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IN VITRO ACTIVITY OF *ORIGANUM VULGARE* ESSENTIAL OIL AGAINST *CANDIDA* SPECIES

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ABSTRACT

The aim of this study was to evaluate the in vitro activity of the essential oil extracted from *Origanum vulgare* against sixteen *Candida* species isolates. Standard strains tested comprised *C. albicans* (ATCC strains 44858, 4053, 18804 and 3691), *C. parapsilosis* (ATCC 22019), *C. krusei* (ATCC 34135), *C. lusitaniae* (ATCC 34449) and *C. dubliniensis* (ATCC MY646). Six *Candida albicans* isolates from the vaginal mucous membrane of female dogs, one isolate from the cutaneous tegument of a dog and one isolate of a capuchin monkey were tested in parallel. A broth microdilution technique (CLSI) was used, and the inoculum concentration was adjusted to 5 x 10^6 CFU mL^{-1}. The essential oil was obtained by hydrodistillation in a Clevenger apparatus and analyzed by gas chromatography. Susceptibility was expressed as Minimal Inhibitory Concentration (MIC) and Minimal Fungicidal Concentration (MFC). All isolates tested *in vitro* were sensitive to *O. vulgare* essential oil. The chromatographic analysis revealed that the main compounds present in the essential oil were 4-terpineol (47.95%), carvacrol (9.42%), thymol (8.42%) and □-terpineol (7.57%). *C. albicans* isolates obtained from animal mucous membranes exhibited MIC and MFC values of 2.72 µL mL^{-1} and 5 µL mL^{-1}, respectively. MIC and MFC values for *C. albicans* standard strains were 2.97 µL mL^{-1} and 3.54 µL mL^{-1}, respectively. The MIC and MFC for non-albicans species were 2.10 µL mL^{-1} and 2.97 µL mL^{-1}, respectively. The antifungal activity of *O. vulgare* essential oil against *Candida* spp. observed *in vitro* suggests its administration may represent an alternative treatment for candidiasis.

Key words: *Candida*, *Origanum vulgare*, essential oil, *in vitro*.

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INTRODUCTION

Candidiasis is a mycosis caused by different *Candida* species, which can promote superficial and systemic opportunist diseases around the world (8, 13, 19, 20). The presence of geriatric domestic animals and immunosuppressed patients, who are more susceptible to fungal infections, has increased the incidence of candidiasis (8, 18, 20). The increasing clinical importance of mycoses in veterinary medicine in addition to the emergence of more severe presentations prompts to the development of new diagnostic procedures and treatments (11, 13, 18, 19).

Most antifungals currently available for the treatment of different clinical forms of this disease have limitations that hinder their use, which makes the search for safe, efficient antimycotic products or molecules necessary (3, 11, 15, 24, 25).

In this context, research involving substances obtained from plants, especially from members of the Lamiaceae family such as *Origanum vulgare*, show promise. Several species of the genus *Origanum* have carvacrol and thymol (phenolic monoterpenes) among their main constituents; these are accompanied by other compounds such as ρ-cimene, □-terpinolene, □-terpinene, α-terpineol, linalool, 4-terpinol, germacrene-D and α-pinene, which are present in lower concentrations and also show antimicrobial activity (1, 2, 4, 5, 11, 12, 14, 21, 22, 23, 29).

Oregano has antioxidant properties and exhibits antimicrobial activity against bacteria and fungi (1, 2, 5, 6, 9, 14, 17). Some studies suggest that oregano exerts a therapeutic effect when administered to rats experimentally infected with *Trichophyton rubrum* (1). The antifungal activity of oregano essential oil against some species of *Aspergillus* such as *A. parasiticus*, *A. niger*, *A. flavus* and *A. ochraceus* was demonstrated in studies where it was shown to inhibit growth and aflatoxin production (4, 10). The effects of oregano essential oil on yeasts of medical importance such as *Candida albicans* isolated from human patients have also been studied (7, 11, 16). However, data concerning the impact of essential oils on yeasts of the genus *Candida* isolated from the vaginal mucous membrane of female dogs and other animals with a diagnosis of candidiasis are scarce to date.

The aim of the current investigation was to study the antimicrobial activities of *Origanum vulgare* essential oil on *Candida* species in order to evaluate its use as a therapy.

MATERIALS AND METHODS

Plant material

Oregano samples (dried leaves from Chile) were purchased from a commercial supplier (Torrenueva Vascos Ltda., Uruguay, tnueva@adinet.uy), which provided a botanical identification certificate.

The moisture content was determined to be 9.7g/100g on a dry basis, and the yield of essential oil (EO) in raw material was 1.282 mL (mL EO/100g of oregano).

Essential oil

Dried oregano leaves were submitted to hydrodistillation for 4 h using a Clevenger apparatus modified according to Brazilian Pharmacopeia IV (1988). After extraction, the oil was dried in anhydrous sodium sulfate, filtered, concentrated under ultrapure N_2 and stored in amber flasks at 4ºC (30).

Chemicals

All chemicals (hexane, dichloromethane) were of analytical grade. Analytical standards (α-pinene, β-pinene, myrcene, α-terpinene, p-cymene, limonene, 1,8-cineole, γ-terpinene, terpinolene, linalool, 4-terpineol, α-terpineol, thymol and carvacrol) were supplied by Sigma. RPMI 1640 medium was purchased from Gibco (Gibco BRL, Grand Island, NY, USA); Tween 80 was acquired from Sigma; Sabouraud dextrose agar was supplied by Oxoid (Basingstoke, UK); McFarland standard was purchased from bioMérieux (Marcy l'Etoile, France); 3-morpholinopropanesulfonic acid (MOPS) buffer and phosphate-buffered saline (PBS) were from Sigma.

Chromatographic analysis

Origanum essential oil was analyzed in a gas chromatograph equipped with a flame ionization detector (GC/FID, model Schimadzu 17A). Chromatographic analysis was carried out in a chromatograph equipped with a DB-5 silica capillary column (methyl siloxane with 5% phenyl groups; dimensions: 30 m x 0.25 mm ∅, 0.25 μm film thickness). Nitrogen was used as the carrier gas with a flow rate of 1.0 mL min^{-1} and a split ratio of 1:50. Both injector and detector temperatures were set to 280ºC. The column temperature was programmed to gradually increase from 40ºC to 145ºC at a rate of 2ºC min^{-1}, rise to 280ºC at a rate of 10ºC min^{-1}, and remain at 280°C for 10 min. An aliquot of oregano essential oil was dissolved in hexane at a concentration of 5,000 μg L^{-1}; 0.5 μL of this solution was injected into the system. The essential oil was analyzed in triplicate. Analytical standards were dissolved in hexane at a concentration of 100 μg L^{-1} and subjected to same conditions used for oregano essential oil.

Antimicrobial activity

***Candida* species isolates**

Six *Candida albicans* isolates from the vaginal mucous membrane of female dogs, one isolate from the cutaneous tegument of a dog and one isolate from the cutaneous tegument of a capuchin monkey (*Cebus apella*), all obtained in Pelotas, Rio Grande do Sul, Brazil, were tested. They were stored at Mycology Laboratory, School of Veterinary Medicine, at Federal University of Pelotas (UFPel). The eight standard strains tested were ATCC 44858, ATCC 4053, ATCC 18804 and ATCC 3691 (*C. albicans*), ATCC 22019 (*C. parapsilosis*), ATCC 34135 (*C. krusei*), ATCC 34449 (*C. lusitaniae*) and ATCC MY646 (*C. dubliniensis*), all supplied by Osvaldo Cruz Foundation (Fiocruz; Rio de Janeiro, Brazil).

Inoculum preparation

Fungal inoculums were prepared from overnight culture (24h) on Sabouraud dextrose agar (SDA). Colonies were directly suspended in saline to obtain turbidity comparable to that of the 0.5 McFarland standards (approximately $1.5x10^6$ CFU/ml).

These initial suspensions were diluted 1:50 with sterile physiological saline solution and further diluted 1:20 in RPMI 1640 medium, providing inoculum containing 1-5 x 10^3 CFU mL^{-1}. Inoculums were dispensed in microtiter plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) at 100 μL per well.

Susceptibility tests

Isolated yeasts were tested using a broth microdilution method, which was performed according to Clinical and Laboratory Standards Institute (CLSI, formerly National Committee for Clinical Laboratory Standards, NCCLS) M27-A2 reference method, with minor modifications. Tween 80 was added to RPMI 1640 medium at a final concentration 1% in order to disperse the essential oil.

Ten serial dilutions of oregano essential oil from stock solutions were prepared in RPMI 1640 medium containing L-glutamine, no bicarbonate and buffered with MOPS at pH 7.0. In order to determine the minimal inhibitory concentrations (MICs) of the yeasts, 100 μL of each essential oil dilution were dispensed per well from columns 1 to 10 of the microtiter plates previously inoculated with the different yeast strains. Columns 11 and 12 contained positive controls (inoculum/medium) and negative controls (essential oil/medium), respectively. The plates were incubated under aerobic conditions at 35ºC for 48 h with shaking. Susceptibility was expressed as minimal inhibitory concentration (MIC), which was defined as the lowest essential oil concentration able to inhibit fungal growth after 24 h of incubation in comparison to the positive control. To determine minimal fungicidal concentration (MFC) values, 10 μL of the yeast-containing suspensions were removed from each well, plated in dishes containing SDA and further incubated to allow detection of yeast proliferation.

STATISTICAL ANALYSIS

MICs and MFCs of different *Candida* isolates were

compared through analysis of variance followed by comparison between geometric means using the Tukey's test with the statistical software package Statistix 6.0. P values ≤ 0.05 were considered significant.

RESULTS AND DISCUSSION

C. albicans strains isolated from animal mucous membranes were sensitive to oregano essential oil, with an average MIC of 2.72 µL mL^{-1} and an average MFC of 5 µL mL^{-1}; whereas, average MIC and MFC values for standard strains were 2.97 µL mL^{-1} and 3.54 µL mL^{-1}, respectively. Average MIC and MFC values for non-albicans *Candida* strains were 2.10 µL mL^{-1} and of 2.97 µL mL^{-1}, respectively (Table 1).

Table 1. Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) values of Origanum vulgare essential oil on Candida spp.

ISOLATES	*MIC %	*MIC (µL mL^{-1})	*MFC (%)	*MFC (µL mL^{-1})
C. albicans (mucous membrane, dog 1)	0.25 - 1	2.5	0.5 – 1	5
C. albicans (mucous membrane, dog 2)	0.25 – 1	2.5	0.5 – 1	5
C. albicans (mucous membrane, dog 3)	0.25 – 1	2.5	0.5 – 1	5
C. albicans (mucous membrane, dog 4)	0.5 – 1	5	1	10
C. albicans (mucous membrane, dog 5)	0.5 – 1	5	0.5 – 1	5
C. albicans (mucous membrane, dog 6)	0.12-1	1.2	0.25 – 1	2.5
C. albicans (skin, dog)	0.25 – 1	2.5	0.5 – 1	5
C.albicans (skin, monkey)	0.25 – 1	2.5	0.5 – 1	5
C. albicans (ATCC 44858)	0.25 – 1	2.5	0.25 – 1	2.5
C. albicans (ATCC 4053)	0.5 – 1	5	0.5 – 1	5
C. albicans (IOC 3691)	0.25 – 1	2.5	0.5 – 1	5
C. albicans (ATCC 18804)	0.25 – 1	2.5	0.25 – 1	2.5
C. dubliniensis (MY 646)	0.25 – 1	2.5	0.5 – 1	5
C. parapsilosis (ATCC 22019)	0.25 – 1	2.5	0.25 – 1	2.5
C. lusitanie (ATCC 34449)	0.12 – 1	1.2	0.12 – 1	1.2
C. Krusei (ATCC 34135)	0.25 – 1	2.5	0.5 – 1	5

*MIC = minimal inhibitory concentration; CFM= minimal fungicidal concentration

The results show differences in susceptibility to oregano essential oil between the fresh *Candida* isolates and standard strains, as well as between different *Candida* species, although these differences were not statistically significant (Fig. 1). Some studies have reported higher MIC values for traditional antifungals used against *C. albicans* (24), which demonstrates the occurrence of resistance to azole antifungals (15, 24, 25). These results are of clinical importance since *C. albicans* is responsible for the great majority of the infections in patients with recurrent candidiasis; in addition, *C. albicans* is the most frequently isolated species in cases of mucocutaneous candidiasis in small animals (8, 13, 19) and is considered the most pathogenic species of the genus *Candida* (7, 11, 24). Recent studies confirm the involvement of non-albicans *Candida* species in clinical cases of yeast infection in humans and animals (18, 20). In addition, the intrinsic resistance of *Candida krusei* to azole derivatives is known (15, 24).

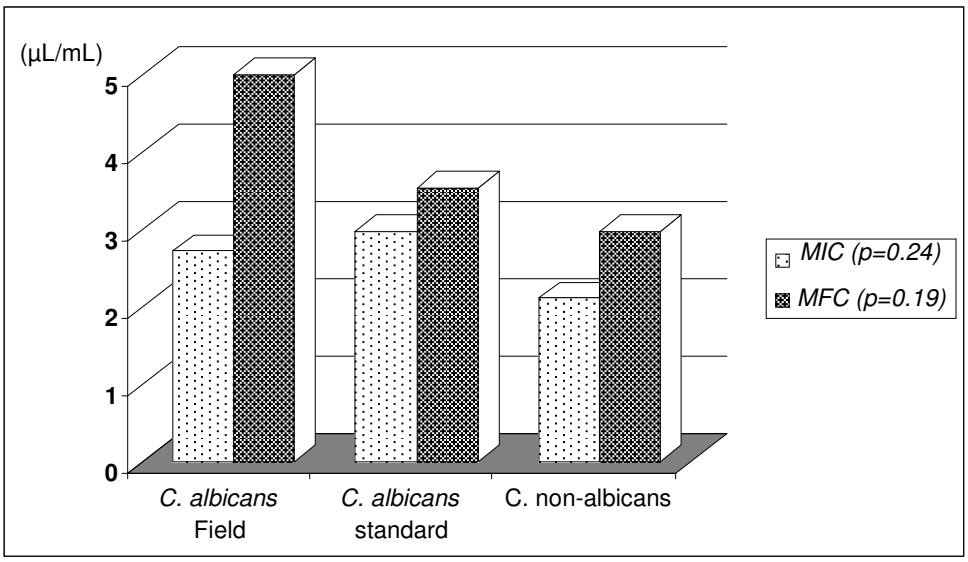

Figure 1. Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) values of *Origanum vulgare* essential oil on *C. albicans* strains isolated from animal mucous membranes, standard *Candida* spp strains and non-albicans *Candida* strains.

Some authors have described the occurrence of clinical resistance and higher MIC values for some antifungals used against different non-albicans *Candida* species including *C. lusitaniae e C. glabrata* (24, 25). Aligiannis *et al*. (2) evaluated the MIC of the essential oils of two species of *Origanum* and found values between 0.28 and 1.27 mg mL^{-1} for bacteria and from 0.65 to 1.27 mg mL^{-1} for fungi, which are similar to the values found in our study (7, 16).

Chromatographic analysis of oregano essential oil revealed the presence of 4-terpineol, γ-terpinene, thymol and carvacrol among its main constituents. The concentration of phenolic monoterpenes was high as compared to those of the other compounds present in the oil (Table 2, Fig. 2). Some monoterpenes and sesquiterpenes could be responsible for the susceptibility of the isolates tested to this essential oil, since the antifungal activity of this oil has been attributed to thymol, carvacrol and eugenol (6, 7, 14, 29).

According to some studies, the composition, quality and content of essential oils present in plants are subject to great variation and are influenced by diverse factors such as the geographical and climatic conditions as well as the conditions used for culture, drying and storage (3, 9, 12, 17).

Inhibition of microorganisms by essential oils seems to rely on different mechanisms of action (2, 14, 26, 27, 28). Toxic effects on membrane structure and function have been generally used to explain the antimicrobial action of essential oils and their monoterpenoid compounds (12, 26, 27).

Some studies suggest that the antimicrobial action of essential oils can be a consequence of a negative effect on enzymes, including those involved in the production of energy and synthesis of structural components of the microorganism, in addition to destruction or inactivation of genetic material (14, 28).

Phenolic compounds present in essential oils may disturb membrane-embedded proteins and inhibit cellular respiration. Also, alterations in the ion transport processes of the cell membrane and modifications in the activity of calcium channels can cause an increase in cell permeability and consequent release of vital intracellular constituents (28).

Table 2. Compounds identified in Origanum vulgare essential oil using chromatographic analysis (GC/FID).

Peak	Retention time (min)	Compounds	MF	C** (%)
1	10.05	α-thujene	$C_{10}H_{16}$	0.25
2	10.36	α-pinene*	$C_{10}H_{16}$	nd
3	12.56	sabinene	$C_{10}H_{16}$	0.33
4	12.68	β-pinene*	$C_{10}H_{16}$	nd
5	13.75	myrcene*	$C_{10}H_{16}$	0.18
6	14.40	α-phellandrene	$C_{10}H_{16}$	2.47
7	15.19	α-terpinene*	$C_{10}H_{16}$	2.83
8	15.67	p-cimene*	$C_{10}H_{14}$	0.71
9	15.94	limonene*	$C_{10}H_{16}$	3.60
10	16.06	1.8-cineole*	$C_{10}H_{18}O$	0.53
11	16.75	cis/trans β-ocimene	$C_{10}H_{16}$	0.08
12	17.97	γ-terpinene*	$C_{10}H_{16}$	4.86
13	18.49	trans sabinene hidrate	$C_{10}H_{18}O$	0.07
14	19.96	terpinolene*	$C_{10}H_{16}$	1.69
15	20.58	cis sabinene hidrate	$C_{10}H_{18}O$	0.08
16	20.87	Linalol*	$C_{10}H_{18}O$	2.89
17	22.18	trans-*p*-menthenol	$C_{10}H_{18}O$	0.12
18	23.11	cis-*p*-menthenol	$C_{10}H_{18}O$	0.05
19	25.20	borneol	$C_{10}H_{18}O$	0.27
20	26.09	4-terpineol*	$C_{10}H_{18}O$	47.95
21	27.03	α-terpineol*	$C_{10}H_{18}O$	7.57
22	27.43	trans-piperitol	$C_{10}H_{18}O$	0.35
23	30.27	methyl thymol eter	$C_{11}H_{16}O$	0.10
24	30.89	methyl carvacrol eter	$C_{11}H_{16}O$	0.71
25	31.88	geraniol/ nerol	$C_{10}H_{18}O$	0.70
26	34.41	thymol*	$C_{10}H_{14}O$	8.42
27	35.00	carvacrol*	$C_{10}H_{14}O$	9.44
28	40.54	geranil/neril acetate	$C_{12}H_{20}O_2$	0.20
29	42.53	β-caryophyllene	$C_{15}H_{32}$	2.92
30	52.37	spathulenol	$C_{15}H_{24}O$	0.59
31	52.67	caryophylene oxide	$C_{15}H_{24}O$	0.05

* Compounds identified by comparison with standard compounds
** C (%) = normalized peak areas using no correction factors
Other compounds were identified based on data from the literature (6, 23)
nd = not detected

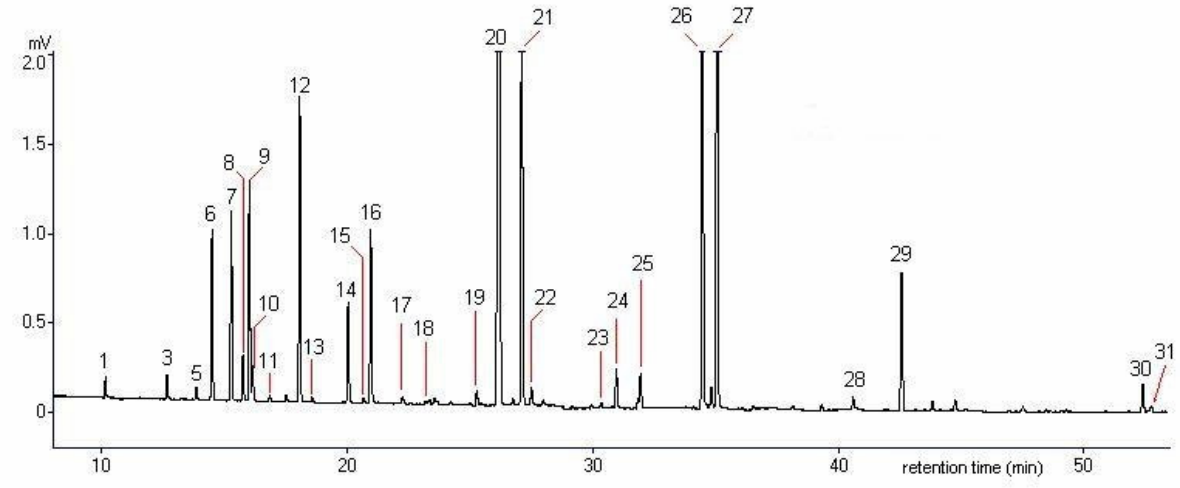

Figure 2. Chromatogram of oregano essential oil obtained by hydrodistillation. Terpineol (peak 20), carvacrol (peak 27), thymol (peak 26) and α-terpineol (peak 21) are the main constituents. See table 2 for a description of the other peaks.

CONCLUSIONS

Based on the results presented herein, it is possible to conclude that the essential oil extracted from *O. vulgare* may represent a good alternative for the treatment of candidiasis due to its appreciable antifungal action against *Candida* spp *in vitro*.

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