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Chemical composition and antifungal activity of the essential oils of *Lavandula viridis* L'Hér

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

In the present work, we report for what we believe to be the first time the antifungal activity and mechanism of action of the essential oils of *Lavandula viridis* from Portugal. The essential oils were isolated by hydrodistillation and analysed by GC and GC/MS. The MIC and the minimal lethal concentration (MLC) of the essential oil and its major compounds were determined against several pathogenic fungi. The influence of subinhibitory concentrations of the essential oil on the dimorphic transition in *Candida albicans* was also studied, as well as propidium iodide and FUN-1 staining of *Candida albicans* cells by flow cytometry following short treatments with the essential oil. The oils were characterized by a high content of oxygen-containing monoterpenes, with 1,8-cineole being the main constituent. Monoterpene hydrocarbons were present at lower concentrations. According to the determined MIC and MLC values, the dermatophytes and *Cryptococcus neoformans* were the most sensitive fungi (MIC and MLC values ranging from 0.32 to 0.64 μl ml^{-1}), followed by *Candida* species (at 0.64–2.5 μl ml^{-1}). For most of these strains, MICs were equivalent to MLCs,

indicating a fungicidal effect of the essential oil. The oil was further shown to completely inhibit filamentation in *Candida albicans* at concentrations well below the respective MICs (as low as MIC/16). Flow cytometry results suggested a mechanism of action ultimately leading to cytoplasmic membrane disruption and cell death. Our results show that *L. viridis* essential oils may be useful in the clinical treatment of fungal diseases, particularly dermatophytosis and candidosis, although clinical trials are required to evaluate the practical relevance of our *in vitro* research.

Introduction

Over the last few decades, there has been an increase in the number of serious human infections in immunocompromised patients caused by fungi (Pfaller *et al.*, 2006). The range of severity of these infections is a consequence of the host reaction to the metabolic products produced by fungi, the virulence of the infecting strain, the site of infection and also environmental factors (Romani, 2007). Nowadays, the increasing impact of these infections, the limitations encountered in their treatment (e.g. resistance, side-effects and high toxicity) and the rising overprescription and overuse of conventional antifungals (Pérez-Parra *et al.*, 2009; Ferris *et al.*, 2002) all stimulate a search for alternative natural drugs.

In recent years, research on aromatic plants, and particularly their essential oils, has attracted many investigators. Essential oils have traditionally been used for centuries for their antifungal properties (Ríos & Recio, 2005). More recently, several studies have confirmed the huge potential of these natural products as antifungal agents (Bakkali *et al.*, 2008; Cavaleiro *et al.*, 2006; Pina-Vaz *et al.*, 2004; Pinto *et al.*, 2006; Zuzarte *et al.*, 2009). Therefore, it is not surprising that essential oils are one of the most promising groups of natural products for the development of broad-spectrum, safer and cheaper antifungal agents.

The genus *Lavandula* provides valuable essential oils mainly for the food (flavouring), perfumery and cosmetic industries, and is also very popular in aromatherapy. However, many other applications can be foreseen, as suggested in several reports on the biological activity of this genus. *Lavandula* oils have been reported to have sedative and antispasmodic properties (Cavanagh & Wilkinson, 2002) as well as acaricidal (Perrucci *et al.*, 1996), antibacterial (e.g. Dadalioğlu & Evrendilek, 2004; Moon *et al.*, 2006), antifungal (e.g. Angioni *et al.*, 2006; Zuzarte *et al.*, 2009) and antioxidant (Matos *et al.*, 2009) activities. More recently, application as a biopesticide has also been suggested by González-Coloma *et al.* (2006).

Lavandula viridis L'Hér is a highly aromatic shrub endemic to the south Iberian Peninsula. It is commonly known as green or white lavender due to its white flowers and green floral bracts, which

are very distinct from those of the other lavenders. Dried leaves of *L. viridis* are used with medical applications in Madeira, Portugal (Upson & Andrews, 2004).

As part of our ongoing study on the valorization of Portuguese lavenders, we now report the chemical composition, antifungal activity and mechanism of action of *L. viridis* essential oils. As far as we know, this is the first report on the antifungal activity of this species.

Methods

Plant material.

Aerial parts of two samples of *L. viridis* were collected from field-growing plants in the flowering stage in the south of Portugal (A, Barranco do Velho region; B, Salir region). Voucher specimens were deposited at the herbarium of the Department of Life Sciences of the University of Coimbra (COI).

Essential oil isolation and analysis.

The essential oils from air-dried plant material were isolated by hydrodistillation for 3 h, using a Clevenger-type apparatus according to the European Pharmacopoeia (Council of Europe, 1997). The oils were preserved in a sealed vial at 4 °C. Oil analyses were carried out by both GC and GC/MS using fused silica capillary columns with two different stationary phases (SPB-1 and SupelcoWax-10) as previously reported (Cavaleiro *et al.*, 2004).

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 a series of n-alkanes, were compared with those of authenticated samples from the database of the Laboratory of Pharmacognosy, Faculty of Pharmacy, University of Coimbra. Mass spectra were compared with reference spectra from a home-made library or from literature data (Adams, 1995; Joulain & König, 1998). Relative amounts of individual components were calculated based on GC peak areas without flame ionization detector response factor correction.

Pure and reference compounds.

Authentic samples of 1,8-cineole (Merck; 99.5% purity), α-pinene (Fluka; 99.0% purity), linalool (Aldrich; 99.0% purity) and camphor (Extrasynthese) were used.

Fluconazole was kindly provided by Pfizer as the pure powder and amphotericin B was from Sigma (80.0% purity).

Fungal strains.

The antifungal activity of the essential oil of sample A was evaluated against yeasts and filamentous fungi: four clinical *Candida* strains isolated from recurrent cases of vulvovaginal and oral

candidosis (*Candida albicans* D5, *Candida albicans* M1, *Candida krusei* H9 and *Candida guilliermondii* MAT23); three *Candida* type strains from the American Type Culture Collection (*Candida albicans* ATCC 10231, *Candida tropicalis* ATCC 13803 and *Candida parapsilopsis* ATCC 90018); one *Cryptococcus neoformans* type strain from the Colección Espanõla de Cultivos Tipo (*Cryptococcus neoformans* CECT 1078); one *Aspergillus* clinical strain isolated from bronchial secretions (*Aspergillus flavus* F44) and two *Aspergillus* type strains from the American Type Culture Collection (*Aspergillus niger* ATCC 16404 and *Aspergillus fumigatus* ATCC 46645); three dermatophyte clinical strains isolated from nails and skin (*Epidermophyton floccosum* FF9, *Trichophyton mentagrophytes* FF7 and *Microsporum canis* FF1) and four dermatophyte type strains from the Colección Espanõla de Cultivos Tipo (*T. mentagrophytes* var. *interdigitale* CECT 2958, *Trichophyton rubrum* CECT 2794, *Trichophyton verrucosum* CECT 2992 and *Microsporum gypseum* CECT 2908). All strains were stored in Sabouraud dextrose broth with 20% glycerol at −80 °C and subcultured on Sabouraud dextrose agar (SDA) or potato dextrose agar (PDA) before each test, to ensure optimal growth conditions and purity.

Antifungal activity.

Broth macrodilution methods based on the Clinical and Laboratory Standards Institute (CLSI) reference protocols M27-A3 (CLSI, 2008a) and M38-A2 (CLSI, 2008b), for yeasts and filamentous fungi, respectively, were used to determine MICs of the essential oils and their major constituents. A macrodilution rather than a microdilution design was used to allow the use of glass test tubes, thus avoiding the interaction of the essential oil with the plastic polymer material of the 96-well microtitre plates. Briefly, inoculum suspensions were prepared at appropriate densities in RPMI 1640 broth (with L-glutamine, without bicarbonate, and with the pH indicator phenol red) from SDA or PDA cultures and distributed into 12×75 mm glass test tubes. Inoculum densities were confirmed by viability counts on SDA. Serial twofold dilutions of the oil were prepared in DMSO and added to the cell suspensions in order to obtain test concentrations ranging from 0.08 to 20.0 µl ml^{-1} (final DMSO concentrations never exceeded 2%, v/v). Oil-free growth controls, as well as sterility and DMSO control tubes, were also included. The test tubes were incubated aerobically at 35 °C for 48 h/72 h (*Candida* species and *Aspergillus*/*Cryptococcus neoformans*) and at 30 °C for 7 days (dermatophytes). MIC values were determined as the lowest concentration of the oil causing full growth inhibition. Quality control was performed by testing fluconazole and amphotericin B with the reference strains *Candida parapsilopsis* ATCC 22019 and *Candida krusei* ATCC 6258 and the results were within the predetermined limits. To measure minimal lethal concentrations (MLCs), 20 µl samples were taken from each negative tube plus the first tube showing growth (to serve as a growth control) after MIC reading to SDA plates and incubated at 35 °C for 48 h/72 h (*Candida* species and *Aspergillus*/*Cryptococcus neoformans*) or at 30 °C for 7 days (dermatophytes). MLC values were determined as the lowest concentration of the oil causing fungal death. All experiments

were performed in triplicate and repeated whenever the results of each triplicate did not agree. A range of values is presented when different results were obtained.

Mechanism of action

Germ tube inhibition assay.

Cell suspensions from overnight SDA cultures of *Candida albicans* strains ATCC 10231, D5 and M1 were prepared in NYP medium [*N*-acetylglucosamine (Sigma; 10^{-3} mol l^{-1}), Yeast Nitrogen Base (Difco; 3.35 g l^{-1}), proline (Fluka; 10^{-3} mol l^{-1}) and NaCl (4.5 g l^{-1}), pH 6.7±0.1] (Marichal *et al.*, 1986) and adjusted to obtain a density of $1.0\pm0.2\times10^{6}$ c.f.u. ml^{-1}. The essential oil was diluted in DMSO and added in 10 µl volumes to 990 µl of the yeast suspensions (final DMSO concentration of 1%, v/v), obtaining a series of subinhibitory concentrations (as low as 1/64 of the MIC). The samples were incubated for 3 h at 37 °C without agitation and 100 cells from each sample were then counted in a haemocytometer. The percentage of germ tubes was determined as the number of cells showing hyphae at least as long as the diameter of the blastospore. Cells showing a constriction at the point of connection of the hypha to the mother cell, typical for pseudohyphae, were excluded. The results are presented as means±standard deviation (SD) of three separate experiments.

Flow cytometry.

Yeast suspensions were prepared in PBS solution with 2% (w/v) D-glucose from overnight SDA cultures of *Candida albicans* ATCC 10231 at 35 °C and adjusted, using a haemocytometer, to a final density of $2.0\pm0.2\times10^{6}$ c.f.u. ml^{-1}. Serial twofold dilutions of the essential oil (final concentrations of 0.64–10.0 µl ml^{-1}) and a single solution of amphotericin B at 2 µg ml^{-1} (four times the respective MIC of 0.5 µg ml^{-1}) in PBS with 2% (w/v) D-glucose were added to the cell suspensions and the mixtures were incubated at 35 °C in a humid atmosphere without agitation for 30 min, 4 h or 24 h. Drug-free control tubes were included in each experiment. After this period, the cells were washed in PBS and resuspended in 500 µl PBS with 2% (w/v) D-glucose for FUN-1 (Invitrogen) staining and PBS for propidium iodide (PI; Sigma) staining. Five microlitres of the FUN-1 and PI solutions in DMSO and PBS, respectively, were added to the cell suspensions to obtain final concentrations of 0.5 µM FUN-1 and 1.0 µg PI ml^{-1}. FUN-1-stained cells were incubated for a further 20 min at 35 °C, away from incident light, while PI-stained samples were read after about 10 min at room temperature. Unstained cell suspensions were included as autofluorescence controls. Flow cytometry was performed using a FACSCalibur (Becton Dickinson Biosciences) flow cytometer with a 488 nm blue argon laser emitting at 15 mW and the results were analysed using CellQuest Pro Software (Becton Dickinson). Intrinsic parameters (forward and side scatter, for relative cell size and complexity analysis) 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 for a minimum of 7500 events per sample using logarithmic scales. Markers (M1) were adjusted to include a maximum of 5% of the cells in monoparametric histograms of the fluorescence intensity of control samples (see Fig. 1 for examples) 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. Results are presented as means±SD of at least three replicate experiments.

Results and Discussion

Chemical compositions of the essential oils

The essential oils were obtained in yields ranging from 0.7 to 1.2% (v/w). A total of 51 compounds were identified, representing 93.2% (sample A) and 95.3% (sample B) of the total volatile oils. The oils were characterized by high contents of oxygen-containing monoterpenes (69.5 and 75.7%), followed by monoterpene hydrocarbons (17.1 and 15.5%). The main constituents of the oils were 1,8-cineole (34.5% and 42.2%), camphor (13.4%), α-pinene (9.0%) and linalool (7.9 and 6.7%). Sesquiterpenic compounds attained only 4.8 and 2.3%.

In a previous study carried out by Garcia-Vallejo (1992), some individual samples of *L. viridis* from the south of Portugal and Spain were analysed. The chemical composition of these samples was very similar to that of our collective samples, 1,8-cineole being the major component in all samples. This fact points to a very high homogeneity in the composition of the essential oils of *L. viridis* from Portugal and Spain.

Antifungal activity of the essential oils

The essential oil was used to evaluate the antifungal activity against several pathogenic strains involved in human diseases. Various degrees of inhibition were registered against all the fungi tested (Table 1).

The highest antifungal activity was observed against dermatophyte strains and *Cryptococcus neoformans*, with MIC and MLC values ranging from 0.32 to 0.64 µl ml^{-1}. For *Candida* strains, MIC and MLC values ranged from 0.64 to 2.5 µl ml^{-1}. The oil was less effective against *Aspergillus* strains (Table 1). The higher susceptibility of dermatophytes has also been reported for other essential oils (Salgueiro *et al.*, 2004; Pinto *et al.*, 2006, 2009; Zuzarte *et al.*, 2009).

For most of the dermatophytes, *Cryptococcus neoformans* and *Candida* strains, the MIC was equivalent to the MLC, indicating a clear fungicidal effect of *L. viridis* essential oil.

The major constituents of the oil (1,8-cineole, camphor, α-pinene and linalool) were also assayed individually for their antifungal activity. 1,8-Cineole and camphor displayed the lowest antifungal activity against all strains but α-pinene proved to be a very active compound, particularly against

dermatophyte strains (Table 1). Since the essential oils are complex mixtures of several compounds, it is difficult to attribute their biological activity to a particular constituent. Usually, major compounds are the ones responsible for the antifungal activity of the essential oils. However, some studies show that minor components may have a crucial role in the biological activity of the oils (Koroch *et al.*, 2007). Our results seem to indicate that the activity of *L. viridis* essential oil is mainly due to the presence of α-pinene in the oil.

Mechanism of action of the essential oil

The essential oil was also found to inhibit filamentation in the tested *Candida albicans* strains at concentrations of 0.08–0.16 μl ml^{-1}, well below the corresponding MICs (Table 2). This marked difference between MICs and filamentation-inhibiting concentrations seems to suggest that different mechanisms may be responsible for these two biological activities. This finding is particularly relevant considering the fact that filamentation has long been shown to be essential for virulence in *Candida albicans* (Mitchell, 1998). In fact, inhibition of the dimorphic transition alone has been suggested to be sufficient to treat disseminated candidosis, thus proving to be a good target mechanism in the development of novel antifungal agents (Saville *et al.*, 2006). Additionally, flow cytometry analyses after FUN-1 staining have revealed a dose-dependent inhibition of cell metabolism after short incubation periods with the oil at concentrations starting from the respective MIC (Fig. 2). The dye FUN-1 freely permeates fungal plasma membranes into the cell and is distributed in the cytoplasm as a bright diffuse green/yellow stain. In normal fungal cells, the dye is metabolized into orange/red cylindrical intravacuolar structures. However, in cells with impaired metabolism, this change does not occur and FUN-1 remains in the cytoplasm in a diffuse pattern, indicating a disorder in cell metabolism (Millard *et al.*, 1997). This change was detected by a reduction of orange fluorescence (FL2 channel) in cells exposed to the essential oil in comparison to untreated controls (Figs 1 and 2). To observe PI staining of the test cells, on the other hand, a 4 h incubation with a concentration of the oil at least two $\log_2$ dilutions above the MIC was required (Fig. 2). The nucleic acid binding fluorescent probe PI penetrates only dead cells showing severe membrane lesions (Pina-Vaz *et al.*, 2001). The observed asymmetry between metabolic inhibition and cell death shows that cells clearly become metabolically inactive in the presence of the essential oil of *L. viridis* before it leads to cell death, thus appearing to exclude a potential mechanism of antifungal action relying on primary leakage of cytoplasmic contents due to direct damage to cell membranes. It is worth pointing out that under the same experimental conditions, the reference fungicidal drug amphotericin B tested at a concentration two $\log_2$ dilutions above the respective MIC did not lead to PI staining (Fig. 2). After 24 h, however, over 90% of the cells presented positive PI staining with amphotericin B treatment (data not shown).

The mechanism of action of essential oils remains somewhat controversial. While some studies suggest that the compounds may penetrate the micro-organism and react with active sites of

enzymes and/or interfere with cellular metabolism, most evidence supports direct disruption of cellular membranes and concentration-dependent pro-oxidant cytotoxic effects (Bakkali *et al.*, 2008). Concerning antifungal activity specifically, the mechanism of action of the oils seems to involve penetration through cell walls and direct damage to both cytoplasmic and mitochondrial membranes (Bakkali *et al.*, 2008). This leads to changes in permeability leading to leakage and ultimately resulting in cell death (Bakkali *et al.*, 2008). Bearing this knowledge in mind, the present results for the specific case of *Candida albicans* treated with the essential oil of *L. viridis* are consistent with a mechanism of action starting from damage to mitochondrial membranes, considering the rapid metabolical arrest appearing earlier and in the presence of lower concentrations of the essential oil than those required to cause cell death. In such a scenario, changes in mitochondrial permeability would disturb electron flow in the electron transport chain, generating free radicals that proceed to damage essential biomolecules (including lipids, proteins and nucleic acids). Given a high enough concentration and/or exposure time, the oil may eventually lead to disruption of cytoplasmic membranes and cell death. Further data are now required to definitively confirm these speculations, however.

The wide-spectrum antifungal activity and high potency of the oil of *L. viridis* support further investigations into the development of this essential oil for clinical use in the management of superficial and/or mucosal fungal infections.

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Fig. 1. Flow cytometry histograms showing fluorescence intensity versus number of events (*Candida albicans* ATCC 10231 cells) in relative units. (a) Orange fluorescence (FL2 channel) intensity of samples stained with FUN-1. (b) Red fluorescence (FL3 channel) intensity of samples stained with PI. Af, Autofluorescence of unstained cells; control, untreated cells; AmB, cells treated with amphotericin B at 2.0 μg ml^{-1}; EO, cells treated with the essential oil of *L. viridis* at 10.0 μl ml^{-1}.

Fig. 2. Percentage (and SD) of M1-gated *Candida albicans* ATCC 10231 cells, analysed by flow cytometry, after treatments with different concentrations of the essential oil of *L. viridis* in comparison with amphotericin B (AmB) and an untreated control. Cells were treated with the compounds for 30 min for staining with FUN-1 and 4 h for staining with PI. *MIC of the essential oil; [a]in μl ml^{-1} for the essential oil and μg ml^{-1} for AmB.

Table 1. Antifungal activity (MIC and MLC) of the essential oil of *Lavandula viridis* (sample A) for *Candida*, dermatophyte and *Aspergillus* strains

Results were obtained from three independent experiments performed in duplicate. When different MIC values were obtained, a range of values is presented. NT, Not tested.

Strain	*L. viridis*		1,8-Cineole		Camphor		α-Pinene		Linalool		Fluconazole		Amphotericin B	
	MIC*	MLC*	MIC*	MLC*	MIC*	MLC*	MIC*	MLC*	MIC*	MLC*	MIC†	MLC†	MIC†	MLC†
Candida albicans ATCC 10231	2.5	2.5	10	10	>20	>20	0.64–1.25	0.64–1.25	5	5	1	>128	NT	NT
Candida albicans D5	1.25	1.25	5–10	5–10	≥20	>20	0.32	0.32	5	5	64	>128	NT	NT
Candida albicans M1	2.5	2.5	10	10	>20	>20	0.64–1.25	0.64–1.25	5	5–10	2	128	NT	NT
Candida tropicalis ATCC 13803	1.25–2.5	1.25–2.5	20	20	>20	>20	1.25	1.25–2.5	5	5	4	>128	NT	NT
Candida krusei H9	1.25–2.5	1.25–2.5	10	10	>20	>20	0.16–0.32	0.32	10	10	64	64–128	NT	NT
Candida guilliermondii MAT23	0.64–1.25	0.64–1.25	10	10	>20	>20	0.64	0.64	5	10	8	8	NT	NT
Candida parapsilopsis ATCC 90018	1.25	1.25	10	10	>20	>20	0.32	0.32	10	10	<1	<1	NT	NT
Cryptococcus neoformans CECT 1078	0.64	0.64	5–10	10	>20	>20	0.08	0.32	5	5	16	128	NT	NT
Trichophyton mentagrophytes FF7	0.32–0.64	0.64	5	5	>20	>20	0.32	0.32–0.64	1.25	2.5	16–32	32–64	NT	NT
Trichophyton mentagrophytes var. *interdigitale* CECT 2958	0.32–0.64	0.64	10–20	10– ≥20	>20	>20	0.32	0.32	2.5	2.5–5	128	≥128	NT	NT
Trichophyton rubrum CECT 2794	0.32	0.32	2.5–5	5	>20	>20	0.08	0.08	1.25	1.25–2.5	16	64	NT	NT
Trichophyton verrucosum CECT 2992	0.32	0.32–0.64	10	10	>20	>20	1.25	1.25	1.25–2.5	1.25–2.5	>128	>128	NT	NT
Microsporum canis FF1	0.32	0.32	5	5	>20	>20	0.16	0.16–0.32	2.5	2.5	128	128	NT	NT
Microsporum gypseum CECT 2905	0.64	0.64	5–10	5	>20	>20	0.16	0.16	1.25–2.5	2.5	128	>128	NT	NT
Epidermophyton floccosum FF9	0.32	0.32	5	5	>20	>20	0.16	0.16	1.25–2.5	2.5	16	16	NT	NT
Aspergillus niger ATCC16404	2.5	20	10	>20	>20	>20	2.5	5	5	≥20	NT	NT	1–2	4
Aspergillus fumigatus ATCC 46645	2.5	5–10	10	10–20	20	>20	1.25	1.25–2.5	2.5	20	NT	NT	2	4
Aspergillus flavus F44	5	10–20	20	20	20	20	1.25	1.25	10	≥20	NT	NT	2	8

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

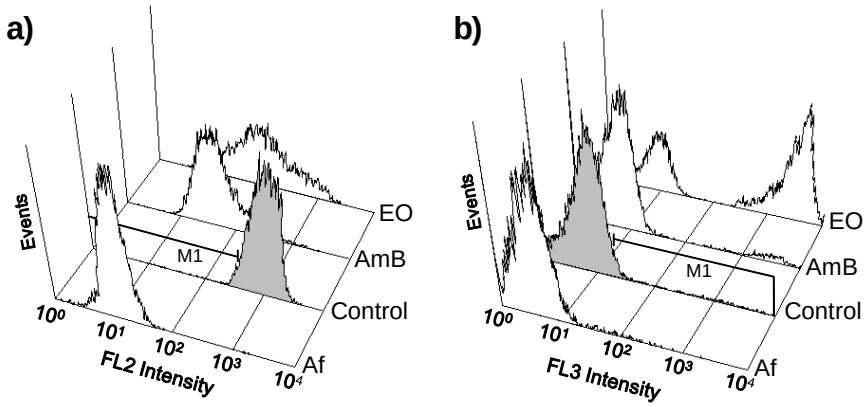
†MIC and MLC were determined by a macrodilution method and expressed in µg ml^{-1} (w/v).

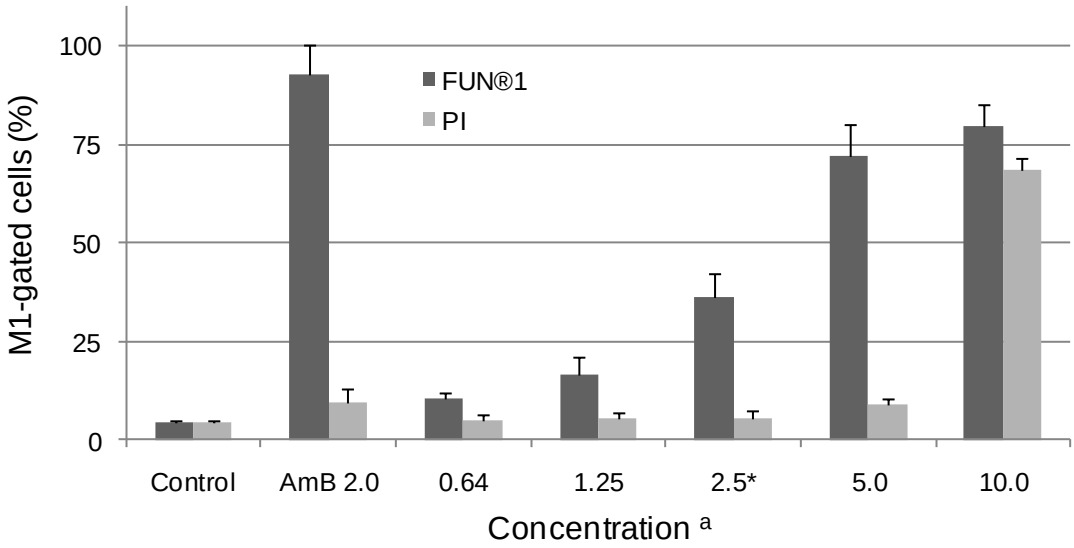
Table 2. Percentage of germ tubes after treatment of three *Candida albicans* strains with subinhibitory concentrations of the essential oil of *L. viridis* for 3 h in a filamentation-inducing medium at 37 °C

Results are presented as mean (±SD) values of three independent experiments. Concentration is in µl ml^{-1} (v/v).

Strain	MIC/16 (concn)	MIC/32 (concn)	MIC/64 (concn)	Control*
C. albicans ATCC 10231	6.7±6.4 (0.16)	54.0±5.6 (0.08)	89.7±2.1 (0.04)	94.0±5.2
C. albicans D5	0.0±0.0 (0.08)	47.5±10.6 (0.04)	66.0±5.7 (0.02)	86.8±9.4
C. albicans M1	0.3±0.6 (0.16)	42.7±10.5 (0.08)	75.0±4.2 (0.04)	87.5±6.6

*Untreated samples including the solvent (1% DMSO) only.


a)
Events
M1
EO
AmB
Control
Af
10⁰
10¹
10²
10³
10⁴
FL2 Intensity
b)
Events
M1
EO
AmB
Control
Af
10⁰
10¹
10²
10³
10⁴
FL3 Intensity


100
75
50
25
0
M1-gated cells (%)
FUN®1
PI
Control
AmB 2.0
0.64
1.25
2.5*
5.0
10.0
Concentration a