Pharmaceutical Biology

ISSN: 1388-0209 (Print) 1744-5116 (Online) Journal homepage: www.informahealthcare.com/journals/iphb20

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To cite this article: Betül Demirci, Fatih Demirci, Ali A. Dönmez, Gerhard Franz, Dietrich H. Paper & K. Hüsnü Can Başer (2005) Effects of *Salvia*. Essential Oils on the Chorioallantoic Membrane (CAM) Assay, Pharmaceutical Biology, 43:8, 666-671, DOI: 10.1080/13880200500383397

To link to this article: https://doi.org/10.1080/13880200500383397

Pharmaceutical Biology
2005, Vol. 43, No. 8, pp. 666–671

Taylor & Francis
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Effects of *Salvia* Essential Oils on the Chorioallantoic Membrane (CAM) Assay

Betül Demirci[1,2], Fatih Demirci[1,2], Ali A. Dönmez[3], Gerhard Franz[2], Dietrich H. Paper[2], and K. Hüsnü Can Başer[1]

[1]Department of Pharmacognosy, Faculty of Pharmacy, Anadolu University, Eskişehir, Turkey; [2]Department of Pharmaceutical Biology, Institute Pharmacy, University of Regensburg, Regensburg, Germany; [3]Department of Biology, Faculty of Science, Hacettepe University, Ankara, Turkey

Abstract

The aerial parts of *Salvia hedgeana* Dönmez, *Salvia huberi* Hedge, *Salvia pisidica* Boiss. & Heldr. *ex* Bentham. were subjected to hydrodistillation. The obtained essential oils were analyzed both by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). The analyses showed that *S. hedgeana* essential oil consisted of β-pinene (30.0%) as a major component, in addition to 65 identified components, representing 89.9% of the total essential oil. The major component of *S. huberi* was identified as 1,8-cineole (20.4%), and 76 further components were characterized representing 87.6%. *S. pisidica* essential oil revealed the occurrence of camphor (21.7%) as the main constituent among another 59 identified components of 76.8% of the total. Furthermore, the biological properties of the analyzed essential oils were tested using the *in vivo* method on the chorioallantoic membrane (CAM) in order to examine the anti-inflammatory and anti-angiogenic activity as well as possible irritant or toxic side effects. All essential oils tested at a concentration of 100 μg/pellet showed no pronounced anti-inflammatory, angiogenic, or membrane-toxic properties.

Keywords: CAM assay, essential oil, GC, GC-MS, *Salvia* species.

Introduction

The genus *Salvia* L. is one of the largest within the Lamiaceae family, which includes almost a thousand species spread throughout the world comprising several centers of diversity (Kintzios, 2000). The same genus was represented with 86 species in Turkey (Davis et al., 1988; Dönmez, 2001). Recently, three new species of this genus in Turkey have been described, resulting in a total of 89 species and altogether 94 taxa. Forty-five of these *Salvia* spp. are endemic (Dönmez, 2001; Demirci et al., 2002). *Salvia hedgeana* Dönmez is also described as a new species for the flora of Turkey (Dönmez, 2001).

Salvia, commonly known as sage, has been used since ancient times as a medicinal and aromatic plant having multiple uses such as condiment, food additive, seasoning, spice, and as herbal tea or its constituent. *Salvia* species, as a whole plant, extract, essential oil, or its preparations, have been reported to possess carminative, diuretic, antispasmodic, antioxidant, anti-inflammatory, analgesic, antipyretic, antiplatelet, antidiaphoretic, antihypertensive, antimicrobial, and antitumor, activities (Moretti et al., 1997; Baytop, 1999; Kintzios, 2000; Perry et al., 2001, 2003; Ulubelen, 2003), and so forth. It is also used in several psychological and neurological conditions (Kintzios, 2000; Perry et al., 2001, 2003).

Economic importance is currently limited only to *Salvia officinalis* L., *Salvia fruticosa* Miller, *Salvia pomifera* subsp. *pomifera* L., and *S. sclarea* L., which are collected from natural stands in Mediterranean countries including Turkey. However, cultivations in Italy, the United Kingdom, and the United States is performed (Kintzios, 2000; Başer, 2002).

Accepted: September 8, 2005

This work was presented at the 34th International Symposium on Essential Oils (ISEO 2003), which was held on September 7–10, 2003, in Würzburg, Germany.

Address correspondence to: Betül Demirci, Department of Pharmacognosy, Faculty of Pharmacy, Anadolu University, 26470 Eskişehir, Turkey. Tel.: +90 (222) 335 05 80 (ext. 3716); Fax: +90 (222) 335 07 50; E-mail: bdemirca@anadolu.edu.tr

DOI: 10.1080/13880200500383397

The main chemical constituents, such as flavonoids, polyphenols, monoterpenes, diterpenes, and triterpenes of *Salvia* species, have been subjected to phytochemical studies published in several reviews (Hegnauer, 1989; Kintzios, 2000; Başer, 2002; Demirci et al., 2002; Lu & Foo, 2002; Ulubelen, 2003). Previous work on the current study material is limited only to *S. pisidica* (Şarer, 1989; Demirci et al., 2002). The relative percentage and enantiomeric distribution of the main component was determined as the enantiomer (+)-camphor in the essential oil of *S. pisidica* using gas chromatography-mass spectrometry (GC-MS) and MD-GC-MS (Demirci et al., 2002). Further, the chemical composition of *S. pisidica* essential oil was studied by GLC followed by liquid-solid chromatography. The major components of the oil were reported as sabinyl acetate, α- and β-thujone, 1,8-cineole, and camphor (Şarer, 1989). To the best of our knowledge, there is, however, no previous report on the constituents of the essential oils of *S. hedgeana* and *S. huberi*.

In the continuation of our biological screenings of essential oils and their constituents, in this study the essential oils from the dried aerial parts of *Salvia hedgeana* Dönmez, *S. huberi* Hedge, and *S. pisidica* Boiss. & Heldr *ex* Bentham (see Table 1 for collection places), all endemic in Turkey, were obtained by hydrodistillation and were further analyzed by GC and GC-MS. The constituents characterized in the essential oils are given in Table 2 with their relative percentages. The *Salvia* essential oils were also evaluated as potential anti-inflammatory and anti-angiogenic agents using the *in vivo* chorioallantoic membrane (CAM) assay.

Materials and Methods

Plant material

Aerial parts of the plants were collected from different regions of Turkey by one of the authors (A.A.D.). Voucher specimens were kept at the Herbarium of Hacettepe University, Department of Biology Faculty of Science. Information concerning the plant material is given in Table 1.

Isolation and analysis of the essential oils

The dried aerial parts were hydrodistilled for 3 h using a Clevenger-type apparatus. The yields were calculated on dry weight basis (v/w). (see Table 1).

The oils were analyzed by GC using a Hewlett Packard (USA) 6890 system. An HP-Innowax FSC column (60 m × 0.25 mm Ø, with 0.25-μm film thickness) was used with nitrogen as carrier gas (1 ml/min). The oven temperature was kept at 60°C for 10 min and programmed to 220°C at a rate of 4°C/min, then kept constant at 220°C for 10 min and programmed to 240°C at a rate of 1°C/min. The injector temperature was set at 250°C. The percentage of the individual components were obtained from electronic integration measurements using flame ionization detection (FID: 250°C). *n*-Alkanes were used as reference points in the calculation of relative retention indices (RRIs). Relative percentages of the characterized components were as cited in Table 2.

GC-MS analysis was performed with 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 conditions were as described above. 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. A library search was carried out using the in-house Başer Library of Essential Oil Constituents.

The biological activity of the essential oils was tested on the chorioallantoic membrane (CAM) assay by modification of the method of D'Arcy and Howard (1967), which was also described earlier (Bürgermeister et al., 2002; Demirci et al., 2004).

The CAM assay

Salvia essential oils were dissolved in a 2.5% (w/v) agarose (Merck, Darmstadt, Germany) solution. For ease of application, pellets of these solutions (10 μl) were prepared and applied dropwise on circular Teflon supports of 3-mm diameter, cooled to room temperature for solidification, and applied onto the CAM.

The fertilized hen's eggs were previously incubated for 65–72 h at 37°C at a relative humidity of 80%. The eggs were positioned in a horizontal position and rotated

Table 1. Information on the plant material.

Salvia ssp.	Collection site	Altitude (m)	Collection period	Essential oil yield[a] (%)	AAD[b]
S. hedgeana	Sivas: Divriği, Uzunkaya village	1545	June 2002	0.2	10887
S. huberi	Erzurum: Oltu, Dutlu village	1285	July 2002	0.2	11058
S. pisidica	Antalya: Korkuteli-Karaman village	1430	August 2002	0.18	10924

[a]Essential oil yields are given on moisture-free basis (v/w).
[b]AAD: Herbarium code of the collector.

Table 2. The composition of the essential oils of *Salvia* species.

No.	Compound	RRI	*S. hedgeana*	*S. huberi*	*S. pisidica*
1	Tricyclene	1014	0.1	0.1	—
2	α-Pinene	1032	4.0	10.1	0.3
3	α-Thujene	1035	0.3	0.1	—
4	Camphene	1076	0.9	2.4	0.2
5	β-Pinene	1118	30.0	12.3	0.1
6	Sabinene	1132	3.2	3.2	0.1
7	Myrcene	1174	1.0	1.0	tr
8	α-Terpinene	1188	0.2	0.1	0.1
9	Limonene	1203	7.9	6.7	0.1
10	1,8-Cineole	1213	23.1	20.4	4.7
11	(*Z*)-β-Ocimene	1246	0.8	tr	—
12	γ-Terpinene	1255	0.4	0.3	0.2
13	(*E*)-β-Ocimene	1266	0.2	tr	—
14	*p*-Cymene	1280	0.3	0.1	0.8
15	Terpinolene	1290	0.1	0.1	0.1
16	1-Octen-3-one	1304	tr	0.1	—
17	3-Octyl acetate	1345	—	—	0.1
18	Octenyl acetate	1386	—	0.1	0.1
19	(*Z*)-3-Hexenol	1391	—	0.1	—
20	3-Octanol	1393	tr	0.2	0.1
21	α-Thujone	1437	—	—	0.2
22	γ-Campholene aldehyde	1439	—	0.1	—
23	β-Thujone	1451	—	—	4.2
24	1-Octen-3-ol	1452	0.2	0.5	—
25	Eucarvone	1465	—	—	0.1
26	*trans*-Sabinene hydrate	1474	0.6	0.7	0.5
27	α-Copaene	1497	0.1	1.1	—
28	α-Campholene aldehyde	1499	—	—	0.6
29	Chrysanthenone	1522	—	—	0.1
30	Camphor	1532	2.2	6.6	21.7
31	β-Bourbonene	1535	0.1	—	—
32	β-Cubebene	1549	—	0.2	—
33	Linalool	1553	0.1	0.1	0.3
34	*cis*-Sabinene hydrate	1556	0.2	0.2	0.4
35	*trans*-*p*-Menth-2-en-1-ol	1571	0.1	tr	0.1
36	Pinocarvone	1586	0.8	tr	0.5
37	β-Ylangene	1589	—	0.3	—
38	Bornyl acetate	1597	—	0.4	3.9
39	β-Elemene	1600	0.3	0.1	—
40	β-Gurjunene	1610	—	0.6	0.1
41	Terpinen-4-ol	1611	0.6	—	0.8
42	β-Caryophyllene	1612	—	1.9	—
43	6,9-Guaiadiene	1617	—	1.2	0.8
44	Aromadendrene	1628	—	—	0.4
45	*trans*-*p*-Mentha-2,8-dien-1-ol	1639	0.1	0.2	—
46	Myrtenal	1648	1.1	0.1	0.5
47	γ-Elemene	1650	—	0.1	—
48	Sabinyl acetate	1658	—	—	12.4
49	(*Z*)-β-Farnesene	1668	0.6	1.1	—
50	*trans*-Pinocarveol	1670	1.2	0.4	—
51	δ-Terpineol	1682	—	0.2	—
52	*trans*-Verbenol	1683	—	—	3.5
53	α-Humulene	1687	1.0	0.9	—
54	α-Terpineol	1706	0.3	0.7	0.2
55	Borneol	1719	0.6	1.9	2.5
56	Germacrene D	1726	1.7	1.1	1.1
57	(*Z*,*E*)-α-Farnesene	1737	—	0.4	—

(*Continued*)

Table 2. Continued.

No.	Compound	RRI	*S. hedgeana*	*S. huberi*	*S. pisidica*
58	Carvone	1751	0.2	—	0.3
59	Bicyclogermacrene	1755	—	0.2	—
60	(*E*,*E*)-α-Farne sene	1758	—	0.4	—
61	Naphthalene	1763	—	—	0.1
62	δ-Cadinene	1773	0.2	0.4	0.1
63	γ-Cadinene	1776	—	0.2	0.6
64	*ar*-Curcumene	1786	—	0.1	—
65	*p*-Methyl acetophenone	1797	0.1	—	tr
66	Cumin aldehyde	1802	—	—	0.6
67	Myrtenol	1804	1.0	0.4	—
68	2-Tridecanone	1815	0.1	—	—
69	(*E*)-β-Damascenone	1838	—	—	0.1
70	*trans*-Carveol	1845	tr	0.1	0.7
71	Calamenene	1853	—	0.6	0.1
72	Germacrene-B	1854	0.3	—	—
73	*p*-Cymen-8-ol	1864	—	—	0.4
74	(*E*)-Geranyl acetone	1868	tr	0.1	—
75	*cis*-Carveol	1882	tr	0.1	—
76	*epi*-Cubebol	1900	0.2	0.1	—
77	α-Calacorene	1941	—	0.1	—
78	*trans*-Jasmone	1948	—	0.1	—
79	Palustrol	1953	0.3	—	—
80	Cubebol	1957	0.5	0.1	0.3
81	*cis*-Jasmone	1969	—	tr	—
82	2-Phenylethyl-2-methylbutyrate	1988	—	0.1	—
83	Isocaryophyllene oxide	2001	—	0.3	—
84	Caryophyllene oxide	2008	0.3	2.5	2.4
85	Salvial-4(14)-en-1 -one	2037	0.2	0.1	—
86	Ledol	2057	0.3	—	—
87	Humulene epoxide-II	2071	0.6	0.8	—
88	*p*-Mentha-1,4-dien-7-ol	2073	—	—	0.4
89	Cubenol	2080	—	—	0.2
90	β-Oplopenone	2092	—	0.2	—
91	Furopelargone B	2109	—	0.2	3.8
92	Spathulenol	2144	—	0.9	0.5
93	Valeranone	2145	0.3	—	—
94	T-Cadinol	2187	—	—	1.4
95	Thymol	2198	0.2	—	—
96	T-Muurolol	2209	0.2	—	—
97	Carvacrol	2239	—	—	0.2
98	α-Eudesmol	2250	0.1	0.1	—
99	β-Eudesmol	2257	0.1	0.4	1.9
100	Caryophylla-2(12),6(13)-dien-5α-ol (caryophylladienol II)	2324	—	—	0.4
101	Isopimaradiene	2349	0.2		—
102	Caryophylla-2(12),6-dien-5β-ol	2392	—	0.4	0.4
103	1-Octadecanol	2607	—	—	—
104	14-Hydroxy-δ-cadinene	2607	0.1	—	—
105	Nonacosane	2900	—	0.1	—
	Monoterpene hydrocarbons		49.4	36.5	2.0
	Oxygenated monoterpenes		32.5	32.7	59.8
	Sesquiterpene hydrocarbons		4.3	11.0	3.2
	Oxygenated sesquiterpenes		3.2	5.9	7.5
	Others		0.5	1.5	4.3
	Total		89.9	87.6	76.8

[a]Sequence of the compounds is listed according to relative retention indices (RRIs).
[b]RRIs, relative retention indices calculated against *n*-alkanes on a polar column. % calculated from TIC data.
[c]tr, trace (<0.1%)

several times. Then the eggs were opened on the snub side. Before opening, 10–15 ml of albumin was aspirated from a hole on the pointed side. At two-thirds of the height (from the pointed side), the eggs were traced with a scalpel, and 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 a further 75 h. If the formed CAM had approximately a diameter of 2 cm, 1 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 used in parallel each time.

For the evaluation of the effects on the CAM, a scoring system was used as described recently (Bürgermeister et al., 2002). Briefly, the score obtained from Eq. (1) was assigned as follows:

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

score ≤ 0.5 = no anti-angiogenic effect; score 0.5 to 1 = weak to medium anti-angiogenic effect; and score ≥ 1 = medium to strong anti-angiogenic effect. Results can be seen in Table 3. As controls, LaPSvS1 [see (Bürgermeister et al., 2002)] suramin (Merck), and sodium dodecyl sulfate (SDS; Merck) 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 at least in triplicate.

Results and Discussion

Overall, more than 100 components of the investigated *Salvia* spp. have been identified using GC and GC-MS as seen in Table 2. Fifty-nine compounds were identified representing 89.9% of the total essential oil of *S. hedgeana*, with β-pinene (30.0%) and 1,8-cineole (23.1%) as the main constituents.

GC-MS analysis of the essential oil of *S. huberi* has shown that 1,8-cineole (20.4%) and β-pinene (12.3%) were the main constituents. Seventy-three compounds were characterized representing 87.6% of the total oil.

S. pisidica essential oil revealed the occurrence of camphor (21.7%) and sabinyl acetate (12.4%) as the main constituents, among 57 identified components of 76.8% of the total components.

The essential oil profiles of *S. hedgeana* suggest that *S. huberi* is hereditary related but quite obviously different in nature. Distinctive differences between these species also exist at morphological and microscopic levels (Dönmez, 2001). When the overall chemical compositions of the essential oils were elaborated, *S. hedgeana* and *S. huberi* contained monoterpene hydrocarbons, whereas *S. pisidica* was composed of oxygenated monoterpenes as major constituents. Interestingly, the essential oil of *S. huberi* showed a remarkable amount of sesquiterpenes with 11%, knowing that *Salvia* spp. generally lack sesquiterpenes and diterpenes (Table 2).

In the light of previous research on the extensive ethnobotanical use and various biological activity experiments, including wound healing, antioxidant, anti-inflammatory, analgesic, antipyretic and antitumor activities of *Salvia* species (Peana & Satta, 1993; Moretti et al., 1997; Baytop, 1999; Kintzios, 2000; Perry et al., 2001, 2003; Kim et al., 2002), the current work was directed to evaluate the potential anti-inflammatory and anti-angiogenic activity of the *Salvia* essential oils using the CAM assay (D'Arcy & Howard, 1967; Bürgermeister et al., 2002; Demirci et al., 2004). The CAM assay can be used for evaluation of natural products and pure compounds as a preferential alternative to animal experiments. The *in vivo* test on the chorioallantoic membrane of the fertilized hen's egg (CAM assay) or

Table 3. Effects of *Salvia* essential oils in the CAM assay.

Test samples	Score*	Irritation (%)	Toxicity Concentration (%)	Concentration (μg/pellet)
S. hedgeana	0.3 ± 0.1	18 ± 10	—	100
S. huberi	0.1 ± 0.2	27 ± 15	—	100
S. pisidica	0.2 ± 0.2	18 ± 10	—	100
Agarose (Blank)	0.2 ± 0.1	—	14 ± 8	2.5%, w/v
Suramin (Positive control)	0.5 ± 0.2	—	—	50
LaPSvS1 (Positive control)	1.2 ± 0.2	—	—	50
Sodium dodecyl sulfate (SDS) (Negative control)	0.1 ± 0.1	85 ± 5	18 ± 5	50

CAM, chorioallantoic membrane.

its modified versions are current methods to determine anti-angiogenic and anti-inflammatory activity and toxic effects of individual compounds or complex plant extracts. Possible side effects such as membrane irritation, embryotoxic and anticoagulant properties of the investigated material in question can also be detected (Luepke, 1985; Reichling et al., 2000; Wilson et al., 2000; Bürgermeister et al., 2002).

The essential oils were initially tested at a concentration of 100 µg/pellet, as generally practiced in the CAM assay (Bürgermeister et al., 2002; Demirci et al., 2004). The obtained results are presented in Table 3. All essential oils tested showed a rather weak anti-angiogenic effect compared with the standard substances suramin and LaPSvS1. However, *S. hedgeana* was slightly more effective than the other two essential oils, which were at a similar activity level. Another observation was that the essential oil of *S. huberi* was remarkably more irritating when compared with the other two tested essential oils, albeit no toxic effects at the tested concentration were observed.

Conclusions

As an overall conclusion, the *Salvia* essential oils tested in the CAM assay did not possess any noteworthy anti-inflammatory and anti-angiogenic properties at the tested concentration. The modified Hühner Embryonen Test-Chorio Allantoic Membrane (HET-CAM) assay is still worthwhile to employ to evaluate the anti-inflammatory effect of the oils as well as other fractions (e.g., polar water-soluble extracts or compounds) of the plant material.

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