Antimycotic Activity of Some Aromatic Plants Essential Oils Against Canine Isolates of *Malassezia pachydermatis*: An *in vitro* Assay

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Abstract: The antifungal activity and the chemical composition of essential oils (EOs) from some Mediterranean autochthonous plants were investigated against *Malassezia pachydermatis*, a fastidious opportunistic yeast usually involved in canine external otitis. Minimum inhibitory concentrations (MICs) of *Anthemis nobilis, Citrus limon, Citrus paradisi, Illicium verum, Lavandula hybrida, Mentha piperita, Ocimum basilicum, Origanum vulgare, Origanum majorana, Rosmarinus officinalis, Salvia sclarea, Thymus serpillum* were assessed by microdilution test; minimum fungicidal activity (MFC) was also determined. *O. vulgare, T. serpillum* and *O. basilicum* showed the lowest MIC and MFC values (0.8%) followed by *C. limon* and *M. piperita* (1%).

EOs from the tested plants showed variable degrees of anti-malassezia activity, putatively related to their chemical composition.

The effectiveness, manageability and pleasant organoleptic properties of *O. vulgare, T. serpillum, O. basilicum, C. limon* and *M. piperita* EOs make them advisable as promising new natural antifungal drugs in the management of *M. pachydermatis* otitis in dog.

Keywords: Essential oils activity, *malassezia pachydermatis, in vitro* sensitivity, microdilution test, gas chromatography-mass spectra, mediterranean plants.

INTRODUCTION

Essential oils (EOs) are concentrated, hydrophobic substances containing volatile aroma compounds extracted by various processes from different parts of plants. Many common EOs are characterized by healing properties well known in folk medicine since ancient times and still widely used today. The therapeutic value of an EO is held in its composition, which represents a complex make-up of many chemical components with different biological activities. The chemical composition and yield of different EOs show wide variation, depending on the herbal source, chemotype of the plant species, and the analytical methods used [1].

Many studies have demonstrated that extracts from aromatic plants traditionally used in popular medicine, exert antiseptic and inhibitory activities upon filamentous fungi and yeasts [2-4]. The phytotherapeutical use of EOs in veterinary medicine is still poorly substantiated by evidence-based studies, being mainly based on anecdotal experiences of veterinarians, aromatherapists and pet owners.

Malassezia pachydermatis is a normal commensal and occasional pathogen of the skin and mucosae in Carnivora, and represents the most frequently isolated yeast in canine external otitis [5]. Most cases of *Malassezia* otitis in the dog are associated with concurrent skin disorders or systemic diseases. Treatment is generally based on topical administration of azoles or nystatin, combined with antibiotics and glucocorticoids [6]. Many dogs affected by *Malassezia* require regular maintenance therapy to prevent relapse, which is common when predisposing factors are not identified or corrected.

The factors involved in the transition from commensalism to parasitism by *M. pachydermatis* in dogs are not fully understood even if it is well stated that the interface between commensalism and pathogenicity in *Malassezia* infections is a fine balance, in which both the regulation of host immune response and the activity of the fungus are strictly involved [7].

Among the multitude of EOs, tea tree oil (*Melaleuca alternifolia*) is the most extensively tested against several microbial agents. Some studies deal with the effectiveness versus *Malassezia* yeasts responsible for both canine [8] and human infections [9, 10]. In veterinary medicine attention should be paid to otologic use of tea tree oil due to its toxicity after instillation into the ear [11], consequently the search for alternative products is recommended.

In recent years, the interest in selecting products from landscape plants that are sustainable has increased and some data are available for malassezia species of human interest

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indicating extract of *Citrus aurantifolia* as the most active against *Malassezia furfur* [12].

Aim of the present study was to evaluate the *in vitro* anti-M. pachydermatis activity of EOs from *Anthemis nobilis*, *Citrus limon*, *Citrus paradisi*, *Illicium verum*, *Lavandula hybrida*, *Mentha piperita*, *Ocimum basilicum*, *Origanum vulgare*, *Origanum majorana*, *Rosmarinus officinalis*, *Salvia sclarea*, *Thymus serpillum* using a microdilution test. All the EOs were supplied by Flora srl (Lorenzana, Pisa, Italy).

Volatile constituents of each EO were identified by GC-MS analysis, in order to investigate the relation between chemical composition and biological activity as antifungal agents. GC-MS analysis was accomplished with a HP-5890 Series II instrument equipped with a HP-Wax and HP-5 capillary columns (both 30 m X 0.25 mm, 0.25 μm film thickness), working with the following temperature program: 60 °C for 10 min, rising at 5 °C/min to 220 °C. The injector and detector temperatures were maintained at 250 °C; carrier gas nitrogen (2 mL/min); detector dual, FID; split ratio 1:30. The volume injected was 0.5 μL.

GC-MS analysis was performed with a Varian CP-3800 gas-chromatography equipped with HP-5 capillary column (30 m X 0.25 mm; film thickness 0.25 μm). Analytical conditions: injector and transfer line temperature 220 °C and 240 °C, respectively; oven temperature programmed 220 °C and 240 °C from 60 °C – 240 °C at 3 °C/min.; carrier gas helium at a flow rate of 1 mL/min.; split ratio 1:30. Mass spectra were recorded at 70 eV. The acquisition mass range was m/z 30-300 at a scan rate of 1 scan/sec.

Identification of the constituents was based on comparison of the retention time with those of authentic samples, comparing their linear indices relative to a series of *n*-alkanes (C8-C23). Further identifications were also made possible by the use of a homemade library of mass spectra built up from pure substances and components of known oils, and MS literature data (NIST 2000, ADAMS).

EOs antifungal activity was tested against five clinical isolates of *M. pachydermatis,* obtained from recurrent cases of canine external otitis. *M. pachydermatis* reference strain CBS 1879 was used as reference strain. Diagnosis of *Malassezia* otitis was based on history, microscopic and otoscopic examination. The ceruminous discharge was collected with a sterile-tipped applicator from the junction of the vertical and horizontal ear canal for fungal culture and the samples were promptly seeded onto Sabouraud Dextrose Agar added with 0.5% of chloramphenicol and cycloheximide (DID, Milano, Italy) and mDixon agar (3.6% malt extract, 0.6% peptone, 2% desiccated ox-bile, 1% Tween 40, 0.2% glycerol, 0.2% oleic acid, 1.2% agar). The plates were incubated at 30°C for 7 days and processed as previously reported [13]. *M. pachydermatis* strains apparently lipid-dependent were identified by serial transfers on a lipid-free culture medium. The Tween assimilation test as described by Guého *et al.,* (1996) [14] and catalase activity were performed as additional tests both to confirm the identification, and to exclude the presence of other *Malassezia* species. Subcultures were inoculated on mDixon agar plates, to obtain pure isolates.

For all *in vitro* studies, *A. nobilis* s. 71565, *C. limon* s. 90660, *C. paradisi* s. 60221, *I. verum* s. 70487, *L. hybrida* s. 80241, *M. piperita* s. 80059, *O. basilicum* s. 70486, *O. vulgare* s. 60326, *O. majorana* s. 80717, *R. officinalis* s.60734, *S. sclarea* s.80193, *T. serpillum* s. 90017 and sweet almond oil s.70312 of EOs were assayed.

Stock solutions of EOs were prepared in sweet almond oil (*Prunus dulcis* Mill. Flora Srl., Lorenzana, Pisa, Italy) and diluted to obtain concentrations ranging from 0,6% to 10%. The dilutions were chosen considering that EOs *in vivo* maximum dilution for dermatologic administration is 20%. Considered both the lack of related reference data and the peculiar morpho-physiological features of external auditory canal, 10% was selected as starting dilution for the assay to avoid the risk of ear swelling and inflammation.

The effectiveness of EOs was assessed by means of a microdilution test carried out as described by Hammer *et al.,* (1997) [9], modified using a semisolid mDixon agar with 0.6% agar instead of solid mDixon. This medium was adopted to ensure the optimal growth of *Malassezia* strains, while the use of a semisolid medium was more suitable to the lipophylic nature of EOs tested, to ensure a better mutual contact between yeast cells and the different EOs during the assays, as previously described [15]. Control cultures were achieved with medium alone, and with medium supplemen-

Table 1. Chemical Composition of the Tested Essential Oils

Chemical Class	Relative Percentage Area (%)										
	O.v.	***O.b.***	***T.s.***	***C.l.***	***M.p.***	***R.o.***	***O.m.***	***I.v.***	***C.p.***	***L.h.***	***S.s***
Hydrocarbon monoterpenes	34,6	2,3	16,2	92,5	6,8	50,5	25,4	4,8	97,1	11,5	2,7
Oxygenated monoterpenes	58,1	68,8	80,3	4,9	86,5	46,1	63,0	95,1	0,9	73,2	91,2
Total monoterpenes	92,7	71,1	96,5	97,4	93,3	96,6	88,4	99,9	98,0	84,7	93,9
Hydrocarbon sesquiterpenes	5,8	19,7	2,2	2,6	5,2	2,3	4,9		1,1	10,2	2,0
Oxygenated sesquiterpenes	0,2	7,9	0,4		0,4	0,2	0,9		0,1	1,5	1,2
Total sesquiterpenes	6,0	27,6	2,6	2,6	5,6	2,5	5,8		1,2	11,7	3,2
Other	1,0				0,3		0,2		0,9	1,8	0,5

O.v: *Origanum vulgare*; **O.b**.: *Ocimum basilicum*; **T.s**.: *Thymus serpillum*; **C.l**.:*Citrus limon*; **M.p**.:*Mentha piperita*; **R.o**.: *Rosmarinum officinalis*; **O.m**.: *Origanum majorana*; **I.v**.:*Illicium verum*; **C.p.**: *Citrus paradisi*; **L.h**.: *Lavandula hybrida*; **S.s**.: *Salvia sclarea*.

Table 2. Main Constituents of the Tested Essential Oils

Compound	LRI[§]	*O.v.*	*O.b.*	*T.s.*	*C.l.*	*M.p.*	*R.o.*	*O.m.*	*I.v.*	*C.p.*	*L.h.*	*S.s.*
α-pinene	939				2,3		23,4		0,7			
β-pinene	979				13,7		3,8					
p-cymene	1026	14,3		5,2				7,6				
limonene	1029				59,2		4,7		3,1	91,7		
1,8-cineole	1033		5,9			6,8	27,5					
γ-terpinene	1060	11,2		4,7	10,8							
linalool	1098	3,2	46,0	2,1							28,9	28,4
camphor	1146						7,1					
menthone	1154					23,6						
menthofuran	1164					11,8						
menthol	1173					33,9						
4-terpineol	1180							16,0				
linalyl acetate	1257										28,6	48,9
E-anethol	1285								93,7			
thymol	1290	45,0						20,2				
carvacrol	1298	4,0		72,0								
eugenol	1356		11,5									
β-cariophyllene	1419										7,1	

[§] Linear Retention Index calculated on the basis of retention time of a mixture of *n*-alkanes (C_8-C_{30}).
O.v: *Origanum vulgare*; **O.b**.: *Ocimum basilicum*; **T.S**.: *Thymus serpillum*; **C.l**.: *Citrus limon*; **M.p**.: *Mentha piperita*; **R.o**.: *Rosmarinum officinalis*; **O.m**.: *Origanum majorana*; **I.v**.: *Illicium verum*; **C.p.**: *Citrus paradisi*; **L.h**.: *Lavandula hybrida*; **S.s**.: *Salvia sclarea*

ted with 50% of sweet almond oil, respectively. Furthermore, the yeasts were tested against ketoconazole by E-test (AB Biodisk, Solna, Sweden), as described by Nijima *et al.,* (2010) [16]. Cultures were incubated at 30 °C for 24-72 h, until a full development of the yeasts in control wells was noticeable. After incubation, yeasts inocula were harvested by centrifugation, gently washed in fresh mDixon broth, then inoculated on mDixon agar plates to monitor killing of the yeasts. All tests were performed in quadruplicate.

All analyzed EOs showed a high percentage of total monoterpene derivatives (> 85%, with the highest value in *I. verum*), while only the EO obtained from *O. basilicum* was characterized by a lower percentage of these compounds (71,1%). In detail, the chemical composition of the tested EOs was characterized by a high amount of oxygenated monoterpenes. The two *Citrus* species analyzed had an opposite profile with a predominance of hydrocarbon monoterpenes (97,1% in *C. paradisi* and 92,5% in *C. limon*). The ratio between the oxygenated and non-oxygenated monoterpenes for the *R. officinalis* essential oil was near to 1. The presence of sesquiterpene derivatives ranged from 0,0% (*I. verum*) to 27,6% (*O. basilicum*). The chemical composition of tested EOs and main constituents are reported in Tables **1** and **2**, respectively.

The plates were daily observed and effects were evaluated at 72 h p.i., when control wells showed a good fungal development. All EOs except *S. sclarea* inhibited the growth of *M. pachydermatis* tested strains at different concentrations (Table **3**).

Table 3. Minimum Inhibition Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of the Tested Essential Oils

Essential Oil	MIC and –MFC (% v/v)
Citrus limon	1
Citrus paradisi	1,3
Illicium verum	1,3
Lavandula hybrida	4
Mentha piperita	1
Ocimum basilicum	0,8
Origanum vulgare	0,8
Origanum majorana	1,3
Rosmarinus officinalis	1,3
Salvia sclarea	>10
Thymus serpillum	0,8

T. serpillum, *O. vulgare* and *O. basilicum* had the highest MICs (0.8%), while 4% *L. hybrida* inhibited yeasts' growth. Washed and re-seeded inocula failed to yield a

growth, showing same values for MIC and MFC for all the strains and for all the oils tested. The MIC for ketoconazole was <0,03 $\mu g/ml^{-1}$ for all yeasts isolates.

Several publications on antifungal activity of EOs are reported in literature. In the present paper EOs obtained from *T. serpillum*, *O. vulgare*, *O. basilicum*, *C. limon* and *M. piperita* showed a good antifungal action against *M. pachydermatis*, inhibiting mycotic development at high dilution. Their good biological activity may be related to the chemical composition. *T. serpillum* was characterized by an high percentage of oxygenated monoterpene derivatives (80,3%), in particular carvacrol was the more represented compound of this EO (72%), while *γ*-terpinene and p-cymene were present in minor percentages (4,7% and 5,2%, respectively). The high biological activity observed in *T. serpillum* EO is in agreement with the literature, even if the chemical composition showed some differences in the amount of thymol. At this regard, Soković *et al.,* (2009) [2] reported a very strong antifungal activity for this plant due to the presence of both thymol and carvacrol. Thymol was the main constituent of *O. vulgare* EO (45%), with lower amounts of p-cimene (14,3%) and *γ*-terpinene (11,2%). Anti-malassezia activity of oregano was assessed by Lee and Lee (2010) [12] also, confirming our results. The EO profile of *O. basilicum* was characterized by an high percentage of linalool (46%) and eugenol (11,5%) while 1,8-cineole and β-pinene were less represented. The *in vitro* activity of *O. basilicum* was assayed by Saggiorato *et al.,* (2009) [17], who reported the efficacy of this EO as a natural fungicide. *C. limon* EO contained an high percentage of the hydrocarbon monoterpene derivative limonene (59,2%). Several Authors have attributed the antifungal activity of *C. limon* to the presence of volatile compounds such as limonene and linalool. This activity may be determined by single major compounds or by synergistic or antagonistic effect of various components [18]. *C. limon* EO therefore contains γ-terpinene and β-pinene, also. Finally the EO obtained from *M. piperita* was characterized by a high percentage of menthol (33,9%) and menthone (23,6%). Soković *et al.,* (2009) [2] reported for these two derivatives a very strong antifungal activity. Even if potential antifungal effects of some compounds from plants have recently attracted attention, there is scanty scientific information about anti-malassezia properties of herbal remedia. To the best of our knowledge, *in vitro* sensitivity of *M. pachydermatis* has been scarcely investigated in veterinary medicine [4, 19]. It is well stated that malassezia acts as an opportunist agent, so identification and correction of predisposing diseases is often essential for successful management, although many dogs with *Malassezia* otitis require regular maintenance therapy. Predisposing factors such as all dermatologic disorders resulting in alteration of skin surface environment (seborrhea, otitis) are responsible for relapsing infection and repeated administration of conventional antifungal drugs may cause side effects. EOs show eudermic, lenitive and anti-inflammatory activities and may contribute to skin healing. Moreover the use of compounds with a pleasant smell is appreciated by the owners, fighting the strong odour of rancid fat, characteristic of malassezia skin disorders. For these reasons the choice of EOs could be an intriguing alternative.

There are few experienced herbal vets, so safe and effective coordination in a holistic manner is not common. Off-the-shelf herbal medicines can therefore be dangerous if there is no skilled professional herbal vet overseeing the entire input given to the patient. Furthermore attention should be paid in EOs' selection in the treatment of external and middle ear infections. It is important to select new compounds with neither residual nor toxic properties and to remember that herbs and conventional medicines can clash dangerously or can summate with a risk of serious overdose.

CONFLICT OF INTEREST

None declared.

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None declared.

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