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Antifungal activity of the essential oil of *Thymus villosus* subsp. *lusitanicus* against *Candida*, *Cryptococcus*, *Aspergillus* and dermatophyte species

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ABSTRACT

Composition and antifungal activity of the essential oil (EO) of *Thymus villosus* subsp. *lusitanicus* and its main components geranyl acetate, terpinen-4-ol, linalool and geraniol against clinically relevant yeasts and moulds were studied. EO from the plant's aerial parts was obtained by hydrodistillation and analyzed by GC and GC–MS. The oil showed high contents of geranyl acetate and terpinen-4-ol and, in lower percentage, linalool and geraniol. Minimum inhibitory concentrations (MICs) were measured according to the reference Clinical and Laboratory Standards Institute (CLSI) broth macrodilution protocols. Minimum fungicidal concentrations (MFCs) were determined by subsequent subculturing of the same cell suspensions in solid medium. The inhibition of germ tube formation and the influence of the EO on cytoplasmic membrane integrity, analyzed by flow cytometry, were also investigated in *Candida albicans*. In addition, the effect of the EO on mitochondrial function was studied. The EO, geranyl acetate and geraniol displayed low MIC and similar MFC values against dermatophytes and *Cryptococcus neoformans* (0.04–0.64 μL/mL), while terpinen-4-ol showed a weaker activity (1.25–2.5 μL/mL). However, *Aspergillus* species are likely to be more resistant than other studied fungi and the fungicidal activity of the EO against these moulds could be due to terpinen-4-ol and geraniol rather than geranyl acetate. Linalool showed week activity against all the tested strains. Regarding *Candida* species, the EO susceptibility profiles seem to be diverse and were not directly correlated with fluconazole susceptibility patterns. Moreover, the inhibition of yeast-mycelium transition was demonstrated at sub-inhibitory concentrations of the EO in *C. albicans* ATCC 10231. Our findings revealed that the fungicidal activity of the EO is probably due, at least in part, to its ability to cause rapid metabolic arrest and plasma membrane disruption. The yield of *T. villosus*' essential oil and its broad fungicidal activity are compatible with an industrial use, particularly for pharmaceutical, therapeutic and food preservation purposes.

1. Introduction

Over the last twenty-five years, the incidence of life-threatening fungal infections has increased dramatically, generally due in great part to the increasing number of immunocompromised patients. In addition, a change in epidemiology of systemic fungal infections has been observed (Richardson and Lass-Flörl, 2008). *Candida* and *Aspergillus* species have been found to be the most common etiological agents in nosocomial bloodstream fungal infections (BSI). The most common species accounting for more than 90% of all *Candida*-associated BSIs are *Candida albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. Krusei*, while *Aspergillus fumigatus*, *A. flavus*, *A. niger*, and *A. terreus* are the most common isolated species in *Aspergillus*-associated BSIs (Pfaller et al., 2006). Considering illness-associated superficial mycoses, on the other hand, dermatophytes are considered as primary etiologic agents that remain a continuing problem worldwide, not only in impaired immunity patients, but in healthy persons as well. This is partially due to the fact that dermatophytosis are frequently resistant to the usual treatment, and an rate of relapse is unacceptably high, especially in case of onychomycosis (Gupta and Cooper, 2008).

For this reason, fungal diseases are currently considered as a serious problem around the world, effective treatments and management strategies being required. Unfortunately, currently

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available effective antifungal agents are limited in number. Moreover, almost all antifungals exhibit therapeutic limitations, with fungistatic mechanism of action, high toxicity, many drug interactions, insufficient bioavailability and facing the development of resistance by the common fungal pathogens or innate resistance by emerging fungal species. Hence, the present situation regarding currently available therapeutic agents and the response rate of fungal infections to treatment are far from adequate (de Pauw and Picazo, 2008). The development of novel antifungal drugs will be necessary for the future.

Aromatic plants and their essential oils have been used throughout history in folk medicine and their medicinal properties have consequently been the object of frequent scientific studies (Edris, 2007). Essential oils (EOs) are complex mixtures of volatile compounds, mainly monoterpenes, sesquiterpenes and phenylpropanoids. It was established that most of the biological activities of aromatic plants are due to the terpenic volatile compounds that take part in their composition (Bakkali et al., 2008). Because of the natural origin of the EOs and their biological activities they have been globally used, not only in the food industry, but also in perfumery, cosmetics, and pharmaceutical industry (Pauli, 2006; Edris, 2007; Bakkali et al., 2008). However, it will be necessary to evaluate their applicability according to the potential use, as particularly their toxicity and safety.

As a complex group of aromatic plants, the genus *Thymus* (Lamiaceae) is widely distributed across the Iberian Peninsula. Several species of *Thymbra* and *Thymus* from Portugal have been studied and their biological activities reported (Figueiredo et al., 2008). *In vitro* antifungal activity studies of thyme and thymbra oils against a wide variety of human pathogenic fungi have been collected from literature (Pina-Vaz et al., 2004; Salgueiro et al., 2004; Pinto et al., 2006; Salgueiro et al., 2006; Vale-Silva et al., 2010). The treatment of dermatomycosis with EOs of *T. vulgaris* and thymol has recently been found to be effective in experimental animal models infected by *Trychophyton* spp. (Sokovic et al., 2008). The results collectively indicate an interesting potential of EOs for use in therapy, particularly for topical application (das Neves et al., 2008; Sokovic et al., 2008).

In the present work, we studied the antifungal activity of the EO of *T. villosus* subsp. *lusitanicus*, as well as its main compounds, geranyl acetate, terpinen-4-ol, linalool and geraniol. We have further studied the effect of the EO at sub-inhibitory concentrations on the yeast-mycelium transition in *C. albicans*. The germ tube formation is considered an important virulence factor in *C. albicans*, which is crucial for the invasion of the host's tissues (Saville et al., 2006). Flow cytometry studies on *C. albicans* treated with the EO were performed to evaluate its effect on fungal membrane integrity. Besides, thiazolyl blue tetrazolium bromide (MTT) assay was also carried out to assess mitochondrial function.

2. Materials and methods

2.1. Fungal organisms

The activity of the EO and its main components (geranyl acetate, terpinen-4-ol, linalool, and geraniol) was evaluated against five *Candida* spp. clinical strains: two of *C. albicans* (M1, D5); one of *C. krusei* (D39); and one of *C. glabrata* (D10R), isolated from recurrent cases of vulvovaginal or oral candidiasis; plus one of *C. dubliniensis* (CD1), isolated from blood; and two American Type Culture Collection (ATCC) reference strains (*C. albicans* ATCC 10231 and *C. parapsilosis* ATCC 90018). One strain of *Cryptococcus neoformans* (Colección Española de Cultivos Tipo, CECT, *C. neoformans* type strain-1078) was also tested. *Candida parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were used for quality control. Regarding the tested moulds, three *Aspergillus* spp. strains, *A. niger* ATCC 16404, *A. fumigatus* ATCC 46645, and an *A. flavus* strain isolated from bronchial secretions (F44) and seven dermatophyte strains, three isolated from nails and skin (*Microsporum canis* FF1, *Trichophyton mentagrophytes* FF7, and *Epidermophyton floccosum* FF9) and four from CECT collection (*M. gypseum* 2905, *T. interdigitale* 2958, *T. verrucosum* 2992, and *T. rubrum* 2794), were included. All strains were stored in Sabouraud dextrose broth with 20% glycerol at −70 °C. To ensure optimal growth conditions and purity all the strains were subcultured in Sabouraud dextrose agar (SDA) or potato dextrose agar (PDA) before each test.

2.2. Plant material and reference products

Aerial parts of the plants were collected at the flowering stage from Beira Litoral (S. Jorge), Portugal. A voucher specimen was deposited at the Herbarium of the Department of Life Sciences of the University of Coimbra (COI).

Authentic samples of geranyl acetate were purchased from DBH (99.5%), terpinen-4-ol, linalool and geraniol from Fluka (>95%).

2.3. Essential oil isolation and analysis

The fresh aerial flowering parts of the plant were used. The essential oils were isolated by hydrodistillation for 3 h, using a *Clevenger*-type apparatus, according to the procedure described in the European Pharmacopoeia (Council of Europe, 1997). Analyses of the oil were carried out by gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS). GC was performed on a Hewlett Packard 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) with a HP GC ChemStation Rev. A.05.04 data handling system, equipped with a single injector and two flame-ionization detectors (FID). A graphpak divider (Agilent Technologies, Part Number 5021-7148) was used for simultaneous sampling in two Supelco (Supelco Inc., Bellefont, PA, USA) fused silica capillary columns with different stationary phases: SPB-1 (polydimethylsiloxane; 30 m × 0.20 mm i.d., film thickness 0.20 μm) and SupelcoWax 10 (polyethyleneglycol; 30 m × 0.20 mm i.d., film thickness 0.20 μm). Oven temperature program: 70–220 °C (3 °C/min), 220 °C (15 min); injector temperature: 250 °C; detector carrier gas: He, adjusted to a linear velocity of 30 m/s; splitting ratio 1:50; detector temperature: 250 °C. GC/MS analyses were performed on a Hewlett Packard 6890 gas chromatograph fitted with a HP1 fused silica column (polydimethylsiloxane; 30 m × 0.25 mm i.d., film thickness 0.25 μm), interfaced with a Hewlett Packard Mass Selective Detector 5973 (Agilent Technologies, Palo Alto, CA, USA) operated by HP Enhanced ChemStation software, version A.03.00. GC parameters as above; interface temperature: 250 °C; MS source temperature: 230 °C; MS quadrupole temperature: 150 °C; ionization energy: 70 eV; ionization current: 60 mA; scan range: 35–350 u; scans/s: 4.51. The volatile compounds were identified by both their retention indices and their mass spectra. Retention indices, calculated by linear interpolation relative to retention times of C_8–C_{23} of *n*-alkanes, were compared with those of authentic products included in CEF/Faculty of Pharmacy, University of Coimbra laboratory database and/or literature data. Mass spectra were compared with reference spectra from a home-made library or from literature data (Adams, 2004; Joulain and Konig, 1998). Relative amounts of individual components were calculated based on GC peak areas without FID response factor correction. The oils were then preserved in a sealed vial at 4 °C for further experiments.

2.4. Antifungal activity

In order to determine minimum inhibitory concentrations (MICs), the antifungal activity of the EO and its main components was tested *via* broth macrodilution methods based on the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) reference documents M27-A3, S3 (CLSI, 2008a,b), and M38-A2 (CLSI, 2008c), for yeasts and filamentous fungi, respectively, with some modifications. Briefly, fungal suspensions from SDA or PDA cultures of the corresponding organisms were prepared in RPMI-1640 medium (with glutamine, without bicarbonate, and with phenol red as a pH indicator). Twofold serial dilutions of the EO isolated from *T. villosus* and its main pure components obtained from DBH and Fluka (geranyl acetate, terpinen-4-ol, linalool, and geraniol) ranging from 0.02 to 20 μL/mL, were prepared in DMSO, in a maximum concentration of 1% (v/v). The tested systems were mixed and incubated in humid atmosphere, without agitation, at 37 °C for 48 h for *Candida* and *Aspergillus* spp., 72 h for *C. neoformans* and at 30 °C for seven days for dermatophytes. MICs were the lowest oil concentrations resulting in 100% growth inhibition. In addition, fluconazole (Pfizer; 0.25–128 mg/L), for yeasts and dermatophytes, or amphotericin B (Sigma; 0.016–16 mg/L), for *Aspergillus*, were used as standard agents. Besides, the MICs of fluconazole and amphotericin B against *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258, quality control strains, were performed and the results were included within the CLSI recommended limits. Sterility and growth controls in RPMI 1640 medium alone and with 1% DMSO (v/v) were included. The minimum fungicidal concentrations (MFC) were determined after 48 h (for yeasts and *Aspergillus* spp.) and 96 h (for dermatophytes) of incubation, by removing 20 μL from all tubes showing no visible growth to SDA plates. The plates were incubated at 37 °C, except for dermatophytes (30 °C). The MLC was defined as the lowest concentration showing 100% growth inhibition, resulting from the subculture of MIC plates. All the experiments were performed in duplicate and repeated independently three times.

2.5. Germ tube inhibition assay

In order to determine the effect of the EO on the yeast-mycelium transition, the suspensions of *C. albicans* strains ATCC 10231, D5, and M1, from overnight cultures in SDA were prepared in NYP medium (N-acetylglucosamine [Sigma; 10^{-3} mol/L], Yeast Nitrogen Base [Difco; 3.35 g/L], proline [Fluka; 10^{-3} mol/L], NaCl [4.5 g/L], and pH 6.7 ± 0.1; Marichal et al., 1986). The suspensions were adjusted to obtain a density of yeast cell suspensions at $1.0 \pm 0.2 \times 10^6$ CFU/mL and distributed into glass test tubes in a volume of 990 μL. Each dilution of the EO was added into the cell suspension tubes, in 10 μL volumes, to obtain appropriate sub-inhibitory concentrations. After incubation at 37 °C without agitation for 3 h, the treated and untreated yeast cells were counted for germ tube formation under light microscope and the percentage of germinating cells was calculated. A cell was considered positive for germ tube formation when the germinating tube was at least as long as the diameter of the blastospore. The results are presented as average ± standard deviation (SD) of three independent experiments.

2.6. Flow cytometry analysis

The suspensions of *C. albicans* ATCC 10231 were prepared in RPMI 1640 medium from overnight SDA cultures and adjusted, using a haemocytometer, to a final density of $2.0 \pm 0.2 \times 10^6$ CFU/mL. Different twofold serial dilutions of EO and a single solution of amphotericin B were added to the cell suspensions to obtain a final concentration of 0.04–1.25 μL/mL and 2 μg/mL, respectively. The reactions were incubated at 37 °C in humid atmosphere without agitation for 2 h. Drug-free control tubes were also included. After incubation, the cells were washed and resuspended in 500 μL of phosphate buffered saline solution (PBS) with 2% D-glucose (w/v) for FUN-1 (Invitrogen, USA) staining and 0.125% (w/v) sodium deoxycholate (Sigma) in case of PI (propidium iodide; Sigma) staining. Five microliters of the FUN-1 and PI solutions in DMSO and PBS, respectively, were added to the cell suspensions in order to obtain final concentrations of 0.5 μM of FUN-1 and 1.0 μg/mL of PI. FUN-1 stained cells were further incubated for 30 min at 37 °C in the dark while PI stained samples were read after 3 h of reaction at room temperature. Unstained cell suspensions were included, as auto-fluorescence controls. Flow cytometry was performed using a FACSCalibur® (Becton Dickinson Biosciences, San Jose, Calif.) flow cytometer with a blue argon laser emitting at 488-nm wavelength at 15 mW and the results were analyzed using CellQuest Pro Software (Becton Dickinson). Intrinsic parameters (forward and side scatter, for relative cell size and complexity analysis, respectively) and fluorescence in the FL2 channel (log yellow/orange fluorescence, bandpass filter 585/42 nm) for FUN-1, and the FL3 channel (log red fluorescence, longpass filter >650 nm) for PI were acquired and recorded, using logarithmic scales, for a minimum of 7500 events/sample. Markers (M1) were adjusted to include a maximum of 5% of the cells in monoparametric histograms of control samples' fluorescence intensity and kept unchanged through the analysis of the remaining samples to quantify the percentages of cells showing altered fluorescence in comparison to the drug-free controls. The results are presented as average ± SD of at least three replicate experiments.

2.7. Effect of the essential oil on mitochondrial function (MTT assay)

Effect of the EO on mitochondrial reductase activity in *C. albicans* ATCC 10231 was determined through MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay based on the method of Lopes et al. (2012) with some modifications. Briefly, yeast cell suspension was prepared and adjusted in RPMI 1640 medium to obtain the final required cell density of $0.5–2.5 \times 10^3$ CFU/mL. The suspension was then distributed into a 12 well plate, in volume of 1 mL/well, and incubated in humid atmosphere, without agitation, at 37 °C. After 18–24 h of incubation, the cells were carefully homogenized, collected and centrifuged at 10,000 rpm for 5 min. The various concentrations of the EO solutions were added into each sample of the cell pellet in volume of 1 mL and the mixtures were transferred to the microwell plate. After exposure to the EO at 37 °C for 1 h, the treated cells were harvested by centrifugation at 10,000 rpm for 5 min and 500 μL of 0.5 mg/mL of MTT (Sigma–Aldrich, St. Louis, MO, USA) in RPMI were added into each treated sample. Then the mixtures were further incubated at 37 °C for 30 min and a water-insoluble purple formazan product occurring in mitochondria of viable cells was solubilized with 300 μL of DMSO. The intensity of the solubilized purple color was measured spectrophotometrically at 510 nm using a Multiskan Ascent plate reader (Thermo Electron Corporation).

3. Results

The yield of the EO was 1.3% (v/w). More than 92% of the whole composition of the volatile essential oil was identified. The quantitative and qualitative composition is given in Table 1, where components are listed in the order of elution on the SPB-1 column. The oil is predominantly composed of oxygen-containing monoterpenes (64.7%) and monoterpene hydrocarbons (24.0%). Geranyl

Table 1
Constituents of the essential oil of *Thymus villosus* subsp. *lusitanicus*.

Compound[a]	RI SPB-1[b]	RI SW 10[c]	%
α-Thujene	922	1029	1.0
α-Pinene	930	1030	1.9
Camphene	943	1077	0.3
Oct-1-en-3-ol	959	1447	0.1
Sabinene	964	1128	1.1
β-Pinene	970	1118	1.0
Myrcene	980	1161	4.1
α-Phellandrene	997	1171	0.2
α-Terpinene	1010	1187	1.3
p-Cymene	1011	1275	5.0
1,8-Cineole	1019	1214	1.6
Limonene	1020	1206	1.3
E-β-Ocimene	1035	1253	0.3
γ-Terpinene	1046	1249	5.5
trans-Sabinene hydrate	1050	1459	2.5
cis-Linalool oxide	1070	1438	0.3
Terpinolene	1076	1288	1.0
Nonanal	1079	1393	t
cis-Sabinene hydrate	1080	1544	0.5
Linalool	1082	1543	**8.9**
Oct-1-en-3-yl acetate	1093		0.1
Campholenal	1102	1487	t
Camphor	1118	1515	0.4
trans-Verbenol	1126	1664	0.1
Borneol	1144	1695	0.1
p-Cymene-8-ol	1158	1845	t
Terpinen-4-ol	1158	1597	**13.5**
trans-Dihydrocarvone	1167	1602	0.1
α-Terpineol	1169	1692	0.8
Nerol	1209	1797	0.4
Citronellol	1210	1764	t
Carvone	1211	1728	0.1
Geraniol	1233	1842	**8.1**
Linalyl acetate	1240	1555	t
Geranial	1242	1730	0.9
Cuminic alchool	1260		0.1
Bornyl acetate	1266	1578	0.3
Thymol	1268	2183	0.1
Eugenol	1324	2159	0.2
α-Terpenyl acetate	1328	1688	0.2
Neryl acetate	1342	1722	0.5
Geranyl acetate	1359	1755	**25.0**
β-Bourbunene	1376	1517	0.2
β-Elemene	1382	1585	t
E-Caryophyllene	1408	1590	0.5
γ-Muurolene	1464	1683	0.1
Germacrene-D	1466	1699	0.1
γ-Cadinene	1498	1751	t
δ-Cadinene	1508	1751	0.5
α-Elemol	1526	2070	t
Spathulenol	1553	2113	0.1
Caryophyllene oxide	1557	1968	t
Viridiflorol	1569	2072	0.2
γ-Eudesmol	1607	2158	0.1
T-cadinol	1615	2153	0.3
T-Muurolol	1615	2172	0.1
β-Eudesmol	1622	2215	0.1
α-Cadinol	1628	2218	0.2
α-Eudesmol	1628	2208	0.3
Monoterpene hydrocarbons			24.0
Oxygen containing monoterpenes			64.7
Sesquiterpene hydrocarbons			1.5
Oxygen containing sesquiterpenes			1.5
Others			0.5
Total identified			92.2

The numbers in bold serve to highlight that these are the four main components and tested on antifungal activity.

[a] Compounds listed in order of elution in the SPB-1 column. t = traces ≤ 0.05%.

[b] RI SPB 1: GC retention indices relative to C_9–C_{23} n-alkanes on the SPB-1 column.

[c] RI SW 10: GC retention indices relative to C_9–C_{23} n-alkanes on the Supelcowax-10 column.

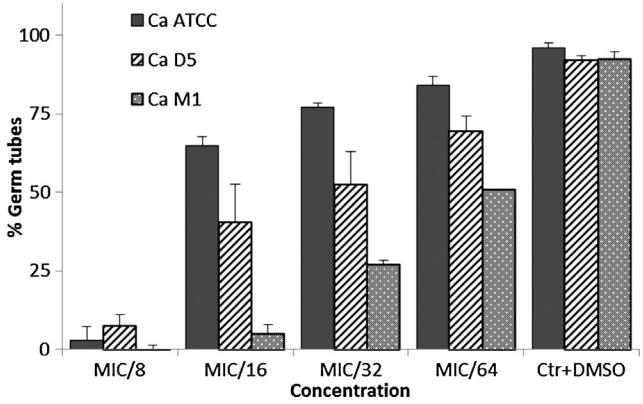

Fig. 1. Percentage of germ tube formation by three strains of *C. albicans* (ATCC 10231, D5 and M1) incubated with sub-inhibitory concentrations (μL/mL) of the essential oil of *Thymus villosus*. Results presented as mean (±SD) values of three independent experiments. Untreated samples included 1% DMSO.

acetate (25.0%) and terpinen-4-ol (13.5%) are the main compounds. In addition, linalool (8.9%), and geraniol (8.1%) are also important compounds.

T. villosus EO exhibited a broad-spectrum antifungal activity, including all tested fungi (animal and human pathogenic species or spoilage fungi): *Candida* spp., *C. neoformans*, *Aspergillus* spp., and dermatophytes (Table 2). Regarding filamentous fungi, the EO showed the higher activity against dermatophytes (0.04–0.16 μL/mL, only *T. verrucosum* showed a MIC of 0.64 μL/mL) and the lower activity against *Aspergillus* species (0.32–2.5 μL/mL, *A. flavus* being the less susceptible strain with a MIC of 2.5 μL/mL). Considering yeast cells, MIC values ranged from 0.16 to 1.25 μL/mL. Strains with higher MIC values to fluconazole (*C. albicans* D5, *C. krusei* D39, and *C. neoformans*) showed the lower MICs for the EO (0.16–0.32 μL/mL). *Cryptococcus neoformans* showed the lowest MIC value for the EO. In addition, the EO revealed fungicidal effect against *Candida* spp. and dermatophytes, with MFC values equal to, or just one $\log_2$ dilution above, the MICs. The exceptions are *A. niger*, showing the highest value of MFC, and *A. fumigatus* which showed a MFC two to three times above the MIC.

Regarding the major components of the EO, terpinen-4-ol also displayed activity against all tested pathogens, with the MICs ranging from 0.64 to 2.5 μL/mL and the MFCs from 1.25 to 2.5 μL/mL. Geranyl acetate revealed the fungicidal effect only on dermatophytes and *C. neoformans*, with the MFC value ranging from 0.16 to 0.64 μL/mL, but not against all *Candida* spp. and *Aspergillus* spp. tested (Table 2). The susceptibility profile was not the same for all the species or strains of *Candida*. Within *C. albicans*, only the D5 strain was susceptible, with a MIC value of 0.32–0.64 μL/mL in opposition to ≥5 μL/mL for ATCC and M1 strains. Among the other *Candida* species, *C. parapsilosis* also showed a MIC ≥5 μL/mL. Linalool was the component with the lowest activity and, in opposition geraniol, showed the highest fungicidal activity against almost the isolates tested.

In addition to fungistatic or fungicidal activity, the EO revealed an important inhibitory effect on germ tube formation, a virulence factor in *C. albicans* pathogenesis (Saville et al., 2006), in all tested strains of *C. albicans* at sub-inhibitory concentrations. At the concentration of 1/8 MIC, germination was completely inhibited in the clinical *C. albicans* strain M1. For the strains ATCC 10231 and D5 the inhibition of filamentation was more than 95% and 90%, respectively, in comparison to untreated control cells (Fig. 1). Different *C. albicans* strains showed differences on susceptibility to inhibition of germ tube formation. The order of susceptibility of *C. albicans* strains was M1 > D5 > ATCC. For a concentration of MIC/64, *C. albicans* M1 showed an inhibitory effect of around 50%.

Table 2
Antimicrobial activity (MIC and MFC) of the essential oil of *Thymus villosus*, geranyl acetate, terpinen-4-ol, linalool, and geraniol against yeast, dermatophyte, and *Aspergillus* strains. Results are from three independent experiments performed in duplicate; when different MIC values were obtained a range of values is presented.

Strains	*Thymus villosus*		Geranyl acetate		Terpinen-4-ol		Linalool		Geraniol		Fluconazole		Amphotericin B	
	MIC[a]	MFC[a]	MIC[a]	MFC[a]	MIC[a]	MFC[a]	MIC[a]	MFC[a]	MIC[a]	MFC[a]	MIC[b]	MFC[b]	MIC[b]	MFC[b]
Candida albicans ATCC 10231	0.64	1.25	≥5	≥5	1.25	2.5	5	5	0.32–0.64	0.64	1	>128	N.T	N.T
C. albicans D5	0.32	0.32	0.32–0.64	0.64–2.5	1.25	2.5	5	5	0.32	0.32	64	>128	N.T	N.T
C. albicans M1	1.25	1.25	≥5	≥5	1.25	2.5	5	5–10	0.32	0.64	2	128	N.T	N.T
C. dubliniensis CD1	0.64	0.64	0.32	≥2.5	1.25	2.5	5	5	0.32	0.32–0.64	1	>128	N.T	N.T
C. krusei D39	0.16–0.32	0.16–0.32	0.32	>2.5	1.25–2.5	2.5	10	10	0.64	0.64	64	64–128	N.T	N.T
C. parapsilosis ATCC 90018	0.64–1.25	1.25	≥5	≥5	1.25	2.5	10	10	0.32	0.64	1	1	N.T	N.T
C. glabrata D10R	0.64	1.25	0.32–0.64	>2.5	2.5	2.5	5	10	0.64	0.64	32	32	N.T	N.T
Cryptococcus neoformans CECT 1078	0.16	0.16–0.32	0.32	0.64	1.25	1.25–2.5	5	5	0.16–0.32	0.32	16	128	N.T	N.T
Trichophyton rubrum CECT 2794	0.04	0.08	0.32	0.32–0.64	1.25	1.25	1.25	1.25–2.5	0.16	0.32	16	64	N.T	N.T
T. mentagrophytes FF7	0.16	0.16	0.32–0.64	0.64	2.5	2.5	1.25	2.5	0.08	0.64	16–32	32–64	N.T	N.T
T. interdigitale CECT 2958	0.16	0.32	0.32	0.32	1.25–2.5	2.5	2.5	2.5–5	0.16	0.32	128	≥128	N.T	N.T
T. verrucosum CECT 2992	0.64	0.64	0.64	0.64	1.25–2.5	2.5	1.25–2.5	1.25–2.5	0.16	0.32	>128	>128	N.T	N.T
Microsporum canis FF1	0.08	0.08–0.16	0.16	0.16–0.32	1.25	1.25	2.5	2.5	0.16	0.32	128	128	N.T	N.T
M. gypseum CECT 2905	0.16	0.16	0.64	0.64	2.5	2.5	1.25–2.5	2.5	0.32	0.32	128	>128	N.T	N.T
Epidermophyton floccosum FF9	0.08	0.16	0.16	0.16	1.25	1.25	1.25–2.5	2.5	0.16	0.32	16	16	N.T	N.T
Aspergillus niger ATCC16404	0.32–0.64	5–10	1.25	≥2.5	0.64–1.25	2.5–5	5	≥20	0.32	1.25	N.T	N.T	1–2	4
A. fumigatus ATCC 46645	0.64–1.25	2.5–5	1.25	≥2.5	0.64–1.25	2.5	2.5	20	0.32	0.64	N.T	N.T	2	4
A. flavus F44	2.5	2.5	>2.5	>2.5	1.25–2.5	2.5	10	≥20	0.32	1.25	N.T	N.T	2	8

N.T: not tested.
[a] MIC and MFC were determined by a macrodilution method and expressed in μL/ml (v/v).
[b] MIC and MFC were determined by a macrodilution method and expressed in μg/mL (w/v).

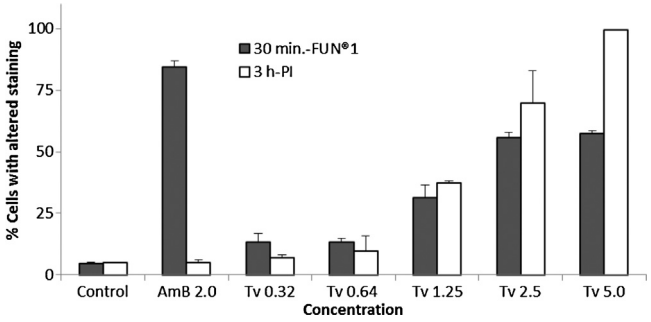

Fig. 2. Percentage (and SD) of M1-gated *C. albicans* ATCC 10231 cells, analyzed by flow cytometry, after 2-h treatments with different concentrations of the essential oil of *T. villosus* in comparison to amphotericin B (AmB) and an untreated control, and staining with FUN-1 and PI. Concentrations in μL/mL for the essential oil (MIC of 0.64 μL/mL) and μg/mL for AmB.

The results of the flow cytometry studies with the model strain *C. albicans* ATCC 10231, treated with the EO for 2 h, show different outcomes according to the two fluorescent probes employed. Only 15% of the cells showed altered FUN-1 staining at the MIC concentration of the EO (0.64 μL/mL) (Fig. 2). The effect was shown to be dose dependent, with 35% of altered staining for twofold the MIC and 55% for four-times the MIC (2.5 μL/mL), which is lower than the 85% obtained with the amphotericin B-treated control (at 2 μg/mL, two $\log_2$ dilutions above the respective MIC of 0.5 μg/mL). The reference compound did not lead to PI staining under the test conditions, while EO-induced staining was found in about 40% of the cells at two times the MIC (1.25 μg/mL and about 70% at 2.5 μL/mL, Fig. 2).

Regarding the effect on mitochondrial enzymes, the treatment of *C. albicans* ATCC 10231 with the EO caused a reduction in activity of reductase enzymes in a dose dependent manner. At the sub-inhibitory concentration of 0.32 μL/mL (1/2 MIC), the mitochondrial activity was significantly decreased when compared to the control, with a p value lower than 0.001 (Fig. 3).

4. Discussion

The EO of *T. villosus* subsp. *lusitanicus* is characterized by high amounts of geranyl acetate (25%) and terpinene-4-ol (13.5%).

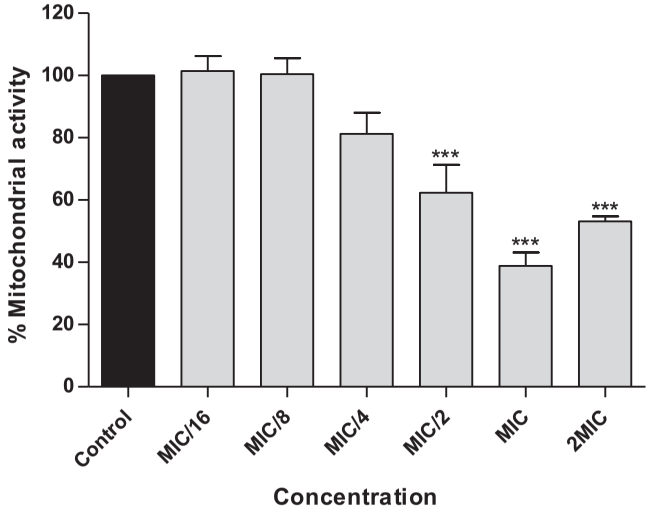

Fig. 3. Mitochondrial activity of *C. albicans* ATCC 10231 cells treated with different concentrations of *Thymus villosus* essential oil. Values are expressed as % of mitochondrial activity relative to control (mean ± SD of three independent assays). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Concentrations lower than MIC/16 presented a mitochondrial activity similar to the untreated cells.

Although in lower %, linalool (8.9%) and geraniol (8.1%) are also important components. Concerning Portuguese *Thymus*, high amounts of geranyl acetate were only reported in the oils of *T. zygis* and *T. pulegioides* (chemotype geraniol/geranyl acetate), whereas important amounts of terpinen-4-ol were only reported for *T. carnosus and T. camphoratus* oils (Salgueiro et al., 1995, 1997; Figueiredo et al., 2008; Gonçalves et al., 2010). The presence of both compounds in high amounts is particular of this Portuguese *Thymus* taxon.

In our previous studies the phenolic *Thymus* EOs with great amounts of thymol and carvacrol revealed higher antifungal activity (Pina-Vaz et al., 2004; Pinto et al., 2006; Vale-Silva et al., 2010). Moreover, the isolated compounds carvacrol and thymol alone have been shown to be at least as active as the total EO (Pinto et al., 2006). Concerning *Thymus villosus*, however, these phenolic compounds are present in very low concentrations therefore the activity of this oil cannot be attributed to them. More likely, the antifungal activity observed for its main compounds and the possible existence of synergistic interactions between the components could justify the observed activity. Despite being found in the highest amount in the EO of *T. villosus* subsp. *lusitanicus*, geranyl acetate exhibited strong fungicidal effect only against dermatophytes and some strains of *C. albicans* whereas geraniol showed the highest activity against all the tested strains. Although being present in low percentage, it might be responsible in part for the activity of the EO. The antifungal activity of geraniol and geranyl acetate was also observed by other groups (Pattnaik et al., 1997; Siti Humeirah et al., 2010; Arputha et al., 2012; Khan and Ahmad, 2012). Terpinen-4-ol showed an intermediate activity and very similar for all fungi tested. Dermatophytes are normally more susceptible than *Aspergillus* spp. and yeasts to EO or their components. However, curiously terpinen-4-ol showed higher activity against *Aspergillus* species. Moreover, the activity verified for the oil of *Malaleuca*-terpinen-4-ol type is justified by the high content of this component (Carson et al., 2006). In spite of having the lowest antifungal activity, for linalool we observed some dermatophyte fungicidal activity. The antifungal activity of linalool against *C. albicans* and dermatophytes was also reported by other researchers (Siti Humeirah et al., 2010). These data revealed that *T. villosus* EO has a broad-spectrum antifungal activity. The fungicidal activity of the EO is possibly due to a synergistic effect of its monoterpene components rather than an individual compound. Dermatophytes showed the highest susceptibility, what can be justified by the activity observed for the four major components against these filamentous fungi.

The yeast-mycelium transition in *C. albicans* is described as an important virulence factor in this species (Saville et al., 2006). The influence of the *T. villosus* subsp. *lusitanicus* EO on its yeast-mycelium transition, at concentrations below the MIC, was in agreement with preceding results (Pina-Vaz et al., 2004; Vale-Silva et al., 2010). The inhibition of morphogenesis gives further confirmation of the potential of the EO for therapy of candidiasis, as this process has long been regarded as a crucial virulence factor in *C. albicans* (Mitchell, 1998). Furthermore, it has recently been suggested by Saville et al. (2006) that this inhibitory activity alone can be used to treat infection in animal models of invasive candidiasis. The ability of geraniol and linalool to interfere with morphological switch at sub-MIC levels was documented by other groups (Hsu et al., 2012; Khan and Ahmad, 2012). These data suggested that the dramatic adverse effect against yeast-mycelium transition of the EO may be due to the synergistic action of its various monoterpene constituents.

In order to clarify the mechanism of fungicidal action, the effect of the EO on membrane integrity of *C. albicans* was determined by staining the EO treated cells with two fluorescent probes and analyzed by flow cytometry. Increased uptake of the nucleic acid stain propidium iodide, to which the cell membrane is normally impermeable (Pinto et al., 2008), was observed. Our results from cell staining with PI and flow cytometry analysis revealed that the antifungal activity of the EO could be due, at least in part, to its capability to interfere with the integrity of the membrane in a dose dependent manner. This possibility is consistent with the result from FUN1 staining study which showed that not only the membrane integrity, but also the metabolism (Millard et al., 1997) of EO-treated cells is compromised. In conclusion, our results revealed that sub inhibitory levels of the EO are able to alter the membrane structure of the treated cells. These results are in agreement with the general statement that EOs cause disruption of the structure of plasma membranes in their different layers, leading to a permeabilization compatible with prompt PI penetration (Nguefack et al., 2004; Bakkali et al., 2008). Moreover, *via* the MTT assay, we also found the involvement of the EO in the activity of mitochondrial reductase enzymes, which leads to impairment of the energy production ability of yeast cells. Several studies showed that monoterpenes exert membrane-damaging effects. Being the most potent fungicidal component in the EO, geraniol was also documented as one of monoterpenes showing the ability to increase the rate of potassium leakage and membrane fluidity in *C. albicans* (Bard et al., 1988). Therefore, it is possible that geraniol might play, at least in part, an important role in membrane structural alteration and dysfunction in the tested yeast strain, ultimately leading to fungal death.

The fungicidal activity displayed by the *T. villosus* subsp. *lusitanicus* EO and some of its main components confirms its potential as an antifungal agent against a wide spectrum of fungal species frequently implicated in human mycoses, particularly candidiasis, cryptococcosis and dermatophytosis. Besides, the inhibition of morphogenesis takes further confirmation of the potential of the EO for therapy of candidiasis, as this process has long been regarded as a crucial virulence factor in *C. albicans* (Mitchell, 1998). Furthermore, it has recently been suggested by Saville et al. (2006) that this inhibitory activity alone can be used to treat infection in animal models of invasive candidiasis.

5. Conclusion

These results demonstrated that *T. villosus* subsp. *lusitanicus* is a potential source of bioactive compounds with beneficial antimicrobial properties. Its essential oil's yield could be compatible with use in the pharmaceutical industry, as well as the food industry, where it can be associated with currently used preservatives for a synergistic action. However, toxicity, safety, and sensorial assays are mandatory before their application. The fungicidal activity displayed by the *T. villosus* subsp. *lusitanicus* EO confirms its potential as an antifungal agent against a wide spectrum of fungal species more frequently implicated in human mycoses, particularly dermatophytosis. The association with other commercial antifungal compounds could bring benefits, by the effect on germ tube formation, and used in candidiasis treatment. After a study concerning topical toxicity, it will be object for further investigation for development of clinically useful therapeutic preparations, particularly for topical application in the management of superficial mycoses.

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