Journal of Medical Microbiology (2006), **55**, 1367–1373 DOI 10.1099/jmm.0.46443-0

Antifungal activity of the essential oil of *Thymus pulegioides* on *Candida*, *Aspergillus* and dermatophyte species

Eugénia Pinto,[1] Cidália Pina-Vaz,[2,3] Lígia Salgueiro,[4] Maria José Gonçalves,[4] Sofia Costa-de-Oliveira,[2] Carlos Cavaleiro,[4] Ana Palmeira,[1] Acácio Rodrigues[2,3] and José Martinez-de-Oliveira[5]

[1]Department of Microbiology/CEQOFF, Faculty of Pharmacy, University of Porto, 4050-Porto, Portugal

[2]Department of Microbiology, Faculty of Medicine, University of Porto, 4200-Porto, Portugal

[3]IPATIMUP – Institute of Pathology and Molecular Immunology, University of Porto, 4200-Porto, Portugal

[4]Department of Pharmacognosy, Faculty of Pharmacy/CEF, University of Coimbra, 3000-Coimbra, Portugal

[5]Department of Obstetrics/Gynaecology, Faculty of Medicine, University of Beira-Interior, 6200-Covilhã, Portugal

Correspondence
Eugénia Pinto
epinto@ff.up.pt

Received 30 November 2005
Accepted 15 June 2006

The composition of the essential oil of *Thymus pulegioides* and its antifungal activity on *Candida*, *Aspergillus* and dermatophyte fungal strains were studied. Essential oil from the aerial parts of the plant was obtained by hydrodistillation and analysed by GC and GC-MS. The oil showed high contents of carvacrol and thymol. The MIC and minimal lethal concentration were used to evaluate the antifungal activity against *Candida* (seven clinical isolates and four ATCC type strains), *Aspergillus* [five clinical isolates, and two Colección Española de Cultivos Tipo (CECT) and two ATCC type strains] and five clinical dermatophyte strains. Antifungal activity was evaluated for the essential oil and for its main components. To clarify its mechanism of action on yeasts and filamentous fungi, flow-cytometric studies of cytoplasmic membrane integrity were performed, and the effect on the amount of ergosterol was investigated. Results showed that *T. pulegioides* essential oil exhibited a significant activity against clinically relevant fungi, mainly due to lesion formation in the cytoplasmic membrane and a considerable reduction of the ergosterol content. The present study indicates that *T. pulegioides* essential oil has considerable antifungal activity, deserving further investigation for clinical applications.

INTRODUCTION

Fungal infections have been increasing in recent years due to a growing number of high-risk patients, particularly immunocompromised hosts. *Candida* is the third- or fourth-most-common isolate in nosocomial bloodstream infections in the USA. In addition, candidosis is the most common invasive fungal infection in critically ill non-neutropenic patients (Eggimann *et al.*, 2003). The mortality rate due to invasive aspergillosis increased by 357 % between 1980 and 1997 in the USA (McNeil *et al.*, 2001). Dermatomycoses are common infections caused by members of the genus *Candida* and by filamentous fungi, particularly the dermatophytes. Superficial candidosis and dermatophytosis can be severe in immunocompromised patients.

Abbreviations: MLC, minimal lethal concentration; PI, propidium iodide.

In spite of the introduction of new antifungal drugs, they are limited in number. The increase of fungal resistance to classical drugs, the treatment costs, and the fact that most available antifungal drugs have only fungistatic activity, justify the search for new strategies (Rapp, 2004).

Aromatic plants have been widely used in folk medicine. It is known that most of their properties are due to their volatile oils. Essential oils from many plants are known to possess antifungal activity (Kalemba & Kunicka, 2003), but only limited information exists about activity toward human fungal pathogens. They have been empirically used as antimicrobial agents, but the mechanisms of action are still unknown.

According to our preliminary results (Pina-Vaz *et al.*, 2004; Salgueiro *et al.*, 2003, 2004), some essential oils show an

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important antifungal activity against yeasts, dermatophyte fungi and *Aspergillus* strains, which could predict therapeutic benefits, mainly for diseases with mucosal, cutaneous and respiratory tract involvement.

Several studies have shown that thyme oils, particularly those of *Thymus vulgaris* and *Thymus zygis* (Bruneton, 1999; Pina-Vaz *et al.*, 2004; Stahl-Biskup & Sáez, 2002), possess antimicrobial activity, those of the phenol type being the most active. The limited occurrence of these phenols in nature is one of the reasons why *Thymus* oils containing thymol and carvacrol have been of great interest for some time.

Thymus pulegioides is widely distributed on the European continent south of the Mediterranean isles. In Portugal, it grows in the northeast, and it is locally used as an antiseptic. Previous results have demonstrated that this species is polymorphic (Salgueiro, 1994; Stahl-Biskup & Sáez, 2002), and that the thymol/carvacrol chemotype is one of the most abundant in Portugal.

The objective of our present research was to evaluate the antifungal activity and investigate the mechanism of action of this specific chemotype and of its main components.

METHODS

Fungal organisms. The antifungal activity of the essential oil and its main components was evaluated against *Candida*, *Aspergillus* and dermatophyte strains: seven clinical *Candida* strains, two of *Candida albicans* (M1, H37), one of *Candida krusei* (H9), one of *Candida tropicalis* (H18), one of *Candida guillermondii* (Mat23) and two of *Candida glabrata* (H16, H30) isolated from recurrent cases of vulvovaginal candidosis, and four ATCC type strains (C. *albicans* ATCC 10231, *C. tropicalis* ATCC 13803, *Candida parapsilosis* ATCC 90018 and *C. krusei* ATCC 6258); five *Aspergillus* clinical strains, one of *Aspergillus niger* (F01), three of *Aspergillus fumigatus* (F05, F07 and F17) and one of *Aspergillus flavus* (F44) isolated from bronchial secretions, as well as two ATCC type strains (*A. niger* ATCC 16404 and *A. fumigatus* ATCC 46645) and two CECT type strains (*A. niger* CECT 2574 and *A. fumigatus* CECT 2071); and five dermatophyte clinical strains (*Microsporum canis* FF1, *Microsporum gypseum* FF3, *Trichophyton rubrum* FF5, *Trichophyton mentagrophytes* FF7 and *Epidermophyton floccosum* FF9) isolated from nails and skin.

C. parapsilosis ATCC 90018 and *C. krusei* ATCC 6258 were used as controls. The fungal isolates were identified by standard microbiology methods and stored in Sabouraud dextrose broth with glycerol at −70 °C.

Plant material and chemicals. Aerial parts of the plants were collected at the flowering stage from Moimenta, Trás-os-Montes (north of Portugal). A voucher specimen was deposited at the Herbarium of the Instituto Botânico of the University of Coimbra (COI).

Thymol (99·5 %) was purchased from DBH, and carvacrol, γ-terpinene and *p*-cymene (all 99·5 %) from Fluka.

Essential oil analysis. Essential oil was isolated by water distillation for 3 h from air-dried material, using a Clevenger-type apparatus, according to the procedure described in the European Pharmacopoeia (Council of Europe, 1997).

Analysis of volatile oil was carried out by GC and GC-MS. Analytical GC was carried out in a Hewlett Packard 6890 gas chromatograph (Agilent Technologies) with a Hewlett Packard GC ChemStation Rev. A.05.04 data-handling system, equipped with a single injector and two flame-ionization detectors (FIDs). A graphpak divider (Agilent Technologies, part no. 5021-7148) was used for simultaneous sampling to two Supelco 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). The oven temperature programme was 70–220 °C (3 °C min^{-1}), 220 °C (15 min); the injector temperature was 250 °C; the carrier gas helium, adjusted to a linear velocity of 30 cm s^{-1}; the splitting ratio 1 : 40; and the detector temperature 250 °C.

GC-MS analyses were carried out in a Hewlett Packard 6890 gas chromatograph fitted with an 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) operated by Hewlett Packard Enhanced ChemStation software, version A.03.00. GC parameters were as above, and other parameters were as follows: interface temperature, 250 °C; MS source temperature, 230 °C; MS quadrupole temperature, 150 °C; ionization energy, 70 eV; ionization current, 60 μA; scan range, 35–350 u; scans per second, 4·51.

The identity of the components was ascertained based on their retention indices, calculated by linear interpolation relative to retention times of a series of *n*-alkanes, and their mass spectra, which were compared with those from our own library and literature data (Adams, 1995; Joulain & Konig, 1998). Relative amounts of individual components were calculated based on GC peak areas without FID response factor correction.

Antifungal activity. MICs, determined by the macrodilution broth method, and minimal lethal concentrations (MLCs) were performed according to reference documents M27-A and M38-A (National Committee for Clinical Laboratory Standards, 1997, 2002) for yeasts and filamentous fungi, respectively.

Serial twofold dilutions in DMSO, ranging from 0·02 to 20 μl ml^{-1}, were tested for essential oil and its main components (thymol, carvacrol, *p*-cymene and γ-terpinene). In addition, the reference antifungal compounds, fluconazole (Pfizer) for yeasts and dermatophytes, or amphotericin B (Sigma) for *Aspergillus*, were used as standard antifungal drugs. Twofold serial dilutions ranging from 0·25 to 128 μg ml^{-1} for fluconazole and 0·016 to 16 μg ml^{-1} for amphotericin B were used.

Quality control determinations of the MICs of fluconazole and amphotericin B were performed by testing *C. parapsilosis* ATCC 90018 and *C. krusei* ATCC 6258. The results obtained were within the recommended limits.

These experiments performed in duplicate were repeated independently three times and yielded essentially the same results. A range of values is presented where different results were obtained. Two growth controls, RPMI medium and RPMI with 2·0 % (v/v) DMSO, were included for each strain.

Mechanism of activity

Lesion of cytoplasmic membrane. Flow cytometry analysis using propidium iodide (PI; Sigma) was performed. PI is a fluorescent probe used to study the effect of drugs on membranes. It only penetrates cells with severe membrane lesions, showing increased red fluorescence (Pina-Vaz *et al.*, 2001). Cells (10^6 ml^{-1}) were incubated with serial concentrations of the oil and its main components

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Table 1. Constituents of the essential oil of *T. pulegioides*

T, Trace (<0·05 %).

Compound*	RI SPB-1†	RI SW 10‡	Percentage of total
Tricyclene	921	1029	0·1
α-Thujene	922	1029	0·5
α-Pinene	930	1030	1·0
Camphene	943	1077	1·5
Oct-1-en-3-ol	959	1447	2·0
Octan-3-one	962	–	3·9
Sabinene	964	1128	0·2
β-Pinene	970	1118	0·5
Myrcene	980	1161	1·2
α-Phellandrene	997	1171	0·1
Δ-3-Carene	1005	1152	T
α-Terpinene	1010	1187	0·9
p-Cymene	1011	1275	7·8
1,8-Cineole	1019	1214	1·4
Limonene	1020	1206	0·6
Z-β-Ocimene	1025	1235	T
γ-Terpinene	1046	1249	8·8
trans-Sabinene hydrate	1050	1459	1·0
n-Octanol	1052	–	1·1
p-Cymene-8-ol	1158	1845	0·1
α-*p*-Dimethylstyrene	1071	–	0·1
Terpinolene	1076	1288	0·2
cis-Sabinene hydrate	1080	1544	0·4
Linalool	1082	1543	0·6
Camphor	1118	1515	3·9
trans-Verbenol	1125	1668	0·1
Borneol	1144	1695	2·9
Terpinene-4-ol	1158	1597	0·2
trans-Dihydrocarvone	1167	1602	T
α-Terpineol	1169	1692	0·1
Cuminaldehyde	1211	1774	T
Neral	1214	1679	T
Thymyl methyl ether	1214	1583	0·7
Carvacryl methyl ether	1223	1601	1·0
Geraniol	1233	1842	0·1
Bornyl acetate	1264	1574	0·1
Thymol	1268	2183	26·0
Carvacrol	1275	2212	21·0
α-Cubebene	1342	1455	0·1
Geranyl acetate	1359	1755	T
α-Copaene	1369	1487	T
β-Bourbonene	1376	1517	0·3
E-Caryophyllene	1408	1590	1·8
E-α-Bergamotene	1427	1580	T
α-Humulene	1442	1665	0·1
E-β-Farnesene	1447	1666	0·1
allo-Aromadendrene	1447	1636	T
γ-Muurolene	1464	1683	0·2
Germacrene-D	1466	1699	1·9
β-Bisabolene	1495	1723	3·0
γ-Cadinene	1498	1751	T
δ-Cadinene	1508	1751	0·2
β-Sesquiphellandrene	1510	1763	T
α-Bisabolene	1529	1767	0·1
Spathulenol	1553	2113	0·1
Caryophyllene oxide	1557	1968	0·2
α-Cadinol	1628	2218	0·1
Monoterpene hydrocarbons	–	–	23·5
Oxygen-containing monoterpenes	–	–	59·8
Sesquiterpene hydrocarbons	–	–	7·8
Oxygen-containing sesquiterpenes	–	–	0·4
Others	–	–	7·0
Total identified	–	–	98·5

*Compounds listed in order of elution from the Supelco SPB-1 column.

†RI SPB-1 signifies GC-retention indices relative to C_9–C_{23} *n*-alkanes on the SPB-1 column.

‡RI SW 10 signifies GC-retention indices relative to C_9–C_{23} *n*-alkanes on the Supelco Supelcowax-10 column.

(0·32–1·25 μl ml^{-1}) for 1 h (*Candida*) or 7 h (*Aspergillus*), and then stained with 1 μg PI ml^{-1} for 30 min. Cells were also incubated with the same compounds, at MLC values, for 5, 10, 15 and 30 min at 30 °C for *C. albicans*, representing yeasts, and 3, 5, 7 and 16 h for *A. fumigatus* and *A. niger*, representing moulds. Scattergram analysis was performed to evaluate morphological changes (size and complexity). The percentage of stained cells at FL3 (620 nm, red), representing dead cells with severe lesions of the membrane, was quantified.

Study of ergosterol amount. For determination of the amount of ergosterol, the strains were incubated in RPMI medium (Sigma) supplemented with 2 % glucose (Difco) for 48 h at 35 °C (yeasts), 3 days at 25 °C (*Aspergillus*) or 5 days at 25 °C (dermatophytes), while shaking. A quantification of ergosterol amount was performed after incubation with and without the essential oil, its main components, or fluconazole as control, at both MIC and subinhibitory concentrations. Ergosterol was isolated from fungal cells by saponification, and the non-saponifiable lipids were extracted with heptane. Ergosterol was identified by its spectrophotometric absorbance profile (230–300 nm) (Arthington-Skaggs *et al.*, 1999).

RESULTS AND DISCUSSION

The oil was obtained from air-dried plant material in a yield of 1·8 % (v/w). The qualitative and quantitative composition of the oil analysed is shown in Table 1. Fifty-seven components representing 98·5 % of the volatile oil were identified. The oil was characterized by high amounts of thymol (26·0 %), and of carvacrol (21·0 %) and its biogenetic precursors, γ-terpinene (8·8 %) and *p*-cymene (7·8 %) (thymol/carvacrol chemotype).

Evaluation of MIC and MLC showed that the oil was active against all the tested strains (Table 2). *T. pulegioides* essential oil exhibited significant antifungal activity. MIC values

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Table 2. Antimicrobial activity (MIC and MLC) of the essential oil of the thymol/carvacrol chemotype of *T. pulegioides* and its major compounds for *Candida*, dermatophyte and *Aspergillus* strains

Results were obtained from three independent experiments performed in duplicate. NT, Not tested.

Strains	Essential oil		Thymol		Carvacrol		*p*-Cymene		γ-Terpinene		Fluconazole		Amphotericin B	
	MIC*	MLC*	MIC*	MLC*	MIC*	MLC*	MIC*	MLC*	MIC*	MLC*	MIC†	MLC†	MIC†	MLC†
Yeast strains														
Candida albicans ATCC 10231	0·64	0·64	0·16	0·32	0·16	0·16–0·32	5–10	>10	2·5–5	5–10	1	>128	NT	NT
C. albicans H37	0·32	0·32–0·64	0·16	0·32	0·16	0·16–0·32	5	>10	5	5–10	64	>128	NT	NT
C. albicans M1	0·32–0·64	0·64	0·16	0·32	0·16	0·32	5–10	>10	2·5–5	5–10	2	128	NT	NT
C. tropicalis ATCC 13803	0·32–0·64	0·32–0·64	0·16–0·32	0·32	0·16	0·16–0·32	5–10	>10	10–20	⩾20	4	>128	NT	NT
C. tropicalis H18	0·64	0·64	0·16–0·32	0·32	0·16	0·16–0·32	5–10	>10	10–20	10–20	2	>128	NT	NT
C. glabrata H16	0·32–0·64	0·32–0·64	0·16	0·32	0·16	0·32	5–10	>10	5	10	16	16	NT	NT
C. glabrata H30	0·64	0·64	0·32	0·32	0·16	0·32	10	>20	5–10	20	32	32	NT	NT
C. krusei ATCC 6258	0·32–0·64	0·32–0·64	0·16–0·32	0·32	0·16	0·16–0·32	5–10	10	5–10	10	64	64–128	NT	NT
C. krusei H9	0·32–0·64	0·32–0·64	0·16–0·32	0·32	0·16	0·16–0·32	5–10	10	5–10	10	64	64–128	NT	NT
C. guilliermondii MAT23	0·32	0·32	0·16	0·16	0·08–0·16	0·16	5	10	1·25–2·5	2·5–5	8	8	NT	NT
C. parapsilosis ATCC 90018	0·64	0·64	0·32	0·32	0·16	0·16–0·32	2·5	>20	5	20	<1	<1	NT	NT
Filamentous fungi, dermatophytes														
E. floccosum FF9	0·16	0·16	0·16	0·16	0·08	0·08	5	5	2·5	2·5	16	16	NT	NT
T. rubrum FF5	0·32	0·32	0·16	0·16	0·08	0·08	1·25	1·25–2·5	5	5	16–32	32	NT	NT
T. mentagrophytes FF7	0·16	0·32	0·16	0·16–0·32	0·04	0·08	5	>5	10	10	16–32	32–64	NT	NT
M. canis FF1	0·16	0·16–0·32	0·08	0·16	0·04	0·08	2·5	2·5	5	5	128	128	NT	NT
M. gypseum FF3	0·16	0·32	0·16	0·32	0·04	0·08–0·16	10	>10	10	10	⩾128	⩾128	NT	NT
Filamentous fungi, *Aspergillus*														
Aspergillus niger ATCC 16404	0·32	0·64	0·16	0·64	0·16	0·16–0·32	>20	>20	>20	>20	NT	NT	1–2	4
A. niger CECT 2574	0·32	0·64	0·16	0·64	0·16	0·16–0·32	>20	>20	20	>20	NT	NT	2	4
A. niger F01	0·32	0·64	0·16	0·64	0·16	0·32	>20	>20	>20	>20	NT	NT	1	2
A. fumigatus ATCC 46645	0·16	0·64	0·16	0·64	0·16	0·32	>20	>20	20	>20	NT	NT	2	4
A. fumigatus CECT 2071	0·16	0·64	0·16	0·64	0·16	0·32	>20	>20	20	20	NT	NT	1–2	4
A. fumigatus F05	0·16	0·64	0·16	0·64	0·16	0·32	>20	>20	10–20	>20	NT	NT	2–4	4–8
A. fumigatus F07	0·16	0·32	0·16	0·64	0·16	0·16	>20	>20	20	>20	NT	NT	2–4	4
A. fumigatus F17	0·16	0·64	0·16	0·64	0·16	0·32	>20	>20	20	>20	NT	NT	2	4–8
A. flavus F44	0·32	0·64	0·32	0·64	0·32	0·32	>20	>20	20	20	NT	NT	2	8

*MIC and MLC were determined by a macrodilution method and expressed in μl ml^{-1} (v/v).
†MIC and MLC were determined by a macrodilution method and expressed in μg ml^{-1} (w/v).

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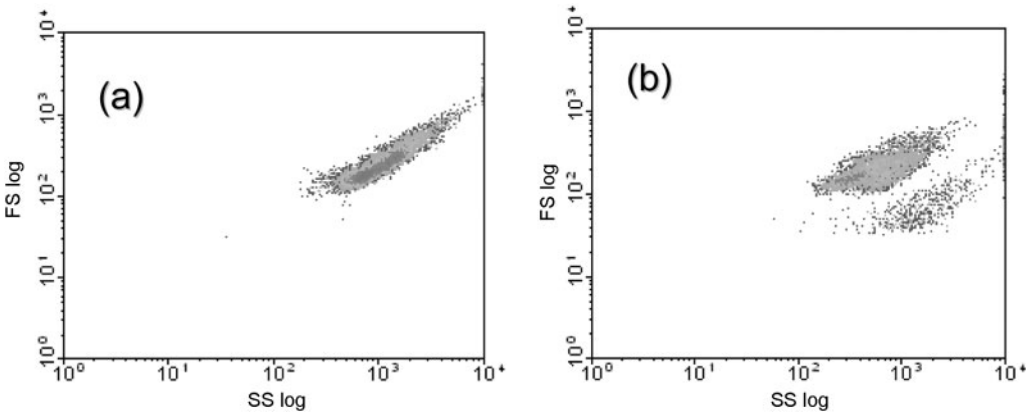

Fig. 1. Scattergram showing cell complexity (side scatter, SS log) versus cell size (forward scatter, FS log). (a) Viable cells of *C. albicans*; (b) cells treated with the essential oil of *T. pulegioides* at MLC value (0·64 μl ml^{-1}), showing significant morphological alterations.

ranged from 0·16 to 0·32 μl ml^{-1} against dermatophyte and *Aspergillus* strains. *Candida* showed the highest MIC values, ranging from 0·32 to 0·64 μl ml^{-1}. For *Candida* and most dermatophyte strains, MIC and MLC values were similar, ranging from 0·16 to 0·64 μl ml^{-1} (Table 2). It is difficult to attribute the activity of a complex mixture to particular constituents. Nevertheless, it is reasonable to speculate that the activity of this oil can be related to the presence of carvacrol and thymol. These compounds were found to be the most active constituents (Table 2) of *T. pulegioides* oil, with MIC values ranging from 0·04 to 0·32 μl ml^{-1} and 0·08 to 0·32 μl ml^{-1}, respectively. The importance of the phenolic hydroxyl groups for the antimicrobial activity of the monoterpenoids has previously been reported (Adam *et al.*, 1998; Aligiannis *et al.*, 2001; Dorman & Deans, 2000; Nostro *et al.*, 2004; Sivropoulou *et al.*, 1996). Other species of the genus *Thymus*, such us *T. zygis* and *T. vulgaris*, with high amounts of phenols, also show a broad spectrum of activity against a variety of pathogenic yeasts and filamentous fungi, including fungi with decreased susceptibility to fluconazole (Dorman & Deans, 2000; Nguefack *et al.*, 2004; Pina-Vaz *et al.*, 2004). Nevertheless, carvacrol proved to be more active against dermatophyte strains, in a similar manner to the essential oil. MIC and MLC values were very similar and the fungistatic and fungicidal properties of the oil were suspected to be associated with high carvacrol and thymol content.

Flow cytometry was used to evaluate the effect of the essential oil on the integrity of fungal cells, using PI as fluorescent marker. The results showed that the oil acts by primary lesion of the membrane. The effect on *Candida* was fungicidal, with severe lesion of the membrane, as PI could penetrate most of the yeast cells (more than 95 %) after 5 min at the MLC value (0·64 μl ml^{-1}). This effect was dose dependent, so that there were >95 % PI-positive cells at a sub-MLC concentration (0·32 μl ml^{-1}). Substantial morphological changes were observed on a scattergram of *C. albicans* cells after 1 h incubation at MLC values (Fig. 1). Previous work carried out with essential oils has revealed anti-*Candida* activity (Pina-Vaz *et al.*, 2004; Salgueiro *et al.*, 2003, 2004). Incubating *Aspergillus* with essential oil of *T. pulegioides*, PI began to stain the cells after 7 h incubation. At MLC values, ~40 % of *A. fumigatus* cells and ~20 % of *A. niger* cells were stained, the effect being dose dependent (Fig. 2). Thymol and carvacrol gave identical cytometric results to *T. pulegioides* essential oil against *Candida* and *Aspergillus*. To understand the mechanism of action on moulds, *Aspergillus* species were studied as typical. *Aspergillus* begins its germination at ~7 h incubation, so it is understandable that it was only after this period that the essential oil was effective and PI could enter the cells. This effect is superior to the effect of most antifungals, as most of them are fungistatic.

Ergosterol is the major sterol component of the yeast cell membrane, and is responsible for maintaining cell function and integrity (Rodriguez *et al.*, 1985). The primary

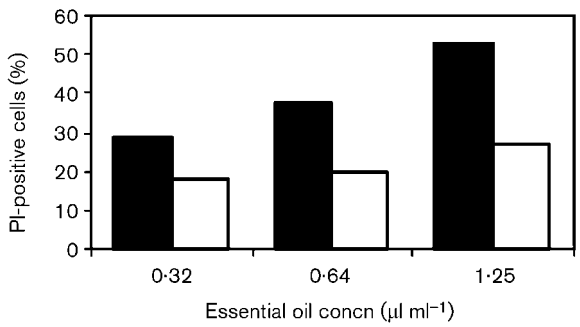

Fig. 2. Number of PI-positive cells (dead cells) of *A. fumigatus* ATCC 46645 (black bars) and *A. niger* ATCC 16404 (white bars) at serial concentrations of the essential oil of *T. pulegioides*.

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mechanism of action by which azole antifungal drugs inhibit yeast cell growth is disruption of normal sterol biosynthetic pathways, leading to a reduction in ergosterol biosynthesis (Kelly *et al.*, 1995). After incubation of *C. albicans* ATCC 10231 at MIC (0·64 μl ml^{-1}) and subinhibitory (0·32 μl ml^{-1}) concentrations of essential oil, a reduction of 80–100 % of ergosterol content was observed. A similar effect was obtained with fluconazole at 1 μg ml^{-1}. For *T. rubrum*, a subinhibitory essential oil concentration (0·08 μl ml^{-1}) reduced ergosterol content by around 70 %. The essential oil of *T. pulegioides* therefore induces considerable impairment of the biosynthesis of ergosterol by *C. albicans* and *T. rubrum*.

The large spectrum of activity of this essential oil acting on *Candida*, *Aspergillus* and dermatophytes agrees with the mechanism of action proposed: cytoplasmic membrane lesion.

In conclusion, the findings of the present study indicate that *T. pulegioides* essential oil has potential as a topical antifungal agent against fungi that are pathogenic to humans. This essential oil is a broad-spectrum agent that inhibites not only dermatophytes, *Aspergillus* and *Candida* species (such as *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. guilliermondii*), but also fluconazole-resistant *C. albicans* isolates, and *C. krusei* and *C. glabrata*, which are intrinsically resistant to fluconazole or whose resistance is easily inducible.

Given the results described above, particularly the possible mechanisms of action, which might induce side-effects in humans, these antifungals require further investigation.

The results presented should stimulate studies on toxicity, improved formulations and the determination of optimal concentrations for clinical applications, as well as comparative studies alongside currently used drugs of the therapeutic efficacy of essential oils to control mucocutaneous infections.

ACKNOWLEDGEMENTS

This work was supported by Fundação para a Ciência e Tecnologia (FCT) and Fundo Europeu de Desenvolvimento Regional (FEDER) (Programa Operacional Ciência, Tecnologia, Inovação-POCTI/40167/2001).

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