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Antifungal activity of *Coriandrum sativum* essential oil, its mode of action against *Candida* species and potential synergism with amphotericin B

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ABSTRACT

The increasing incidence of drug-resistant pathogens and toxicity of existing antifungal compounds has drawn attention towards the antimicrobial activity of natural products. The aim of the present study was to evaluate the antifungal activity of coriander essential oil according to classical bacteriological techniques, as well as with flow cytometry. The effect of the essential oil upon germ tube formation, seen as an important virulence factor, and potential synergism with amphotericin B were also studied. Coriander essential oil has a fungicidal activity against the *Candida* strains tested with MLC values equal to the MIC value and ranging from 0.05 to 0.4% (v/v). Flow cytometric evaluation of BOX, PI and DRAQ5 staining indicates that the fungicidal effect is a result of cytoplasmic membrane damage and subsequent leakage of intracellular components such as DNA. Also, concentrations bellow the MIC value caused a marked reduction in the percentage of germ tube formation for *C. albicans* strains. A synergetic effect between coriander oil and amphotericin B was also obtained for *C. albicans* strains, while for *C. tropicalis* strain only an additive effect was observed. This study describes the antifungal activity of coriander essential oil on *Candida* spp., which could be useful in designing new formulations for candidosis treatment.

Introduction

Essential oils are mixtures of compounds obtained from spices, aromatic herbs, fruits, and flowers and characterized by their aroma (Cristani et al. 2007). Essential oils and their main components have many applications in popular medicine, food, beverages, preservation, cosmetics as well as in the fragrance and pharmaceutical industries (Tavares et al. 2008). The antimicrobial properties of essential oils have been known for a long time, and various researches have been conducted into their antimicrobial activities using various bacteria and fungi (Matasyoh et al. 2009). Considering the increased pathogen resistance, investigations into the antimicrobial activities, mode of action and potential uses of essential oils and its components have gained a new impulse.

Opportunistic fungi are a group of pathogens that are responsible for causing infections in a growing group of vulnerable patients, such as patients treated with immunosuppressors, those with HIV infections or other acquired immunodeficiency conditions and chemotherapy patients (Pappas 2010). *Candida* species are the most common cause of invasive fungal infections in humans , with candidemia being the fourth most common cause of nosocomial bloodstream infections in the United States and other developed countries (Fridkin 2005). The increased fungal resistance to classical drugs, their toxicity, and the costs involved justify the search for new approaches. From those new approaches, essential oils (EOs) are one of the most promising groups of natural compounds for usage in the prevention and treatment of fungal infection.

Coriander (*Coriandrum sativum* L.) essential oil is among the most used essential oils worldwide (Burdock and Carabin 2009). Coriander oil is approved for food uses by the FDA, which granted it the GRAS status, by FEMA and the Council of Europe (CoE) (Burdock and Carabin 2009). So far, many of the studies concerning the antimicrobial activity of coriander oil were performed on bacteria (Singh et al. 2002) due to the use of the oil as food additive. However, this oil or some of their components can be used in other formulations such as washing solutions for the treatment of infections (Kunicka-Styczynska et al. 2010). So, it is important to unveil coriander oil antifungal activity as well as the mode of action of this oil on yeast cells.

In this work, we propose to study the antifungal effect of coriander oil against *Candida* species and to use flow cytometry to evaluate the oil effects on cellular functions such as membrane permeability, membrane potential and intracellular DNA. The potential synergetic effect of coriander oil with a common antifungal, amphotericin B, was also studied.

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Materials and methods

Fungal strains

The antifungal activity of commercial coriander oil extracted by steam distillation (Sigma–Aldrich, St. Louis, MO) was tested against three reference *Candida* strains: *Candida albicans* ATCC 90028, *Candida albicans* 24433 and *Candida tropicalis* ATCC 750.

Prior to susceptibility testing, each strain was inoculated on Sabouraud agar to ensure optimal growth and purity.

Determination of minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) of fungal strains

A microdilution broth susceptibility assay for yeasts was used, as recommended by CLSI for the determination of the minimum inhibitory concentration (M27-A2). Geometric dilutions ranging from 0.00156 to 3.2% (v/v) of the essential oil were prepared in DMSO. Final DMSO concentration never exceeded 2%. Growth conditions and sterility of the medium were checked. The plates were incubated under normal atmospheric conditions at 37 °C for 48 h. The MIC was defined as the lowest concentration of oil which prevented visible growth. Minimum lethal concentrations (MLC) were determined according to the method previously described (Pinto et al. 2009).

Amphotericin B was used as positive control for these experiments. Each experiment was repeated at least three times at each test concentration and the modal MIC values were selected.

Checkerboard assay

Two 96-well plates were prepared: the first one was used to serial two-fold dilutions of coriander oil in horizontal orientation, and the second one, was used to make antimicrobial dilutions of amphotericin B in the vertical orientation. Both dilutions were made in RPMI (50 μl per well). Using a multichannel pipette, 50 μl of amphotericin B was transferred to the first plate, and 100 μl of yeast suspension was added to each well and incubated for 48 h at 37 °C. The used concentrations of amphotericin B and coriander oil were selected on the basis of MIC values previously determined.

The results of the combined effects of amphotericin B and coriander oil were calculated and expressed in terms of a fractional inhibitory concentration index (FICI), equal to the sum of FIC of the coriander oil and FIC of amphotericin B. The fractional inhibitory concentration (FIC) was defined as the MIC of oil and amphotericin B in combination divided by the MIC of oil and amphotericin B used alone. The results were considered as a synergistic effect when the FICI was $\leq$0.5, as no interaction if the FICI was between 0.5 and 4 or as antagonistic when it was >4 (Hemaiswarya et al. 2008).

GC/MS analysis of coriander oil

Chromatographic analysis was performed using a Simadzu GC-17A gas chromatograph equipped with a QP 5000 mass-selective detector (Shimadzu, Kyoto, Japan). A capillary column DB-5 (30 m × 0.25 mm i.d., 25 μm film thickness) was used. The temperature program was initiated at a temperature of 50 °C that was maintained for 5 min; after, the temperature was elevated from 50 to 180 °C at a rate of 2 °C/min and this temperature was maintained for 20 min. The temperatures of the injection port and transfer line were set at 230 °C. The split injection mode (ratio 1:40) was adopted, and the carrier gas was helium at a constant flow rate of 1.6 ml/min. The mass spectrometer was operated in the electron ionization mode with an electron energy value of 70 eV. The identity of the components was ascertained based on their retention indices and their mass spectras which were compared with those obtained from available libraries.

Germ tube formation assays

Germ tube inhibition assays were performed according to the method previously described (Pinto et al. 2009). For these assays, coriander oil was diluted in DMSO and added to the yeast suspensions (final DMSO concentration of 2%, v/v) to obtain appropriate coriander oil concentrations (1/8, 1/4, 1/2 and 1 time the MIC value for each strain). Oil-free control suspensions with and without 2% (v/v) DMSO were included for each yeast cell. *C. tropicalis* strain was excluded from this assay due to its inability to form germ tubes (Gunasekaran and Hughes 1977).

The results are presented as mean ± standard deviation of three independent experiments.

Exposure of yeast cells to coriander oil

Incubation of yeast cells with the appropriated coriander oil concentrations, as well as control cultures were performed according to the following protocol. Exponentially grown yeast cells cultured in YPD broth at 35 °C and 200 rpm, were harvested by centrifugation at 5000 rpm and ressupended in YPD broth to yield a cell concentration of 1–2 × 10^7 CFU/mL. Cultures were then centrifuged at 5000 rpm for 5 min, washed twice in YPD broth and resuspended in YPD broth. This cell suspension was used to inoculate tubes containing the appropriate concentrations of coriander oil (1/4, 1/2 and 1 time the MIC value for each strain) and 2% DMSO in YPD broth to obtain a cell density of about 1–2 × 10^6 CFU/mL. The inoculated tubes were incubated at 35 °C and 200 rpm. The contact time established for flow cytometric experiments was 30 min. At the end of 30 min, samples were centrifuged at 5000 rpm for 10 min, washed and resuspended in 1 ml of sterile MOPS (0.1 M, pH = 7.0) buffer.

In all cases, control experiments were carried out in parallel; cells were incubated in YPD broth without 2% DMSO and with 2% DMSO and treated under the same conditions.

Staining procedures for flow cytometry experiments

For membrane permeability assessment, suspensions of yeast cells (1 × 10^6 CFU/ml) were incubated with 1 μg/ml propidium iodide (PI) (Sigma–Aldrich, St. Louis, MO) (Pina-Vaz et al. 2001).

For membrane potential evaluation, suspensions of yeast cells (1 × 10^6 CFU/ml) were incubated with 2 μg/ml bis-1,3-dibutylbarbutiric acid (BOX) (Molecular Probes®, Invitrogen, part of Life Technologies, Carlsbad, CA) (Liao et al. 1999).

Ethanol-fixed yeast cells were used as positive controls for PI and BOX staining while exponentially growing cells were used as negative controls for these staining protocols.

In the case of DNA staining, suspensions of cells (1 × 10^6 CFU/ml) were fixated with 70% of ethanol (Stover et al. 1998) before staining. Afterwards, cells were washed twice in PBS and stained with 7.5 μg/ml DRAQ5 (Biostatus Limited, Leicestershire, UK) in PBS buffer for 10 min in the dark at room temperature. Stained cells were centrifuged again, washed once in PBS (pH = 7.0) and ressuspended in PBS (pH 7.0), sonicated for 5 s in the "hot spot" of a sonication bath to prevent cell clumps and analysed (Herrero et al. 2006).

Flow cytometry

Yeast samples were analysed on a BD Biosciences FACSCalibur. Acquisition was performed with CellQuest™ Pro Software. Light scatter measurements, FL-1 and FL-3 fluorescence measurements were acquired logarithmically, while FL-4 fluorescence was

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Table 1
Antimicrobial activity of coriander (*Coriandrum sativum* L.) essential oil alone and in combination with amphotericin B against *Candida* strains. Each experiment was repeated at least three times at each test concentration and the modal MIC, MLC and FIC values were selected.

	Candida albicans ATCC 90028				*Candida albicans* ATCC 24433				*Candida tropicalis* ATCC 750			
	MIC	MLC	FIC	FICI	MIC	MLC	FIC	FICI	MIC	MLC	FIC	FICI
Coriander essential oil (% (v/v))	0.2	0.2	0.25	0.375	0.4	0.4	0.125	0.185	0.05	0.05	0.25	1
Amphotericin B (μg/ml)	0.5	0.5	0.125		0.5	0.5	0.06		0.25	0.5	0.125	

acquired in a linear scale. Threshold levels were set on SSC to eliminate particles much smaller than intact cells. Yeast cells were gated according to FSC/SSC parameters. Sample acquisition was operated at low flow rate setting (12 μl sample/min) with eventual sample dilution required. A total of 10,000 events were acquired for each sample for PI and BOX staining, whereas, for DRAQ5 staining, a total of 30,000 events was acquired. Data analysis was performed using FCS Express version 3 Research Edition (De Novo Software™, Los Angeles, USA).

Results and discussion

Antifungal activity of coriander oil

Coriander essential oil and some of its components are known to exhibit broad antimicrobial activity (Burdock and Carabin 2009; Matasyoh et al. 2009). Data for coriander oil susceptibility testing by broth microdilution are shown in Table 1. All *Candida* strains studied were inhibited by coriander oil, with different degrees of inhibition. *C. albicans* ATCC 24433 exhibited the highest MIC (0.2%) and *C. tropicalis* ATCC 750 exhibited the lowest MIC value determined (0.05%). These values are similar to those obtained while testing other essential oils against *Candida* species (Hammer et al. 2003, 2004; Bagg et al. 2006). Coriander oil fungicidal activity is expressed by means of the MLC value. Fungicidal activity of coriander oil was obtained against all *Candida* strains used in this study and MLC values are equal to the values obtained for the MIC. The coriander oil tested in this work appears to have higher antifungal activity against *Candida* strains (0.43–3.35 μg/ml, given that the oil density is 0.868 g/ml) that a recently reported study (Begnami et al. 2010) using an essential oil extracted from *Coriandrum sativum* leaves (125–500 μg/ml). Another relevant fact is that the coriander oil used in our study was able to inhibit *C. tropicalis* growth, which was not verified by other authors using other coriander essential oil (Begnami et al. 2010). The GC/MS analysis of the coriander oil (Table 2) showed that the main component was linalool (64.38%) and the other major components (>4%) identified were α-pinene, p-cymene, camphor and geranyl acetate. Studies with this oil main constituent, linalool, against *Candida* strains, have demonstrated that MLC values were higher than MIC values for this component (Khan et al. 2010). However, since in our study, MIC values are equal to MLC values, this could indicate that coriander oil activity is not solely due to linalool, but it is the result of a more complex interaction between all the oil components. This fungicidal activity could be related to the alcohol constituents of coriander oil, since Begnami and collaborators (Begnami et al. 2010) have shown that the alcohol-containing fractions possess higher anti-*Candida* activity. However, The fact that coriander oil was more effective against *C. tropicalis* than *C. albicans* could have clinical relevance since *C. tropicalis* strains are, generally, more virulent than *C. albicans* strains (Wingard et al. 1982).

Germ tube formation

Hyphae result from the germination of blastospores and are characterized by parallel walls at the point of germ tube emergence, absence of constrictions, and the development of true septa (Vale-Silva et al. 2007). It is widely believed that germ tube formation plays an important role in the pathogenesis of *Candida albicans* (Mitchell 1998); thus, the capacity of a compound to inhibit germ tube formation could be an important factor to assess its antifungal activity.

Germ tube formation by *C. albicans* strains was strongly inhibited by coriander oil and this effect started at concentrations lower than their MIC values (Fig. 1). For the two *Candida albicans* strains inhibition of germ tube formation was almost total for an oil concentration of 1/4 × MIC value. The results obtained for oil concentrations of 1/2 × MIC and 1 × MIC are very similar and almost full inhibition of germ tube formation was attained. Furthermore, the percentage of germ tube formation was not higher than 23% when *C. albicans* strains were treated with an oil concentration as low as 1/8 × MIC value. The percentage of germ tube formation in the positive controls (with and without 2% DMSO) was not higher than 60%, which is a slightly lower value when compared to those obtained by other studies (Vale-Silva et al. 2007), in the same conditions but with different strains. This might be related to the fact

Table 2
Chemical composition (% of compound) of commercial coriander essential oil isolated by steam distillation from the seeds of *Coriandrum sativum*. T, trace (<0.05%).

Compound	Percentage of total (%)
Tricyclene	T
Alpha-tujene	T
Alpha-pinene	4.04
Camphene	0.83
Beta-pinene	0.28
6-Methyl-5-hepten-2-one	T
Myrcene	0.32
6-Methyl-5-hepten-2-ol	T
p-Cymene	4.54
Limonene	1.78
1,8-Cineole	0.08
Trans-linalool oxide (furanoid)	3.12
Cis-linalool oxide (furanoid)	2.76
Linalool	64.38
Camphor	4.88
Borneol	0.47
Trans-linalool oxide (pyranoid)	0.33
Terpinen-4-ol	0.14
p-Cymenol	0.12
Alpha-terpineol	0.33
3E-hexenylbutanoate	0.36
3-Decanol	0.1
Trans-carveol	0.06
Nerol	0.06
Neral	0.06
Geraniol	1.56
Geranial	0.21
1-Decanol	0.14
Isobornyl acetate	0.14
8-Hidroxylinalool	0.12
Geranyl formate	T
Cis-pinocarvyl acetate	0.12
Neryl acetate	0.06
Geranyl acetate	5.82
Dodecanal	0.05
Cyclogeranyl acetate	0.06
Total identified	97.5

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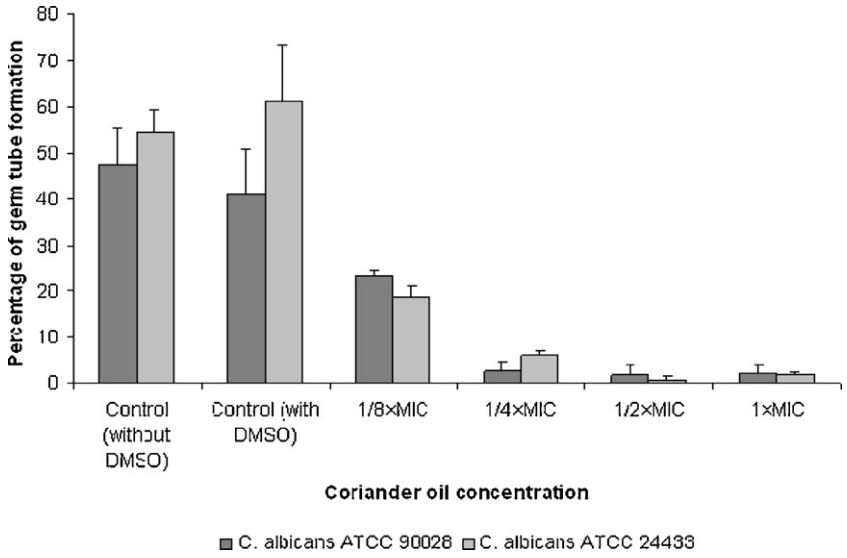

Fig. 1. Percentage germ tube formation by the two strains of *C. albicans* incubated without DMSO, with 2% DMSO alone (positive control) or with subinhibitory and inhibitory concentrations (1/8 × MIC to 1 × MIC) of coriander oil in DMSO (final DMSO concentration of 2%). The mean results and standard deviations (error bars) of three independent assays are presented.

that these collection strains could be less virulent, and therefore exhibited lower ability to form germ tubes.

Overall, coriander oil proved to be effective in inhibiting germ tube formation on both *C. albicans* strains tested even at sub-MIC concentrations which could be advantageous in reducing *C. albicans* pathogeneicity that appears to be related to hyphal growth and germ tube formation.

Membrane permeability, membrane potential and intracellular DNA

Flow cytometry was used to assess the effect of coriander oil on membrane integrity and membrane potential of fungal cells. Dead or dying cells with injured membranes are able to incorporate PI, which binds to the nucleic acids (Pina-Vaz et al. 2001) resulting in increased red (FL-3) fluorescence. Our results showed that the percentage of PI-positive cells seems to be oil-dependent since higher oil concentrations caused higher membrane permeability (Fig. 2). PI penetrates over 99% of the yeast cells following a short incubation period of 30 min with an oil concentration of 1 × MIC (Fig. 2), meaning that the structure of the cell membrane was disrupted by the oil.

BOX has an high voltage sensitivity and can enter depolarized cells (Liao et al. 1999). For *Candida* species, the effect observed for BOX staining was similar to that obtained for PI staining with a concentration-dependent effect (Fig. 3). After the 30 min of incubation, cells incubated with an oil concentration of 1 × MIC (Fig. 3) showed over 97% of stained cells, indicating that membrane potential was compromised.

This similarity between membrane integrity and membrane potential values may result from the fact that when membrane integrity is affected, the cell is no longer capable of maintaining its cellular functions (Cristani et al. 2007). Permeation to PI, particularly following short incubation periods, such as 30 min, indicates that the mode of action of the oil involves a lesion of the cell membrane that results from direct damage to the cell membrane instead of a metabolic impairment leading to secondary membrane damage. These findings are further supported by other reports found in the literature, not only for fungal cells but also for bacterial cells (Hammer et al. 2004).

Flow cytometric measurement of DNA content, may contribute to unveil unknown antimicrobial susceptibility mechanisms (Gottfredsson et al. 1995). As a result, in this work we further investigated the effects of coriander oil on intracellular DNA con-

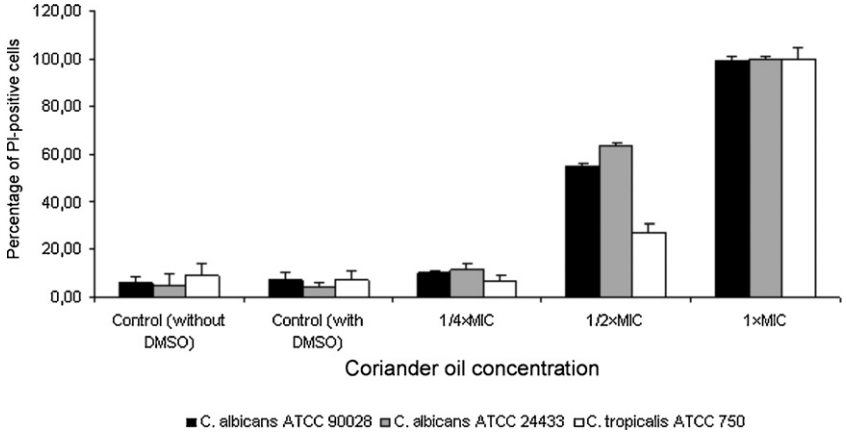

Fig. 2. PI staining of *Candida* strains incubated without DMSO, with 2% DMSO alone (positive control) or with subinhibitory and inhibitory concentrations (1/8 × MIC to 1 × MIC) of coriander oil in DMSO (final DMSO concentration of 2%). The mean results and standard deviations (error bars) of two independent assays are presented.

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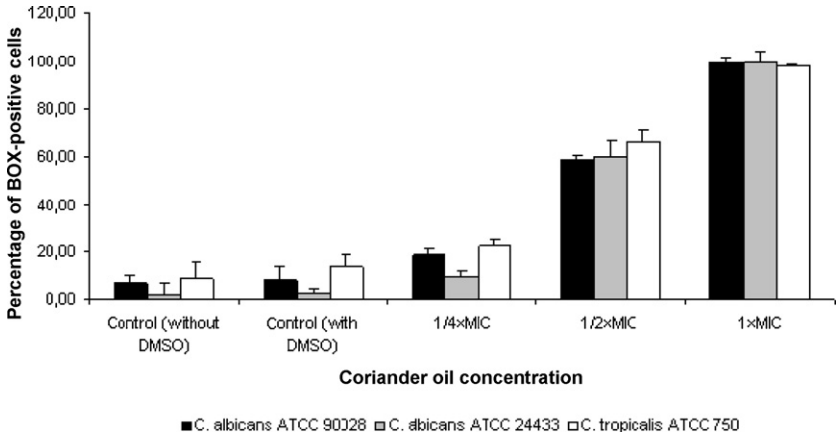

Fig. 3. BOX staining of *Candida* strains incubated without DMSO, with 2% DMSO alone (positive control) or with subinhibitory and inhibitory concentrations (1/8 × MIC to 1 × MIC) of coriander oil in DMSO (final DMSO concentration of 2%). The mean results and standard deviations (error bars) of two independent assays are presented.

tent using a recently developed fluorescent stain, DRAQ5, which has been described to stain yeast and bacteria cells (Herrero et al. 2006; Silva et al. 2010). Our results showed that DNA distribution histograms are very similar in the control and in oil-treated cells (Fig. 4), which could indicate that coriander oil does not interfere with DNA synthesis. Despite the similarity in DNA distributions, the fluorescence intensity values are slightly different for control cultures and cultures incubated with coriander oil. Cultures incubated with coriander oil at 1/2 × MIC exhibited higher fluorescence values than control cultures, indicating that, in response to cell damage by coriander oil, cells are synthesizing more DNA in order to repair damaged functions. Cells incubated with coriander oil at 1 × MIC had the lowest fluorescence intensity values, probably indicating DNA leakage from the cell, as a result of increased membrane permeability. These results corroborate the results obtained for flow cytometric assessment of membrane permeability by PI.

The correlation between loss of membrane potential, membrane permeability and leakage of intracellular DNA indicate that coriander oil kills *Candida* spp. by damaging the cytoplasmic membrane, leading to an impairment of all cellular functions (Cristani et al. 2007). Some authors proposed that essential oil activity may, in part, be related with their hydrophobicity, responsible for their partition into the lipid bilayer of the cell membrane, leading to an alteration of permeability and a consequent

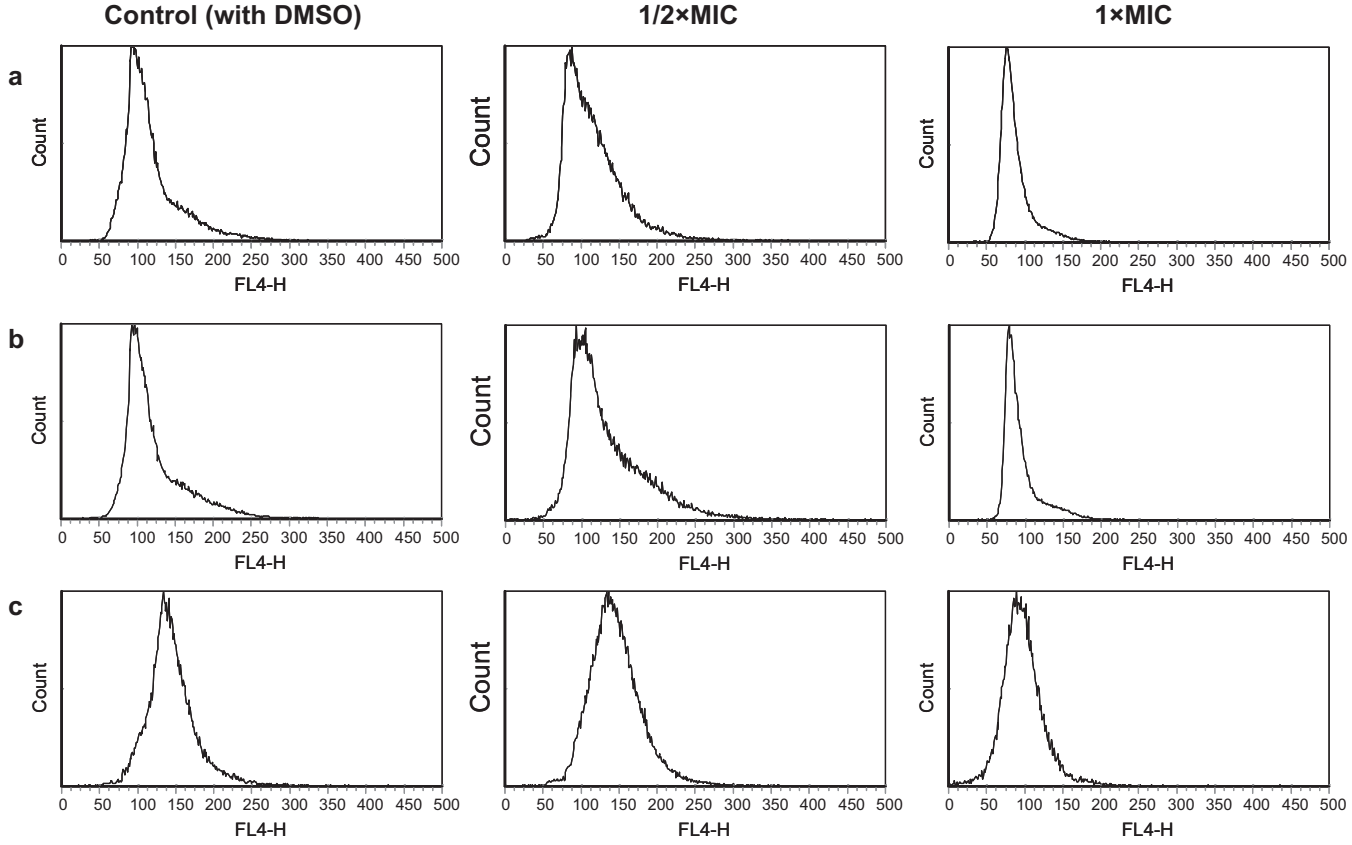

Fig. 4. Histograms of FL-4 channel fluorescence showing the distribution of DRAQ5 staining of *Candida* strains: (a) *C. albicans* ATCC 90028, (b) *C. albicans* ATCC 24433 and *C. tropicalis* ATCC 750 incubated without DMSO, with 2% DMSO alone (positive control) or with subinhibitory and inhibitory concentrations (1/8 × MIC to 1 × MIC) of coriander oil in DMSO (final DMSO concentration of 2%, v/v).

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leakage of cell contents (Burt 2004). The results obtained herein are congruent with the results obtained by other investigations. For instance, it was demonstrated that *Ocimum sanctum* essential oil and two of its main components, methyl chavicol and linalool, target the structural and functional integrity of the cytoplasmic membrane in *Candida* (Khan et al. 2010). Also, other researches have also shown that the fungicidal activity of *Thymus* oils resulted primarily from an extensive damage of the cell membrane, as shown by propidium iodide incorporation (Pina-Vaz et al. 2004).

Synergetic effect of coriander oil plus amphotericin B

The combination between plant essential oils and antimicrobial drugs has been referred as a strategy for combating microbial development due to the production of an additive or synergistic effect (Wagner and Ulrich-Merzenich 2009). Since amphotericin B is toxic to cells and there are reports of *Candida* strains resistant to amphotericin B (Han 2007); in many cases, this antifungal cannot be given in sufficient high doses to treat the infection and is often co-administered with other antifungals. So, to reduce amphotericin B dosage, bellow toxic levels, while producing a fungicidal effect, a new strategy could be employed using its synergism with natural antimicrobial agents. Therefore, considering the antifungal activity of coriander oil demonstrated in this work, its potential synergism with amphotericin B was studied. The synergistic effect of coriander essential oil in combination with amphotericin B was studied against *C. albicans* and *C. tropicalis*. The MIC of amphotericin B alone against *C. albicans* was lowered from 0.5 to 0.06 and 0.03 μg/ml to *C. albicans* ATCC 90028 and 24433, respectively. The FIC index (Table 1) showed a marked synergism between the oil and amphotericin B against *C. albicans* (FICI = 0.375 and 0.185), however when the combination was tested against *C. tropicalis* ATCC 750 an additive effect was observed (FICI = 1). Considering that amphotericin B antifungal mode of action in *Candida* spp. is similar to that of coriander oil and it is related with the permeation of cellular membrane causing leakage of intracellular components, a possible explanation for the results obtained could be related with the inhibition of the formation of germ tubes. Since coriander oil caused inhibition of germ tube formation in *Candida albicans* strains, this fact can potentiate amphotericin B known effect on germ tube formation (Ogletree et al. 1978) which cannot be observed in *Candida tropicalis* strain due to its inability to form germ tubes.

The findings of the present study indicate that coriander essential oil has interesting potential as therapeutic option against *Candida* cells due to its fungicidal ability, inhibition of germ tube formation and synergetic effect with amphotericin B. The flow cytometric assessment of some cellular functions demonstrated that coriander oil primary mode of action seems to be bacterial cell permeabilization and consequent intracellular DNA leakage.

Coriander oil has GRAS status and it is already extensively used in both food and cosmetic industry. If the results of this work are considered, one can foresee numerous additional applications for this oil in either already existing medical accessories or other formulations for use in clinical practice to prevent candidal infections.

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