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Investigation of the *Origanum onites* L. Essential Oil Using the Chorioallantoic Membrane (CAM) Assay

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The in vivo test on the chorioallantoic membrane of the fertilized hen's egg (CAM assay) is a current method to determine antiangiogenic, antiinflammatory activity and toxic effects of individual compounds or complex plant extracts. The method is 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 detected. For the evaluation, the essential oil obtained by hydrodistillation of the aerial parts of *Origanum onites* L., a common spice and medicinal plant, was tested for its effect in the chorioallantoic membrane (CAM) assay. The essential oil composition was revealed by means of gas chromatography-mass spectrometry (GC-MS). Eighty three components were identified, representing 99.1% of the total oil. Carvacrol, thymol, *p*-cymene, and γ-terpinene were found as major components and were also individually tested in the CAM assay. Along with the monoterpenes carvacrol and thymol, their methyl ether derivatives were also examined for comparison of their physiological action. Neither the essential oil nor its components showed any pronounced antiinflammatory or antiangiogenic property in the CAM assay, at 10−250 μg/pellet. However, the irritant effect of the essential oil was linked to thymol in a dose−response fashion, up to 10 μg/pellet, where it was still showing irritation.

KEYWORDS: Chorioallantoic membrane (CAM)-assay; *Origanum onites* L.; essential oil; monoterpenes; GC-MS

INTRODUCTION

The genus *Origanum* L. of Lamiaceae family within the Menthae tribe consists of 22 species or 32 taxa, of which 21 are endemic with a variation of ecotypes and chemotypes native to Southeast Europe, Turkey, and Syria (*1*−*3*).

Origanum are among the species recognized as "oregano" (= kekik) in Turkey. Aerial parts of *Origanum* species are aromatic and fragrant which are used as spice, condiment, or herbal tea in addition to its medicinal uses since ancient times. The aromatic oregano water, rich in carvacrol and water soluble compounds, is consumed for gastrointestinal disorders, to reduce blood cholesterol and glucose levels, and also against cancer (*4*−*6*). Oregano oil, mainly rich in carvacrol, is used as a pain killer in rheumatism by rubbing externally on the painful limbs (*3*−*7*).

Dried *Origanum* species are used for the production of the essential oil (*Origanum* oil = kekik yağı) and an aromatic water or hydrosol (*Origanum* water = kekik suyu). Turkey has, in recent years, become a major supplier of oregano herb for various demands. *Origanum onites* (Syn. *O. smyrnaeum* L.) tops the list of commercial *Origanum* species of Turkey and composes over 80% of all the oregano exports of Turkey. This species is mainly wild crafted but also cultivated (*3*−*5*).

During our research into the essential oils of *O. onites*, carvacrol (67−82%) was found as main constituent in thirteen samples. However, four samples from Antalya and Muğla provinces in Southern Turkey yielded oils rich in linalool (80−92%), as well. Mixed chemotypes were also encountered in three samples containing carvacrol (36−66%) and linalool (15−52%) as main constituents. Chemical composition of *O. onites* essential oils of different origins has also been previously investigated and reviewed (*2*, *3*, *7*−*10*, and references herein).

The fertilized hen's egg test (Hühner-Embryonen-Test) on the chorio allantoic membrane, known as HET-CAM or CAM test/assay and relevant modifications (*11*−*13*), is utilized to determine antiangiogenic, antiinflammatory and anti-irritant properties of individual compounds or complex plant extracts. It can be used as an in vivo model for testing natural compounds in minute amounts for revealing various modes of action and

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Table 1. The Essential Oil Composition of *Origanum onites* L.

no.	compound[a]	RRI[b]	%[c]	no.	compound[a]	RRI[b]	%[c]
1	tricyclene	1014	tr	43	α-terpineol	1706	0.3
2	methyl 2-methylbutyrate	1018	tr	44	borneol	1719	2.6
3	α-pinene	1032	0.7	45	germacrene D	1726	0.3
4	α-thujene	1035	0.2	46	carvenone	1737	0.2
5	camphene	1076	0.4	47	β-bisabolene	1741	1.2
6	β-pinene	1118	0.1	48	carvone	1751	0.2
7	δ–3-carene	1159	0.1	49	*cis*-piperitol	1758	tr
8	myrcene	1174	1.5	50	geranyl acetate	1765	tr
9	α-terpinene	1188	1.4	51	δ-cadinene	1773	0.1
10	limonene	1203	0.3	52	γ-cadinene	1776	0.1
11	1,8-cineole	1213	0.1	53	β-sesquiphellandrene	1783	tr
12	β-phellandrene	1218	0.2	54	(*E*)-α-bisabolene	1784	tr
13	(Z)–3-hexenal	1225	tr	55	nerol	1808	tr
14	(*Z*)-β-ocimene	1246	tr	56	*p*-mentha-1(7),5-dien-2-ol	1823	tr
15	γ-terpinene	1255	5.2	57	*trans*-carveol	1845	tr
16	(*E*)-β-ocimene	1266	tr	58	calamenene	1849	tr
17	*p*-cymene	1280	8.1	59	geraniol	1857	tr
18	terpinolene	1290	0.3	60	*p*-cymen-8-ol	1864	0.1
19	3-octanol	1393	tr	61	thymol acetate	1867	tr
20	β-thujone	1451	tr	62	carvacryl acetate	1890	0.3
21	α,*p*-dimethylstyrene	1452	0.1	63	4-isopropyl salicylaldehyde	1940	0.1
22	1-octen-3-ol	1452	0.3	64	α-calacorene	1941	tr
23	*trans*-sabinene hydrate	1474	0.1	65	caryophyllene oxide	2008	0.5
24	furfural	1479	tr	66	(*E*)-nerolidol	2050	tr
25	dihydroachillene	1547	0.1	67	humulene epoxide II	2071	tr
26	linalool	1553	0.1	68	cubenol	2080	tr
27	*cis*-sabinene hydrate	1556	0.1	69	globulol	2098	0.1
28	linalyl acetate	1565	0.2	70	cumin alcohol	2113	tr
29	*trans*-*p*-menth-2-en-1-ol	1571	0.1	71	spathulenol	2144	0.3
30	bornyl acetate	1590	tr	72	*cis*-*p*-menth-4-en-1,2-diol	2157	0.1
31	*trans*-β-bergamotene	1594	tr	73	isothymol (= *2-isopropyl-4-methyl phenol*)	2181	0.1
32	β-caryophyllene	1612	2.4	74	eugenol	2186	tr
33	carvacrol methyl ether	1614	0.1	75	T-cadinol	2187	0.3
34	aromadendrene	1628	0.3	76	thymol	2198	11.6
35	*cis*-*p*-menth-2-en-1-ol	1638	tr	77	isocarvacrol (= *4-isopropyl-2-methyl phenol*)	2221	0.1
36	*cis*-isodihydrocarvone	1645	tr	78	carvacrol	2239	57.4
37	phenylacetaldehyde	1663	tr	79	3-isopropyl-5-methyl phenol	2300	tr
38	*trans*-pinocarveol	1670	tr	80	caryophylla-2(12),6(13)-dien-5α-ol (= *caryophylladienol II*)	2324	0.1
39	α-humulene	1687	0.1	81	manoyl oxide	2376	0.1
40	*trans*-piperitol (= *trans-p-menth-1-en-3-ol*)	1689	tr	82	caryophylla-2(12),6-dien-5α-ol (= *caryophyllenol I*)	2389	0.2
41	*p*-mentha-1,8-dien-4-ol (= *limonen-4-ol*)	1700	tr	83	caryophylla-2(12),6-dien-5β-ol (= *caryophyllenol II*)	2392	0.1
42	γ-muurolene	1704	tr	83	total		99.1
	total				**99.1**		

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

mechanisms related to angiogenesis and inflammation. Furthermore, possible side effects such as membrane irritation, lysis, coagulation, and toxic properties of the compounds or compound complexes in question can be detected (*11*−*14*). The CAM assay and/or modified versions were previously used in screening and testing of various natural products, including essential oils, as well as cosmetics or other chemicals as an alternative to animal experiments (*11*−*17*).

Utilizing the CAM assay (*11*, *17*), an economically important product of Turkey, namely the *Origanum onites* L. essential oil, was examined. In addition, the major components carvacrol, thymol, *p*-cymene, and γ-terpinene, identified by GC-MS, were also tested. Along with the monoterpenes carvacrol and thymol, their structurally related methyl ether derivatives were also evaluated in the CAM assay for comparison.

MATERIALS AND METHODS

Plant Material and Distillation of the Essential Oil. The plant material was collected in the surroundings of Antalya: Kemer−Kaya village, Turkey, on 26.5.1998. Voucher specimens are kept at the Herbarium of the Faculty of Pharmacy (ESSE 13022), at the Anadolu University. Air dried aerial parts of the plant material were cut and crushed into small pieces and then subjected to hydrodistillation for 3 h using a Clevenger-type system. The essential oil yield was calculated based on moisture free basis as 2.35% (w/v). Azeotropic distillation method was used to determine moisture content of the plant material.

Analysis of Essential Oil. The oil was analyzed by GC-MS using a Hewlett-Packard GCD system. An HP−Innowax FSC column (60 m × 0.25 mm i.d., with 0.25-μm film thickness) was used with helium as carrier gas (1 mL/min). GC oven temperature was kept at 60 °C for 10 min and programmed to 220 °C at a rate of 4 °C/min, kept constant at 220 °C for 10 min, and then programmed further to 240 °C at a rate of 1 °C/min. The split ratio was adjusted to 50:1. The injector temperature was at 250 °C. Mass spectra were recorded at 70 eV, and the mass range was from 35 to 425 *m*/*z*.

Identification of Components. The components were identified by comparison of their mass spectra both with the Wiley GC/MS Library and the in-house Baser Library of Essential Oil Constituents. Relative percentage amounts of the separated compounds were calculated automatically from peak areas of the total ion chromatogram. *n*-Alkanes were used as reference points in the calculation of relative retention indices (RRI). The compounds identified in the oil are listed in **Table 1**.

Performance of the CAM Assay. The essential oil and the commercially available pure test compounds (95−99%, Fluka, Sigma-Aldrich, Taufkirchen; Merck, Darmstadt; Frutarom, Haifa) were dis-

Table 2. Chorioallantoic Membrane (CAM) Assay (Antiangiogenic Effect) Results of *Origanum onites* Essential Oil and Monoterpenes

test samples	score #	irritation (%)	toxicity[a] (%)	conc (μg/pellet)
Origanum onites L.	0.3 ± 0.1	100		250
	0.2 ± 0.1	90 ± 5		125
	0.2 ± 0.1	90 ± 5		50
	0.2 ± 0.1			10
carvacrol	0.1 ± 0.1			125
	0.2 ± 0.1			50
	0.2 ± 0.1			10
thymol	0.2 ± 0.1	100	20 ± 10	125
	0.4 ± 0.1	95 ± 5	15 ± 10	50
	0.3 ± 0.1	30 ± 0	8 ± 0	10
γ-terpinene	0.4 ± 0.1		10	50
p-cymene	0.1 ± 0.2			50
carvacrol methyl ether	0.2 ± 0.1			50
thymol methyl ether	0.2 ± 0.1			50
agarose	0.2 ± 0.2		14 ± 8	(blank)
suramin	0.5 ± 0.2			(positive control)
sodium dodecyl sulfate (SDS)	0.1 ± 0.1	85 ± 5	18 ± 5	(negative control)

[a] 25% ≤ nontoxic #. See eq 1 for score calculation.

solved or emulsified in a 2.5% (w/v) agarose (Merck) solution to reach the final concentrations of 1−25 mg/mL (10−250 μg/pellet), as seen in **Table 2**. For ease of application, pellets of these solutions (10 μl) were prepared and applied dropwise on circular Teflon supports of 3-mm diameter and cooled to room temperature for solidification, immediately.

The fertilized chick eggs (Brüterei Süd, Regensburg) were incubated previously for 65−72 h at 37 °C and a relative humidity of 80%. During this period, the eggs were positioned in a horizontal position and rotated several times. After the incubation, the eggs were opened on the snub side. However, prior to opening, approximately 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 after that the shells were removed carefully with forceps. The opened cavity was covered with film, and the eggs were incubated at 37 °C at a relative humidity of 80% for a further 72 h. If the formed CAM had a diameter of approximately 2 cm, one of the freshly prepared sample pellets (1 pellet/egg) was placed onto it. The eggs were incubated under the same conditions for one further day and then evaluated using a conventional stereo-microscope, where necessary with contrast material. For every test substance, 10−15 eggs were utilized. As standard controls, suramin (Merck) and sodium dodecyl sulfate (SDS) (Merck) at the concentration of 50 μg/pellet were also tested. As a blank, CAMs treated only with agarose pellets were included. A scoring system was used for the evaluation of CAM and the resulting effect, which can briefly be explained as score < 0.5 = no antiangiogenic effect; score 0.5−1.0 = weak to medium antiangiogenic effect; score > 1.0 = medium to strong antiangiogenic effect. Each experiment was performed at least in triplicate, according to the modified method of D'Arcy and Howard (*11*), as recently described (*14*). The formulas for calculation of the scoring values (**Table 2**) are given in eq 1.

$$\text{ave. score} = \frac{\text{no. of eggs (score 2)} \times 2 + \text{no. of eggs (score 1)} \times 1}{\text{total no. of eggs (score 0, 1, 2)}} \quad (1)$$

RESULTS AND DISCUSSION

The plant material was collected from Antalya, and water was distilled to yield 2.35% essential oil on dry basis. The 83 components of the investigated *Origanum onites* essential oil were identified by means of GC-MS, representing 99.1% of the total oil. Carvacrol (57.4%), thymol (11.6%), *p*-cymene (8.1%), and γ-terpinene (5.2%) were identified as the major components. Oxygenated monoterpenes composed the majority of the components found in the essential oil. These results resemble those of previous investigations (*2*, *5*, *9*, *10*). To the best of our knowledge, the CAM assay was performed for the first time on *Origanum onites* essential oil and some of its individual constituents. This assay was utilized to explore the antiinflammatory and antiangiogenic potential and to evaluate the safety of the oregano essential oil, which is in consumption from the point of irritation and toxicity.

Our results in the CAM assay suggested no pronounced antiangiogenic or antiinflammatory effect of the essential oil or its major components at the tested concentrations (10−250 μg/pellet). It was observed that it rather caused irritation comparable to that of the standard sodium dodecyl sulfate (SDS). The oregano oil concentration of 250 μg/pellet showed strong irritation up to 10 μg/pellet when applied. Consequently, the major essential oil components were questioned for the irritation and were applied in pure form to the CAM using the same conditions, which interestingly indicated that thymol even in low concentrations was responsible for the strong irritation, however, with negligible toxicity as seen in **Table 2**. It was also shown that thymol was less irritating when the applied concentration was decreased, showing the dose response of the CAM. Interestingly, the major component carvacrol (57.4%), the structural isomer of thymol did not show any irritation at the same applied concentrations. Neither thymol methyl ether nor carvacrol methyl ether showed toxicity or irritation effects when applied in the same concentration, showing the link and importance to the position of the hydroxyl function on the monoterpene. Furthermore, it should be mentioned that thymol is used more than carvacrol in different herbal formulations (*18*). Unfortunately, none of the tested compounds showed any antiinflammatory or antiangiogenic effects. None of the components scored comparably to the standard suramin, although some effects have been observed that still would suggest weak to moderate anti-angiogenic effect.

Certain essential oils and some of their components were earlier investigated from a membrane irritation viewpoint using a modified CAM assay by Reichling et al. (*14*), specifically for cosmetic applications. Only γ-terpinene and *p*-cymene were the comparable pure substances that were also examined in our system. *p*-Cymene and γ-terpinene were found to promote distinctive irritation, where γ-terpinene was more irritating than *p*-cymene. Interestingly, the essential oil of *Thymus vulgaris* L. was reported to be very irritative, among other tested essential oils. This work also demonstrates the irritation through decomposed or oxidized essential oils, which is also a well-known phenomena or characteristic for some essential oils (*19*, *20*). When compared to our common tested substances *p*-cymene and γ-terpinene, which were not irritating in our CAM system, the concentration and application fashion and time could be discussed.

Natural products and essential oils are still of potential interest and will remain in application for their antiinflammatory properties, which was and is subject to research (*19*−*22*).

In conclusion, the CAM assay is a versatile tool for the in vivo mechanistic evaluation of antiangiogenic and antiinflammatory natural products (*11*−*13*). Essential oils and their components can be conveniently examined using the CAM assay. Additionally, possible side effects, like membrane irritation and toxic properties of the test compounds can be observed in the same system. Thus, essential oils whether consumed, used, applied internally or externally can be justified and evaluated for their unwanted effects as well. Investigations with other essential oils are under progress to substantiate these findings.

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