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ARTICLE

Antifungal activity of *Ferulago capillaris* essential oil against *Candida*, *Cryptococcus*, *Aspergillus* and dermatophyte species

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Abstract This study evaluates the composition, antifungal activity and mechanism of action of the essential oil of *Ferulago capillaris* (Link ex Spreng.) Cout. and its main components, limonene and α-pinene, against clinically relevant yeasts and moulds. Essential oil from the plant's aerial parts was obtained by hydrodistillation and analysed by gas chromatography (GC) and gas chromatography/mass spectrometry (GC-MS). Essential oil showed high contents of limonene (30.9 %) and α-pinene (35.8 %). Minimum inhibitory concentrations (MICs) were measured according to the reference Clinical and Laboratory Standards Institute (CLSI) broth macrodilution protocols. Cell suspensions were subcultured in solid medium and the minimum fungicidal concentrations (MFCs) were rendered. The effect of essential oil on germ tube formation, mitochondrial function and ergosterol biosynthesis was investigated. Essential oil and α-pinene displayed low and similar MIC and MFC values against tested organisms (0.08 to 5.0 μL/mL), while limonene showed a weaker activity (0.32 to 20 μL/mL). Essential oil inhibited germ tube formation at sub-inhibitory concentrations on *Candida albicans*. The exposure of *C. albicans* to the essential oil resulted in impairment of mitochondrial functions in a dose-dependent manner. No difference in ergosterol content was observed in essential oil-treated *C. albicans*. *F. capillaris* and α-pinene display a broad fungicidal activity. The fungicidal activity of *F. capillaris* on *C. albicans* can be related to an induced oxidative stress which affects enzymes activity and the membrane potential of mitochondria. The essential oil of *F. capillaris* was shown to have potential for use in the development of clinically useful therapeutic preparations, particularly for topical application in the management of superficial mycoses.

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Introduction

During the last few decades, fungal infections have been considered a serious health and life-threatening disease, particularly among immunocompromised patients. As the numbers of these patients gradually grow, the incidence of opportunistic fungal infections will increase. In addition, many pathogenic fungi are also responsible for a wide range of superficial infections affecting apparently healthy individuals [1]. The increasing impact of these infections, incidence of drug-resistant pathogens and toxicity of available antifungal drugs, at least in part, are major encouraging factors that lead to heightened interest in the study of alternative natural products such as essential oils [2–4]. Essential oils are natural products formed by several volatile compounds, mainly terpenic compounds. Monoterpenes (2 units of isoprene) and sesquiterpenes (3 units of isoprene) are usually the main compounds found in essential oils [5].

The Apiaceae family includes a high number of aromatic plants that are known to possess antimicrobial properties, particularly due to their essential oils contents. Being a perennial genus of the Apiaceae family, the genus *Ferulago* is represented by 40 species around the world. These species

have been used in folk medicine for their sedative, tonic, digestive and anti-parasitic effects and for the treatment of ulcers, snake bites, haemorrhoids, headache and diseases of the spleen [6–10]. The antimicrobial activity has previously been reported for some *Ferulago* species, such as *F. thyrsiflora*, *F. sylvatica*, *F. nodosa*, *F. bernardii*, *F. longistylis* and *F. angulata* subsp. *carduchorum* [10–13].

In Portugal, the genus *Ferulago* is represented by only one taxon, *Ferulago capillaris* (Link ex Spreng.) Cout. [14], which is an endemic plant from the Iberian Peninsula. Concerning phytochemical studies, only the chemical composition of the root extract of this taxon has been documented [15]. Considering the potential use of this plant in the treatment of cutaneous infections, the objective of the present work was to characterise the chemical composition of the essential oil by gas chromatography (GC) and gas chromatography/mass spectrometry (GC-MS) and then measure the minimum inhibitory and lethal concentrations (MICs and MLCs, respectively) for the oil and their main constituents against a collection of human pathogenic species (including *Candida* spp., *Cryptococcus neoformans*, *Aspergillus* spp. and several dermatophyte isolates). The effect of the essential oil on the germ tube formation, cell membrane composition and on mitochondrial function in *C. albicans* were also studied.

Materials and methods

Plant material

Aerial parts (umbels with mature seeds) of *F. capillaris* were collected in the flowering stage in Central Portugal (Guarda). A voucher specimen was deposited at the herbarium of the Faculty of Pharmacy, University of Coimbra.

Essential oil isolation and analysis

The essential oil from air-dried plant material was isolated by hydrodistillation for 3 h, using a Clevenger-type apparatus according to the European Pharmacopoeia [16]. The oils were preserved in a sealed vial at 4 °C. Oils 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 [17].

The volatile compounds were identified by both their retention indices and their mass spectra. Retention indices, calculated by linear interpolation relative to the 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 the literature data [18, 19]. Relative amounts of individual components were calculated based on GC peak areas without FID response factor correction.

Reference compounds

Authentic samples of limonene (Fluka, 99.0 % purity) and α-pinene (Fluka, 99.0 % purity), were used. Fluconazole was kindly provided by Pfizer as the pure powder and amphotericin B was obtained from Sigma (80 % purity).

Fungal organisms

The antifungal activity of *F. capillaris* essential oil and its major constituents were evaluated against 17 isolates of medically important fungi (*Candida*, *Cryptococcus*, *Aspergillus* and dermatophyte strains): four *Candida* type strains from the American Type Culture Collection (ATCC) (*C. albicans* ATCC 10231, *C. tropicalis* ATCC 13803, *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 90018); four clinical isolates of *Candida* (*C. albicans* D5, *C. albicans* M1, *C. glabrata* D10R and *C. dubliniensis* CD1); two ATCC type strains and one clinical strain of *Aspergillus* spp. (*A. niger* ATCC 16404, *A. fumigatus* ATCC 46645 and *A. flavus* F44); two type strains of dermatophytes from Colección Española de Cultivos Tipo (CECT) (*Trichophyton rubrum* CECT 2794, *Microsporum gypseum* CECT 2905) and three clinical isolates (*Trichophyton mentagrophytes* FF7, *Microsporum canis* FF1 and *Epidermophyton floccosum* FF9); one CECT type strain of *C. neoformans* 1078. All strains were stored in Sabouraud dextrose broth with 20 % glycerol at −80 °C and subcultured in Sabouraud dextrose agar (SDA) before each test, to ensure optimal growth conditions and purity.

Antifungal susceptibility test

In order to determine the MICs of *F. capillaris* essential oil and its major constituents (limonene and α-pinene), broth macrodilution methods based on the Clinical and Laboratory Standards Institute (CLSI) reference protocols M27-A3 and M38-A2 [20, 21], for yeasts and filamentous fungi, respectively, were used. Briefly, the yeast cell or spore suspensions were prepared in 0.85 % NaCl and diluted at appropriate densities in RPMI 1640 broth (with L-glutamine, without bicarbonate and with phenol red as the pH indicator) from SDA or potato dextrose agar (PDA) cultures, and distributed into 12×75-mm glass test tubes. Serial two-fold dilutions of the oil and their main components were prepared in dimethyl sulfoxide (DMSO) and added to the cell suspensions in order to obtain test concentrations ranging from 0.08 to 20.0 μL/mL (the final DMSO concentration never exceeded 1 % v/v). In addition,

reference antifungal compounds, fluconazole for yeasts and dermatophytes and amphotericin B for *Aspergillus* spp. were used as standard antifungal drugs. Quality control was performed by testing fluconazole and amphotericin B with the reference strains *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258, and the results were within the predetermined limits (data not shown). Oil-free and 1 % DMSO growth controls, as well as a sterility control, were included. The tubes were incubated aerobically at 35 °C for 48 h/72 h (*Candida* spp. and *Aspergillus* spp./*C. neoformans*) or at 25 °C for 7 days (dermatophytes). The MIC values were determined as the lowest concentration of the oil that revealed 100 % growth inhibition. To determine the MLCs, 20 μL of samples from each negative tube and the first tube exhibiting growth (to serve as a growth control) were taken and spotted onto SDA plates and incubated at 35 °C for 48 h/72 h (*Candida* spp. and *Aspergillus* spp./*C. neoformans*) or at 25 °C for 7 days (dermatophytes). The MLC values were determined as the lowest concentration of the oil which results in fungal death. All experiments were performed in duplicate and repeated three times, yielding essentially the same results (a range of values is presented when different results were obtained).

Assessment of ergosterol biosynthesis

In order to investigate an effect of *F. capillaris* essential oil on ergosterol biosynthesis, *C. albicans* ATCC 10231 was grown in RPMI medium supplemented with 2 % glucose (Difco) and incubated at 37 °C in a shaking water bath. After incubation with and without the essential oil at the sub-inhibitory concentrations of 0.04–0.16 μL/mL or 0.25 μg/mL of fluconazole (as a control), yeast cells were collected by centrifugation at 4,000 rpm for 5 min, washed twice with sterile distilled water and the pellet was dried at 50 °C. To isolate ergosterol, the yeast powder was mixed with 25 % ethanolic KOH and incubated at 85 °C for 1 h in a water bath. The saponified mixture was further extracted with 1:2 mL portion of distilled water and hexane. The hexane extract was evaporated and the residue was suspended in 5 mL of methanol before analysis by high-performance liquid chromatography (HPLC) [22].

Germ tube inhibition assay

Cell suspensions of each isolate of *C. albicans* (*C. albicans* ATCC 10231, *C. albicans* D5 and *C. albicans* M1) from 18–24-h SDA cultures 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) and NaCl (4.5 g/L), pH 6.7±0.1] [23] and adjusted to obtain a density of $(1.0\pm0.2)\times10^{6}$ CFU/mL. *F. capillaris* essential oil was diluted in DMSO and added in 10- to 990-μL volumes of the yeast suspensions (final DMSO concentration of 1 %, *v/v*) to obtain appropriate sub-inhibitory concentrations (1/8, 1/16, 1/32 and 1/64 of the MIC values). Drug-free control suspensions with and without DMSO were included for each *C. albicans* strain. After 3 h of incubation at 37 °C, 100 cells from each sample were counted using a haemocytometer and the percentage of germ tubes was determined. Germ tubes were considered positive when they were at least as long as the blastospore. Protuberances showing a constriction at the point of connection to the mother cell, typical for pseudohyphae, were excluded. The results are presented as means ± standard deviation of three separate experiments.

Effect of the essential oil on mitochondrial function

Assessment of mitochondrial reductase enzymes

To measure the effect of *F. capillaris* essential oil on the mitochondrial reductase activity of fungi, Thiazolyl Blue Tetrazolium Bromide (MTT: [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide]) assay was performed according to the method of Lopes et al., with some modifications [24]. Briefly, *C. albicans* ATCC 10231 cell suspensions were prepared in NaCl 0.85 % and the turbidity was adjusted to 0.5 McFarland standard. A 1:50 followed by a 1:20 dilution was performed in RPMI culture medium. 500 μL of RPMI was added to the same volume of the previous cell suspension into a 12-well plate and incubated overnight (18–24 h at 37 °C). After the incubation period, cells were carefully homogenated, transferred to Eppendorf tubes and centrifuged at 10,000 rpm for 5 min. The supernatant was removed and 1 mL of the test compound was added to each Eppendorf tube, in the required concentration. The mixture was homogenised, transferred to the 12-well plate and incubated for 1 h at 37 °C. After the exposure time, cell suspensions were centrifuged, the supernatant removed and 500 μL of MTT (Sigma-Aldrich, St. Louis, MO, USA) solution (0.5 mg/mL prepared in RPMI) were added to each well and left incubating for 30 min at 37 °C. The yellow tetrazolium salt MTT converted by mitochondrial dehydrogenases of metabolically active cells to an insoluble purple formazan product was then solubilised with 300 μL of DMSO. The extent of the reduction to formazan within the cells was quantified by measuring the absorbance at 510 nm in a Multiskan Ascent plate reader (Thermo Electron Corporation).

Monitoring of the mitochondrial membrane potential

In order to observe the effect of *F. capillaris* essential oil on the mitochondrial membrane potential of fungal cells, rhodamine 123 uptake assay was performed according to the protocol of Ludovico et al., with some modifications [25]. Briefly, an inoculum suspension was prepared in phosphate-buffered saline (PBS) from 18-h SDA culture of *C. albicans* ATCC 10231 and the cell density was adjusted to that

produced by a 2.0 McFarland standard. After incubation with each concentration of the essential oil ranging from half to twice the MIC or with PBS (as a control) at 37 °C for 30 min, 5 μL of 0.5 mM in DMSO of rhodamine 123 (Sigma-Aldrich, St. Louis, MO, USA) was added in each tube of 1 mL treated suspension and further incubated at 37 °C for 10 min. The cell pellet was collected by centrifugation at 10,000 rpm for 10 min, resuspended in 1 mL of PBS and then transferred to a 96-well microplate. Sodium azide (Sigma-Aldrich, St. Louis, MO, USA) was used as a mitochondrial respiratory chain inhibitor. The fluorescence intensity was measured in a fluorescence Microplate Reader (Synergy™ HT, BioTek Instruments, Winooski, VT, USA) operated by Gen5 software, with excitation and emission wavelengths of 485/20 nm and 528/20 nm, respectively.

Hemolytic activity

Human red blood cells from healthy individuals were used to test the haemolytic activity of *F. capillaris* essential oil, limonene and α-pinene according the protocol described by Ahmad et al. [26]. Amphotericin B was used as a commercially antifungal drug.

Statistical analysis

Data were analysed by using GraphPad Prism software (GraphPad Software, San Diego, CA, USA) (version 5.02 for Windows). One-way analysis of variance (ANOVA), using the Dunnett multiple comparison test, was carried out on data obtained from three independent assays performed in duplicate for each sample. Levels of statistical significance at $p<0.05$, $p<0.01$ and $p<0.001$ were used.

Results

Essential oil composition and antifungal activity

The essential oil was obtained in a yield of 0.6 % (*v/w*). The oil was analysed by GC and GC-MS, and its qualitative and quantitative compositions are presented in Table 1, where compounds are listed according to their elution on a polydimethylsiloxane column. Forty-four constituents were found, representing 98.0 % of the oil. Monoterpene hydrocarbons were shown to be the main group of constituents, with α-pinene and limonene being the main compounds (35.8 % and 30.9 %, respectively). Sesquiterpenic compounds only attain 2.8 % of the oil.

The *F. capillaris* essential oil was evaluated against a wide range of human pathogenic fungi, including *Candida* spp., *C. neoformans*, *Aspergillus* spp. and dermatophytes, and revealed a broad spectrum of antifungal activity

Table 1 Constituents of the essential oil of *Ferulago capillaris* from Portugal

Compound[a]	RI SPB-1[b]	RI SW 10[c]	%
α-Thujene	922	1029	0.2
α-Pinene	930	1030	35.8
Camphene	943	1077	1.7
Sabinene	964	1128	0.6
β-Pinene	970	1118	1.5
Myrcene	980	1161	5.2
α-Phellandrene	997	1171	0.1
p-Cymene	1012	1272	2.0
α-Terpinene	1010	1187	t
Limonene	1020	1206	30.9
β-Phellandrene	1020	1215	6.0
Z-β-Ocimene	1025	1235	4.0
E-β-Ocimene	1035	1250	0.9
γ-Terpinene	1046	1249	0.5
trans-Sabinene hydrate	1050	1459	t
Z-Linalool oxide	1055	1439	1.1
Cymenene	1070	1439	1.0
Terpinolene	1076	1288	1.0
Linalool	1082	1543	0.3
E-p-2-Menthen-1-ol	1121	1621	0.4
Epoxyterpinolene	1125		0.6
Terpinene-4-ol	1158	1597	t
p-Cymene-8-ol	1159	1845	0.5
Myrtenal	1160	1621	0.4
α-Terpineol	1169	1692	0.1
Carvone	1212	1728	0.2
Geraniol	1233	1842	t
Linalyl acetate	1240	1555	t
Geranyl acetate	1359	1755	t
α-Copaene	1369	1487	0.4
β-Bourbonene	1378	1514	0.3
β-Elemene	1382	1585	0.1
E-Caryophyllene	1408	1590	0.3
γ-Elemene	1422	2136	0.1
α-Humulene	1442	1665	t
Allo-aromadendrene	1494	1638	0.1
Germacrene-D	1466	1699	0.3
β-Bisabolene	1495	1723	t
γ-Cadinene	1498	1751	t
δ-Cadinene	1508	1751	0.3
Germacrene B	1541	1818	0.3
Spathulenol	1554	2113	0.2
Caryophyllene oxide	1557	1968	0.2
α-Cadinol	1628	2218	t
Monoterpene hydrocarbons			91.4
Oxygen-containing monoterpenes			3.8
Sesquiterpene hydrocarbons			2.4
Oxygen-containing sesquiterpenes			0.4

Table 1 (continued)

Compound[a]	RI SPB-1[b]	RI SW 10[c]	%
Total identified			98.0

[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

(Table 2). Based on the geometric mean of MICs, a total of six tested *Candida* spp. isolates, including *C. albicans* ATCC 10231, *C. albicans* M1, *C. dubliniensis* CD1, *C. tropicalis* ATCC 13803, *C. glabrata* D10R and *C. parapsilosis* ATCC 90018, were considered as fluconazole-susceptible strains (MIC≤8 μg/mL), whereas the other two isolates of *Candida* spp. (*C. krusei* ATCC 6258 and *C. albicans* D5) were fluconazole-resistant strains (MIC≥64 μg/mL) [27]. Fortunately, the activity of *F. capillaris* essential oil could be observed in both fluconazole-susceptible and fluconazole-resistant strains of *Candida* spp., with MICs ranging from 0.16 to 1.25 μL/mL, with *C. tropicalis* and *C. parapsilosis* being less susceptible (MIC of 1.25 μL/mL). *C. neoformans* showed high susceptibility, with MIC values of 0.16 μL/mL. Similar results were observed for the five strains of tested dermatophytes, with MIC values of 0.32–0.64 μL/mL. Unfortunately, all the tested strains of *Aspergillus* spp. were less susceptible to the essential oil than the others. Interestingly, the MIC values of *F. capillaris* against all the tested organisms are almost equal to the MLC values (Table 2).

The antifungal activity of both major components, namely, α-pinene (35.8 %) and limonene (30.9 %), was also investigated against all the tested microorganisms. Limonene possesses less inhibitory activity against all the tested fungi when compared to the essential oil. Although α-pinene revealed less inhibitory activity against some tested strains, when compared to essential oil, it exhibited a potent effect against all dermatophytes and some pathogenic yeasts, *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 90018 and *C. neoformans* CECT 1078 at an MIC range of 0.08–0.32 μL/mL.

Germ tube inhibition assay

Being considered as fungicidal, the essential oil of *F. capillaris* was subjected to further investigations for

Table 2 Antimicrobial activity [minimum inhibitory and lethal concentrations (MICs and MLCs), respectively] of *Ferulago capillaris* essential oil and its components, limonene and α-pinene, against yeasts, dermatophytes and *Aspergillus* strains

Strains	*Ferulago capillaris*		Limonene		α-Pinene		Fluconazole		Amphotericin B	
	MIC[a]	MLC[a]	MIC[a]	MLC[a]	MIC[a]	MLC[a]	MIC[b]	MLC[b]	MIC[b]	MLC[b]
Candida albicans ATCC 10231	0.32–0.64	0.32–0.64	0.64–1.25	1.25–2.5	0.64–1.25	0.64–1.25	1	>128	N.T.	N.T.
Candida albicans D5	0.16–0.32	0.16–0.32	0.64	0.64	0.32	0.32	64	>128	N.T.	N.T.
Candida albicans M1	0.64	0.64–1.25	0.64	0.64	0.64–1.25	0.64–1.25	2	128	N.T.	N.T.
Candida dubliniensis CD1	0.16	0.16	0.64	0.64	0.64	0.64	1	>128	N.T.	N.T.
Candida tropicalis ATCC 13803	1.25	1.25	2.5	2.5	1.25	1.25–2.5	4	>128	N.T.	N.T.
Candida krusei ATCC 6258	0.16	0.16	0.64	0.64	0.16–0.32	0.16–0.32	64	64–128	N.T.	N.T.
Candida glabrata D10R	0.64	0.64	2.5	2.5	0.64–1.25	1.25	8	8	N.T.	N.T.
Candida parapsilosis ATCC 90018	1.25	1.25	1.25–2.5	1.25–2.5	0.32	0.32	<1	<1	N.T.	N.T.
Cryptococcus neoformans CECT 1078	0.16	0.32	0.32	0.64	0.08	0.32	16	128	N.T.	N.T.
Trichophyton mentagrophytes FF7	0.64	0.64	2.5	2.5	0.32	0.32–0.64	16–32	32–64	N.T.	N.T.
Microsporum canis FF1	0.32	0.64–0.32	0.64–1.25	1.25	0.16	0.16–0.32	128	128	N.T.	N.T.
T. rubrum CECT 2794	0.32	0.32	0.64	0.64	0.08	0.08	16	64	N.T.	N.T.
M. gypseum CECT 2905	0.64	0.64	1.25	1.25	0.16	0.16	128	>128	N.T.	N.T.
Epidermophyton floccosum FF9	0.64	0.64	1.25	1.25	0.16	0.16	16	16	N.T.	N.T.
Aspergillus niger ATCC 16404	1.25	2.5	5	20	2.5	5	N.T.	N.T.	1–2	4
A. fumigatus ATCC 46645	0.64–1.25	1.25	5	5–10	1.25	1.25–2.5	N.T.	N.T.	2	4
A. flavus F44	1.25	1.25	5–10	10	1.25	1.25	N.T.	N.T.	2	8

N.T. not tested. The results were obtained from three independent experiments performed in duplicate

[a] MICs and MLCs were evaluated by the macrodilution method and are expressed in μL/mL (*v/v*)

[b] MICs and MLCs were evaluated by the macrodilution method and are expressed in μg/mL (*w/v*)

filamentation inhibitory activity against one ATCC type strain and two clinical isolates of *C. albicans*, namely, *C. albicans* ATCC 10231, *C. albicans* D5 and *C. albicans* M1, respectively. A lowest concentration of the essential oil that almost completely inhibited germ tube formation in all strains of *C. albicans* was 1/16 times of the essential oil's MIC (Table 3).

Effect of the *F. capillaris* essential oil on ergosterol biosynthesis

In order to clarify the mechanism of action of the essential oil, the inhibition of ergosterol synthesis activity was investigated. In this study, the effect of *F. capillaris* essential oil on the ergosterol amount in *C. albicans* ATCC 10231 was compared to fluconazole drug and 1 % DMSO (as a control). After incubation of the yeast with sub-inhibitory concentrations (0.04 to 0.16 μL/mL) of essential oil, no difference in the amount of ergosterol content was observed in essential oil-treated organisms compared to a control, whereas a reduction of the ergosterol content of over 60 % was found in fluconazole-treated (0.25 μg/mL) *C. albicans* (Fig. 1).

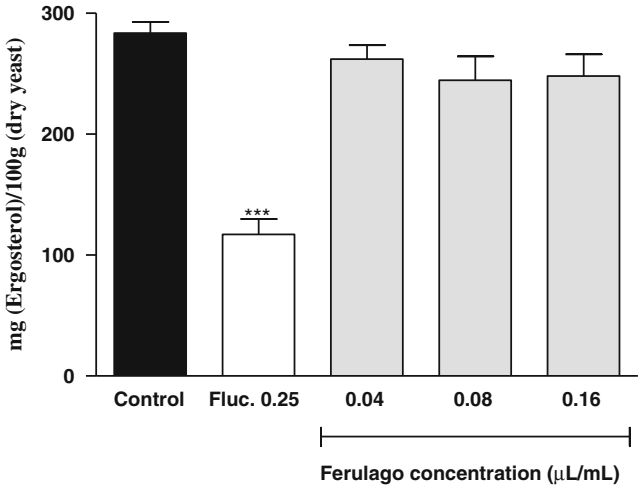

Fig. 1 Ergosterol concentration on *Candida albicans* ATCC 10231 cells treated with *Ferulago capillaris* essential oil. Values of each group were determined by high-performance liquid chromatography–diode array detector (HPLC-DAD) (detection wavelength 280 nm) and expressed as mg of ergosterol/100 g of dry yeast (mean ± standard deviation of three independent assays). Fluconazole (0.25 μg/mL) was used as a control. *$p<0.05$, **$p<0.01$, ***$p<0.001$

Effect of the *F. capillaris* essential oil on mitochondrial activity

In order to determine the metabolic activity of *C. albicans* ATCC 10231, the formazan product obtained by the action of mitochondrial reductases on MTT was performed [28]. After exposure of the tested yeast cells with various concentrations of the *F. capillaris* essential oil, over 50 % relative reduction in the activity of mitochondrial reductase enzymes was observed at essential oil concentrations ranging from 1/8 (0.08 μL/mL) to one times the MIC (0.64 μL/mL) when compared to a control (Fig. 2). Taking into account that the antifungal effect of the essential oil could be related, at least in part, by the disruption of mitochondrial enzymes activity, the membrane potential was investigated by the accumulation of rhodamine 123.

Surprisingly, the highest concentrations of the essential oil, such as twice the MIC (1.25 μL/mL), only caused a slight decrease in the level of rhodamine 123 accumulation, whereas the lowest concentrations of the oil were more influential on the rhodamine uptake ability of the treated *C. albicans*.

Table 3 Influence of sub-inhibitory concentrations of the essential oil of *Ferulago capillaris* on the germ tube formation of three *Candida albicans* strains. The results are expressed as the mean ± standard deviation of a minimum of three independent experiments performed in duplicate

		C. albicans ATCC 10231	*C. albicans* D5	*C. albicans* M1
Control[a]		96.0±3.6	86.0±5.4	89.3±3.3
F. capillaris	MIC/64	91.7±5.1	83.3±6.17	82.0±3.6
	(Conc.[b])	(0.01)	(0.005)	(0.01)
	MIC/32	77.5±9.2	76.0±9.5	66.7±11.0
	(Conc.)	(0.02)	(0.01)	(0.02)
	MIC/16	1.0±1.0	2.3±2.2	3.0±3.3
	(Conc.)	(0.04)	(0.02)	(0.04)
	MIC/8	0.3±0.6	0.0±0.0	0.3±0.6
	(Conc.)	(0.08)	(0.04)	(0.08)

[a] Untreated samples including 1 % DMSO

[b] Absolute concentration in μL/mL

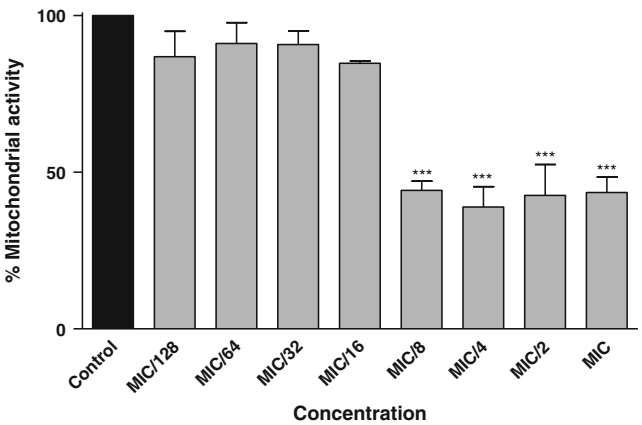

Fig. 2 Mitochondrial activity of *Candida albicans* ATCC 10231 cells treated with different concentrations of *Ferulago capillaris* essential oil. Values are expressed as % of mitochondrial activity relative to the control (mean ± standard deviation of three independent assays). *$p<0.05$, **$p<0.01$, ***$p<0.001$. Concentrations lower than MIC/128 (0.005 μL/mL) presented a mitochondrial activity similar to the untreated cells

However, the reduction in rhodamine 123 accumulation was reversed again when the concentration of the oil was lower than 1/32 times the MIC (Fig. 3).

Hemolytic activity

At 2.5 μL/mL (MIC × 4), *F. capillaris* oil, limonene and α-pinene showed 18 %, 9 % and 14 % of haemolysis, respectively. Amphotericin B at 320 μg/mL showed 45 % of haemolysis. These results indicate that the tested compounds have low cytotoxic activity, even for concentrations four times the MIC.

Discussion

F. capillaris oil is characterised by a large amount of monoterpene hydrocarbons, with the cyclic terpene hydrocarbons α-pinene and limonene being the main compounds (35.8 % and 30.9 %, respectively). The pinenes (α-pinene and β-pinene) are the most common naturally occurring bicyclic terpene hydrocarbons and they occur in varying ratios in several essential oils. Limonene is a monocyclic terpene hydrocarbon, with the *p*-menthadiene structure. It is a liquid with lemon-like odour and occurs abundantly in many *Citrus* essential oils. High amounts of both α-pinene and limonene were only identified in the oil of *F. isaurica* from Turkey [29]. High amounts of other compounds like myrcene, α- and β-phellandrene, (*Z*)-β-ocimene, trimethylbenzaldehyde derivates, spathulenol and ferulagone were found in other *Ferulago* species [12]. Some of these compounds were not present in *F. capillaris* oil from Portugal.

Due to the recognised antimicrobial activity of essential oils, *F. capillaris* essential oil was evaluated against a wide range of human pathogenic fungi. The activity of *F. capillaris* essential oil could be observed in both fluconazole-susceptible and fluconazole-resistant strains of *Candida* spp., revealing that its antifungal effect against the tested yeast strains is independent of the fluconazole susceptibility profile of those microorganisms. This could imply that the antifungal mechanism of *F. capillaris* essential oil may not be interrupted by the fluconazole resistance mechanism of the tested fungi. The MIC values of *F. capillaris* essential oil against all the tested organisms are almost equal to the MLC values (Table 2), suggesting that the antifungal activity of this oil could be fungicidal rather than fungistatic. The major components of the essential oil, namely, α-pinene and limonene, were also investigated against all the tested microorganisms. Limonene was slightly less active than α-pinene, particularly against dermatophyte and *Cryptococcus*, and presented a similar activity against the majority of the strains of *Candida* and *Aspergillus*. Both of the isolated compounds were less active than the essential oil, except for dermatophytes, with α-pinene being more active. These data suggest that a fungicidal effect of the *F. capillaris* essential oil against almost all the tested fungi may be due to, at least in part, a combinatory effect of its phytochemical components. As a result, it could be implied that the essential oil from *F. capillaris* possesses a promising potency to be objected for further investigation and development as an antifungal drug, particularly for the treatment of candidiasis, cryptococcosis and ring worm.

Of the genus *Candida*, only the species *C. albicans* and *C. dubliniensis* display the capability to undergo dimorphic transition by producing a germ tube [30]. The inhibition of the germ tube formation of yeast is considered to be the mechanism of action of several fungistatic compounds, as it contributes to the reduction of the microorganism virulence [30]. Our results suggest that the *F. capillaris* essential oil possesses not only growth inhibitory effect but also exhibits morphological transformation inhibitory activity against *C. albicans* (Table 3). On the other hand, this result could imply that the essential oil possesses a potency to be a glucan synthase

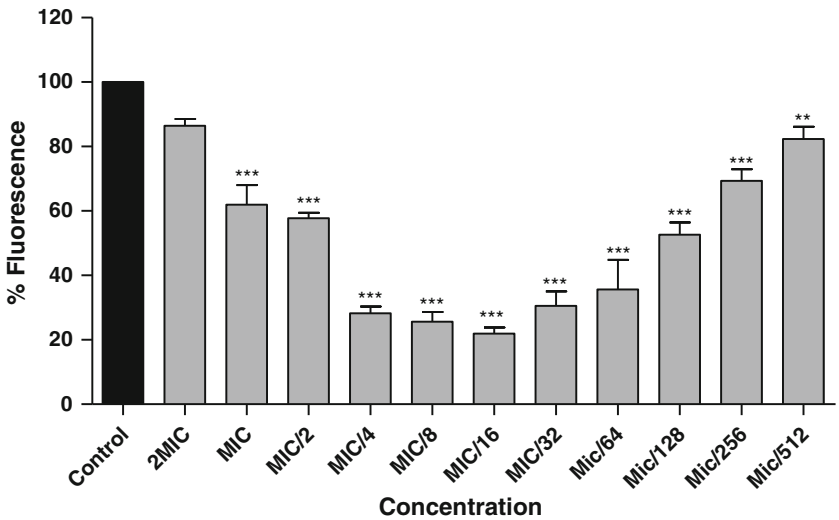

Fig. 3 Rhodamine 123 accumulation in *Candida albicans* ATCC 10231 cells treated with different concentrations of *Ferulago capillaris* essential oil. Values are expressed as % of fluorescence relative to the control (mean ± standard deviation of three independent assays). *$p<0.05$, **$p<0.01$, ***$p<0.001$. Concentrations lower than MIC/512 presented a relative fluorescence similar to the untreated cells

inhibitor [31–33]. In addition, the treatment of *C. albicans* with sublethal concentrations of the *F. capillaris* essential oil could reduce the pathogenicity of *C. albicans* by the inhibition of dimorphic transition, which is one of the *C. albicans* virulence mechanisms involved in pathogenesis [30] and its inhibition was described to be sufficient to treat disseminated candidiasis [34]. The potent capacity to inhibit this process, at a concentration 16 times lower than the MIC, can be very useful in the treatment of candidiasis.

Being a major component of sterol in the yeast cell membrane, ergosterol is responsible for maintaining cell function and integrity [35]. The primary mechanism of action by which azole antifungal drugs inhibit yeast cell growth is the disruption of normal sterol biosynthetic pathways, leading to a reduction in ergosterol biosynthesis, through the inhibition of the ergosterol biosynthetic enzyme lanosterol 14α-demethylase [36, 37]. No difference was observed in the ergosterol amount of yeast treated with sub-inhibitory concentrations of *F. capillaris* (Fig. 1), indicating that a disruption of ergosterol biosynthesis may not be involved in the mechanism of action of the essential oil. However, we observed that *C. albicans* cells treated with sub-inhibitory concentrations of fluconazole, the most commonly used azole for the prevention of candidiasis, presented a reduction of 60 % of ergosterol biosynthesis. This observation suggests that *C. albicans* may belong to the 14α-demethylation-tolerant species. According to the previous literature, a very significant inhibition of ergosterol biosynthesis is expected in tolerant species, while in fungi intolerant to 14α-demethylation, the MIC of the azole drug required for complete inhibition should be equal to its MIC [38].

Yeast exposure to *F. capillaris* essential oil leads to a reduction in mitochondrial reductase enzymes activity, for concentrations ranging from 1/8 to 1 MIC (Fig. 2). These data indicated that the inhibitory mechanism of the essential oil against *C. albicans* may be due to, at least in part, a disruption of the mitochondrial enzyme activity, which leads to impairment in the energy production ability of yeast cells. In order to confirm this possibility, the membrane potential activity of mitochondria was further investigated by rhodamine 123 uptake assay. In order to ascertain the effect of the *F. capillaris* essential oil on the mitochondrial membrane potential of *C. albicans* ATCC 10231, the accumulation of rhodamine 123, which occurs only in energised mitochondria, was used as an investigation tool [39]. The obtained results implied that the exposure of *C. albicans* to the *F. capillaris* essential oil results in the impairment of mitochondrial functions in a dose-dependent manner (Fig. 3). According to the results, it appears that there are two different mechanisms regulating the mitochondria membrane potential, measured by the accumulation of rhodamine 123. The change in fluorescence intensity is related to the inner mitochondrial membrane depolarisation, namely, the loss of membrane potential. In the absence of membrane potential, mitochondria loses the ability to sequester calcium, which leads to a loss of selectivity of the inner mitochondrial membrane, leading to a mitochondrial membrane permeability transition dependent on calcium. This change in permeability is involved in the process of injury and cell death [40]. The increase in fluorescence intensity observed between the concentrations MIC/16 and MIC/512 may be related to the change in permeability to calcium in the mitochondria. Thus, the mitochondrial membrane is more depolarised at a higher essential oil concentration (MIC/16) and, as the essential oil concentration decreases (MIC/512), the mitochondrion recovers the membrane potential and the fluorescence values become similar to the control. The effect observed for higher essential oil concentrations is more unexpected and can be associated with cellular defence mechanisms against apoptosis. Anti-apoptotic proteins are responsible for regulating the programmed cell death, the integrity of mitochondria and the release of cytochrome c into the cytoplasm. In cases of severe cellular injury, these proteins block cell death by reducing the oxidative stress. The expression of this protein is capable of inhibiting the generation of reactive oxygen species and intracellular acidification, as well as stabilising the mitochondrial membrane potential [41]. Given this assumption, it can be hypothesised that, for the highest concentration (2MIC), there is increased expression of anti-apoptotic proteins, and, therefore, a quicker stabilisation of the membrane potential. Since the concentration of essential oil decreases (from 2MIC to MIC/8), the cellular injury and the expression of anti-apoptotic proteins decrease, so that the membrane potential is not established as quickly. Taking into account such hypotheses, there are two different mechanisms that could explain the behaviour of mitochondria in *C. albicans* ATCC 10231 when exposed to the action of *F. capillaris* essential oil: for higher concentrations (from 2MIC to MIC/8), an anti-apoptotic mechanism can take place, and for lower concentrations (from MIC/16 to MIC/512), the most common mechanism may be related to the change in calcium permeability.

In conclusion, it could be implied that the essential oil from *F. capillaris* possesses a promising potency to be objected for further investigation and development as an antifungal drug, particularly for the treatment of candidiasis, cryptococcosis and ring worm. The potent capacity to inhibit dimorphic transition of *C. albicans*, at concentrations 16 times lower than the MIC, can be very useful in the treatment of candidiasis. *F. capillaris* showed a reduced cytotoxic effect. These data indicate the possibility to use this essential oil in therapeutic, particularly on superficial infections. Concerning their mechanism of action, the disruption of ergosterol biosynthesis does not seem to be involved. On the other hand, the exposure of *C. albicans* to the *F. capillaris* essential oil results in changes in mitochondrial metabolic activity in a dose-dependent manner, which can be one of the mechanisms that justifies the effectiveness of this agent in causing cell death.

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Conflict of interest No conflict to disclose.

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