopy (GC/MS). To evaluate the essential oil and its potential anti-angiogenic and anti-inflammatory activity, the CAM-assay was employed.

Methods and Materials

Plant material

Aerial parts of the plant were collected from a step area in Kayseri, Erciyes Mountain, at about 2000 m, on July 24, 2002, by one of us (M.Y.D.) and M. G. Halıcı. A voucher specimen (MYD1621) was kept at the Herbarium of Erciyes University, Faculty of Science and Letters.

Isolation of the essential oil

The aerial parts were dried in shade at room temperature. The essential oil was obtained by water distillation using a Clevenger type apparatus for 3 h having a yield of 0.05% (w/v).

Gas chromatography mass spectrometry

The essential oil was analysed by gas chromatography/mass spectrometry using a Hewlett-Packard GCD system. Innowax FSC column (60 m × 0.25 mm, 0.25 μm film thickness) was used with helium as carrier gas. GC oven temperature was kept at 60 °C for 10 min and programmed to 220 °C at a rate of 4 °C/min, and then kept constant at 220 °C for 10 min and programmed to 240 °C at a rate of 1 °C/min. Split flow was adjusted at 50 ml/min. The injector temperature was 250 °C. Mass spectra were recorded at 70 eV. Mass range was from *m/z* 35 to 425. *n*-Alkanes were used as reference points in the calculation of relative retention indices (RRI). Relative percentages of the characterized components were as listed in Table I. Library search was carried out using both “Wiley GC/MS Library” and “Baser Library of Essential Oil Constituents”.

CAM-assay

Sample preparation: The essential oil (10 mg/ml) was dissolved in a 2.5% (w/v) agarose (Merck, Darmstadt) solution. For ease of application pellets of these solutions (10 μl) were prepared and applied drop wise on circular teflon supports of 3 mm diameter, cooled to room temperature for solidification and applied on to the chorioallantoic membrane (CAM).

Preparation of the test medium: The fertilized hen’s eggs were previously incubated for 65–72 h at 37 °C and a relative humidity of 80%. The eggs were positioned in a horizontal position and ro-

Table I. The composition of the essential oil of *Phlomis linearis*.

RRI	Compound	%
1479	δ-Elemene	tr
1497	α-Copaene	0.3
1528	α-Bourbonene	tr
1535	β-Bourbonene	1.4
1553	Linalool	0.6
1589	β-Ylangene	0.5
1600	β-Elemene	0.7
1612	β-Caryophyllene	24.2
1650	γ-Elemene	0.1
1668	(Z)-β-Farnesene	6.6
1687	α-Humulene	3.5
1704	γ-Muurolene	0.4
1706	α-Terpineol	0.2
1726	Germacrene D	22.3
1740	α-Muurolene	1.4
1755	Bicyclogermacrene	1.1
1773	δ-Cadinene	1.0
1776	γ-Cadinene	tr
1808	Nerol	tr
1827	(E,E)-2,4-Decadienal	0.2
1838	(E)-β-Damascenone	0.3
1857	Geraniol	0.5
1868	(E)-Geranyl acetone	0.5
1900	Nonadecane	0.2
1958	(E)-β-Ionone	0.6
2001	Isocaryophyllene oxide	0.8
2008	Caryophyllene oxide	9.2
2045	Humulene epoxide-I	tr
2046	Norbourbonene	0.1
2071	Humulene epoxide-II	0.7
2095	Hexyl benzoate	0.2
2131	Hexahydrofarnesyl acetone	5.8
2179	3,4-Dimethyl-5-pentylidene-2(5H)-furanone	0.6
2198	Thymol	0.5
2209	T-Muurolol	0.3
2239	Carvacrol	1.2
2255	α-Cadinol	1.1
2300	Tricosane	0.6
2324	Caryophylla-2(12),6(13)-dien-5α-ol (Caryophylladienol II)	0.4
2384	Hexadecanol	0.9
2389	Caryophylla-2(12),6-dien-5α-ol (Caryophyllenol I)	0.1
2392	Caryophylla-2(12),6-dien-5β-ol (Caryophyllenol II)	1.1
2400	Tetracosane	0.1
2500	Pentacosane	1.3
2600	Hexacosane	tr
2622	Phytol	0.1
2655	Benzyl benzoate	0.2
2700	Heptacosane	1.7
2900	Nonacosane	0.8
	Total	94.5

RRI: Relative retention indices calculated against *n*-alkanes.

%: calculated from TIC.

tr: Trace (< 0.1%).

tated several times. Then the eggs were opened on the snub side. Before the opening 10–15 ml of albumin were aspirated from a hole on the pointed side. At two third of the height (from the pointed side) the eggs were traced with a scalpel and after that the shells were removed with forceps. The cavity was covered with film and the eggs were incubated at 37 °C at a relative humidity of 80% for further 75 h. If the formed CAM had approximately a diameter of 2 cm one pellet (1 pellet/egg) was placed on it. The eggs were incubated for one further day and then evaluated under the stereomicroscope. For every test compound 10–15 eggs were utilized.

Evaluation and scoring: For the evaluation of the effects, a scoring system was used (see Tables II and III). In brief; score: 0.5, no anti-angiogenic effect; score: 0.5–1, weak to medium anti-angiogenic effect; score: 1, medium to strong anti-angiogenic effect. As controls suramin (Merck, Darmstadt) and sodium dodecyl sulphate (SDS) (Merck, Darmstadt) at the concentration of 50 µg/pellet were also tested. As blank, CAMs treated with solidified agarose-solution in pellet form (2.5%, w/v) were also included. Each experiment was performed in triplicate (D'Arcy and Howard, 1967; Bürgermeister *et al.*, 2002).

Results and Discussion

The chemical compositions of various *Phlomis* species have been investigated. However, to the best of our knowledge this is the first investigation of the volatile fraction of *P. linearis*. The hydrodistillation of the aerial parts yielded a yellowish essential oil with a pleasant odour, however in a poor yield. Subsequent GC/MS analysis resulted in detection and identification of 49 components

representing 94.5% of the total oil composition (Table I). Sesquiterpene hydrocarbons were the major volatiles with β -caryophyllene (24.2%), germacrene D (22.3%), and (*Z*)- β -farnesene (6.6%), respectively. Oxygenated sesquiterpenes were present mostly as epoxides like caryophyllene oxide (9.2%). Noteworthy was the presence of various caryophyllene derivatives in different amounts that probably contribute to the characteristic odour of the oil. Oxygenated monoterpenes and monoterpene hydrocarbons were the minor portion of the whole oil which certainly also have impacts to the odour.

Due to the ethno-botanical use and various biological activities of *Phlomis* species we have also subjected the essential oil for potential anti-angiogenic and anti-inflammatory activity evaluation using the CAM-assay. At 100 µg/pellet the essential oil showed a very weak angiogenic effect compared to standard substances (Table II and III). When applied to the CAM the essential oil showed at this concentration neither toxic, nor irritant effects.

The essential oil composition of *Phlomis olivieri* Benth. from Iran was very recently reported, sesquiterpenes were among the major components with germacrene D (28.1%) and β -caryophyllene (16.1%) of fifteen identified compounds (Mirza and Nik, 2003). *Phlomis lanata* Willd. essential oil was analyzed to reveal forty-eight compounds representing 97% of the oil where α -pinene (25.4%), limonene (15.7%) and *trans*-caryophyllene (8.8%) were found as major components (Couladis *et al.*, 2000). Another study was on the essential oils from the leaves of *Phlomis fruticosa* L. Forty components were identified, β -caryophyllene (12%), (*E*)-methyl isoeugenol (15.3%) and α -asarone

Table II. Effects of *Phlomis linearis* essential oil in the CAM-assay.

Test Sample	Score*	Irritation (%)	
<i>Phlomis linearis</i> oil	0.2 ± 0.2	–	100 µg/pellet
Agarose	0.2 ± 0.2	–	Blanc (2.5%, w/v)
Suramin	0.5 ± 0.2	–	Positive control (50 µg/pellet)
Sodium dodecyl sulphate (SDS)	0	85 ± 5	Negative control (50 µg/pellet)

* Formula for scoring:

$$\text{Average score} = \frac{\text{Number of eggs (score 2)} \times 2 + \text{number of eggs (score 1)} \times 1}{\text{Total number of eggs (score 0, 1, 2)}}$$

Table III. Score values for the evaluation of the anti-angiogenic effect on the CAM.

Score	Effect	Observation/Interpretation
0	no	Normal embryo formation.
0.5	very weak	No capillary free area.
1	weak medium	Area with reduced density of capillaries around the pellet not larger than its own area. Small capillary free area or area with significantly reduced density of capillaries. Effects not larger than double the size of the pellet.
2	strong	Capillary free area around the pellet at least double the size of the pellet.

(10.9%) as major compounds, respectively (Sokovic *et al.*, 2002a). *Phlomis fruticosa* L. from two different localities in Yugoslavia were analyzed and investigated for their antimicrobial activities. Here, α -pinene (39–57%) was found to be the major constituent in both samples (Ristic *et al.*, 2000).

Phlomis species are widely distributed in Turkey, however, composition of the essential oil has not been investigated thoroughly. This is also the case for their biological activities. Using the CAM-assay other essential oils and major common components will be also tested in further work. Our studies on medicinal and aromatic *Phlomis* species are ongoing.

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