AN UPDATE ON ANTIFUNGAL ACTIVITY OF ESSENTIAL OILS AGAINST *MALASSEZIA PACHYDERMATIS*

Carla PAVLOV-ENESCU[1], Andra-Cristina BOSTĂNARU [1], Mihai MAREȘ[1],

pavlov.carla@gmail.com

Abstract
In recent years, the lipophilic yeast *Malassezia pachydermatis* is considered to be the most significant opportunistic pathogen associated with dermatitis and otitis externa in veterinary medicine. At the same time, various findings have shown the capacity of clinical isolates to acquire azole-resistance, therefore the development of new alternative treatment strategies are highly demanded. In the last decade, plant-based antimicrobials have known a resurrection, and special attention was given to essential oils (EOs). EOs are complex mixtures of small lipophilic molecules, of which one up to three compounds constitute the main phytochemical markers. EOs arose as candidates for the alternative treatment of *Malassezia*-related diseases. This review highlights the antifungal potential of EOs and their bioactive compounds against *M. pachydermatis* based on literature reports (*in vitro* and *in vivo* retrospective studies). A search was conducted using three databases (PubMed, Web of Science, Google Scholar), and all relevant articles from the period 2015-2021 were extracted. The findings showed most of EOs *had* significant *antifungal activity* against *M. pachydermatis,* especially through bioactive compounds such as monoterpenes and sesquiterpenes, on their own or by synergism with conventional antifungal drugs or other compounds, such as fluconazole and Tween 80. EOs with promising antifungal activity against *M. pachydermatis* include winter savory, lemongrass, oregano, cinnamon and oregano. The review emphasizes the importance of Eos as novel antifungal agents. EOs could be considered as an alternative to conventional antifungals, as they act concurrently towards different fungal targets due to their multicomponent nature.

Key words: antifungal, essential oils, *Malassezia pachydermatis*.

INTRODUCTION

In present times, worldwide, thousands of plant species are being used to obtain modern medication and at the same time, being used in traditional medicine.

Increasingly aggressive microorganisms, with a multi-drug resistant profile, urged the reduction of chemicals being used as antimicrobial agents and focused medical professionals on substances derived from plants, such as hydro-alcoholic extracts or essential oils.

The versatility of such substances is enormous as the same plant can provide a pool of substances with a very broad spectrum of action due to their different chemical structure. The term “Essential Oil” (EO) was coined in the 16th century by the Swiss reformer of medicine, Paracelsus von Hohenheim. Approximately 17,000 plant species produce essential oils. These are belonging mainly to a few families, and the most representative are *Lamiaceae, Asteraceae, Myrtaceae*, and *Lauraceae* (*Bruneton, 1999)*. Currently, about 4000 EOs are known, the number growing exponentially every day. Most of these EOs are being used in the food and beauty industry as flavours and fragrances. Plant EOs represent a complex mixture of compounds, are known for their antiseptic and medicinal properties (sedative, analgesic, anti-spastic, anti-carcinogenic) and, furthermore, due to their antimicrobial and antioxidant potential, are used as natural additives in foods and food products. Many thousands of compounds belonging to the family of terpenes have so far been identified in essential oils, mainly of terpenoidic nature *(Bilia et al., 2014)*

Terpenoids represent groups of hydrocarbons which have as base structure the isoprene (C_5H_8). They represent the largest group of phytochemicals with the highest antimicrobial potential. They are classified in 8 categories based on the number and structure of the isoprene units. Studies from 2017 have showed that 67% of the terpenoids that exhibit bioactivity are represented by monoterpenes ($C_{10}H_{16}$) and sesquiterpenes ($C_{15}H_{24}$). As for the antimicrobial activity of terpenoids, the mechanism of action is not fully understood, but recent studies report that the majority of terpenoids inhibit two crucial survival processes of microorganisms: oxidative

[1] Faculty of Veterinary Medicine, University of Life Sciences, Iași

phosphorylation and oxygen absorption. Antifungal components can also lead to dysfunction of fungal mitochondria at the same time disrupting the formation of the fungal cell membranes.

Malassezia spp. represents a group of lipid-dependent fungi, commensals on the skin of humans and animals. Through mechanisms still incompletely elucidated, they become pathogenic and are involved in a variety of skin conditions, both in human medicine - pityriasis versicolor (*M. furfur)* and veterinary medicine - complications in atopic dermatitis *(M. pachydermatis).*

Despite the extraordinary advancement of mycological knowledge in the last two decades in the study of these yeasts, the pathogenetic mechanism and virulence factors are not fully clarified. The ability of this group of microorganisms to colonize and infect is determined by complex interactions between the fungal cell and the host, through virulence factors. Current *in vitro* and in *vivo* studies mention the presence of *M. pachydermatis* strains with increased tolerance to azoles, due to point mutations in the ERG11 gene.

Recent studies *(Chiavassa et al., 2014, Watanabe et all., 2014)* have reported the sporadic presence of *M. pachydermatis* strains with increased tolerance to azoles in vitro. In 2019, Angileri et al. isolated a strain from a Toy-Poodle that did not respond to azole treatment, which resulted in a 7x higher MIC of itraconazole. In 2018, Rui Kano et al. isolated the first multi-resistant azole strain with mutations in the ERG11 gene, which has a MIC of 320 μg / ml for itraconazole and over 32 μg / ml for ketoconazole. This justifies the importance of alternative therapeutic research, which has caused an increased interest in studying the antifungal potential of EOs.

In the field of veterinary medicine, EOs have been used as prevention against ectoparasites, they have shown a positive effect in atopic dermatitis, chronic dermatitis, pyoderma and malodor in canines. EOs can also be used as ingredients in mouth rinses for their antimicrobial properties or in the treatment of abscesses.

This review highlights the antifungal potential of EOs and their bioactive compounds against *M. pachydermatis* based on literature (*in vitro* and *in vivo* retrospective studies).

MATERIALS AND METHODS

A search was conducted using three databases (PubMed, Web of Science, Google Scholar), and all relevant articles from the period 2015-2021 were extracted.

RESULTS AND DISCUSSION

In vitro tests

Authors reported different assays to evaluate the antifungal properties. Broth microdilution assay was the most used, followed by the agar disk diffusion test and vapor phase methods. This review focused on species with implication in veterinary medicine, but some authors included in their studies species involved in human pathology. The strains tested in the studies are represented by clinical isolates. All of the authors cited in this review evaluated the antimycotic activity of different essential oils against *M. pachydermatis*. The literature related to the last five years shows a great variety of essential oils originating from different plant genera (i.e. *Thymus, Artemisia, Malaleuca, Cinnamomun, Ocimum, Zataria, Rosmarinus, Origanum, Syzigium, Foenicolum, Thapsia, Tachyspermum, Myrtus*). Different assays were used to evaluate the antifungal proprieties. Agar diffusion test was the most used, followed by broth microdilution assay.

Váczi, P. et al. tested the antifungal activity of 14 selected EOs at 3 different concentrations: 0.5%, 5% and 30% against 18 clinical isolates and one reference strain of *M. pachydermatis*. The isolates were collected from canine patients diagnosed with otitis externa. Out of the tested EOs, clove, cinnamon and oregano showed a 100% antifungal efficacy at 30% concentration, whereas at the concentration of 5% the efficacy was less significant (38%, 33% and 5%, respectively). The main active compound in the previously mentioned EOs is represented by eugenol (77%). Satureja inhibited the growth at 30% concentration with an efficacy of only 16%. The remaining 10 EOs tested did not exhibit any inhibition zone greater or equal to 15 mm which was defined in the methodology used, therefore they were considered ineffective. Regarding all the EOs, the 0.5% concentration was not effective inhibiting the growth of *M. pachydermatis*.

Table 1

Effectiveness of the EOs against *M. pachydermatis* according to Váczi, P. et al.

EO	5% concentration			30% concentration		
	Isolates		Reference strain	Isolates		Reference strain
	Inhibition zones (mm)	% of efficacy (no of sensitive strains/no of samples tested)	Inhibition zones (mm)	Inhibition zones (mm)	% of efficacy (no of sensitive strains/no of samples tested)	Inhibition zones (mm)
Clove	16,94	38% (7/18)	18	41,67	100% (18/18)	44
Cinnamon	15,44	33% (6/18)	15	40,14	100% (18/18)	50
Oregano	13	5	14	38,8	100%(18/18)	52
Satureja	7,49	0	0	23,26	16% (3/18)	12
Cedar	3,22	0	8	11,94	0	12
Chamomile	3,61	0	6	11,17	0	12
Bergamot	0	0	0	8,44	0	10
Lavender	0	0	0	6,55	0	8
Grapefruit	0	0	0	10,78	0	10
Sage	0	0	0	10,06	0	14
Tea tree	0	0	0	8,44	0	10
Juniper	0	0	0	5,55	0	8
Pine	0	0	0	4,44	0	6
Yarrow	0	0	0	0	0	0

Table 2

Effectiveness of the EOs against M. pachydermatis according to Bismarck et al.

EO	Inhibition zone radius (median)			
	Aromatogram	Vapour assay	EO 20% solution	EO 10% solution
Winter savory	>40	>40	10.75	4.5
Lemon grass	>40	>40	19.5	8
Rose geranium	>40	>40	7	0
Oregano	>40	>40	14.5	6
Palmarosa	>40	>40	12.5	6.75
Indian melissa	>40	>40	7	0
Thyme thymol 19%	37.5	>40	0	n.t.
Cinnamon leaf	26	24	9	4
Clove	25	22.5	8	5
Thyme	19.75	21.5	0	n.t.
Coriander seed	19	17	0	n.t.
Thyme linalool	16.5	13	0	n.t.
Manuka	15	10.5	5.5	0
Tea tree	10	7.5	0	n.t.
Lavandin super	9	9.5	0	n.t.
Fennel	8.75	0	0	n.t.
Lavender fine	8	7.25	0	n.t.
Lemon	7.75	4.5	0	n.t.
Clary sage	7.5	5.5	0	n.t.
Angelica root	7.5	0	0	n.t.
Ravintsara	6.75	0	0	n.t.
Neroli	0	0	0	n.t.

n.t – not tested;

Bismark et al., tested 22 EOs on 15 canine clinical isolates using two different assays – Agar disk diffusion with 20%, respectively 10% EO concentration and vapor assay. Out of the 22 EOs tested, winter savory, lemon grass, oregano, palmarosa, and cinnamon leaf oil showed excellent in vitro activity. The activity of antifungal agents was tested simultaneously using agar disc diffusion assay. The efficacy of the EOs was classified based on the inhibition radius as follows: not sensitive <8mm, slightly sensitive 8-13.9mm, moderately sensitive 14.0-19.9 mm, very sensitive >20mm, extremely sensitive if no growth.

Ebani et al., conducted the first study that tested simultaneously the efficacy of EOs against *Staphylococcus spp*. and *Malassezia spp*. strains isolated from canine patients. The antifungal activity was conducted by using Agar Disk Diffusion Method.

Five clinical isolates of *M. pachydermatis* were tested, these strains were isolated from the skin of dogs diagnosed with atopic dermatitis. The antifungal activity of selected EOs was assessed by microdilution method, diluted in dimethyl sulfoxide (DMSO, Oxoid Ltd.), at concentrations of 5%, 4.5%, 4%, 3.5%, 3%, 2.5%, 2%, 1.5%, 1%, 0.75%, and 0.5%. MIC was established as the lowest concentration of EO where no fungal growth was yielded. *Commiphora myrrha* and *Litsea cubeba* were not effective at 5% dilution. *Aloysia tryphilla* was the most active with MICs of 0.87 and 1.03 mg/mL, followed by *S. montana* with MIC of 1.8 mg/mL and *Cinnamomum zeylanicum* with 3.06 and 4.08 mg/mL. *Malassezia yeasts* showed a marked variability in their susceptibility to EOs. For these reasons, a sensitivity assay of the fungal isolates is recommended, as suggested by Bismarck et al.

Table 3

MIC values of the tested EOs expressed in mg/mL against selected *M. pachydermatis* isolates according to Ebani et al.

EO	UNIT	Strain 1	Strain 2	Strain 3	Strain 4	Strain 5
Aloysia triphylla	mg/mL	1.03	0.87	1.03	0.87	1.03
Cinnamomum zeylanicum	mg/mL	3.06	3.06	4.08	3.06	4.08
Commiphora myrrha	mg/mL	n.e.	n.e.	n.e.	n.e.	n.e.
Cymbopogon citratus	mg/mL	7.13	7.13	7.13	7.13	7.13
Litsea cubeba	mg/mL	n.e.	n.e.	n.e.	n.e.	n.e.
Melissa officinalis	mg/mL	3.55	3.55	3.55	3.55	2.66
Origanum vulgare	mg/mL	7.73	7.73	6.76	7.73	6.76
Satureja montana	mg/mL	1.8	1.8	1.8	1.8	1.8
Thymus vulgaris	mg/mL	8.7	8.7	7.73	7.73	7.73
Cloramphenicol	µg/mL	0.02	0.02	0.02	0.02	0.02

Aiemsaard J. et al. in 2020, evaluated the antifungal activity of the essential oil in the betel vine against 17 strains of *M. pachydermatis* isolated from lesions of canine dermatitis, using the microdilution assay and time kill assay. The predominant EO in the betel vine was eugenol (32.82%). The results were promising, the EO having a high antifungal activity against all tested isolates, with MIC and MFC values equal to 0.66 – 1.13 µg/ml. The time kill assay showed that the highest activity was achieved by 8 times the MIC, which eradicated more than 99.9% of the microorganisms within 30 minutes, followed by 4 times the MIC, which eradicated tested microorganisms by 90% in less than 30 minutes. In a different study, in 2019, the same authors tested clove EO and eugenol on 17 strains of *M. pachydermatis* isolated from canine patients and found no significant differences between the two EOs MPICs, MPFCs, MBICs and MBECs, suggesting that eugenol was the major component of the clove EO, which justifiably led the authors to continue with the aforementioned study. On the other hand, they found that the yeast biofilms were 2 times more tolerant to the EOs tested, a much lower figure than previously reported in 2013 by Figueredo et al.

Table 4

MPIC50 and MPFC50 for 17 planktonic M. pachydermatis isolates according to Aiemsaard J. et al.

Antifungal agent	MPIC50 (mg/mL)	MPFC50 (mg/mL)
Clove EO	0.156	0.312
Eugenol	0.156	0.312
Ketoconazole	0.019	0.038

$MPIC_{50}$ – minimum planktonic inhibitory concentration for 50% of tested isolates; $MPFC_{50}$ - minimum planktonic fungicidal concentration for 50% of tested isolates.

Table 5

MBIC50 and MBEC50 for biofilms of 6 M. pachydermatis isolates according to Aiemsaard J. et al

Antifungal agent	MBIC50 (mg/mL)	MBEC50 (mg/mL)
Clove EO	0.312	0.624
Eugenol	0.312	0.624
Ketoconazole	0.038	0.076

MBIC50 – minimum inhibitory concentration for 50% of tested isolates; MBEC50 – minimum biofilm eradication concentration for 50% of tested isolates.

Jowenna Xiao Feng Sim et al. tested the in vitro efficacy of the main phenolic constituents of oregano oil, thyme oil against 20 *M. pachydermatis* clinical isolates associated with canine otitis externa. The antimicrobial susceptibility testing was evaluated by using broth microdilution with spot-planting assay to determine the MIC, MBC and MFC. To confirm the fungicidal activity of the EOs, a time-kill kinetics assay was performed. Oregano oil (carvacrol) and thyme oil (thymol) showed promising antifungal activity against *M. pachydermatis* isolates. They were more sensitive to oregano oil (MIC90 = 0.06%; 563–585 lg/mL), in detriment to thyme oil (MIC90 = 0.125%; 1,146 lg/mL). Additionally, *M. pachydermatis* was reported to have the same MFC values as their MIC values.

Table 6

MIC50, MIC90, MFC50 and MFC90 of tested EOs on 20 isolated from dogs with Malassezia otitis externa according to Jowenna Xiao Feng Sim et al.

Concentration, % values (μg/mL)				
Value	**Oregano**	**Carvacrol**	**Thyme**	**Thymol**
MIC_{50}	0.06 (563)	0.06 (585)	0.125 (1146)	0.09 (800)
MIC_{90}	0.06 (563)	0.06 (585)	0.125 (1146)	0.09 (800)
MFC_{50}	0.06 (563)	0.06 (585)	0.125 (1146)	0.09 (800)
MFC_{90}	0.06 (563)	0.06 (585)	0.125 (1146)	0.09 (800)

In 2015, A.R. Khosravi et al. evaluated the antifungal efficacy of medicinal EOs on 84 strains isolated from canine patients, from various parts of the body (ear, mouth, interdigital, groin). The authors collected plants from different regions of Iran, specifically *Zataria multiflora*, *Thymus kotschyanus*, *Mentha spicata*, *Artemisia sieberi*, *Rosmarinus officinalis* and *Heracleum persicum*. EOs were isolated by water distillation, according to the European Pharmacopoeia. The MICs of the 6 EOs, were evaluated by broth microdilution method. Out of the tested oils, two of them were more active than the other: *Z. multiflora* (MIC90 value: 60 μg/mL) and *A. sieberi* (MIC90 value: 80 μg/mL), having the strongest antifungal proprieties against *M. pachydermatis* and other species, containing a high percentage of phenolic compounds, such as thymol and carvacrol.

Table 7

MIC90 of some essential oils against M. pachydermatis, according to A.R. Khosravi et al.

Agent	MIC90 (μg/mL)
Zataria multiflora	60
Thymus kotschyanus	100
Mentha spicata	150
Artemisia sieberi	80
Rosmarinus officinalis	360
Heracleum persicum	580

In vivo studies

Unfortunately, in vivo studies in the veterinary field at this time are scarce. From the aforementioned databases, only two such studies were retrieved.

Nardoni S. et al., in 2017, tested in vivo, five mixture of essential oils that were obtained from plants found in the Mediterranean area. The EOs themselves showed promising results in previously reported in vitro studies: *Citrus paradisi*, *Salvia sclarea*, *Ocimum basilicum*, *Rosmarinus officinalis*, *Citrus limon*, *Anthemis nobilis*, *Lavandula hybrida* and *Thymus vulgaris*.

The study centered on testing 25 atopic dogs, diagnosed with Malassezia otitis externa, by treating them once daily for 2 weeks. The mixture comprised by C. limon 1%, S. sclarea 0,5%, R. officinalis 1%, A. nobilis 0,5% showed the best results in all treated dogs. There was a complete clinical resolution of symptoms, although the number of blastopores did not decrease. All of the mixtures were tested comparatively with ketoconazole with a MIC of < 0.03 μg/ml−1. Individually, out of all the EOs tested, *T. vulgaris* achieved the lowest MIC (0.05%). The authors mentioned caution when using some EOs topically as some of them can contain irritant compounds (i.e. *O. basilicum*, citrale, limonene) and also discourage the use of high EOs concentrations, especially in atopic patients, as it can cause skin adverse effects. Their concern is based on the fact that 2 patients had adverse effects to one of the mixtures containing 2% Citrus spp. The aforementioned mixture, with the highest grade of the success contains EOs characterized by eudermic action (S. sclarea and R. officinalis).

Rita C.S.M. Neves et al., in 2019, conducted a study on 28 dogs with clinical signs of otitis externa, subsequently diagnosed as being fungal otitis externa, identifying *M. pachydermatis* as the primary pathogen. The aim of the study was to compare the effects of tea tree EO with common antifungal topic treatments. The authors used a 5% tea tree EO lotion in the right ears, while using a 0.15% nystatin lotion in all the left ears, as positive control. They concluded there was no significant difference between the two treatments. Not all dogs treated with tea tree EO have healed completely in the 14 days trial, but a significant improvement of the clinical and cytological findings was obvious on all patients, less inflammation, pruritus, discharge and a lower quantification of microorganisms and inflammatory cells.

CONCLUSIONS

Chemical drugs are being associated with the rapid emergence of multi-drug resistant microorganisms while traditional medicinal plants represent a very important reservoir of active substances that can be used in as aid in treatment of infectious diseases, due to their already known antimicrobial effects. The studies reviewed in this summary show that EOs have a great antimicrobial potential and should be used alone, or in addition with antifungal agents.

Due to different study designs, test methods and of course, the EOs used in the assays, a comparison of results is not always possible. Testing more than one isolate reveals different susceptibility of *M. pachydermatis* to EOs, indicating that an EO might help on one strain isolated from a specific patient, but might not be the best option for a different individual. More in vivo studies need to be conducted to evaluate the risk-benefit ratio of EOs in treating Malassezia related infections.

Further studies to determine the suitable concentration and formulation of EOs and in vivo testing of the antifungal efficacy against *M. pachydermatis* infections are required.

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