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Susceptibility of *Microsporum canis* arthrospores to a mixture of chemically defined essential oils: a perspective for environmental decontamination

Abstract: The zoophilic dermatophyte *Microsporum canis* has cats as natural reservoir, but it is able to infect a wide range of hosts, including humans, where different clinical features of the so-called ringworm dermatophytosis have been described. Human infections are increasingly being reported in Mediterranean countries. A reliable control program against *M. canis* infection in cats should include an antifungal treatment of both the infected animals and their living environment. In this article, a herbal mixture composed of chemically defined essential oils (EOs) of *Litsea cubeba* (1%), *Illicium verum*, *Foeniculum vulgare*, and *Pelargonium graveolens* (0.5% each) was formulated and its antifungal activity assessed against *M. canis* arthrospores which represent the infective environmental stage of *M. canis*. Single compounds present in higher amounts in the mixture were also separately tested in vitro. *Litsea cubeba* and *P. graveolens* EOs were most effective (minimum inhibitory concentration (MIC) 0.5%), followed by EOs of *I. verum* (MIC 2%) and *F. vulgare* (MIC 2.5%). Minimum fungicidal concentrations (MFC) values were 0.75% (*L. cubeba*), 1.5% (*P. graveolens*), 2.5% (*I. verum*) and 3% (*F. vulgare*). MIC and MFC values of the mixture were 0.25% and 0.5%, respectively. The daily spray of the mixture (200 µL) directly onto infected hairs inhibited fungal growth from the fourth day onwards. The compounds present in higher amounts exhibited variable antimycotic activity, with MIC values ranging from >10% (limonene) to 0.1% (geranial and neral). Thus, the mixture showed a good antifungal activity against arthrospores present in infected hairs. These results are promising for

a further application of the mixture as an alternative tool or as an adjuvant in the environmental control of feline microsporosis.

Keywords: antifungal activity; essential oils; *Microsporum canis*.

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1 Introduction

Dermatophytosis due to *Microsporum canis* represents the most common fungal skin infection in cats [1]. This zoophilic dermatophyte is able to infect a wide range of hosts, including humans, where different clinical features have been reported. The incidence of skin disease due to *M. canis* has increased in the recent years, in particular in Mediterranean countries, being the most prevalent agent of *tinea capitis* in children [2]. *Tinea faciei* and *tinea corporis* have also been reported recently, mostly as result of contact with pets [3]. Infected cats have been proven to contaminate the environment by shedding arthroconidia (arthrospores) which are asexual spores derived from fragmentation of fungal hyphae that are able to invade hairs and scales [4]. These fungal elements, embedded in hairs and skin debris are highly resistant at room temperature, may be viable up to 18 months and are thus responsible for infection and/or reinfection [5]. For these reasons, a reliable control program should include an antifungal treatment of both animals and their living environment. Aggressive removal of contaminated material followed by thorough application of commercial ready-to-use disinfectants is strongly suggested, and several commercially available sporocidal products have been successfully checked [6]. Users are advised to read labels to avoid toxicity risk for pets and children and to follow the

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manufacturer's recommendations regarding target surfaces [7]. Furthermore, these products frequently present a variety of environmental concerns [8]. Hence, there is a growing interest of consumers in ingredients from natural sources; phytochemicals would be considered as alternatives to synthetic disinfectants/antimycotics also because many of them are relatively non-toxic to mammals and the environment. Essential oils (EOs) are gaining increasing interest because of their relatively safe status, their wide acceptance by consumers and their exploitation for potential multipurpose functional use. EOs are complex mixtures comprising many single compounds, chemically mostly of terpenoid origin, and have been used for many thousands of years in food preservation, pharmaceuticals, alternative medicine and natural therapies [9]. Antifungal activity of EOs has been demonstrated both against pathogenic fungi causing mycoses [10–12] and against fungi causing food spoilage and producing mycotoxins [13].

Essential oils from a number of plants exhibited good anti- *M. canis* activity both in vitro and in topical treatment of naturally infected cats [11], while no information was available on their environmental use. For these reasons, the aims of this article were to formulate an herbal mixture and to assess its antifungal activity against *M. canis* arthrospores with special reference to an environmental application.

2 Materials and methods

2.1 Essential oils

Essential oils from star anise (*Illicium verum*), bergamot (*Citrus bergamia*), cinnamon (*Cinnamomum zeylanicum*), fennel (*Foeniculum vulgare*), geranium (*Pelargonium graveolens*), litsea sambal (*Litsea cubeba*), spearmint (*Mentha spicata*), sandal (*Santalum album*) and incense (*Boswellia sacra*) were selected for this study based on their efficiency as antiseptics [14–20], pleasant smell, and safety for both human and animals. The nine selected EOs were examined for both their composition by GC-MS analysis and antimycotic activity by in vitro tests.

2.2 GC-MS analysis

Volatile constituents of each EO were analyzed by GC-MS as previously reported [21]. Briefly, a CP-3800 gas chromatograph equipped with HP-5 capillary column and a Varian Saturn 2000 ion trap mass detector were employed. Analytical conditions were as follows: injector and transfer line temperature, 220 and 240 °C, respectively; oven temperature, programmed from 60 to 240 °C at 3 °C/min; carrier gas, helium at 1 mL/min; injection, 0.2 mL (10% hexane solution);

split ratio, 1:30. Identification of the constituents was based on comparison of the retention times with those of authentic samples, comparing their linear retention indices relative to the series of *n*-hydrocarbons, and on computer matching against commercial and home-made library mass spectra built up from pure substances and components of known oils and MS literature data [22, 23].

2.3 Source of infected hair samples

Shortly before starting the in vitro tests, *M. canis* ringworm was diagnosed in a symptomatic, male, European short hair, 2-month-old kitten by the hair-brushing technique and colony identification in culture. In some cases of feline ringworm caused by *M. canis*, a metabolite (pteridine) is produced within infected hairs that lets them fluoresce yellow-green under ultraviolet light. Therefore, the kitten was examined under Wood's lamp to allow the identification of hairs containing infectious arthrospores. A number of entirely fluorescent, and thus certainly infected hairs, were cut by the use of scissors. After collection, all infected hairs were kept within the same Petri dish and stored in the dark at room temperature (22–25 °C) until tests were performed.

2.4 In vitro susceptibility studies

The antimycotic activity of each EO listed previously was evaluated by a microdilution test on the collected hairs, using the technique described by Mugnaini et al. [11] slightly modified. Briefly, all tests, including EOs or mixtures (see the following), were carried out in triplicate in 24 well plates. Stock solutions at 10% (v/v) of the listed EOs in 95% ethanol were diluted into a semisolid malt extract with 1% agar as culture medium to obtain concentrations ranging from 3% to 0.05%. Such dilutions were arbitrarily chosen considering that a concentration of 3% is the highest for in-home use. In detail, 3%, 2.5%, 2%, 1.5%, 1%, 0.75%, 0.5%, 0.25%, 0.2%, 0.1% and 0.05% dilutions were checked. Each well was seeded with an inoculum of infected hairs (n=5) obtained as mentioned. The viability of inocula was evaluated by seeding control wells containing culture medium with and without ethanol. Plates were incubated at 25 °C for up to 10 days, or until mycotic growth was visually detected in control wells, to determine minimum inhibitory concentration (MIC) values. To further evaluate if lack of growth was due to a fungistatic or fungicidal effect and to assess minimum fungicidal concentration (MFC) values of the EOs, inocula which did not grow were washed with distilled water, subcultured onto malt extract agar and incubated at 25 °C for 4 weeks.

After preliminary results on the effectiveness of each single EO, the most effective were selected to compose a mixture for a further trial. Therefore, inocula, obtained as described, were also tested against a mixture composed of 1% *L. cubeba*, 0.5% *I. verum*, 0.5% *F. vulgare* and 0.5% *P. graveolens*, in 95% ethanol. The mixture was formulated on the basis of the antimycotic efficiency of each ingredient when tested alone, as well as of pleasant smell, considering that, when infected subjects are present, the whole environment where the animals are allowed to roam should be treated. In order to reproduce conditions similar to environmental usage, the mixture was checked at dilutions of 50%, 25%, 10%, 5%, 1%, 0.5%, 0.25% and 0.1%. Minimum inhibitory concentration and MFC values were assessed.

Single components present in amounts of >10% in EOs selected to formulate the mixture and/or known for their antifungal activity were separately tested in vitro as well. So anethole, citronellol, geranial, neral, geraniol, fenchone and limonene were assayed against *M. canis* obtained as described. All tests were carried out in triplicate. The viability of the inocula was evaluated as described.

In addition, the mixture was assayed by direct spraying of 200 µL onto 50 infected hairs, once a day for 1 week. After each spraying, hairs were left to air dry and five of them subcultured onto Sabouraud medium supplemented with chloramphenicol (0.05 g/L) and cycloheximide (0.4 g/L).

3 Results

3.1 GC-MS analysis

The chemical composition of the tested oils is reported in Tables 1 and 2.

3.2 In vitro susceptibility studies

Essential oils showing antimycotic activity at dilutions ≤3% were *L. cubeba* and *P. graveolens* (MIC 0.5% each), followed by *I. verum* (MIC 2%) and *F. vulgare* (MIC 2.5%). Their MFC values were 0.75% (*L. cubeba*), 1.5% (*P. graveolens*), 2.5% (*I. verum*) and 3% (*F. vulgare*).

The whole mixture composed of 1% *L. cubeba*, 0.5% *I. verum*, 0.5% *F. vulgare* and 0.5% *P. graveolens*, in 95% ethanol was effective with MIC and MFC values of 0.25% and 0.5%, respectively.

The main constituents present in each EO selected to formulate the mixture showed variable antimycotic activity, ranging from MIC >10% (limonene) to 0.1% (geranial and neral). MFC was equal to MIC for citronellol and geraniol, while all other tested compounds, including the mixture, were fungicidal at concentrations higher than the MIC. More detailed results are reported in Table 3. The

mixture sprayed on infected hairs inhibited fungal growth from the fourth day of spraying onwards.

4 Discussion

In this article, the antifungal activity of a mixture composed of 1% *L. cubeba*, 0.5% *I. verum*, 0.5% *F. vulgare* and 0.5% *P. graveolens*, in 95% ethanol against the parasitic form of *M. canis* (arthrospores) in infected cat hairs was investigated.

Considering that arthrospores, the infective element, are present on the broken hairs, and contamination of the environment from these sources is common, this work represents the first attempt to assess the antifungal role of EOs in environmental decontamination from *M. canis*. Our results can be only partially compared with those of Mugnaini et al. [11], who topically administered a mixture composed by 5% *O. vulgare*, 5% *R. officinalis* and 2% *T. serpyllum* essential oil, respectively, in sweet almond oil to seven *M. canis* infected cats with satisfactory results. In fact, in this article EOs were dissolved in 95% ethanol, which is a solvent appropriate for environmental usage and not suitable for in vivo administration.

Litsea cubeba showed the lowest MIC and MFC values, probably due to its high content of geranial (36.8%) and neral (31.9%) which exhibited a strong antifungal activity as single compounds. *P. graveolens* also gave good results, probably due to the presence of geraniol and citronellol accounting for 13.7% and 44.4% of the total oils. The MIC and MFC shown by *I. verum* were 2% and 2.5%, respectively. These values were slightly higher in *F. vulgare*. The difference could be due to the lower amount of anethole present in the fennel EO.

Essential oils selected for the mixture are known to have antiseptic properties. All EOs showed antifungal effects and have previously been tested mostly against plant pathogens and postharvest pathogenic fungi, except

Table 1: Major classes of constituents of the tested essential oils (area percentage, %) determined by GC-MS.

Class of constituents compounds	<i>Boswellia sacra</i>	<i>Litsea cubeba</i>	<i>Mentha spicata</i>	<i>Santhalam album</i>	<i>Pelargonium graveolens</i>	<i>Illicium verum</i>	<i>Cinnamomum zeylanicum</i>	<i>Foeniculum vulgare</i>	<i>Citrus bergamia</i>
Monoterpene hydrocarbons	84.29	4.42	7.08	0.00	0.00	3.03	0.00	8.23	38.80
Oxygenated monoterpenes	6.76	50.37	76.69	0.00	86.16	0.00	18.68	19.37	57.74
Sesquiterpenes hydrocarbons	3.74	39.57	10.52	5.63	7.77	0.00	0.00	0.00	1.62
Oxygenated sesquiterpenes	0.00	0.33	1.52	88.79	4.14	0.00	0.38	0.00	0.46
Phenylpropanoids	0.25	0.00	0.00	0.00	0.00	94.20	80.14	67.90	0.00
Others	0.00	1.38	0.18	0.00	1.19	0.00	0.00	1.92	0.12
Total	95.04	96.07	95.99	94.42	99.26	97.23	99.20	97.42	98.74

(Table 2: Continued)

Compound	LRIS	<i>Boswellia sacra</i>	<i>Litsea cubeba</i>	<i>Mentha spicata</i>	<i>Santhalam album</i>	<i>Pelargonium graveolens</i>	<i>Illicium verum</i>	<i>Cinnamomum zeylanicum</i>	<i>Foeniculum vulgare</i>	<i>Citrus bergamia</i>
Menthone	1154			16.46		1.07				
<i>neo</i> -3-Thujanol	1154	0.20								
Citronellal	1155		1.70							
Isomenthone	1164					3.48				
<i>neo</i> -Menthol	1166			11.18						
Menthol	1178			39.03						
4-Terpineol	1180		0.30	0.83						0.15
Isomenthol	1183			0.77						
<i>neo</i> - <i>iso</i> -Menthol	1187			0.21						
α -Terpineol	1192		0.75	0.31		0.25		0.29		0.28
Myrtenol	1195									0.11
Methyl chavicol (= estragol)	1198								2.89	
Safranal	1200								00.16	
<i>trans</i> - <i>para</i> -Menthane-2-one	1205								00.11	
γ -Terpineol	1207									0.11
Octanol acetate	1214									0.12
<i>trans</i> -Carveol	1221									0.15
Nerol	1228		0.86							0.18
<i>cis</i> -Carveol	1229									
Citronellol	1231					44.46				
<i>cis</i> - <i>para</i> -Mentha-1(7),8-dien-2-ol	1231									0.15
Pulegone	1237			3.29						
(E)- β -Ocimene	1238	0.13								
Isobornyl formate	1239	2.54								
Neral	1242		31.97							0.15
Cuminaldehyde	1244					0.18				
Carvone	1248							0.79		0.23
(Z)-Anethole	1253	0.06								0.15
<i>trans</i> -Sabinene hydrate acetate	1256									
Piperitone	1258			0.59						
<i>para</i> -Anisaldehyde	1258								1.92	
Geraniol	1259		1.48			13.73				34.47
Linalool acetate	1260									
<i>neo</i> -Menthyl acetate	1274			0.36						0.26
Geranial	1276		36.89			0.70				
Citronellyl formate	1280		0.48			7.26				
(E)-Anethole	1283						94.20			
Safrole	1291							1.36		

(Table 2: Continued)

Compound	LRIS	<i>Boswellia sacra</i>	<i>Litsea cubeba</i>	<i>Mentha spicata</i>	<i>Santhalam album</i>	<i>Pelargonium graveolens</i>	<i>Illicium verum</i>	<i>Cinnamomum zeylanicum</i>	<i>Foeniculum vulgare</i>	<i>Citrus bergamia</i>
Menthyl acetate	1294			6.94						
Geranyl formate	1298					1.92				0.48
α -Cubebene	1351		0.25							
α -Terpinyl acetate	1352									
Citronellyl acetate	1353					0.44				0.28
neo-iso-Dihydro carveol acetate	1359									
Eugenol	1361							64.77		0.65
Neryl acetate	1368									
α -Ylangene	1372	0.31								
α -Copaene	1376	1.52				0.26				
β -Bourbonene	1383	0.39		0.5		0.73				
Geranyl acetate	1384					0.68				
β -Elemene	1392		0.23	0.13						
Methyl eugenol	1407	0.19								
β -Cedrene	1418	0.17								
α -Santalene	1418				0.58					
β -Caryophyllene	1418			2.09		0.68				0.23
β -Copaene	1429	0.12	2.21							
β -Gurjunene	1432									
trans- α -Bergamotene	1437	0.12								0.53
α -Guaiene	1440					0.28				
Aromadendrene	1445	0.10				2.86				
epi- β -Santalene	1449				0.58					
Isoeugenol	1449							14.01		
Citronellyl propanoate	1450					0.72				
α -Humulene	1456		0.22	0.09		0.21				
allo-Aromadendrene	1461					0.22				
β -Santalene	1461				1.42					
9-epi-(E)-Caryophyllene	1467			0.23						0.11
β -Chamigrene	1475				0.16					
γ -Muurolene	1477	0.13								
Geranyl propanoate	1478				0.25	0.68				
γ -Curcumene	1481			0.67						
Germacrene D	1481	0.88				0.20				
ar-Curcumene	1484									
β -Selinene	1485					0.34				
Valencene	1493				0.25					
Bicyclogermacrene	1495		0.15							

(Table 2: Continued)

Compound	LRIS	Boswellia sacra	Litsea cubeba	Mentha spicata	Santhalam album	Pelargonium graveolens	Illicium verum	Cinnamomum zeylanicum	Foeniculum vulgare	Citrus bergamia
Viridiflorene	1495					0.63				
α -Muurolene	1499					0.25				
β -Dihydro agarofuran	1500				0.86					
<i>cis</i> -Dihydro agarofuran	1503				0.33					
β -Bisabolene	1509				0.33					
α -Alaskene	1511				0.28					0.76
<i>trans</i> - γ -Cadinene	1513					0.19				
(<i>Z</i>)- γ -Bisabolene	1515					0.20				
7- <i>epi</i> - α -Selinene	1519				0.40					
δ -Cadinene	1523					0.72				
β -Sesquiphellandrene	1524				0.38					
Kessane	1528									
Citronellyl butyrate	1532					1.19				
Europelargone A	1540					0.18				
Selina-3,7(11)-diene	1542				1.00					
α -Agarofuran	1548				0.63					
Elemol	1553				4.69					
Geranyl butyrate	1564				0.28					
(<i>E</i>)-Nerolidol	1566					1.11				
Spathulenol	1577			0.27		0.25				
Caryophyllene oxide	1582		0.33	0.69		0.85				
(<i>E</i>)-2-Phenyl ethyl tiglate	1585					1.19				
Viridiflor	1591			0.56						00.46
Caryophyllene oxide	1592							0.17		
Guaiol	1597				0.62					
Geranyl-2-methyl butyrate	1607					0.26				
Humulene epoxide II	1607					0.22				
1,10- <i>di-epi</i> -Cubanol	1614					0.22		00.21		
Citronellyl pentanoate	1625					0.33				
<i>epi</i> -10- γ -Eudesmol	1627				4.87					
1- <i>epi</i> -Cubanol	1630					0.20				
Eremoligenol	1632				0.41					
γ -Eudesmol	1634				5.16					
Hinesol	1638				0.49					
Cubanol	1641					0.33				
β -Eudesmol	1649				5.52					
4- α -hydroxy-dihydro agarofuran	1653					0.22				
α -Cadinol	1655					0.37				
(<i>Z</i>)-Citronellyl tiglate	1658					1.15				

(Table 2: Continued)

Compound	LRIS	Boswellia sacra	Litsea cubeba	Mentha spicata	Santhalum album	Pelargonium graveolens	Illicium verum	Cinnamomum zeylanicum	Foeniculum vulgare	Citrus bergamia
Valerianol	1658				14.35					
7-epi- α -Eudesmol	1664				5.92					
(E)-Citronellyl tiglate	1668					0.22				
(Z)- α -Santalol	1672				27.09					
(Z)-trans- α -Bergamotol	1691				2.00					
Geranyl tiglate	1696					1.19				
(Z)- β -cis-Santalol	1705				2.13					
(Z)- β -trans-Santalol	1710				10.75					
(E)- β -Santalol	1740				0.59					
6S,7R-Bisabolone	1750				0.50					
Dirimenol	1759				0.44					
(Z)-Lanceol	1768				1.16					

SLRI, Linear Retention Index calculated on the basis of the retention times of a mixture of *n*-alkanes (C8–C30).

Table 3: Minimal inhibitory (MIC) and fungicidal (MFC) concentrations, respectively, of the mixture of EOs and their major constituents.

Compound	MIC, %	MFC, %
Mixture	0.25	0.5
<i>Foeniculum vulgare</i>	2.5	3
<i>Illicium verum</i>	2	2.5
<i>Litsea cubeba</i>	0.5	0.75
<i>Pelargonium graveolens</i>	0.5	1.5
Anethole	1	5
Citronellol	0.25	0.25
Geranial	0.1	0.25
Geraniol	0.25	0.25
Limonene	>10	>10
Neral	0.1	0.25
Fenchone	0.25	0.5

for *P. graveolens* [24] and *I. verum* [11] that were assayed also against several *Trichophyton* species and *M. canis*, respectively, with antifungal activities agreeing to those found in the present study. *L. cubeba* was effective against *Aspergillus niger*, *Alternaria alternata*, *Fusarium moniliforme* and *Fusarium solani* [25], while *F. vulgare* inhibited several fungi responsible for food spoilage [26]. Furthermore *P. graveolens* and *I. verum* were active against plant pathogens [17, 27].

Most of the represented components, except for limonene, provided good antimycotic activity in agreement with literature data [28–30], in particular the isomers of citral (neral and geranial) are the main components of *L. cubeba* EO. Citral has a strong influence on fungi [31], being able to injure the wall and the membrane of *Aspergillus flavus* spores. This action results in a decrease of elasticity, and in a changed aggregation of protein-like macromolecules.

Tests of the sensitivity of dermatophytes to EOs described in the literature have been performed with fungal mycelia cultured from clinical isolates, which is not the form of spreading in the environment as represented by arthrospores [7]. In the present work, *M. canis* arthrospores embedded in infected hairs were employed to reproduce an in-field situation to set up an effective mixture for environmental usage.

The EO mixture showed MIC and MFC values lower than that obtained for *L. cubeba*, the most effective ingredient. These findings could be ascribed to synergism of the different active components.

Conventional treatment of infected environments has been exhaustively investigated by Moriello et al. [7], who reported complete inhibition of *M. canis* by commercial disinfectants at concentrations ranging from 3.2% for

disinfectants containing lactic acid, to 0.22% for disinfectants based on quaternary ammonium derivatives. Thus, the mixture prepared by us, being effective at a concentration of 0.25%, showed antimycotic activity comparable to that of the most effective commercial product.

The direct application of the mixture on infected hairs inhibited fungal growth at day 4, with a total amount of 800 μ L sprayed on the specimens. This volume was lower than that required to obtain the same results with some commercial disinfectants [7]. The mixture sprayed on soft furnishing did not stain (data not shown), unlike some conventional disinfectants such as sodium hypochlorite and lime sulphur. Furthermore this last product is not available worldwide.

P. graveolens and *L. cubeba* at concentrations used in the present work do not act as potential contact allergens [32] and the other selected oils are not reported to induce cutaneous adverse reactions in contrast with common household cleaning products [33]. These preliminary results seem to be promising for a further application of the EO's mixture as a suitable tool to inactivate the environmental form of *M. canis*.

Conflict of interest statement: None declared.

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