Antifungal in vitro Activity of Essential Oils against Clinical Isolates of *Malassezia pachydermatis* from Canine Ears: A Report from a Practice Laboratory

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Keywords

Essential oils · *Malassezia pachydermatis* · Otitis externa · Veterinary medicine

Abstract

Background: The yeast *Malassezia pachydermatis* is a commensal but also the most isolated pathogenic yeast in canine otitis externa. Essential oils (EOs) exhibit antimicrobial activity and thus could be an alternative for treatment of otitis. **Objective:** The aim of this study was to analyse the in vitro efficacy of EOs against *M. pachydermatis* isolates from canine ears. **Materials and Methods:** Twenty-two EOs were tested for their in vitro activity against 15 *M. pachydermatis* isolates by agar disc diffusion. In addition, diluted EOs were tested and volatile antifungal activity was analysed by vapour assay. **Results:** Most EOs showed in vitro efficacy against *M. pachydermatis*. A different susceptibility pattern of tested isolates was found. Overall, the most potential EO was lemon grass. Also, cinnamon leaf, clove, manuka, Indian melissa, oregano, palmarosa, and winter savory oil exhibited a strong antifungal activity. Volatile activity was proven by the inhibiting effect in vapour assay. **Conclusion:** EOs show in vitro activity against *M. pachydermatis*. This study gives scientific background for the use of EOs in veterinary medicine. Diverse susceptibility patterns of different *M. pachydermatis* strains emphasise the helpfulness of an aromagram as one lead for the best choice of an EO for therapy.

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Antimykotische In-vitro-Wirksamkeit ätherischer Öle gegen *Malassezia pachydermatis* isoliert aus Hundeohren: Ein Bericht aus dem Routinelabor

Schlüsselwörter

Ätherische Öle · *Malassezia pachydermatis* · Otitis externa · Veterinärmedizin

Zusammenfassung

Einleitung: Der Hefepilz *Malassezia pachydermatis* gilt als Kommensale, ist aber gleichzeitig auch der am häufigsten isolierte Hefepilz bei caniner Otitis externa. Ätherische Öle könnten aufgrund ihrer antimikrobiellen Eigenschaften als alternative Therapie der Otitis verwendet werden. **Zielsetzung:** Ziel dieser Studie war die Untersuchung der antifungalen In-vitro-Wirksamkeit ätherischer Öle gegen *M. pachydermatis*-Isolate aus Hundeohren. **Material und Methoden:** Die In-vitro-Aktivität von 22 ätherischen Ölen wurde mittels Agar-Plättchen-Diffusionstest gegen 15 *M. pachydermatis*-Isolate untersucht. Zusätzlich wurden Verdünnungen der ätherischen Öle getestet und die volatile Wirkung mittels "vapour assay" analysiert. **Ergebnisse:** Die meisten ätherischen Öle zeigten in vitro eine Wirksamkeit gegen *M. pachydermatis*. Es konnte eine unterschiedliche Empfindlichkeit einzelner *M. pachydermatis*-Isolate gefunden werden. Lemongrasöl erwies sich als das po-

tenteste ätherische Öl. Ebenfalls zeigten Zimtblätter-, Nelken-, Manuka-, indisches Melissen-, Oregano-, Palmarosa- und Bergbohnenkrautöl eine starke antifungale Wirksamkeit. Die volatile Wirkung konnte mittels "vapour assay" gezeigt werden. **Schlussfolgerung:** In vitro zeigen ätherische Öle eine Wirksamkeit gegen den Hefepilz *M. pachydermatis*. Diese Studie bietet eine wissenschaftliche Basis zur Anwendung ätherischer Öle in der Veterinärmedizin. Die unterschiedliche Empfindlichkeit der verschiedenen *M. pachydermatis*-Isolate gegenüber einzelner ätherischer Öle zeigt einmal mehr die Nützlichkeit eines Aromatogramms als Anhaltspunkt bei der Auswahl des geeigneten ätherischen Öls für die Therapie.

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Introduction

The yeast *Malassezia pachydermatis* is a commensal of the skin and mucosae of dogs. As an opportunistic secondary pathogen it is also the most significant yeast in canine otitis externa [1–3]. The mechanisms of the transition of *M. pachydermatis* from a commensal to a pathogen are not fully understood [4]. Generally, treatment of Malassezia-associated otitis externa is based on topical application of an antifungal drug combined with antibiotics to control bacterial infection and glucocorticoids to reduce inflammation [4, 5].

Standardised antifungal susceptibility testing of *M. pachydermatis* has not been established yet, but studies show that Malassezia isolated from animals with dermatitis lesions show less susceptibility to some fungal agents than isolates from healthy animals [6]. One possibly resistant isolate of *M. pachydermatis* to the antifungals ketoconazole and itraconazole was also described [7]. In vitro cross-resistance of Malassezia among azoles was observed [8]. In human medicine, general emergence of antifungal resistance is already known [9]. Besides, the interest in natural plant products in veterinary medicine is increasing [10–12]. Thus, essential oils (EOs) could be a candidate for alternative treatment of otitis externa. EOs are plant secondary metabolites which are secreted and stored in glandular trichomes, modified epidermal hairs of the plant, but also in pockets or reservoirs and even in intercellular spaces. EOs are known to protect the plant against enemies, such as pathogenic microorganisms, insects acting as plaque vectors, and herbivores. On the other hand, they can be beneficial for attracting insects necessary for dispersion of pollens and seeds [13, 14]. EOs are products made by water or steam distillation, mechanical processing, or dry distillation of plant material [15]. They are complex mixtures of volatile lipophilic natural compounds with a specific odour. They mainly consist of terpenes and/or phenylpropanoids [16]. Al-

ready in ancient civilisations like Egypt, India, and China EOs were used for healing. Modern medical aromatherapy was founded by René-Maurice Gattefossé, who started utilising EOs in medical treatment in modern times. Inhalation, local treatment, and baths are the major applications of EOs in human medical aromatherapy [14].

In veterinary medicine, EOs have the potential to be used as repellents against ectoparasites [17, 18]. They also have behaviour-modulating properties [19, 20]. In addition, supplements containing EOs were shown to have a positive effect on atopic dermatitis [21, 22], chronic dermatitis [23], pyoderma [24], nasal hyperkeratosis [25], and malodour in dogs [26]. EOs can also be used as ingredients in mouth rinses for their antimicrobial properties [27, 28] or in the treatment of abscesses [29].

The antimicrobial activity of EOs can be tested in vitro by agar disc diffusion – related to susceptibility testing of antibiotics or antifungals. This so-called aromatogram is a reproducible and affordable laboratory screening test [30, 31].

Studies analysing EO activity against *M. pachydermatis* isolated from canine ears are scarce. Existing in vivo studies analysed the clinical outcome of otitis and the effectivity of single EOs like tea tree oil [32] or the effectivity of a single EO mixture [33] against Malassezia among other microbial agents. A few in vitro studies exist. For example, the activity of 12 EOs against 1 reference strain of *M. pachydermatis*, but also other pathogens, is described [34]. The activity of 11 EOs against 5 clinical isolates and 1 reference strain of *M. pachydermatis* or the activity of cinnamon oil against 20 *M. pachydermatis* isolates from canine otitis was analysed [35, 36]. Eighteen *M. pachydermatis* isolates from healthy canine ears and 1 reference strain were screened against 14 EOs [37]. Possible synergistic effects between the azole derivative clotrimazole and 5 EOs against *M. pachydermatis* isolated from different localisations were analysed by Bohmova et al. [38]. Khosravi et al. [39] analysed the activity of 6 EOs against *Malassezia* spp. isolated from different locations of dogs with atopic dermatitis.

The aim of this study was in vitro analyses of the antifungal activity of 22 EOs against 15 clinical *M. pachydermatis* isolates from canine ears deriving from veterinary routine diagnostics to offer scientific background for the veterinary use of EOs in the treatment of Malassezia-associated otitis externa. To the authors' knowledge, we analysed for the first time a unique panel of 22 EOs by different methods using agar disc diffusion and analyses of volatile activity by vapour assay against a higher number of clinical isolates of *M. pachydermatis* isolated from canine ears. Some of the used EOs overlap with other studies, the other included EOs might bear new potential. Analyses of a larger number of fungal isolates may reveal differences in susceptibility patterns.

Fig. 1. Experimental setup for aromagram (agar disc diffusion) (A) in comparison to vapour assay (B). Pictures 4A versus 4B show the manifestation of inhibition zones after incubation. EO, essential oil.

Materials and Methods

M. pachydermatis Isolates

In this study, 15 *M. pachydermatis* strains isolated from ear swabs of dogs were tested for their in vitro susceptibility to EOs. The ear swabs were sent in from veterinarians for routine diagnostics in August 2016. Swabs were streaked out on Sabouraud glucose agar with chloramphenicol and cycloheximide (BD Mycosel Agar; Becton Dickinson GmbH, Heidelberg, Germany). Agar plates were incubated at 36°C up to 7 days or until growth of typical *M. pachydermatis* colonies was detected. Afterwards, identification was confirmed by MALDI-TOF mass spectrometry (microflex LT; Bruker Daltonik GmbH, Bremen, Germany). Processing of samples for MALDI-TOF analysis was performed using the ethanol-formic acid extraction method according to the manufacturer's standard operating procedure (Bruker Daltonik GmbH). Colony material was suspended in 300 µL of deionised water (Sigma Aldrich, Taufkirchen, Germany) and mixed by vortexing. Nine hundred microlitres of ethanol (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) was added and vigorously mixed. Afterwards, samples were centrifuged and supernatant was discarded, followed by a similar second centrifugation step. Residual ethanol was carefully removed with a pipette and the cell pellet was dried at room temperature for 3 min. Twenty microlitres of 70% formic acid (Carl Roth GmbH + Co. KG) was added to the pellet and vigorously mixed by pipetting. Subsequently, 20 µL of acetonitrile (Fluka Chemie GmbH, Buchs, Switzerland) was added and gently mixed. Afterwards, the sample was centrifuged and 1 µL of the supernatant was pipetted on a target plate and dried at room temperature. The dried spot was covered with 1 µL of IVD HCCA matrix solution (Bruker Daltonik GmbH) and dried at room temperature. MALDI-TOF mass spectrometry identification results were automatically classified using the log score values generated by the MALDI Biotyper software procedure (Bruker Daltonik GmbH). All *M. pachydermatis* isolates included in this study had score values >2.

Malassezia isolates were chosen randomly from identified routine samples under the predetermined condition that they were isolated from canine ears. This random selection resulted in *M. pachydermatis* isolates deriving from dogs of different breeds and mix breeds of both sexes (male: 6; male neutered: 3; female: 4; female neutered: 2), aged from 2 months up to 14 years (mean 7.38 years, standard deviation [SD] 3.46) from different countries (Germany: 11; Switzerland: 1; Austria: 1; Netherlands: 1; Norway: 1).

Aromagram (Agar Disc Diffusion Test)

M. pachydermatis colonies were resuspended in sterile 0.9% NaCl solution and adjusted to McFarland 0.5. Afterwards, 100 µL of *M. pachydermatis* suspension were streaked out on a Sabouraud glucose agar with chloramphenicol and cycloheximide (BD Mycosel Agar; Becton Dickinson GmbH). Subsequently, sterile blank filter paper discs with 6 mm diameter (BBL™ Sensi-Disc™ Susceptibility Test Disc, Blanc Disc; Becton Dickinson GmbH) were placed in the middle on the inoculated agar plate and loaded with 10 µL of the EO to be analysed, meaning that 1 EO per agar plate was tested. Plates were kept at room temperature for 30 min to favour diffusion of EOs over bacterial growth [40, 41] and were then incubated for 4 days at 36°C. After incubation, the radius of the inhibition zones of yeast growth was determined (Fig. 1). Each of the 15 *Malassezia* isolates was tested in duplicate against all 22 EOs; for each *M. pachydermatis* isolate two agar plates per EO were assessed. Thus, two inhibition zone radii per isolate were determined and the mean was calculated and used for classification of EO activity and further descriptive statistical analyses. Growth controls without EO were performed for each *Malassezia* isolate in duplicate. The in vitro efficacy of EOs was classified as follows: not sensitive for an inhibition radius <8.0 mm, slightly sensitive for an inhibition radius of 8.0–13.9 mm, moderately sensitive for an inhibition radius of 14.0–19.9 mm, very sensitive for an inhibition radius ≥20 mm, and extremely sensitive if total growth on the agar plate was inhibited. The in vitro activity of the 22 commercially available pure EOs listed in Table 1 was analysed against 15 *M. pachydermatis* isolates. EOs can be arranged according to their ef-

Table 1. Analysed EOs (listed are all tested EOs with their common and scientific name, plant species, manufacturer's labelling, and manufacturer)

EO common name	Scientific name	Plant species	Manufacturer's labelling		plant part	country; cultivation	preparation		Manufacturer
			ingredients (INCI)						
Angelica root	<i>Angelicae aetheroleum</i>	<i>Angelica archangelica</i> L.	<i>Angelica archangelica</i> root oil, limonene, linalool		root	France; controlled organic cultivation	steam distillation		PRIMAVERA® LIFE GmbH, Oy-Mittelberg, Germany
Cinnamon leaf	<i>Cinnamomi aetheroleum</i>	<i>Cinnamomum verum</i> J.S. PRESL	benzyl benzoate, eugenol, limonene, linalool, cinnamaldehyde, cinnamic alcohol		leaf	Madagascar; n.s.	steam distillation		distributor: Aromaland Inh. Gerda Foltis, Röttingen-Aufstein, Germany
Clary sage	<i>Salviae sclareae aetheroleum</i>	<i>Salvia sclarea</i> L.	<i>Salvia sclarea</i> oil, linalool, geraniol, limonene, eugenol		herb	France; controlled organic cultivation	steam distillation		TAOASIS®, TAOASIS GmbH, Detmold, Germany
Clove	<i>Caryophylli aetheroleum</i>	<i>Syzygium aromaticum</i> (L.) MERR. et L.M. PERCY	<i>Syzygium aromaticum</i> oil, eugenol, geraniol, benzyl salicylate		flower	India; controlled organic cultivation	steam distillation		TAOMED®, TAOASIS GmbH, Detmold, Germany
Coriander seed	<i>Coriandri aetheroleum</i>	<i>Coriandrum sativum</i> L.	<i>Coriandrum sativum</i> fruit oil, geraniol, limonene, linalool		seed	France; controlled organic cultivation	steam distillation		PRIMAVERA® LIFE GmbH, Oy-Mittelberg, Germany
Fennel	<i>Foeniculi aetheroleum</i>	<i>Foeniculum vulgare</i> var. <i>dulce</i> (MILL.) BATT. et TRAB.	<i>Foeniculum vulgare</i> oil (var. dulce), limonene, linalool		seed	Hungary; controlled organic cultivation	steam distillation		TAOASIS®, TAOASIS GmbH, Detmold, Germany
Lavandin super	<i>Lavandulae hybridae aetheroleum</i>	<i>Lavandula hybrida</i> REVERCH	<i>Lavandula hybrida</i> oil, geraniol, limonene, linalool		herb	Italy; controlled organic cultivation	steam distillation		PRIMAVERA® LIFE GmbH, Oy-Mittelberg, Germany
Lavender fine	<i>Lavandulae aetheroleum</i>	<i>Lavandula angustifolia</i> MILL	<i>Lavandula angustifolia</i> oil, geraniol, limonene, linalool		herb	France; conventional cultivation	steam distillation		PRIMAVERA® LIFE GmbH, Oy-Mittelberg, Germany
Lemon	<i>Limonis aetheroleum</i>	<i>Citrus limon</i> L.	<i>Citrus limon</i> peel oil, citral, limonene, linalool		peel	Italy; controlled organic cultivation	cold extraction		PRIMAVERA® LIFE GmbH, Oy-Mittelberg, Germany
Lemon grass	<i>Cymbopogonis citrati aetheroleum</i>	<i>Cymbopogon citratus</i> (DC.) STAPF	<i>Cymbopogon citratus</i> oil		leaf	India; controlled organic cultivation	steam distillation		Baldini, TAOASIS GmbH, Detmold, Germany
Manuka	<i>Leptospermi scoparii aetheroleum</i>	<i>Leptospermum scoparium</i> J.R. et G. FORST	<i>Leptospermum scoparium</i> leaf oil, limonene, linalool, farnesol, geraniol		leaf	New Zealand; wild collection	steam distillation		TAOASIS®, TAOASIS GmbH, Detmold, Germany
Indian melissa	<i>Cymbopogonis nardi aetheroleum</i>	<i>Cymbopogon nardus</i> (L.) RENDLE	<i>Cymbopogon nardus</i> oil, geraniol, citronellol, limonene, citral, linalool, eugenol		herb	Nepal; controlled organic cultivation	steam distillation		TAOMED®, TAOASIS GmbH, Detmold, Germany
Neroli	<i>Aurantii flos aetheroleum</i>	<i>Citrus aurantium</i> L. ssp. <i>amara</i>	<i>Citrus aurantium amara</i> flower oil, geraniol, farnesol, limonene, linalool		flower	Morocco; controlled organic cultivation	steam distillation		PRIMAVERA® LIFE GmbH, Oy-Mittelberg, Germany
Oregano	<i>Origanii aetheroleum</i>	<i>Origanum vulgare</i> L.	<i>Origanum vulgare</i> oil, linalool, limonene, geraniol, citral, eugenol		herb	Albania; controlled organic cultivation	steam distillation		TAOASIS®, TAOASIS GmbH, Detmold, Germany
Palmarosa	<i>Cymbopogonis martinii aetheroleum</i>	<i>Cymbopogon martinii</i> (ROXB.) J.F. WATSON	<i>Cymbopogon martinii</i> oil, geraniol, linalool, farnesol, citral, limonene, citronellol		grass	Nepal; controlled organic cultivation	steam distillation		TAOMED® TAOASIS GmbH, Detmold, Germany
Ravintsara	<i>Cinnamomi camphorae aetheroleum</i>	<i>Cinnamomum camphora</i> (L.) J.S. PRESL	<i>Commiphora camphora</i> leaf oil, limonene		branch	Madagascar; controlled organic cultivation	steam distillation		PRIMAVERA® LIFE GmbH, Oy-Mittelberg, Germany
Rose geranium	<i>Oleum geranii</i>	<i>Pelargonium graveolens</i> L'HERIT. ex AIT	<i>Pelargonium roseum</i> asperum oil, citronellol, geraniol, linalool, limonene		leaf	Egypt; controlled organic cultivation	steam distillation		TAOASIS®, TAOASIS GmbH, Detmold, Germany
Tea tree	<i>Melaleuca aetheroleum</i>	<i>Melaleuca alternifolia</i> CHEEL	<i>Melaleuca alternifolia</i> leaf oil, limonene		leaf	Australia; selective cultivation	steam distillation		TAOASIS®, TAOASIS GmbH, Detmold, Germany
Thyme	<i>Thymi aetheroleum</i>	<i>Thymus vulgaris</i> L.	<i>Thymus vulgaris</i> oil, linalool, geraniol		flowering plant	Spain; controlled organic cultivation	steam distillation		TAOMED®, TAOASIS GmbH, Detmold, Germany
Thyme linalool	<i>Thymi aetheroleum c.t. linalool</i>	<i>Thymus vulgaris</i> L.c.t. linalool	alcohol, <i>Thymus vulgaris</i> flower/leaf oil, limonene, linalool		herb	France; controlled organic cultivation	steam distillation		PRIMAVERA® LIFE GmbH, Oy-Mittelberg, Germany
Thyme thymol 19%	<i>Thymi aetheroleum c.t. thymol</i>	<i>Thymus vulgaris</i> L.c.t. thymol	alcohol, <i>Thymus vulgaris</i> flower/leaf oil, limonene, linalool		herb	Spain; controlled organic cultivation	steam distillation		PRIMAVERA® LIFE GmbH, Oy-Mittelberg, Germany
Winter savory	<i>Saturejae montanae aetheroleum</i>	<i>Satureja montana</i> L.	<i>Satureja montana</i> oil		herb	Croatia; controlled organic cultivation	steam distillation		TAOASIS®, TAOASIS GmbH, Detmold, Germany

EO, essential oil; INCI, International Nomenclature of Cosmetic Ingredients.

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Table 2. Range, median, and IQR of inhibition zone radii of all tested EOs for different experimental setups

EO	Inhibition zone radius											
	aromatogram (<i>n</i> = 15)			vapour assay (<i>n</i> = 15)			EO 20% solution (<i>n</i> = 5)			EO 10% solution (<i>n</i> = 5)		
	range, mm	median, mm	IQR	range, mm	median, mm	IQR	range, mm	median, mm	IQR	range, mm	median, mm	IQR
Winter savory	28–>40	>40.00	0	>40	>40.00	0	8.5–13	10.75	2.25	4–7.5	4.50	1.00
Lemon grass	22.5–>40	>40.00	0	>40	>40.00	0	17–23	19.50	2.75	6.5–8	8.00	0.50
Rose geranium	22–>40	>40.00	0	22.5–>40	>40.00	0	6.5–8	7.00	0.50	0	0	0
Oregano	21–>40	>40.00	0	>40	>40.00	0	8–17	14.50	2.50	5–7	6.00	1.00
Palmarosa	17.5–>40	>40.00	4.50	16–>40	>40.00	0	9.5–17	12.50	3.00	5–7.5	6.75	0.25
Indian melissa	22.5–>40	>40.00	9.75	18–>40	>40.00	0	4.5–8	7.00	1.00	0	0	0
Thyme thymol 19%	20–>40	37.50	14.00	13–>40	>40.00	1.75	0	0	0	n.t.		
Cinnamon leaf	25–32	26.00	3.50	10–35	24.00	6.63	8–13	9.00	1.50	4–5.5	4.00	0.25
Clove	25–30	25.00	4.88	18–32	22.50	6.38	6–16	8.00	1.25	0–6	5.00	1.00
Thyme	9.5–26.5	19.75	5.50	11.5–30	21.50	9.75	0	0	0	n.t.		
Coriander seed	19–25	19.00	3.88	12.5–35	17.00	6.50	0	0	0	n.t.		
Thyme linalool	8–26	16.50	5.88	4–25.5	13.00	5.63	0	0	0	n.t.		
Manuka	0–20	15.00	2.75	4.5–19	10.50	5.25	4–6.5	5.50	0.75	0	0	0
Tea tree	6–17.5	10.00	3.75	4–24	7.50	4.63	0	0	0	n.t.		
Lavandin super	9–22.5	9.00	4.38	4–17.5	9.50	6.25	0	0	0	n.t.		
Fennel	8.5–12	8.75	1.63	0–7.5	0	5.50	0	0	0	n.t.		
Lavender fine	8.5–15.5	8.00	3.13	0–17	7.25	4.00	0	0	0	n.t.		
Lemon	8–11	7.75	1.50	0–8	4.50	6.25	0	0	0	n.t.		
Clary sage	7.5–16.5	7.50	1.38	0–11	5.50	6.38	0	0	0	n.t.		
Angelica root	7.5–10	7.50	2.13	0–5	0	0	0	0	0	n.t.		
Ravintsara	5–9	6.75	1.13	0–6.5	0	5.50	0	0	0	n.t.		
Neroli	0	0	0	0	0	0	0	0	0	n.t.		

EO, essential oil; IQR, interquartile range; n.t., not tested.

fectivity by the median of all inhibition zone radii means of the tested 15 isolates; in case of equal medians, the higher sum of all inhibition zone radii of each EO counted for higher effectivity (Fig. 1). The chromatographic profiles of EOs specified by manufacturer or product specifications are shown in online supplementary Table 1 (see www.karger.com/doi/10.1159/000504316).

Antifungal Susceptibility Testing

The activity of antifungal agents was tested simultaneously using agar disc diffusion assay. *M. pachydermatis* colonies were resuspended in sterile 0.9% NaCl solution and adjusted to McFarland 0.5. Afterwards, 100 µL of *M. pachydermatis* suspension were streaked out on a Sabouraud glucose agar with chloramphenicol and cycloheximide. Instead of blank filter paper disc with EOs, an antifungal ring (MASTRING_LAB 504/1; Mast Group Ltd., Merseyside, UK) was placed on the agar. The antifungal ring included among others following agents: amphotericin B (20 µg), nystatin (100 µg), natamycin (10 µg), and 5-fluorocytosine (1 µg). The diameter of the inhibition zones of yeast growth was determined. Antifungal susceptibility testing of each *Malassezia* isolate was performed in duplicate, and the mean of duplicate for each isolate was calculated for further descriptive statistical analyses.

Dilution of EOs

All EOs were diluted in sesame oil as a carrier oil and tested in a 20% solution for their growth-inhibiting activity in the aromatogram as mentioned above. Growth controls of each *Malassezia* isolate without EO were performed in duplicate to exclude an antifungal activity of the carrier oil; additionally, pure sesame oil was

used as negative control. The diluted EOs were tested against 5 *M. pachydermatis* isolates, which were chosen randomly.

Afterwards, all EOs which showed a growth-inhibiting effect in the 20% solution were tested for their antifungal activity in a 10% solution. This included cinnamon leaf, clove, lemon grass, manuka, Indian melissa, oregano, palmarosa, rose geranium, and winter savory oil. The aromatogram of diluted EOs was performed in duplicate; for each tested *M. pachydermatis* isolate two agar plates per EO were assessed. Thus, two inhibition zone radii per isolate were determined and the mean was calculated for classification of EO activity and further descriptive statistical analyses.

Vapour Assay

To analyse the antifungal activity of the volatile components of EOs, the agar disc diffusion test was modified according to Serban et al. [42] and Maruzzella and Sicurella [43]. *M. pachydermatis* colonies were resuspended in sterile 0.9% NaCl solution and adjusted to McFarland 0.5. Afterwards, 100 µL of *M. pachydermatis* suspension were streaked out on a Sabouraud glucose agar with chloramphenicol and cycloheximide. The filter paper disc was placed in the centre of the plastic lid of the agar plate and not on the agar itself and loaded with 10 µL of pure EOs. Therefore, the EOs had no direct contact with the agar. Afterwards, plates were incubated standing on their lid and not on their bottom. Thus, only volatile components could exhibit antimicrobial activity. Analysis was performed similar to the agar disc diffusion test by measuring inhibition zone radii (Fig. 1). The vapour assay was performed in duplicate; for each tested *M. pachydermatis* isolate two agar plates per EO were assessed. Thus, two inhibition zone radii per isolate were determined and the mean was calculated for classification

Fig. 2. Agar disc diffusion: in vitro activity of EOs against *M. pachydermatis* ($n = 15$) using agar disc diffusion. Susceptibility of *M. pachydermatis* is classified by the size of the zone of inhibition radius in mm (mean of duplicates): extremely sensitive, no growth at all; very sensitive, ≥ 20 mm; moderately sensitive, 19.9–14.0 mm; slightly sensitive, 13.9–8.0 mm. *M. pachydermatis* isolates classified as not susceptible (inhibition zone radius ≤ 7.9 mm) are not depicted (missing bars). EOs are arranged according to their effectivity in decreasing order from left to right. The median of all inhibition zone radii of the tested 15 isolates was taken for arrangement; in case of equal medians, the higher sum of all inhibition zone radii of each EO counted for higher effectivity. EO, essential oil.

and further descriptive statistical analyses. Growth controls of each *Malassezia* isolate without EO also incubated on the lid were performed in duplicate.

Statistical Analyses

Statistical analyses was performed using R [44]. The Shapiro-Wilk test was used to analyse data for normality ($p < 0.05$). For nonparametric data the mean and interquartile range (IQR) is stated, for parametric data mean and SD.

Results

Aromatogram

Agar disc diffusion tests were performed in duplicate and variant inhibition zone radii were detected (the SD of duplicates ranged from 0.0 to 3.5 mm). Growth controls showed no inhibition zones. The mean inhibition zone radii of EOs against *M. pachydermatis* are listed in Table 2 (column “Aromatogram”). The tested antifungals, except for 5-flucytosine, showed inhibition zones. The inhibition zone diameter ranges for amphotericin B were 10–24 mm (median: 19.0; IQR: 2.0), for nystatin 8–24 mm (median: 21.0; IQR: 3.5), and for natamycin 8–20 mm (median: 16.0; IQR: 4.0).

The in vitro efficacy of EOs against *M. pachydermatis* growth showed different degrees and can be arranged by decreasing effectivity (Fig. 2). Winter savory, lemon grass, and rose geranium oil inhibited total growth of *M. pachydermatis* on the agar plate in 14 of 15 cases, oregano oil in 13 of 15 cases. Palmarosa, Indian melissa, thyme thymol 19%, cinnamon leaf, clove, thyme, coriander seed, and thyme linalool oil had an inhibitory effect against all isolates as well by decreasing order.

Manuka, tea tree, lavandin super, fennel, lavender fine, lemon, clary sage, and angelica root oil did not exhibit an-

tifungal activity in all cases. Manuka oil was active in 14 of 15 cases, followed by tea tree, lavandin super, and fennel oil. Lavender fine, lemon, clary sage, and angelica root oil had mostly a slight inhibitory effect. Ravintsara oil had only a slight inhibitory effect against 1 isolate of *M. pachydermatis*. Neroli oil had no inhibitory effect at all.

Dilution of EOs

Dilution of EOs was performed in duplicate and variant inhibition zone radii were detected (the SD of duplicates ranged from 0.0 to 2.0 mm). Growth controls and negative control with carrier sesame oil led to no inhibition zone. The mean inhibition zones of diluted EOs against *M. pachydermatis* are listed in Table 2 (columns “EO 20% solution” and “EO 10% solution”). In a 20% solution some EOs still had a growth-inhibiting effect on *M. pachydermatis* (Fig. 3). Among the diluted EOs, lemon grass was the most potent one. Also effective by decreasing order were oregano, palmarosa, winter savory, cinnamon leaf, clove, Indian melissa, and rose geranium oil. Manuka oil led to very small inhibition zones which were < 8.0 mm and thus classified as not effective. Thyme thymol 19%, thyme, coriander seed, thyme linalool, tea tree, lavandin super, fennel, lavender fine, lemon, clary sage, angelica root, ravintsara, and neroli oil showed no inhibition of growth in a 20% solution.

All EOs which showed an inhibitory effect in the 20% solution, i.e., cinnamon leaf, clove, lemon grass, manuka, Indian melissa, oregano, palmarosa, and winter savory, were additionally tested in a 10% solution in sesame oil (Fig. 4). Lemon grass oil was the most effective in the 10% solution. Cinnamon leaf, clove, oregano, palmarosa, and winter savory oil led to smaller inhibition zones. Manuka, Indian melissa, and rose geranium oil had no growth-inhibiting effect in the 10% solution.

Fig. 3. Dilution of EOs: in vitro activity of a 20% EO solution against *M. pachydermatis* ($n = 5$) using agar disc diffusion. Susceptibility of *M. pachydermatis* is classified by the size of the zone of inhibition radius in mm (mean of duplicates): extremely sensitive, no growth at all; very sensitive, ≥ 20 mm; moderately sensitive, 19.9–14.0 mm; slightly sensitive, 13.9–8.0 mm. *M. pachydermatis* isolates classified as not susceptible (inhibition zone radius ≤ 7.9 mm) are not depicted (missing bars). EO, essential oil.

Fig. 4. Dilution of EOs: in vitro activity of a 10% EO solution against *M. pachydermatis* ($n = 5$) using agar disc diffusion. Susceptibility of *M. pachydermatis* is classified by the size of the zone of inhibition radius in mm (mean of duplicates): extremely sensitive, no growth at all; very sensitive, ≥ 20 mm; moderately sensitive, 19.9–14.0 mm; slightly sensitive, 13.9–8.0 mm. *M. pachydermatis* isolates classified as not susceptible (inhibition zone radius ≤ 7.9 mm) are not depicted (missing bars). EO, essential oil.

Fig. 5. Vapour assay: in vitro activity of EOs against *M. pachydermatis* ($n = 15$) using vapour assay. Susceptibility of *M. pachydermatis* is classified by the size of the zone of inhibition radius in mm (mean of duplicates): extremely sensitive, no growth at all; very sensitive, ≥ 20 mm; moderately sensitive, 19.9–14.0 mm; slightly sensitive, 13.9–8.0 mm. *M. pachydermatis* isolates classified as not susceptible (inhibition zone radius ≤ 7.9 mm) are not depicted (missing bars). EO, essential oil.

Vapour Assay

For analysis of volatile components of EOs the vapour assay was performed. Tests were performed in duplicate and variant inhibition zone radii were detected (the SD ranged from 0.0 to 3.5 mm). The mean inhibition zones of diluted EOs against *M. pachydermatis* are listed in Table 2 (column “Vapour assay”). The inhibition zones of vapour assay had the same manifestation as observed in the aromatogram (Fig. 1, picture 4A vs. 4B), although they may differ in size (Fig. 2 vs. Fig. 5; Table 2, column “Aromatogram” vs. column “Vapour assay”).

Eleven of the 22 tested EOs had an antifungal effect against all tested *M. pachydermatis* isolates in the vapour

assay (Fig. 5). Winter savory, lemon grass, and oregano oil inhibited overall growth on agar plates in all cases. Rose geranium, Indian melissa, palmarosa, and thyme thymol 19% oil also inhibited total growth of isolates in the majority of cases. Cinnamon leaf, clove, thyme, and coriander seed oil showed volatile activity by decreasing order against all *M. pachydermatis* isolates. Thyme linalool, manuka, and lavandin super oil were active against the majority of isolates, while tea tree, lavender fine, and clary sage oil were effective in some cases. Lemon, ravintsara, fennel, angelica root, and neroli oil had no volatile inhibiting effect.

Discussion and Conclusion

This study showed an in vitro activity of several EOs against clinical isolates of the yeast *M. pachydermatis* from canine ears. Especially, winter savory, lemon grass, oregano, palmarosa, and cinnamon leaf oil showed excellent in vitro activity.

EOs exhibit their antifungal activity by different mechanisms at the level of the cell membrane and also within the cell. The structure and function of fungal cell membranes is disrupted and their formation is inhibited. Antifungal components can also lead to dysfunction of fungal mitochondria. In addition, inhibition of biofilm development and anti-quorum sensing of EOs was shown [15].

The antifungal activity of EOs was tested using the agar disc diffusion method (aromatogram). The aromatogram is a measurable method to test the in vitro activity of EOs [30]. The zone of inhibition of fungal growth depends on the sensitivity of *Malassezia* against the active components of the EOs, but also on the quantity of components which diffuse into the agar. This holds also true for routinely used agar disc diffusion susceptibility testing of antibiotics [45]. The hydrophobic properties of EOs could prevent uniform diffusion through the agar medium [46]. In general, agar disc diffusion provides qualitative results. Since the amount of diffused active components in the agar medium is not known, determination of a minimum inhibitory concentration (MIC) is not possible. Fungistatic or fungicide effects cannot be identified either because inhibition of yeast growth does not necessarily mean fungal death [47].

Up to now, no standardised method for susceptibility testing of EOs exists. Results of studies on the antimicrobial activity of EOs are not easily compared because different methods are used (agar disc diffusion [34, 37], broth dilution [48], microdilution [35, 49–51]). While in agar disc diffusion the activity of an EO depends on diffusion of active components in the agar, in microdilution in ideal circumstances direct contact between all active components of the EO and the yeast is given. Thus, EOs which are not effective in agar disc diffusion might still have an antimicrobial effect in microdilution.

Even if the method itself is the same, for example agar disc diffusion, factors such as volume of EOs, thickness of the agar layer, solvent, or composition of EOs differ from study to study. The composition of EOs varies due to different plant parts used for extraction, extraction method, natural variation (varieties, subspecies, chemotypes), region of growth, climatic conditions, or time point of harvesting [52–59]. Thus, no constant composition of an EO can be expected. Different composition of an EO can lead to different bioactivity [57, 59, 60]. For the best prediction of EO activity the aromatogram should be performed

with exactly the same EO used for therapy, meaning the same charge number. Nevertheless, the aromatogram can be used as a diagnostic tool before therapeutic treatment and can guide clinicians in their choice of an EO [31]. It also can be used as a screening method for potentially active oils [46, 61, 62]. Important is the standardisation of the method used to achieve the best comparability.

The aromatogram (agar disc diffusion) is a reproducible [31], practical, and affordable test for commercial veterinary routine diagnostic laboratories. The used study design is adapted to routine diagnostics and thus might not always keep up with university research procedures. Nevertheless, aromatograms were performed in duplicate, which is comparable to other published studies [36–38]. In addition, a higher effort was made to gain better insight into the efficacy of EOs by testing EO dilutions as well as performing the vapour assay, which is not part of daily routine.

Despite the fact that agar disc diffusion might not be the optimal in vitro test, the important conclusion can be drawn that EOs have an inhibiting effect on *M. pachydermatis* isolated from canine ears. Thus, EOs could be a promising candidate for local treatment of *M. pachydermatis*-associated otitis externa.

Although the antifungal activity of EOs is known [15], to our knowledge explicit data on in vitro antifungal activity against clinical isolates of *M. pachydermatis* from canine ears are scarce. As already mentioned, comparison between studies is difficult, and moreover the panel of tested EOs differs from study to study. Subsequently, we only focused on the EOs relevant for comparison with our analyses. One study tested clove, lavender, oregano, winter savory, and tea tree oil for their antifungal activity against *M. pachydermatis* isolated from healthy canine ears ($n = 18$) and 1 type strain at three different concentrations (0.5, 5.0, 30%) [37]. Despite methodological differences in agar disc diffusion regarding the agar media used (Sabouraud dextrose agar with chloramphenicol vs. Sabouraud glucose agar with chloramphenicol and cycloheximide in our study), McFarland standard of inoculum (1.0 vs. 0.5 in our study), EO amount (15 vs. 10 μL in our study), and EO concentration (0.5, 5.0, 30% EO diluted in liquid paraffin vs. 100, 20, 10% EO diluted in sesame oil in our study), the results obtained support each other. Similar to our data, diluted clove and oregano oil showed good activity. Diluted lavender and tea tree oil did not show any activity in both studies. Slightly different results were obtained for winter savory oil, which was active in the 20% solution in our study, but only showed weak activity in the 30% solution used by Váczi et al. [37].

Another study showed good in vitro activity of lavender, lemon, and oregano oil [35]. Different concentrations of those EOs diluted in sweet almond oil (0.6–10%) were tested by microdilution in semisolid mDixon media

against 5 clinical isolates of *M. pachydermatis* from canine otitis externa and 1 type strain. Oregano oil with a MIC of 1.3% in microdilution showed inhibition of growth at a 10% concentration in the agar disc diffusion used in our study. In contrary, lemon and lavandin oil were active in microdilution (MIC: 1 and 4%, respectively), but had no effect when used diluted in agar disc diffusion. Clary sage oil in a 10% solution exhibited no antifungal activity in microdilution in the study by Pistelli [35] nor tested with disc diffusion in our study. Cinnamon EO was effective against 20 *M. pachydermatis* isolates from canine otitis externa tested by microdilution [36]. MIC values of 0.008% indicate strong activity, which is in accordance with the antifungal activity of the 20% solution of this EO in our study.

Besides these studies on *M. pachydermatis* isolates from canine ears, data about isolates of unknown localisation, dermatitis, or type strains exist. Clary sage, oregano, lemon grass, tea tree, and thyme oil were tested with agar disc diffusion (in the same procedure as used in our study, except that Müller-Hinton agar was used) against 1 clinical isolate of *M. pachydermatis* from unknown localisation. They showed comparable activity as obtained in our study, whereas the activity of clove and lemon oil was weaker [34]. Lavender and oregano oil tested by microdilution displayed antifungal activity against 5 clinical yeast isolates from dogs with *Malassezia*-associated dermatitis (MIC: 4.0 and 0.8%, respectively) [49]. Tea tree oil was shown to have in vitro activity against 5 clinical *M. pachydermatis* isolates from canine dermatitis and 4 canine type strains tested by microdilution [50]. A skin cream containing tea tree oil was active tested by microdilution against 1 type strain of *M. pachydermatis* [51]. Tea tree, oregano, and clove oil showed synergistic or additive activity with antifungal clotrimazole against 19 *M. pachydermatis* isolates from different locations (ear, skin interdigital space, perineal area) tested by microdilution [38], emphasising possible benefits of complementary treatment with EOs to conventional medicine.

In addition, some in vivo studies prove that treatment of otitis externa with EOs is definitely a therapeutic option. Application of a 5.0% tea tree oil lotion into canine ears led to a similar effect as treatment with synthetic antifungal nystatin lotion [32]. An EO mixture of bergamot (3%), lavender (2%), tea tree (1%), and roman chamomile (1%) oil applied twice daily for 2 weeks had a comparable clinical effect to treatment with amoxicillin-clavulanic acid, ciprofloxacin, and ketoconazole. In this study, bacterial and fungal cell count decreased dramatically in the EO-treated group, but only slightly in the control group [33]. Experimentally induced otitis media in rats could be cured in a high proportion of animals by the use of basil oil [63]. Treatment of human otitis patients with ear drops containing clove, lavender, and herb-Robert

showed a healing effect comparable to that of ciprofloxacin ear drops (0.3%) [64].

Also, in cases of *Malassezia*-associated canine dermatitis, EO-based skin creams showed promising effects in vivo. A skin cream containing 1% lavender, 0.5% oregano, and other EOs (0.5–1%) led to good and long-lasting clinical improvement in dermatitis, whereas conventionally treated dogs had signs of recurrence [49].

Most otitis is polymicrobial and caused by *Staphylococcus* spp. and *M. pachydermatis*, but other bacteria can also be involved [1, 3, 32]. In addition, it is discussed whether combined antifungal and antibacterial therapy of otitis externa prevents the switch from yeast to bacterial infection or vice versa if antifungal or antibacterial monotherapy is used [4]. Thus, another positive effect of EOs in treatment could be their additional antibacterial effect. This was already shown in vitro against several bacterial veterinary isolates [34, 36, 48, 65, 66]. In addition, EOs can exhibit anti-inflammatory potential [67], which might be another positive attribute for treatment of otitis externa.

The volatile antimicrobial properties of EOs, proven in this and other studies [42, 43, 68], could be beneficial for their dispersion in the ear canal. This applies especially for winter savory, lemon grass, rose geranium, oregano, palmarosa, and Indian melissa oil in our study. To our knowledge, this study describes the volatile activity of EOs against *M. pachydermatis* isolated from canine ears for the first time. Others proved the volatile activity of EOs against *Malassezia* by using different experimental setups. Celery EO was proven to exhibit antimicrobial volatile activity against *M. furfur* by a modified chamber assay [69]. *Artemisia annua* EO had volatile activity against *Malassezia* spp.; MICs were lower than those obtained in liquid phase [70].

Several EOs only showed in vitro activity in the agar disc diffusion assay when used undiluted. For therapeutic local application, EOs should not be used undiluted. Due to its popularity, tea tree oil is probably the most investigated EO for compatibility. Instillation of 100% tea tree oil into the middle ear of guinea pigs led to ototoxicity, whereas a 2% solution was tolerated well [71]. Also, intratympanic injection of 3% diluted tea tree oil had no ototoxic side effects in a chinchilla animal model [72]. Treatment of otocariasis and otitis externa in dogs with a 5% tea tree oil solution also led to no adverse reaction [32, 73]. A cotton tip wetted in a 50% tea tree oil solution was even applied as a lid scrub (6 strokes, repeated 3 times total) for treatment of ocular demodex [74]. A 10% tea tree oil cream for treatment of canine dermatitis was also tolerated well [75]. Additionally, other EOs were shown to lack sensitisation ability and thus safe if used diluted: angelica root, clove oil (Madagascar clarified), coriander, lavender (Yugoslavia), lemon (Sicilian), and lemon grass

oil were shown to be non-sensitisers in a modified Draize test, including intradermal injection and topical application in guinea pigs [76]. In the same study, citronella oil from Guatemala (0.5%) with a high content of citronellal (20%) led to a sensitising effect after two injections, whereas citronella oil from Ceylon (1.0%) with a low content of citronellal (5%) did not. Thus, also the composition of the EO used should be taken into consideration before application. Lans et al. [77] documented and validated ethnoveterinary medicine used in British Columbia, Canada. Ear infections in pets were treated with products containing tea tree, thyme, clove, and oregano oil. If used diluted, some EOs showed no adverse side effects applied topically and thus could be a safe alternative for treatment. Nevertheless, the compatibility of the EO intended to be used for therapy should always be clarified before treatment, as should be done with any other drug. There are several records of adverse reactions to EOs [78]. On the other hand, many commercially available ear formulations contain antimicrobial agents which may be ototoxic to the middle and internal ear [5, 79].

It is clear that due to different study designs, test methods, and EOs used a comparison of results from different studies is not always possible. The advantage of this study is the broad spectrum of tested EOs against a higher number of clinical isolates of *M. pachydermatis*. Testing more than 1 isolate reveals different susceptibility of *M. pachydermatis* to EOs. This indicates that an EO might help against a *M. pachydermatis* strain isolated from one patient, but might not be the best option for another individual. Thus, no general prediction of effectiveness for

one EO can be made, and it is advisable to perform a mycological examination, including an aromagram, before treatment.

Statement of Ethics

The authors have no ethical conflict to disclose. The research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki.

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Author Contributions

D. Bismarck: conception of the work, design of the work, interpretation of data, drafting of the work, final approval of the version published. A. Dusold: acquisition, analysis and interpretation of data, critical revision of the manuscript for intellectual content, final approval of the version published. A. Heusinger: design of the work, interpretation of data, critical revision of the manuscript for intellectual content, final approval of the version published. E. Müller: conception of the work, critical revision of the manuscript for intellectual content, final approval of the version published.

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