TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2022) 46: 549-558

doi:10.55730/1300-0128.4225

Activities and mechanisms of oregano, marjoram and rosemary essential oils against *Malassezia pachydermatitis* isolates from canine and feline otitis

Stefanie Bressan WALLER[1,*], Márcia Kutscher RIPOLL[1], Anna Luiza SILVA[1], Emanoele Figueiredo SERRA[1], Tábata Pereira DIAS[2], Vittória Bassi DAS NEVES[2], Luciéle Pereira DE MELO[1], Patrícia LINDEMANN[1], Otávia de Almeida MARTINS[1], Angelita dos Reis GOMES[1], Renata Osório DE FARIA[1], Mário Carlos Araújo MEIRELES[1], João Roberto Braga DE MELLO[3], Marlete Brum CLEFF[2]

[1]Department of Veterinary Preventive Medicine, Faculty of Veterinary Medicine, Federal University of Pelotas (UFPEL), Pelotas, RS, Brazil
[2]Department of Veterinary Clinics, Faculty of Veterinary Medicine, Federal University of Pelotas (UFPEL), Pelotas, RS, Brazil
[3]Department of Pharmacology, Institute of Basic Health Sciences, Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil

Received: 20.04.2022 • **Accepted/Published Online:** 18.07.2022 • **Final Version:** 03.08.2022

Abstract: *Malassezia pachydermatis* is an opportunistic yeast found in the ear canal of small animals; however, the current azole-based therapy applied to it has failed to achieve clinical success due to the antifungal resistance. This issue has encouraged the studies in natural products, such as *Origanum vulgare* (oregano), *Origanum majorana* (marjoram) and *Rosmarinus officinalis* (rosemary) essential oils, although their mechanism of action remains unclear. *Malassezia pachydermatis* specimens deriving from otitis cases in dogs (n = 22) and cats (n = 2) were subjected to CLSI M27-A3. Sorbitol protection and ergosterol effect were analyzed to investigate their mechanism of action. Fungistatic (MIC) and fungicidal (MFC) activities were observed for oregano (MIC_{90}/MFC_{90}: 0.625 mg/mL); marjoram (MIC_{90}/MFC_{90}: 2.5 mg/mL) and rosemary MIC_{90}/MFC_{90} > 2.5 mg/mL). Oregano showed superior antifungal effect even at lower MIC and MFC values. All three oils acted on cell wall and at complexation to fungal ergosterol. By gas chromatography (GC-FID), carvacrol was the major compound found in oregano (73.9%); 1,8-cineole was for marjoram and rosemary (20.9% and 49.4%, respectively). These findings support the potential use of these essential oils to treat canine and feline otitis caused by *Malassezia pachydermatis*.

Key words: Malasseziosis, antifungal, *Origanum vulgare*, *Origanum majorana*, *Rosmarinus officinalis*, mechanism of action

1. Introduction

Infections caused by *Malassezia* sp., mainly the one caused by *Malassezia pachydermatis*, are acknowledged as severe mycosis affecting the ear canals and skin of small animals [1]. This fungal species is a significant commensal and opportunistic pathogen capable of causing otitis and dermatitis in dogs and cats [1,2] besides affecting their oral cavity [3]. Typical lesions observed in veterinary practice are described as ceruminous otitis externa [4] and seborrheic dermatitis with pruritus, erythema, brown greasy and foul odor [2,5].

The current therapy used to treat *M. pachydermatis* infections comprises the application of antiseptic substances and topical/systemic antifungals [6], such as miconazole-chlorhexidine shampoos and oral itraconazole [2]. However, the emergence of antifungal resistance to azoles [1,7], such as fluconazole [8] and itraconazole [9], has made it hard to achieve clinical cure, a fact that reinforces the need of finding new active compounds, such as the ones observed in natural products.

Among the medicinal plants of interest are *Origanum vulgare* L. (oregano), *O. majorana* L. (marjoram) and *Rosmarinus officinalis* L. (rosemary); they belong to family Lamiaceae, which is well-known for the antifungal properties of its botanical species [10,11]. Although the anti-*Malassezia pachydermatis* activity of oregano essential oil is well-known among the aforementioned plants [4,6,12–19] studies conducted with marjoram [10,20] and rosemary [6,14] remain scarce. Moreover, the antifungal action mechanism of these natural products has not yet been described in *M. pachydermatis*.

Thus, the aim of the current study was to evaluate the activity in vitro of oregano, marjoram and rosemary essential oils against *M. pachydermatis* isolated from otitis and dermatitis cases diagnosed in small animals, as well as their antifungal action mechanisms and chemical composition.

* Correspondence: waller.stefanie@yahoo.com.br

2. Materials and methods

2.1. Essential oils

Three commercial essential oils with certified quality were purchased at *Ferquima – Indústria e Comércio de Produtos Alimentícios* Ltda., São Paulo, SP, Brazil. All essential oils were extracted from dried *Origanum vulgare* L., *Origanum majorana* L., and *Rosmarinus officinalis* L. shoots collected in Moldova, Egypt, and Tunisia, respectively. All products were stored in amber vials and kept under refrigeration until the experiments were performed.

2.2. Chemical composition

Chemical analysis was based on high-resolution gas chromatography with flame ionization detector (GC-FID). It was carried out in HP 7820A (Agilent) device equipped with HP-5 column (30 m × 0.32 mm × 0.25 mm) at initial temperature of 70 °C, which increased by 3 °C/min up to 240 °C. Injector and FID detector's temperature was 250 °C and 260 °C, respectively. Hydrogen was used as carrier gas at flow rate of 3 mL/min and split ratio 1:30. The investigated oils were diluted in chloroform (1%), and 1 µL of this dilution was injected into the chromatographic equipment. Chemical compounds were identified by comparing their mass spectra to the Kovats retention index (R.I.). Data were collected and processed in EZChrom Elite Compact software (Agilent).

2.3. Fungal isolates

Twenty-four clinical *Malassezia pachydermatis* isolates deriving from the mycology collection of *Centro de Diagnóstico e Pesquisa em Micologia Veterinária* (Federal University of Pelotas, Pelotas, RS, Brazil) were used in tests conducted in vitro. These fungal isolates were cultured from external otitis cases diagnosed in dogs (n = 22) and cats (n = 2), whose clinical data are summarized in Table 1. All *M. pachydermatis* specimens were previously identified based on their macroscopic and microscopic morphological features, as well as on their ability to grow in culture medium free from lipid supplementation (Sabouraud dextrose agar, SDA, Acumedia, Michigan, USA) at 35 °C. Control strains (*Candida albicans* ATCC 14053, *C. lusitaniae* ATCC 34449, and *C. krusei* ATCC 34135) were also used to assure the quality of the assays; this experiment used 27 fungal isolates, in total.

2.4. Antifungal susceptibility test

Antifungal susceptibility test in vitro was performed based on the broth microdilution method; according to the CLSI M27-A3 standard protocol [21], with adaptations for natural products [12]. Each isolate deriving from young colonies growth in SDA (at 35 °C, for 48 h) was prepared, in separate, for fungal inoculum, and adjusted to absorbance of 0.080–0.100 (530 nm) in spectrophotometer (Spectrum Instruments Co., Shanghai, Chine), in order to find final inoculum of 1–5 × 10^6 CFU/mL. The 1:100 (v/v) dilution of these suspensions was prepared in saline solution; this procedure was followed by 1:20 (v/v) dilution in SDA liquid medium.

A two-fold concentration was prepared in SDA liquid medium and added with a drop of 1% Tween 80 to enable essential oil homogenization. Then, serial dilution was performed on 96-well microplates to find concentrations ranging from 2.5 to 0.078 mg/mL. Subsequently, suspensions of the adjusted fungal inoculum (100 µL) were added to each well. Negative (200 µL of culture medium) and positive (100 µL of culture medium added with 100 µL of inoculum suspension) controls were used for each fungal isolate.

Results were expressed as minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC). Microplates were incubated in rotatory shaker at 34 °C, for 48 h, to find MIC values. Furthermore, 10 µL of the content of each well without fungal growth was transferred to Petri dishes added with SDA medium and incubated at 35 °C, for 72 h, to enable visualizing fungal growth and finding MFC values. All tests were performed in duplicate.

2.5. Mechanism of action through sorbitol protection and ergosterol effect tests

Sorbitol protection and ergosterol effect tests were performed [22,23] with *Malassezia pachydermatis* (n = 2) derived from canine otitis to observe the mechanism of action. MIC was measured in the presence and absence of sorbitol (0.8 mol/L, Sigma-Aldrich, St. Louis, USA) and ergosterol (50–200 mg/mL, Sigma-Aldrich, St. Louis, USA) in RPMI-1640 culture medium added with L-glutamine, without sodium bicarbonate, and buffered to pH 7.0 by using MOPS (0.165 mol/L) in fungal inoculums [22,23]. Moreover, this substance was previously dissolved in dimethylformamide, and then dissolved in RPMI culture medium, for the ergosterol effect test.

Solutions based on the essential oils deriving from all three plant species, which ranged from 16-fold the MIC values to MIC/32 —i.e. from 2.5 to 0.005 mg/mL (*O. vulgare*), from 10 to 0.0195 mg/mL (*O. majorana*), and from 20 to 0.039 mg/mL (*R. officinalis*)— were prepared.

Anidulafungin (Ecalta, Pfizer/Wyelth, Kalamazoo, Michigan, USA) was used as positive control in the sorbitol test, whereas amphotericin B (Sigma Aldrich, San Luis, Missouri, USA) was in the ergosterol test. Antifungals were prepared based on stock solutions ranging from 1.072 to 2 µg/mL (for anidulafungin) and from 32 to 0.0625 µg/mL (for amphotericin B). Both tests were carried out in triplicate and incubated at 35 °C. MIC values used in the sorbitol protection test were checked at three different reading times: the first check was conducted within 48 h; the second, within 96 h; and the third one, within 168 h. MIC values used in the ergosterol effect were checked at two different reading times: the first one, within 48 h; and

Table 1. Clinical data of dogs and cats with otitis by *Malassezia pachydermatis*, which the clinical isolates were used in the present study. *

Animal case	Ear canal	Breed	Sex	Age	Otologic signs	Time of infection (months)
Dog 1	Bilateral	Poodle	F	Senior	Pruritus, cerumen blackish	> 12
Dog 2	Left	Pug	F	Senior	Pruritus, cerumen blackish	> 12
Dog 3	Left	Mongrel	F	Senior	Pruritus, cerumen blackish, hematoma, polyps	≤ 1
Dog 4	Bilateral	Lhasa Apso	M	Senior	Pruritus, cerumen blackish	6 to 12
Dog 5	Right	Chow-Chow	M	Senior	Pruritus, cerumen blackish	1 to 3
Dog 6	Bilateral	Cocker	M	Adult	Pruritus, cerumen blackish	3 to 6
Dog 7	Right	Maltese	M	Young	Pruritus, cerumen blackish	≤ 1
Dog 8	Left	Pug	F	Young	Pruritus, cerumen blackish	1 to 3
Dog 9	Bilateral	Shar-pei	M	Young	Pruritus	≤ 1
Dog 10	Bilateral	Mongrel	M	Adult	Pruritus, cerumen blackish	> 12
Dog 11	Bilateral	Mongrel	F	Adult	Pruritus, cerumen blackish	> 12
Dog 12	Bilateral	Mongrel	F	Adult	Pruritus, cerumen blackish	> 12
Dog 13	Bilateral	Mongrel	F	Adult	Pruritus, cerumen blackish	> 12
Dog 14	Bilateral	Mongrel	F	Senior	Pruritus, cerumen blackish, lichenification	≤ 1
Dog 15	Left	Mongrel	F	Adult	Pruritus, cerumen blackish, lichenification	3 to 6
Dog 16	Left	Mongrel	M	Senior	Alopecia and hyperpigmentation	N.i.
Dog 17	Left	Lhasa Apso	M	Adult	Pruritus and exudation	6 to 12
Dog 18	Bilateral	Poodle	F	Adult	Pruritus, cerumen blackish	≤ 1
Dog 19	Left	Labrador	F	Senior	Pruritus, cerumen blackish	3 to 6
Dog 20	Right	Mongrel	F	Adult	Pruritus, cerumen blackish	≤ 1
Dog 21	Bilateral	Chihuahua	F	Senior	Pruritus, cerumen blackish	3 to 6
Dog 22	Left	Mongrel	M	Adult	Pruritus, cerumen blackish	3 to 6
Cat 1	Left	Siamese	F	Young	Pruritus, cerumen blackish	≤ 1
Cat 2	Bilateral	Mongrel	F	Young	Pruritus, cerumen blackish	≤ 1

*Data provided from records of mycology collection of *Centro de Diagnóstico e Pesquisa em Micologia Veterinária* (Federal University of Pelotas, Pelotas, RS, Brazil); F, Female; M, Male; Young (≤ 2 years old); adult (from 2.1 to 7 years old); senior (≥ 7.1 years old); N.i. – no informed. In cats, the low "n" sample was due to the lower number of otitis by *M. pachydermatis* in this animal species compared to dogs.

the second one, within 168 h.

The increased MIC values observed for amphotericin B and anidulafungin at the second reading, in comparison to the ones observed at the first reading, as well as the presence of ergosterol and sorbitol in them, respectively.

2.6. Statistical analysis

Analysis of variance and geometric mean comparisons were performed through Kruskal–Wallis test, which was followed by Dunn' test. Data were analyzed in BioEstat statistical software (version 5.3); *p* value of 0.05 was considered significant.

3. Results

All three essential oils presented prevalence of phenolic compounds. Carvacrol (**22** - 73.9%) was the major compound observed in oregano oil (Figure 1a) among all 16 identified compounds; it was followed by γ-terpinene (**13** - 3.6%) and thymol (**21** - 3.0%). On the other hand, the major compounds found in marjoram oil (Figure 1b) among all 22 identified compounds comprised 1,8-cineole (**11** - 20.9%), terpinen-4-ol (**17** - 20.4%), and γ-terpinene (**13** - 8.5%). Finally, 1,8-cineole (**11** - 49.4%), camphor (**16** - 17.8%) and α-pinene (**2** - 12.2%) prevailed among all 19 compounds identified in rosemary oil (Figure 1c). Chromatograms, as well as compounds' identification and quantification results, are shown in Figure 1.

In the anti-*Malassezia pachydermatis* activity, all isolates deriving from otitis cases diagnosed in dogs and cats were susceptible to essential oils extracted from all three plants belonging to family Lamiaceae (Table 2). MIC

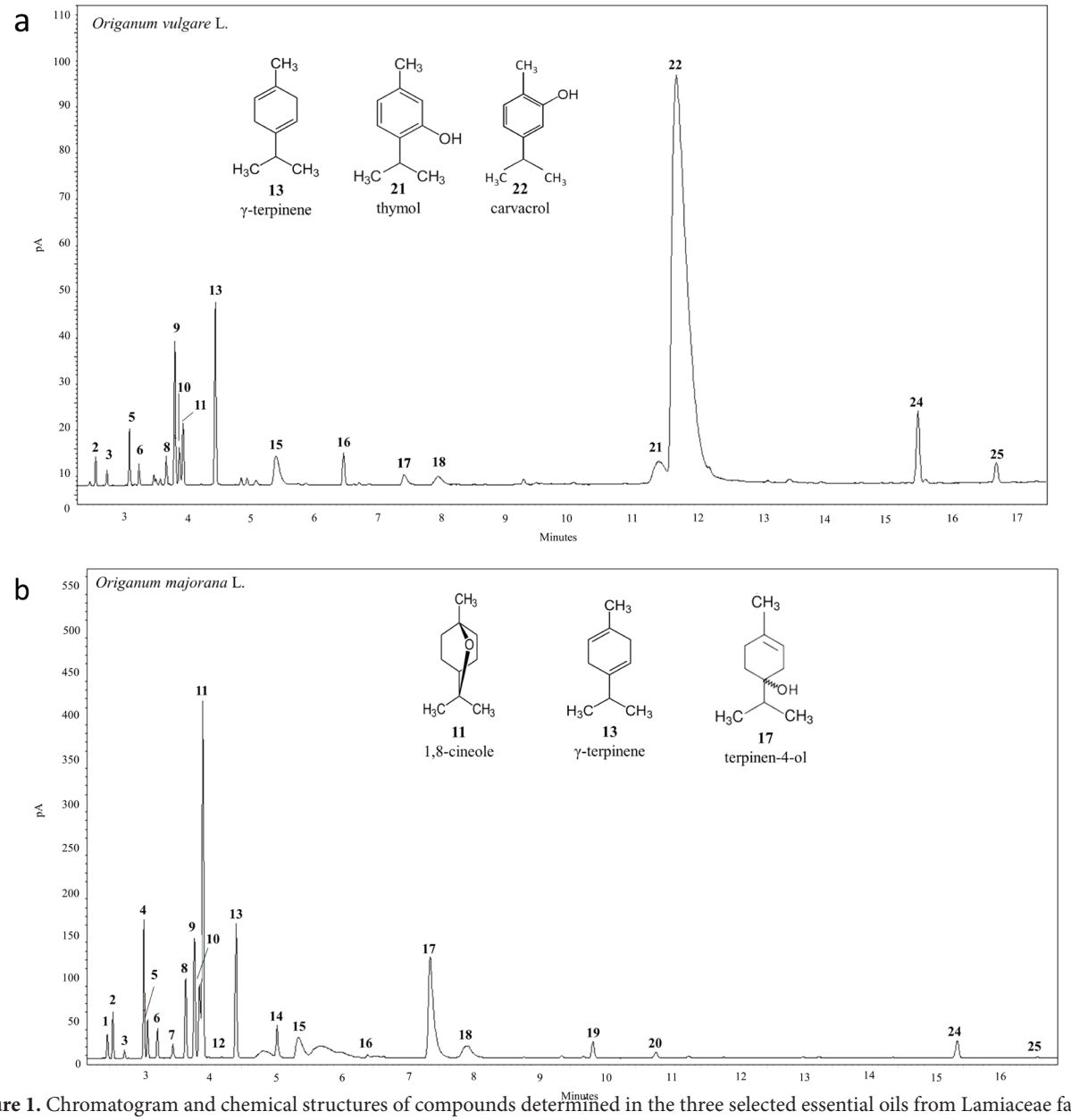

Figure 1. Chromatogram and chemical structures of compounds determined in the three selected essential oils from Lamiaceae family and their respective peaks, Kovats retention index (R.I.) and concentrations (%). In *Origanum vulgare* L. (**a**), 16 compounds were identified, as the following: α-pinene (**2** – 941; 0.4%), camphene (**3** – 946; 0.2%), β-pinene (**5** – 975; 0.9%), myrcene (**6** – 984; 0.4%), α-phellandrene (**7** – 1003; 0.1%), α-terpinene (**8** – 1015; 0.6%), *p*-cymene (**9** – 1019; 2.5%), limonene (**10** – 1023; 0.7%), 1,8-cineole (**11** – 1027; 1.1%), γ-terpinene (**13** – 1089; 3.6%), linalool (**15** – 1099; 2.1%), camphor (**16** –1142; 0.8%), terpinen-4-ol (**17** – 1175; 0.7%), α-terpineol (**18** – 1188; 0.8%), thymol (**21** – 1286; 3.0%), carvacrol (**22** – 1293; 73.9%), β-caryophyllene (**24** – 1421; 2.8%), humulene (**25** – 1453; 0.9%), others (4.5%). In *Origanum majorana* L. (**b**), 22 compounds were identified, as the following: α-thujeno (**1** – 932; 1.1%), α-pinene (**2** – 932; 2.0%), camphene (**3** – 941; 0.4%), sabinene (**4** – 946; 6.7%), β-pinene (**5** – 975; 1.9%), myrcene (**6** – 984; 1.6%), α-phellandrene (**7** – 1003; 0.9%), α-terpinene (**8** – 1015; 4.6%), *p*-cymene (**9** – 1019; 7.0%), limonene (**10** – 1023; 5.3%), 1,8-cineole (**11** – 1027; 20.9%), β-ocimene (**12** – 1056; 0.1%), γ-terpinene (**13** – 1089; 8.5%), trans-sabinene hydrate (**14** – 1094; 2.3%), linalool (**15** – 1099; 4.4%), camphor (**16** – 1142; 0.2%), terpinen-4-ol (**17** – 1175; 20.4%), α-terpineol (**18** – 1188; 4.7%), linalyl acetate (**19** – 1261;1.8%), bornyl acetate (**20** – 1285; 0.8%), β-caryophyllene (**24** – 1421; 2.2%), humulene (**25** – 1453; 0.2%), others (2.1%). In *Rosmarinus officinalis* L. (**c**), 19 were identified, as the following: α-thujeno (**1** – 932; 0.2%), α-pinene (**2** – 941; 12.2%), camphene (**3** – 946; 1.1%), β-pinene (**5** – 975; 9.4%), myrcene (**6** – 984; 0.5%), α-phellandrene (**7** – 1003; 0.1%), α-terpinene (**8** – 1015; 0.1%), *p*-cymene (**9** – 1019; 0.4%), limonene (**10** – 1023; 2.0%), 1,8-cineole (**11** – 1027; 49.4%), γ-terpinene (**13** – 1089; 0.1%), linalool (**15** – 1099; 1.0%), camphor (**16** – 1142; 17.8%), terpinen-4-ol (**17** – 1175; 0.2%), α-terpineol (**18** – 1188; 0.5%), bornyl acetate (**20** – 1285; 0.3%), α-copaene (**23** – 1378; 0.3%), β-caryophyllene (**24** – 1421; 3.7%), humulene (**25** – 1453; 0.2%), others (0.4%).

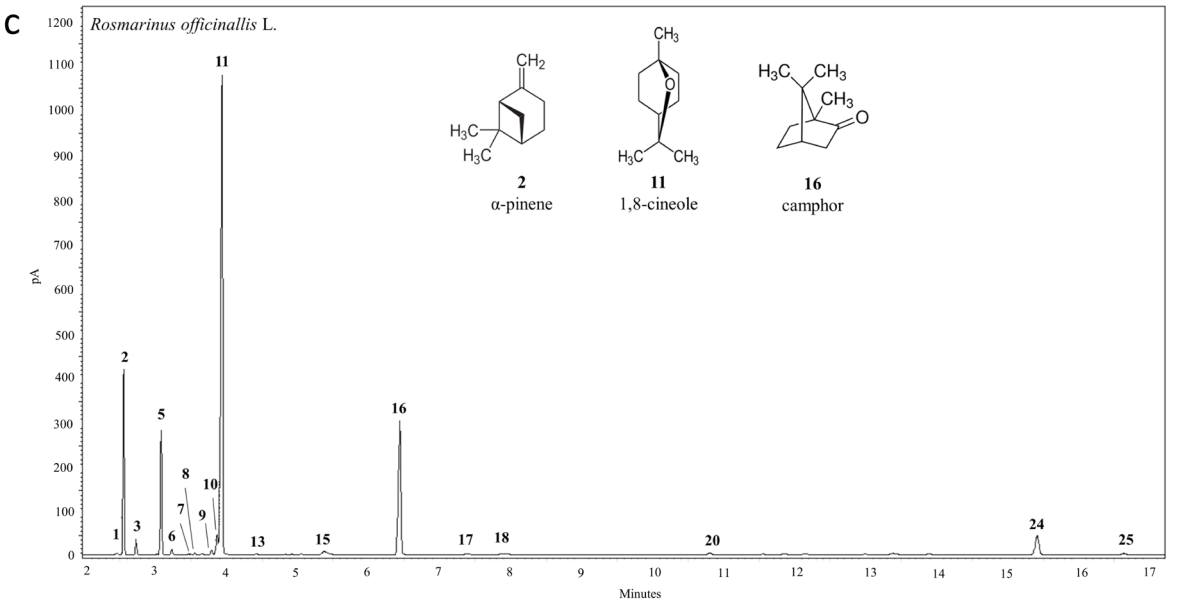

Figure 1. (Continued).

Table 2. Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of three aromatic essential oils (mg/mL) belonging to the Lamiaceae family —*Origanum vulgare* L. (oregano), *O. majorana* L. (marjoram), and *Rosmarinus officinalis* L. (rosemary)— against pathogenic isolates of *Malassezia pachydermatis* from canine and feline otitis.

Origin of the *M. pachydermatis* isolates (*n*)*		***Origanum vulgare* L.**		***Origanum majorana* L.**		***Rosmarinus officinalis* L.**	
		MIC	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**
Canine (22)	Range	≤ 0.078 – 0.625	≤ 0.078 – 0.625	≤ 0.078 – 2.5	≤ 0.078 – 2.5	≤ 0.078 – > 2.5	≤ 0.078 – > 2.5
	50%	≤ 0.078	≤ 0.078	≤ 0.078	0.625	0.156	0.156
	90%	0.625	0.625	2.5	2.5	> 2.5	> 2.5
Feline (2)	Range	≤ 0.078	≤ 0.078	≤ 0.078	≤ 0.078 – 1.25	≤ 0.078	≤ 0.078
ATCC 14053 (1)	Value	0.156	0.156	0.3125	0.3125	0.156	0.156
ATCC 34449 (1)	Value	0.312	0.312	0.156	0.156	0.312	0.312
ATCC 34135 (1)	Value	0.625	0.625	0.156	0.156	0.625	0.625
Overall (27)	Range	≤ 0.078 – 0.625^{A}	≤ 0.078 – 0.625^{a}	≤ 0.078 – 2.5^{A}	≤ 0.078 – 2.5^{b}	≤ 0.078 – > 2.5^{A}	≤ 0.078 – > 2.5^{ab}
	50%	≤ 0.078	≤ 0.078	≤ 0.078	0.3125	0.156	0.156
	90%	0.625	0.625	2.5	2.5	> 2.5	> 2.5

*50%, MIC/MFC values in which 50% of overall isolates were inhibited/killed; 90%, MIC/MFC values in which 90% of overall isolates were inhibited/killed; the following control standard strains were used: ATCC 14053, *Candida albicans*; ATCC 34449, *C. lusitaniae*; ATCC 34135, *C. krusei*; for statistical analysis, the uppercase letters indicate a difference between the columns of MIC, while lowercase letters indicate a difference between the columns of MFC ($p < 0.05$).

and MFC values recorded for oregano ranged from ≤ 0.078 to 0.625 mg/mL. This may indicate the oregano's inhibitory and fungicidal activity; 90% of *M. pachydermatis* isolates were inhibited (MIC_{90}) and killed (MFC_{90}) by oregano at concentration of 0.625 mg/mL.

MIC and MFC values recorded for marjoram ranged from ≤ 0.078 to 2.5 mg/mL; MIC_{90} and MFC_{90} values recorded for this plant were 2.5 mg/mL, for both. On the other hand, MIC and MFC values recorded for rosemary ranged from ≤ 0.078 to > 2.5 mg/mL; 50% of *M. pachydermatis* isolates were inhibited and killed by rosemary at concentration of 0.156 mg/mL. However, not all fungal isolates were sensitive to the tested concentrations of rosemary; therefore, it was not possible estimating the MIC_{90} and MFC_{90} values (> 2.5 mg/mL).

There was not statistically significant difference in MIC values ($p > 0.05$) among treatments, although oregano was superior to marjoram and rosemary for requiring the lowest essential oil concentrations to inhibit 90% of the all isolates. With respect to fungicidal activity, oregano differed from marjoram ($p = 0.0313$) because it required lower MFC values to kill the overall tested isolates. No significant difference in fungicidal activity was observed for the remaining treatments ($p > 0.05$).

The cases of dogs 21 and 22 were characterized as otitis externa (signs of pruritus and blackened earwax) for an infection period of 3 to 6 months. These canine *Malassezia pachydermatis* isolates were selected for the mechanism of action assays (sorbitol protection test and exogenous ergosterol effect test). Based on the sorbitol protection test (Table 3), the incidence of this osmotic protector has influenced MIC values recorded for all three essential oils, similar to that of the positive control (anidulafungin). Oregano, marjoram and rosemary oils recorded 2- to 4-fold increase in MIC value at the second (96 h) and third (168 h) readings in comparison to that of the first (48 h) reading. All essential oils acted on fungal cell wall.

Exogenous ergosterol (Table 4) showed a significant increase in MIC values at reading times and concentrations, as for amphotericin B. Oregano displayed an increase by 2- and 16-fold in MIC values observed for *M. pachydermatis* from "Dog 21" and "Dog 22", respectively, as the ergosterol concentration increased. Moreover, the comparison of the second (168 h) to the first (48 h) reading showed up to a 4-fold increase in the MIC values of oregano for both *M. pachydermatis* isolates. Similarly, a 2- to 4-fold (for marjoram) and a 4- to 8-fold (for rosemary) increase in MIC values were noted for marjoram and rosemary oils, according to the increase in ergosterol concentrations and reading time. These findings have evidenced that oregano, marjoram and rosemary oils appear to have acted at fungal ergosterol complexation level in the plasmatic membrane.

Table 3. Minimal inhibitory concentration (MIC) of *Origanum vulgare* L. (oregano), *O. majorana* L. (marjoram), and *Rosmarinus officinalis* L. (rosemary) essential oils and anidulafungin in the absence and presence of the osmotic protector sorbitol against *Malassezia pachydermatis*.

MIC values and reading times of the following products:		***Malassezia pachydermatis* isolates (*n* = 2) ***	
		"Dog 21"	**"Dog 22"**
Oregano oil (mg/mL)			
48 h	S (–)	0.039	0.156
	S (+)	0.312	0.156
96 h	S (–)	0.039	0.156
	S (+)	0.625	0.312
168 h	S (–)	0.039	0.156
	S (+)	0.625	0.625
Marjoram oil (mg/mL)			
48 h	S (–)	0.039	0.313
	S (+)	0.156	0.313
96 h	S (–)	0.039	0.313
	S (+)	0.625	0.313
168 h	S (–)	0.039	0.313
	S (+)	0.625	0.625
Rosemary oil (mg/mL)			
48 h	S (–)	0.313	0.625
	S (+)	0.625	0.625
96 h	S (–)	0.313	0.625
	S (+)	0.625	0.625
168 h	S (–)	0.313	0.625
	S (+)	2.5	1.25
Anidulafungin (µg/mL)			
48 h	S (–)	268	67
	S (+)	268	536
96 h	S (–)	268	67
	S (+)	536	1072
168 h	S (–)	268	67
	S (+)	536	1072

* Fungal isolates from canine cases of otitis ("Dog 21" and "Dog 22", according to Table 1) by *Centro de Diagnóstico e Pesquisa em Micologia Veterinária* (Federal University of Pelotas - UFPEL, Southern Brazil); S (–), absence of sorbitol; S (+), presence of sorbitol.

Table 4. Minimal inhibitory concentration (MIC) of *Origanum vulgare* L. (oregano), *O. majorana* L. (marjoram), and *Rosmarinus officinalis* L. (rosemary) essential oils and amphotericin B in the absence and presence of the exogenous ergosterol against *Malassezia pachydermatis*.

MIC values and reading times of the following products:		***Malassezia pachydermatis* isolates (*n* = 2) ***	
		"Dog 21"	**"Dog 22"**
Oregano oil (mg/mL)			
48 h	E (–)	0.156	0.019
	E (50)	0.156	0.078
	E (100)	0.312	0.078
	E (150)	0.312	0.156
	E (200)	0.312	0.156
168 h	E (–)	0.156	0.019
	E (50)	0.625	0.312
	E (100)	0.625	0.312
	E (150)	0.625	0.312
	E (200)	0.625	0.312
Marjoram oil (mg/mL)			
48 h	E (–)	1.25	0.313
	E (50)	2.5	0.625
	E (100)	2.5	1.25
	E (150)	2.5	1.25
	E (200)	5	1.25
168 h	E (–)	1.25	0.313
	E (50)	2.5	1.25
	E (100)	2.5	1.25
	E (150)	5	2.5
	E (200)	5	2.5
Rosemary oil (mg/mL)			
48 h	E (–)	1.25	0.156
	E (50)	1.25	0.625
	E (100)	2.5	0.625
	E (150)	5	1.25
	E (200)	5	1.25
168 h	E (–)	1.25	0.156
	E (50)	5	2.5
	E (100)	5	2.5
	E (150)	10	5
	E (200)	10	5
Amphotericin B (µg/mL)			
48 h	E (–)	0.5	0.5
	E (50)	4	0.5
	E (100)	16	1
	E (150)	16	4
	E (200)	32	16
168 h	E (–)	0.5	0.5
	E (50)	4	4
	E (100)	16	16
	E (150)	> 32	32
	E (200)	> 32	> 32

* Fungal isolates from canine cases of otitis ("Dog 21" and "Dog 22", according to Table 1) by *Centro de Diagnóstico e Pesquisa em Micologia Veterinária* (Federal University of Pelotas - UFPEL, Southern Brazil); E (–), absence of ergosterol; E (50–200), presence of ergosterol in concentrations ranging from 50 to 200 µg/mL.

4. Discussion

The antifungal property of plant species belonging to family Lamiaceae is consolidated in the literature [11]. Here, oregano oil highlighted compared to rosemary and marjoram oils due to the lowest MIC values (MIC_{90} and MFC_{90} of 0.625 mg/mL). Studies with this essential oil against *M. pachyermatis* isolated from dogs in Australia [19] and Brazil [12] showed similar findings in MIC and MFC values of oregano, in which they were 563 µg/mL [19] and from ≤ 0.87 to 7 mg/mL [12].

Marjoram also stood out as anti-*Malassezia pachydermatis* agent; it recorded MIC_{90} and MFC_{90} values of 2.5 mg/mL. Although few studies have been conducted with this plant species, its antifungal activity at marjoram concentrations of 1.3% and 1.0% was acknowledged in *M. pachydermatis* isolates from canine external otitis in Italy [15] and Bulgaria [24], respectively.

MIC_{50} and MFC_{50} values recorded for rosemary were 0.156 mg/mL, although it was necessary using concentrations of it higher than 2.5 mg/mL to affect 90% of *M. pachydermatis* isolates. According to studies conducted in Iran [14] and Italy [15], rosemary oil concentrations of 360 µg/mL and 1.3% (v/v) were active against canine *M. pachydermatis* isolates, respectively.

In addition, experimental studies conducted in vivo with murine model, based on using these three plant species, have evidenced their potential to be used as antifungal agents, as shown in the intravaginal use of 3% oregano oil to treat vaginal candidiasis caused by *C. albicans* [25], and in the oral use of marjoram (80 mg/kg)

[26] or rosemary (250 mg/kg) [22] oil to treat cutaneous sporotrichosis caused by *Sporothrix brasiliensis*.

Moreover, the topical use of a mix comprising 5% oregano, 5% rosemary and 2% serpentine thyme (*Thymus serpillum*) oils has shown promising results in dermatophytosis caused by *Microsporum canis* in cats [27]. Most specifically, a mix of essential oils comprising 1% rosemary, 1% lemon (*Citrus limon*), 0.5% clary sage (*Salvia sclarea*) and 0.5% chamomile (*Anthemis nobilis*) provided excellent results against malasseziosis affecting dogs with otitis caused by *M. pachydermatis* who were subjected to topic treatment on a daily basis (200 µL/ear), for two weeks [4]. If one takes into consideration that oregano, marjoram and rosemary oils are well-known natural products with satisfactory antifungal properties, further studies should be conducted to evaluate their effectiveness in animal otitis cases caused by *M. pachydermatis*.

Cell wall integrity is disrupted when cell wall compounds, such as chitin and β-glucans, are inhibited, whereas the binding of antifungal molecules to the fungal ergosterol in the cell membrane impairs the integrity of this structure by making it permeable [28]. The current study was the first to perform the sorbitol protection and exogenous ergosterol effect tests in *M. pachydermatis* in order to investigate the action of oregano, marjoram and rosemary oils on fungal cell wall and plasma membrane, respectively.

The current findings have shown that all essential oils acted at ergosterol complexation and cell wall levels, and it suggested cell membrane disruption and cell wall formation inhibition in *M. pachydermatis*, respectively. Similar to the findings herein recorded for plasma membrane, oregano oil has disintegrated *Saccharomyces cerevisiae* membrane, and it led to fast ion leakage into the extracellular medium [29]. Marjoram and rosemary oil binding to the ergosterol of *Aspergillus flavus* [30] and *S. brasiliensis* [22,26] isolates resulted in loss of ions and molecules, and led to isolates' death. In addition, rosemary oil induced apoptosis-like cell death in *A. flavus* [31].

Marjoram [26] and rosemary [22] oils did not affect *S. brasiliensis*' cell wall; this outcome differed from that observed for *M. pachydermatis* in the current study. Although cell wall components in both fungal genera remain poorly investigated, it is hypothesized that the diversified quantification of components can explain the action of these oils in *M. pachydermatis* cell wall, but not in *S. brasiliensis*. It may happen because *Malassezia* species have rigid cell walls with relatively high chitin and chitosan amounts (up to 25%) [32]; in addition, β-(1,6)-glucan is more abundant (70%) than β-(1,3)-glucans (5%), among all β-glucans polymers [33].

On the other hand, *Sporothrix* species have bilayered cell wall structure [34], which is mainly composed of linear β-glucans, such as β-(1,3)-glucans (up to 66%), β-(1,6)-glucans (26% to 29%) and β-(1,4)-glucans (5% to 10%), besides presenting minor components such as chitin (7% to8%) [35] and peptido-rhamnomannan (10% to 15%) [34]. Thus, oregano, marjoram and rosemary oils may have acted on *M. pachydermatis* cell wall due to higher chitin, chitosan and/or β-(1,6)-glucans concentrations in them. Studies should be carried out to help better understanding the target-specific antifungal action of these natural products in the cell wall of different fungal genera.

With respect to the chemical composition of the tested oils, carvacrol (**22** - 73.9%) was the prevalent compound in oregano; it was followed by γ-terpinene and thymol, which recorded minor concentrations. Similar findings were observed for essential oils extracted from oregano plants grown in Brazil [12] and Italy [13,15,16]. Thymol is known as antifungal compound capable of inhibiting *M. pachydermatis* at MIC values ranging from 10 to 640 µg/mL [18], and from 400 to 800 µg/mL [19]. Although carvacrol was found at lower concentrations, this compound has also shown activity against *M. pachydermatis* at MIC values ranging from 10 to 320 µg/mL [18] and to 585 µg/mL [19]. Moreover, the synergistic activity of both compounds with nystatin and miconazole [18] highlights the potential antifungal property of these phytochemicals.

On the other hand, 1,8-cineole, also known as eucalyptol, was the major compound found in marjoram and rosemary (**11** – 20.9% and 49.4%, respectively); this outcome was similar to other studies conducted in Brazil [22,26], Iran [14], and Italy [15]. This compound also appears to play an important role in antifungal action since it decreased conidia formation, disrupted the fungal cell membrane of *Aspergillus* spp., and hindered fungal cell wall integrity [36]. However, thymol was reported as major component in marjoram oil of Italian origin [13,15]; this finding differs from the one recorded for the herein analyzed sample, and it pointed towards the influence of geographic and climatic conditions, among other factors, on the chemical composition of essential oils.

Findings in the current study have evidenced the promising use of oregano, marjoram and rosemary essential oils to treat canine and feline otitis caused by *M. pachydermatis*. Antifungal activity was observed for all three plants, with emphasis on oregano oil due to its fungistatic and fungicidal activities against all tested strains and to its lower MIC and MFC values; it was followed by marjoram and rosemary oils. The mechanism of action of both natural products was linked to their complexation with fungal ergosterol, both in the plasma membrane and in the fungal cell wall. These actions may be associated with their chemical composition; carvacrol and 1,8-cineole were the major compounds observed for oregano and marjoram/rosemary oils, respectively. The current findings support the potential use of these essential oils to treat otitis caused

by *M. pachydermatis*. Further studies should be conducted to assess their effectiveness in vivo and safe application in canine and feline malasseziosis.

Acknowledgments
The authors would like to thank Prof. Dr. Vany Ferraz (Laboratory of Chromatography, Federal University of Minas Gerais, Brazil) for chemical analysis. S.B. Waller thank National Council for Scientific and Technological Development (CNPq) for granting scholarship (Process no. 164145/2020-6). The authors also are grateful to the Brazilian institutions CAPES, FAPERGS, and CNPq for student and research scholarships.

Conflict of interest
The authors declare that they have no conflicts of interest.

References

1. Peano A, Johnson E, Chiavassa E, Tizzani P, Guillot J et al. Antifungal resistance regarding *Malassezia pachydermatis*: where are we now? Journal of Fungi 2020; 6: 93. doi: 10.3390/jof6020093
2. Guillot J, Bond R. *Malassezia* Yeasts in Veterinary Dermatology: An Updated Overview. Frontiers in Cellular and Infection Microbiology 2020; 10: 79. doi: 10.3389/fcimb.2020.00079
3. Santin R, Mattei AS, Waller SB, Madrid IM, Cleff MB et al. Clinical and mycological analysis of dog's oral cavity. Brazilian Journal of Microbiology 2013; 44: 139-143. doi: 10.1590/S1517-83822013005000018
4. Nardoni S, Pistelli L, Baronti I, Najar B, Pisseri F et al. Traditional Mediterranean plants: characterization and use of an essential oils mixture to treat *Malassezia* otitis externa in atopic dogs. Natural Product Research 2017; 31: 1891-1894. doi: 10.1080/14786419.2016.1263853
5. Crosaz O, Legras A, Vilaplana-Grosso F, Debeaupuits J, Chermette R et al. Generalized dermatitis associated with *Malassezia* overgrowth in cats: A report of six cases in France. Medical Mycology Case Reports 2013; 2: 59-62. doi. 10.1016/j.mmcr.2013.01.005
6. Váczi P, Conková E, Marcincaková D, Sihelská Z. Effectiveness of essential oils on *Malassezia pachydermatis*. Folia Veterinaria 2015; 59: 12-17.
7. Rhimi W, Theelen B, Boekhout T, Aneke CI, Otranto D et al. Conventional therapy and new antifungal drugs against *Malassezia* infection. Medical Mycology 2021; 59: 215-234. doi: 10.1093/mmy/myaa087
8. Cafarchia C, Iatta R, Immediato D, Puttilli MR, Otranto D. Azole susceptibility of *Malassezia pachydermatis* and *Malassezia furfur* and tentative epidemiological cut-off values. Medical Mycology 2015; 53: 743-748. doi: 10.1093/mmy/myv049
9. Kano R, Aramaki C, Murayama N, Mori Y, Yamagishi K et al. High multi-azole-resistant *Malassezia pachydermatis* clinical isolates from canine *Malassezia* dermatites. Medical Mycology 2020; 58: 197-200. doi: 10.1093/mmy/myz037
10. Ebani VV, Nardoni S, Bertelloni F, Najar B, Pistelli L et al. Antibacterial and Antifungal Activity of Essential Oils against Pathogens Responsible for Otitis Externa in Dogs and Cats. Medicines 2017; 4: 21. doi: 10.3390/medicines4020021
11. Waller SB, Cleff MB, Serra EF, Silva AL, Gomes AR et al. Plants from Lamiaceae family as source of antifungal molecules in humane and veterinary medicine. Microbial Pathogenesis 2017; 104: 232-237. doi: 10.1016/j.micpath.2017.01.050
12. Santin R, Giordani C, Madrid IM, Matos CB, Freitag RA et al. Antifungal activity of *Origanum vulgare* essential oil against *Malassezia pachydermatis*. Arquivo Brasileiro de Medicina Veterinária e Zootecnia 2014; 66: 367-373. doi: 10.1590/1678-41626476
13. Nardoni S, Mugnaini L, Pistelli L, Leonardi M, Sanna V et al. Clinical and mycological evaluation of an herbal antifungal formulation in canine *Malassezia* dermatitis. Journal de Mycologie Médicale 2014; 24: 234-240.
14. Khosravi AR, Shokri H, Fahimirad D. Efficacy of medicinal essential oils against pathogenic *Malassezia* sp. isolates. Journal de Mycologie Médicale 2016; 26: 28-34. doi: 10.1016/j.mycmed.2015.10.012
15. Pistelli L, Mancianti F, Bertoli A, Cioni PL, Leonardi M et al. Antimycotic activity of some aromatic plants essential oils against canine isolates of *Malassezia pachydermatis*: an *in vitro* assay. The Open Mycology Journal 2012; 6: 17-21. doi: 10.2174/1874437001206010017
16. Vinciguerra V, Rojas F, Tedesco V, Giusiano G, Angiolella L. Chemical characterization and antifungal activity of *Origanum vulgare*, *Thymus vulgaris* essential oils and carvacrol against *Malassezia furfur*. Natural Product Research 2019; 33: 3273-3277. doi: 10.1080/14786419.2018.1468325
17. Bohmova E, Conkova E, Harcarova M, Sihelska Z. Interactions between clotrimazole and selected essential oils against *Malassezia pachydermatis* clinical isolates. Polish Journal of Veterinary Sciences 2019; 22: 173-175. doi: 10.24425/pjvs.2019.127082
18. Schlemmer KB, Jesus FPK, Tondolo JSM, Weiblen C, Azevedo MI et al. *In vitro* activity of carvacrol, cinnamaldehyde and thymol combined with antifungals against *Malassezia pachydermatis*. Journal de Mycologie Médicale 2019; 29: 375-377. doi: 10.1016/j.mycmed.2019.08.003
19. Sim JXF, Khazandi M, Chan WY, Trott DJ, Deo P. Antimicrobial activity of thyme oil, oregano oil, thymol and carvacrol against sensitive and resistant microbial isolates from dogs with otitis externa. Veterinary Dermatology 2019; 30: 524-159. doi: 10.1111/vde.12794

20. Kim JY, Olivry T, Son WG. Characteristics of *Malassezia pachydermatis* isolated from fogs and antifungal effect of essential oils. Journal of Veterinary Clinics 2012; 29:141-147.

21. Clinical and Laboratory Standard Institute – CLSI. Reference method for broth dilution antifungal susceptibility testing of yeasts M27-A3 Guideline. 2008; 3rd Edition. CLSI, Wayne, PA.

22. Waller SB, Cleff MB, Dalla Lana DF, de Mattos CB, Guterres KA et al. Can the essential oil of rosemary (*Rosmarinus officinalis* Linn.) protect rats infected with itraconazole-resistant *Sporothrix brasiliensis* from fungal spread? Journal de Mycologie Médicale 2021; 31: 101199. doi: 10.1016/j.mycmed.2021.101199

23. Dalla Lana DF, Giuliani LM, Reolon JB, Lopes W, Vainstein MH et al. Nanoemulsion improves the antifungal activity of allylic thiocyanates against yeasts and filamentous pathogenic fungi. ChemistrySelect 2018; 3: 11663-11670. doi: 10.1002/slct.201802204

24. Rusenova N, Parvanov P. Antimicrobial activities of twelve essential oils against microorganisms of veterinary importance. Trakia Journal of Sciences 2009; 7: 37-43.

25. Cleff MB, Wendisch I, Cabana AL, Rodrigues MR, de Mello JRB et al. Experimental vaginal candidiasis: Assessment of *Origanum vulgare* for its treatment. African Journal of Microbiology Research 2011; 5: 4207-4211. doi: 10.5897/AJMR11.744

26. Waller SB, Cleff MB, de Mattos CB, da Silva CC, Giordani C et al. *In vivo* protection of the marjoram (*Origanum majorana* Linn.) essential oil in the cutaneous sporotrichosis by *Sporothrix brasiliensis*. Natural Product Research 2019; 35: 2977-2981. doi: 10.1080/14786419.2019.1678617

27. Mugnaini L, Nardoni S, Pinto L, Pistelli L, Leonardi M et al. *In vitro* and *in vivo* antifungal activity of some essential oils against feline isolates of *Microsporum canis*. Journal de Mycologie Médicale 2012; 22: 179-184. doi: 10.1016/j.mycmed.2012.04.003

28. Lagrouh F, Dakka N, Bakri Y. The antifungal activity of Moroccan plants and the mechanism of action of secondary metabolites from plants. Journal de Mycologie Médicale 2017; 27: 303-311. doi: 10.1016/j.mycmed.2017.04.008

29. Konuk HB, Erguden B. Antifungal activity of various essential oils against *Saccharomyces cerevisiae* depends on disruption of cell membrane integrity. Biocell 2017; 41: 13-18.

30. Chaudhari AK, Singh VK, Deepika SD, Prasad J, Dwivedy AK et al. Improvement of *in vitro* and *in situ* antifungal, AFB_1 inhibitory and antioxidant activity of *Origanum majorana* L. essential oil through nanoemulsion and recommending as novel food preservative. Food and Chemical Toxicology 2020; 143: 111536. doi: 10.1016/j.fct.2020.111536

31. Oliveira RC, Carvajal-Moreno M, Mercado-Ruaro P., Rojo-Callejas F, Correa B. Essential oils trigger an antifungal and anti-aflatoxigenic effect on *Aspergillus flavus* via the induction of apoptosis-like cell death and gene regulation. Food Control 2020; 110: 107028. doi: 10.1016/j.foodcont.2019.107038

32. Muszewska A, Piłsyk S, Perlinska-Lenart U, Kruszewska JS. Diversity of cell wall related proteins in human pathogenic fungi. Journal of Fungi 2017; 4: 6. doi: 10.3390/jof4010006

33. Stalhberger T, Simenel C, Clavaud C, Eilsink VGH, Jourdain R et al. Chemical organization of the cell wall polysaccharide core of *Malassezia restricta*. Journal of Biological Chemistry 2014; 289: 12647-12656. doi: 10.1074/jbc.M113.547034

34. Lopes-Bezerra LM, Walker LA, Niño-Vega G, Mora-Montes HM, Neves GWP et al. Cell walls of the dimorphic fungal pathogens *Sporothrix schenckii* and *Sporothrix brasiliensis* exhibit bilaminate structures and sloughing of extensive and intact layers. PLoS Neglected Tropical Diseases 2018; 12: e0006169. doi: 10.1371/journal.pntd.0006169

35. Previato JO, Gorin PAJ, Haskins RH, Travassos LR. Soluble and insoluble glucans from different cell types of *Sporothrix schenckii*. Experimental Mycology 1979; 3: 92-105. doi: 10.1016/S0147-5975(79)80021-3

36. Moreira ACP, Lima EO, Wanderley PA, Carmo ES, Souza EL. Chemical composition and antifungal activity of *Hyptis suaveolens* (L.) Poit leaves essential oil against *Aspergillus* species. Brazilian Journal of Microbiology 2010; 41: 28-33. doi: 10.1590/S1517-83822010000100006