Antifungal activity of the clove essential oil from *Syzygium aromaticum* on *Candida*, *Aspergillus* and dermatophyte species

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The composition and antifungal activity of clove essential oil (EO), obtained from *Syzygium aromaticum*, were studied. Clove oil was obtained commercially and analysed by GC and GC-MS. The EO analysed showed a high content of eugenol (85.3 %). MICs, determined according to Clinical and Laboratory Standards Institute protocols, and minimum fungicidal concentration were used to evaluate the antifungal activity of the clove oil and its main component, eugenol, against *Candida*, *Aspergillus* and dermatophyte clinical and American Type Culture Collection strains. The EO and eugenol showed inhibitory activity against all the tested strains. To clarify its mechanism of action on yeasts and filamentous fungi, flow cytometric and inhibition of ergosterol synthesis studies were performed. Propidium iodide rapidly penetrated the majority of the yeast cells when the cells were treated with concentrations just over the MICs, meaning that the fungicidal effect resulted from an extensive lesion of the cell membrane. Clove oil and eugenol also caused a considerable reduction in the quantity of ergosterol, a specific fungal cell membrane component. Germ tube formation by *Candida albicans* was completely or almost completely inhibited by oil and eugenol concentrations below the MIC values. The present study indicates that clove oil and eugenol have considerable antifungal activity against clinically relevant fungi, including fluconazole-resistant strains, deserving further investigation for clinical application in the treatment of fungal infections.

INTRODUCTION

The incidence of both community-acquired and nosocomial fungal infections has significantly increased over the past few decades, accompanying the growing number of high-risk patients, particularly those with impaired immunity.

The majority of the clinically used antifungals suffer from various drawbacks in terms of toxicity, drug–drug interactions, lack of fungicidal efficacy, cost and emergence of resistant strains caused by the frequent use of some of them. In spite of the recent introduction of new antifungal drugs, they are still limited in number. Hence, there is a great demand for novel antifungal agents, justifying the intense search for new drugs that are more effective and less toxic than those already in use (Kauffman, 2006; Rapp, 2004).

Aromatic plants have been used in folk medicine as antimicrobial agents since ancient times (Cowan, 1999; Grayer & Harborne, 1994). The essential oils (EOs) from many plants are known to possess antibacterial and antifungal activity (Bakkali *et al.*, 2008; Burt, 2004; Dorman & Deans, 2000; Kalemba & Kunicka, 2003). EOs have been empirically used as antimicrobial agents, but the spectrum of activity and mechanisms of action remain unknown for most of them. Although only limited consistent information exists about activity toward human fungal pathogens, some EOs have shown important antifungal activity against yeasts, dermatophyte fungi and *Aspergillus* strains, which could predict therapeutic benefits, mainly on diseases involving mucosae, the skin and the respiratory tract (Cavaleiro *et al.*, 2006; Pina-Vaz *et al.*, 2004; Pinto *et al.*, 2006). They constitute, in this way, complementary or alternative therapeutic options that are increasing in popularity, yet they still have scant scientific credibility.

Syzygium aromaticum is widely cultivated in Indonesia, Sri Lanka, Madagascar, Tanzania and Brazil. The clove oil from *S. aromaticum* and eugenol have been described as having useful antiseptic, analgesic and anaesthetic effects (Chaieb *et al.*, 2007a) and are largely used in dental medicine. Previous studies have reported antifungal activity for clove oil and eugenol against yeasts and filamentous fungi, such as several food-borne fungal species (López *et al.*, 2005; Velluti *et al.*, 2004) and human pathogenic fungi (Chaieb *et al.*, 2007b; Gayoso *et al.*, 2005). Clove oil and eugenol have also been tested as antifungal agents in animal models (Ahmad *et al.*, 2005; Chami *et al.*, 2004a, b). In order to further clarify the spectrum of antifungal activity and its relationship to chemical composition, some general considerations must be established regarding the study of the antimicrobial activity of EOs and the compounds isolated from them. Of the highest relevance is the definition of common parameters, such as the techniques employed, growth medium and micro-organisms tested. Standardization of both the methods of analysis of the EO and the assays for *in vitro* testing is required so that research in this area can be systematic and objective and the interpretation of results validated.

The limited knowledge concerning antimicrobial activity and the mechanism of action of plant extracts has led us to address such issues, although it is known that the main antifungal action of phenolic compounds, such as eugenol, appears to be exerted on the cellular membrane (Carson *et al.*, 2006; Cox *et al.*, 2001).

The objective of our present research was to evaluate the antifungal activity and investigate the mechanism of action of clove oil and its main component, eugenol.

METHODS

Fungal organisms. Clinical isolates and collection strains of *Candida*, *Aspergillus* and dermatophyte species were used: five *Candida* clinical strains, isolated from recurrent cases of oral candidosis, and four ATCC type strains; one clinical strain of *Aspergillus flavus* isolated from bronchial secretions, and two *Aspergillus* ATCC type strains (*Aspergillus fumigatus* and *Aspergillus niger*); five dermatophyte clinical strains, *Microsporum canis*, *Microsporum gypseum*, *Trichophyton rubrum*, *Trichophyton mentagrophytes* and *Epidermophyton floccosum* isolated from nails and skin (Table 1). *Candida parapsilosis* ATCC 90018 and *Candida krusei* ATCC 6258 were used for quality control. The fungal isolates were identified by standard microbiology methods and stored in

Table 1. Antimicrobial activity (MIC and MFC) of the *S. aromaticum* EO and eugenol against *Candida*, dermatophyte and *Aspergillus* strains

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

	EO		Eugenol		Fluconazole		Amphotericin B	
	MIC*	MFC*	MIC*	MFC*	MIC†	MFC†	MIC†	MFC†
Yeasts								
ATCC								
C. albicans ATCC 10231	0.64	0.64–1.25	0.64	0.64–1.25	1	>128	NT	NT
C. krusei ATCC 6258	0.64	0.64	0.64	0.64	64	64–128	NT	NT
C. tropicalis ATCC 13803	0.64	0.64	0.64	0.64	4	>128	NT	NT
C. parapsilosis ATCC 90018	0.32–0.64	0.64–1.25	0.32	0.64	<1	<1	NT	NT
Clinical isolates								
C. albicans D5	0.64	0.64	0.32–0.64	0.64–1.25	64	>128	NT	NT
C. albicans D1	0.64	0.64–1.25	0.64	0.64–1.25	2	128	NT	NT
C. krusei D39	0.64	0.64	0.64	0.64	64	64–128	NT	NT
C. tropicalis D42	0.64	0.64	0.64	0.64	2	>128	NT	NT
C. glabrata D10R	0.64	0.64–1.25	0.64	0.64–1.25	32	32	NT	NT
Filamentous fungi								
Dermatophytes								
Epidermophyton floccosum FF9	0.16	0.32	0.16	0.32	16	16	NT	NT
Trichophyton rubrum FF5	0.16	0.32	0.16	0.32	16–32	32	NT	NT
Trichophyton mentagrophytes FF7	0.16	0.32	0.16	0.32	16–32	32–64	NT	NT
Microsporum canis FF1	0.08–0.16	0.16–0.32	0.08	0.16	128	128	NT	NT
Microsporum gypseum FF3	0.16	0.32	0.16	0.32	≥128	≥128	NT	NT
Aspergillus species								
A. flavus F44	0.64	1.25	0.32–0.64	1.25	NT	NT	2	8
A. fumigatus ATCC 46645	0.32	1.25	0.32	1.25	NT	NT	2	4
A. niger ATCC 16404	0.32	1.25	0.32	1.25	NT	NT	1–2	4

*MIC and MFC were determined by a macrodilution method and expressed in $\mu l\ ml^{-1}$ (v/v).
†MIC and MFC were determined by a macrodilution method and expressed in $\mu g\ ml^{-1}$ (w/v).

Sabouraud dextrose broth (Becton Dickinson) with glycerol at −70 °C. Prior to antifungal susceptibility testing, each isolate was inoculated on Sabouraud dextrose agar (SDA; Becton Dickinson) to ensure optimal growth characteristics and purity.

EO and chemicals. The antifungal activity of the clove EO and its main component, eugenol, was evaluated. A commercial clove oil from *S. aromaticum* was acquired from the company 'Segredo da Planta', Portugal. Eugenol was purchased from Fluka, fluconazole from Pfizer, and amphotericin B from Sigma. Fluconazole was dissolved in sterile distilled water at $12\,800\ \mu g\ ml^{-1}$, amphotericin B was dissolved in 100 % DMSO (Sigma) at $1600\ \mu g\ ml^{-1}$, and individual aliquots of the solutions were kept at −70 °C. The EO and eugenol were kept at 4 °C throughout the experimental period and appropriate volumes were diluted in 100 % DMSO, at a maximum concentration of $20\ \mu l\ ml^{-1}$ (v/v), for each experiment.

EO analysis. Analysis of volatile oil was carried out by GC and GC-MS. Analytical GC was carried out in a Hewlett Packard 6890 (Agilent Technologies) gas chromatograph with a HP 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 (polydimethyl siloxane, $30\ m \times 0.20\ mm$ i.d., film thickness $0.20\ \mu m$) and SupelcoWax-10 (polyethylene glycol, $30\ m \times 0.20\ mm$ i.d., film thickness $0.20\ \mu m$). The parameters were: oven temperature program 70–220 °C (3 °C min^{-1}), 220 °C (15 min); injector temperature 250 °C; carrier gas helium, adjusted to a linear velocity of 30 cm s^{-1}; splitting ratio 1 : 40; detector temperature 250 °C.

GC-MS analyses were carried out in a Hewlett Packard 6890 gas chromatograph fitted with a HP1 fused silica column (polydimethyl siloxane, $30\ m \times 0.25\ mm$ i.d., film thickness $0.25\ \mu m$), interfaced with a Hewlett Packard mass selective detector 5973 (Agilent Technologies) operated by HP Enhanced ChemStation software, version A.03.00. GC parameters were as above, while the other parameters were: 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 s^{-1} 4.51.

The identity of the compounds was achieved from their retention indices on both SPB-1 and SupelcoWax-10 columns and from their mass spectra. Retention indices, calculated by linear interpolation relative to retention times of C_8–C_{22} *n*-alkanes, were compared with those of authentic samples included in our own laboratory database. Acquired mass spectra were compared with corresponding data of components of reference oils and commercially available standards from a home-made library or from literature data (Adams, 2004; Joulain & Konig, 1998). Relative amounts of individual components were calculated based on GC peak areas without FID response factor correction.

Antifungal activity. Broth macrodilution protocols based on the CLSI reference documents M27-A3 (CLSI, 2008a) and M38-A2 (CLSI, 2008b), with minor modifications, were used to determine MIC values for yeasts and filamentous fungi, respectively. Briefly, twofold serial dilutions, in DMSO, with final test concentrations ranging from 0.02 to $20\ \mu l\ ml^{-1}$ (v/v) for clove oil and eugenol were tested. Inocula were prepared according to the guidelines. For dermatophytes, inocula were prepared by counting conidia harvested from ~7-day-old potato dextrose agar cultures in a haemocytometer, followed by adjustment to the appropriate final density (Shehata *et al.*, 2008). Results were read after 48 h incubations for *Candida* and *Aspergillus* species and 4 day incubations for dermatophytes, and MICs were defined as the lowest test concentrations causing complete growth inhibition. In addition, the reference antifungal compounds

fluconazole, for yeasts and dermatophytes, and amphotericin B, for *Aspergillus*, were used as standard drugs. Twofold serial dilutions ranging from 0.25 to $128\ \mu g\ ml^{-1}$ for fluconazole and 0.031 to $16\ \mu g\ ml^{-1}$ for amphotericin B were used. Quality control determinations of the MIC values of fluconazole and amphotericin B were performed by testing *C. parapsilosis* ATCC 90018 and *C. krusei* ATCC 6258 and the results obtained were within the recommended limits.

To determine minimum fungicidal concentration (MFC) values, after reading the corresponding MIC values, 20 μl samples from all optically clear tubes (complete growth inhibition) plus the last tube showing growth were subcultured on SDA Petri dishes. The dishes were incubated at 35 °C for a minimum of 3 days, until growth was clearly visible in the control samples, and MFC values were determined as the lowest concentration of the clove oil and eugenol for which there was no visible growth.

The experiments were performed in duplicate and repeated independently three times, yielding essentially the same results (a range of values is presented when different results were obtained). Two growth controls, RPMI 1640 and RPMI 1640 with 1.0 % (v/v) DMSO, were included for each strain.

Mechanism of activity

Germ tube inhibition assay. Cell suspensions from overnight SDA cultures of *C. albicans* strains ATCC 10231, D5 and D1, at 37 °C, 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] and adjusted to obtain a density of $(1.0 \pm 0.2) \times 10^6$ c.f.u. ml^{-1}. Clove oil and eugenol were diluted in DMSO and added in 10 μl volumes to 990 μl of the yeast suspensions (final DMSO concentration of 1 %, v/v) to obtain appropriate subinhibitory concentrations (1/2, 1/4 and 1/8 of the MIC values). Drug-free control suspensions with and without 1 % (v/v) DMSO were included for each *C. albicans* strain. After a 3 h 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.

Metabolic activity and lesion of cytoplasmic membrane. Flow cytometry analysis using propidium iodide (PI; Sigma) and FUN 1 (Invitrogen) was performed. *C. albicans* ATCC 10231 suspensions were prepared in Sabouraud broth from overnight SDA cultures and incubated in a water bath at 35 °C, with moderate agitation, until mid-exponential phase. The suspensions were centrifuged, washed and resuspended in PBS with 2 % (w/v) D-glucose (PBS-2 %G) at a density of $(2.0 \pm 0.2) \times 10^6$ c.f.u. ml^{-1}, using a haemocytometer. *Aspergillus* conidia suspensions were prepared in PBS-2 %G from 3–4-day SDA cultures of *A. fumigatus* ATCC 46645 at 35 °C and adjusted, using a haemocytometer, to a final density of $(2.0 \pm 0.2) \times 10^6$ c.f.u. ml^{-1}. Serial twofold dilutions of the EO and eugenol (0.04–1.25 μl ml^{-1}) and amphotericin B (2 μg ml^{-1}) were then added to the cell suspensions. Yeast suspensions were incubated at 35 °C, in a humid atmosphere without shaking, for 15 min. Conidia suspensions were incubated in a water bath at 35 °C with moderate agitation for 2 and 6 h. Drug-free control tubes were included in every experiment (maximum employed DMSO concentrations alone showed no influence on the results). After the incubation, the cells were washed and resuspended in 500 μl PBS-2 %G for staining. Five microlitres of the FUN 1 and PI solutions in DMSO and PBS, respectively, were added to the cell suspensions in order to obtain a final concentration of 0.5 μM FUN 1 and 1.0 μg PI ml^{-1}. The samples were then incubated at 35 °C, in the dark, for 30 min. Unstained cell

suspensions were always included as autofluorescence controls. Flow cytometry was performed using a FACSCalibur flow cytometer (Becton Dickinson Biosciences) with a blue argon laser emitting at a wavelength of 488 nm, at 15 mW, and the results were analysed using CellQuest Pro Software (Becton Dickinson). Intrinsic parameters (forward and side scatter, for cell size and complexity analysis, respectively) and fluorescence in the FL2 channel (log yellow/orange fluorescence, bandpass 585/42 nm filter) for FUN 1 and the FL3 channel (log red fluorescence, longpass >650 nm filter) for PI were acquired and recorded, using logarithmic scales, for a minimum of 7500 events per sample. For data analysis, quadrants were adjusted in raw data density plots of fluorescence intensity of control samples in order to include a maximum of 5 % of the cells in the upper right quadrant and then used in the analysis of the remaining samples to quantify the percentages of cells showing altered fluorescence in comparison to the drug-free controls (see Fig. 1 as an example). The results are presented as mean $\pm$ standard deviation of at least three replicate experiments performed on separate days.

Determination of the amount of ergosterol. For determination of the amount of ergosterol, *C. albicans*, *Candida tropicalis*, *Candida glabrata* and *C. krusei* were incubated in RPMI medium (Sigma) supplemented with 2 % glucose (Difco) for 48 h at 35 °C, with moderate shaking. Quantification of ergosterol was performed after incubation with and without the EO and eugenol at the subinhibitory concentrations of 0.32 µl ml^{-1} or 1 µg ml^{-1} of fluconazole (as a control). Ergosterol was isolated from yeast cells by saponification and the nonsaponifiable 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 qualitative and quantitative composition of the clove EO from *S. aromaticum* is shown in Supplementary Table S1 in JMM Online. A total of 19 components, representing 97.3 % of the volatile oil, were identified. The oil was characterized by high amounts of a phenylpropanoid compound, eugenol (85.3 %). In view of the known activities of the constituents and the variability of the oils, it is important that the composition is known.

The clove oil exhibited wide-spectrum antifungal activity. Evaluation of MIC and MFC values showed that the EO was active against all the tested strains (Table 1). The highest level of activity was observed against five different species of dermatophytes, showing a MIC value of 0.16 µl ml^{-1}. Further MIC values ranged from 0.32 to 0.64 µl ml^{-1} against *Aspergillus* and *Candida* strains. For *Candida* strains, MIC and MFC values were similar (Table 1). It is difficult to attribute the activity of natural and complex mixtures like EOs to a particular constituent, nevertheless it is reasonable to assume that the activity of this oil can be related to the presence of a high concentration (85.3 %) of eugenol. Eugenol was found to be an active constituent of clove oil, with MIC values ranging from 0.08 to 0.64 µl ml^{-1} (Table 1). The importance of the phenolic hydroxyl groups for the antimicrobial activity of EOs has been previously reported (Aligiannis *et al.*, 2001; Dorman & Deans, 2000; Nostro *et al.*, 2004). This EO and eugenol showed a broad spectrum of activity against a variety of pathogenic yeasts and filamentous fungi, including fungi with decreased susceptibility to fluconazole (Table 1). Nevertheless, eugenol proved to be more active against dermatophyte strains, as did the EO. Also, the MIC and MFC values were similar for *Candida* strains, and the fungistatic and fungicidal properties of the oil are probably associated with its high eugenol content.

Researchers normally adapt experimental methods to better represent possible future applications of the findings in their particular field. The major problem with this type of research is the lack of uniformity in the criteria selected to study the activity. Considering that the outcome of a test can be affected by factors such as the method used to extract the EO from plant material, the exact composition of the EO, the type of organism tested, the volume of inoculum, growth phase, culture medium used, pH of the media, and incubation time/temperature, comparison of published data is complicated (Burt, 2004; Janssen *et al.*, 1987; Kalemba & Kunicka, 2003; Ríos & Recio, 2005). Although there was still a degree of discrepancy between the methods used in the different studies, the MICs reported were often relatively similar. Data were also largely limited to *C. albicans*, which was a commonly chosen model organism (Kalemba & Kunicka, 2003).
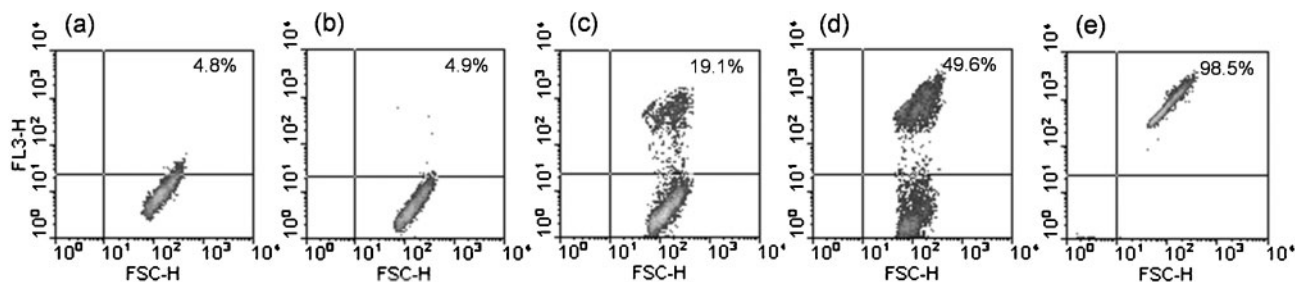
Fig. 1. Sequence of density plots showing *C. albicans* ATCC 10231 cell size (forward scatter–FSC-H log) versus red fluorescence (FL3 channel–FL3-H log) analysed by flow cytometry, and the respective percentages of PI-stained cells (upper right quadrant), for a series of samples treated with increasing concentrations of eugenol. (a) Untreated control cells; (b–e) cells treated with eugenol at 0.32 µl ml^{-1} (b), 0.64 µl ml^{-1} (c), 1.25 µl ml^{-1} (d) and 2.5 µl ml^{-1} (e).

Previous studies have shown the activity of clove oil in fungi. In this work, however, we tried to correlate the comparative data for different types of fungi with medical importance (clinical and ATCC isolates) and we have used the standardized technique from the CLSI to compare the results with those obtained with commercial antifungal drugs. Nevertheless, our results are, in general, in agreement with the data obtained by other authors (Ayoola *et al.*, 2008; Chaieb *et al.*, 2007a, b; Gayoso *et al.*, 2005).

While *C. albicans* is still the most common fungal pathogen among hospitalized patients, non-*albicans Candida* species are becoming increasingly common (Fridkin, 2005; Tortorano *et al.*, 2006). Unfortunately, many of these fungal species are resistant to the currently available therapeutic options. In view of the fact that the spectrum of activity of EOs is quite variable, it was decided to investigate the activity using a large number of species and strains, including strains showing resistance to fluconazole. Resistance to conventional antifungal compounds has not been demonstrated to influence susceptibility to clove EO or eugenol, suggesting that cross-resistance does not occur.

Germ tube formation, generally regarded as an important mechanism of pathogenicity of *C. albicans* (Calderone & Fonzi, 2001; Hammer *et al.*, 2000; Pinto *et al.*, 2008), was strongly inhibited by clove oil and eugenol and this effect started at concentrations lower than their MIC value ($0.64 \, \mu l \, ml^{-1}$; Table 2). The effect was quite different for *C. albicans* ATCC 10231 and the other two tested strains, however. The inhibition was practically total with $0.16 \, \mu l \, ml^{-1}$ (MIC/4) of both compounds for D5 and D1 strains, while $0.32 \, \mu l \, ml^{-1}$ (MIC/2) was required to cause full inhibition in the ATCC strain. Moreover, the registered percentage of germ tubes was not higher than 40 % when the clinical isolates were treated with oil concentrations as low as $0.08 \, \mu l \, ml^{-1}$ (MIC/8). For the ATCC strain, however, higher concentrations were required to cause a comparable effect. A difference was also registered between the EO and eugenol, with the latter showing higher inhibition, in agreement with its suspected responsibility for the activity of the total EO. Curiously, as noted above, a greater inhibition of the filamentation in *C. albicans* at concentrations well below the MIC was found for the clinical isolates than for the ATCC strain. Most antifungal agents are described as germ tube formation inhibitors (Ellepola & Samaranayake, 1998). This attribute may well constitute a relevant therapeutic advantage, considering the importance of filamentation in the evolution from commensality to pathogenicity in *C. albicans*. In fact, eugenol has previously been reported as an effective antifungal agent in the treatment and prophylaxis of animal models of oral and vaginal candidiasis (Chami *et al.*, 2004a, b). The animals in the study were locally administered twice a day with 0.5 ml eugenol solution at concentrations converting to almost $4.0 \, \mu l \, ml^{-1}$ (corresponding to about $20 \, \mu l \, kg^{-1}$ per day). This concentration showed no acute toxicity, while achieving clear therapeutic efficacy, even if not as pronounced as the one shown by carvacrol, as seen by both microbiological and histological observations. These reports thus provide further support for eugenol's potential in the treatment of mucocutaneous candidiasis.

Flow cytometry was used to investigate the effect of clove oil and eugenol on the integrity of fungal cells. PI is a nucleic acid binding fluorescent probe commonly employed to evaluate the effect of drugs on cell membranes. Cells with severe membrane lesions leading to inherent loss of viability will internalize PI, resulting in an increase in red fluorescence (Pina-Vaz *et al.*, 2001). Our results show that PI penetrates over 95 % of the yeast cells following a short incubation period with $2.5 \, \mu l \, EO \, ml^{-1}$ (Fig. 2a), meaning that the structure of the cell membrane was disrupted by the drug. A similar exposure to eugenol, at the same concentration, induced permeation to PI in 99.2 % of the cells, while $1.25 \, \mu l \, ml^{-1}$ affected over 50 % of

Table 2. Percentage germ tube formation by three strains of *C. albicans* incubated with 1 % DMSO alone (positive control) or with subinhibitory concentrations ($<0.64 \, \mu l \, ml^{-1}$, MIC value) of the clove EO and eugenol in DMSO (final DMSO concentration of 1 %, v/v)

Mean ($\pm$SD) results of three consecutive experiments.

|	*C. albicans* D1	*C. albicans* D5	*C. albicans* ATCC 10231
Positive control	77.0 ± 11.3	80.5 ± 12.0	93.0 ± 9.3
Clove oil $\mu l \, ml^{-1}$			
0.08	40.3 ± 11.9	30.5 ± 7.1	92.3 ± 6.7
0.16	5.8 ± 5.0	2.3 ± 2.6	75.0 ± 7.1
0.32	0.0 ± 0.0	0.0 ± 0.0	1.8 ± 2.9
Positive control	79.0 ± 2.8	89.0 ± 8.5	94.5 ± 8.4
Eugenol $\mu l \, ml^{-1}$			
0.08	26.5 ± 10.5	19.3 ± 7.8	67.0 ± 2.8
0.16	2.3 ± 2.9	0.0 ± 0.0	19.7 ± 13.3
0.32	0.0 ± 0.0	0.3 ± 0.4	1.5 ± 1.9
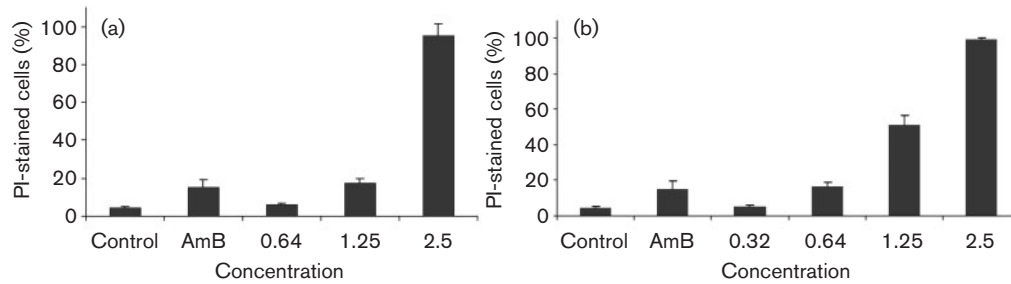
Fig. 2. Percentage of PI-stained *C. albicans* ATCC 10231 cells analysed by flow cytometry after treatment with serial concentrations of (a) clove oil and (b) eugenol for 15 min and comparison with the percentage after amphotericin B (AmB) treatment and with that of an untreated control.

the cells (Fig. 2b). It is worth noting that under these experimental conditions, 2 µg amphotericin B ml^{-1} (MIC 0.5 µg ml^{-1}) induced cell death in fewer than 20 % of cells (Fig. 2). To test the effect of clove oil on moulds, *A. fumigatus* was used as an example. Under our experimental conditions, with maximum incubation times of 6 h, neither the oils nor amphotericin B produced any appreciable PI staining (data not shown). Germination of *A. fumigatus* conidia only begins after around 7 h of incubation, possibly explaining why the conidia remained impermeable to PI after 6 h.

FUN 1 is a membrane-permeant fluorescent probe which flows through freely into the cell and initially appears in the cytoplasm as a bright diffuse green/yellow stain. In normal fungal cells, FUN 1 is metabolically converted into orange/red cylindrical intravacuolar structures. However, in cells with impaired metabolism, the dye remains in the cytoplasm in a diffuse pattern, thus indicating a disorder in the cells' metabolic vitality state (Millard *et al.*, 1997). For *Candida*, the effect observed for FUN 1 staining (data not shown) was similar to that observed for PI staining. For *Aspergillus*, no PI staining was found, as noted above. With FUN 1, however, a clear dose-dependent effect, as well as an influence of the incubation time, were observed (Fig. 3).

Eugenol at 0.64 µl ml^{-1} led to a metabolic arrest in over 90 % of the conidia after a 6 h incubation, while 1.25 µl ml^{-1} yielded the same result when read after 2 h (Fig. 3b). The results for EO were about 20 % lower than for eugenol (Fig. 3a), showing a direct correspondence to the proportion of eugenol (about 85 %) in the EO.

Permeation to PI, particularly following short incubation periods, as was the case in this study, indicates that the mechanism of action of the drug involves a primary lesion of the cell membrane leading to cell death. This way, it can be concluded that the observed dose-dependent fungicidal activity of clove oil and eugenol on yeasts, with severe lesion of the membrane, results from direct damage to the cell membrane rather than from metabolic impairment leading to secondary membrane damage. Furthermore, the effect on the cell membrane was shown to be higher than that resulting from treatment with amphotericin B, a fungicidal ergosterol-binding antifungal agent. Such a finding is in accordance with the biochemical nature of the assayed compounds. As for other EOs, clove oil is likely to have an influence on biological membranes, as supported by other reports found in the literature, not only in fungi but also in bacteria (Bakkali *et al.*, 2008; Carson *et al.*, 2006; Di Pasqua *et al.*, 2007).
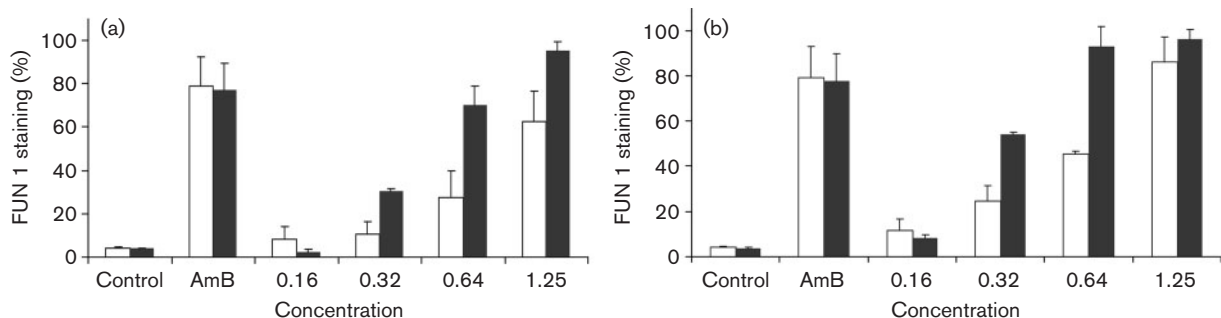
Fig. 3. FUN 1 staining of *A. fumigatus* ATCC 46645 conidia. Percentage of metabolically inactive conidia after treatment with serial concentrations of (a) clove oil and (b) eugenol for 2 h (white bars) and 6 h (black bars) and comparison with the percentage after amphotericin B (AmB) treatment and with that of an untreated control.

In order to determine the target of clove oil and eugenol in the cell membrane, we evaluated the effect of the compounds on the content of ergosterol. Ergosterol is specific to fungi and is the major sterol component of the fungal cell membrane, being responsible for maintaining cell function and integrity (Rodriguez et al., 1985). The primary mechanism of action by which azole antifungal drugs inhibit fungal cell growth is the disruption of normal sterol biosynthetic pathways, leading to a reduction in ergosterol biosynthesis (Kelly et al., 1995). Yeast cells of C. albicans growing in the presence of subinhibitory (0.32 µl ml^{-1}) concentrations of the EO or eugenol revealed a lower quantity of ergosterol in their membrane, in comparison to the control (Fig. 4a). Clove oil and eugenol considerably impair the biosynthesis of ergosterol by C. albicans, a similar effect being observed for C. glabrata and C. tropicalis strains (data not shown). For C. krusei, which is resistant to fluconazole, the same effect was observed, while the quantity of ergosterol was not affected by 1 µg fluconazole ml^{-1} (Fig. 4b).

The large spectrum of activity of clove oil and eugenol in acting on Candida, Aspergillus and dermatophytes, the fungicidal effects observed, the flow cytometry data, and the impairment in the biosynthesis of ergosterol all agree with the mechanism of action proposed: lesion of the cytoplasmic membrane. Burt (2004) proposed that different modes of action are involved in the antimicrobial activity of EOs. The activity may, in part, be due to their hydrophobicity, responsible for their partition into the lipid bilayer of the cell membrane, leading to an alteration of permeability and a consequent leakage of cell contents (Burt, 2004). As typical lipophiles, EOs pass through the cell wall and cytoplasmic membrane, disrupt the structure of the different layers of polysaccharides, fatty acids and phospholipids, and permeabilize them. Cytotoxicity appears to be characterized by such membrane damage. In bacteria, permeabilization of the membranes is associated with ion loss and reduction of membrane potential, collapse of the proton pump and depletion of the ATP

pool. Recent investigations on the antimicrobial action of some EOs showed disruption of the bacterial and fungal membrane (Bakkali et al., 2008; Cox et al., 2000, 2001; Di Pasqua et al., 2007; Hammer et al., 2004). All these reports suggest that this antimicrobial mechanism is due to membrane damage and our results further confirm this point of view. All of these data confirm that clove EO compromises the structural and functional integrity of cytoplasmic membranes.

In conclusion, the findings of the present study indicate that the clove oil from S. aromaticum has interesting potential as a therapeutic option against fungi that are pathogenic to humans. Clove EO is a broad-spectrum agent which inhibited not only dermatophytes, Aspergillus and Candida species (such as C. albicans, C. tropicalis and C. parapsilosis), but also fluconazole-resistant C. albicans isolates, C. krusei, which is intrinsically resistant to fluconazole, and C. glabrata, whose resistance is easily inducible.

Clove oil might be useful in the clinical management of candidosis, particularly mucocutaneous presentations such as vulvovaginal candidosis, considering its fungicidal activity and the inhibition of germ tube formation.

Bearing in mind that about 90 % of onychomycosis infections are caused by dermatophytes and the high sensitivity of this group of fungi to these compounds, as well as the activity against other agents involved in these infections (Candida spp. Aspergillus spp.), they could also be candidates to use in topical treatment of this kind of infection.

Given the results described above, particularly the possible mechanisms of action, which can induce side effects in humans, these antifungals need further investigation. Studies on toxicity, improved formulations, and determination of optimal concentrations for clinical applications, and comparative studies of the therapeutic efficacy of EOs with drugs currently in use to control fungal infections, should be encouraged.
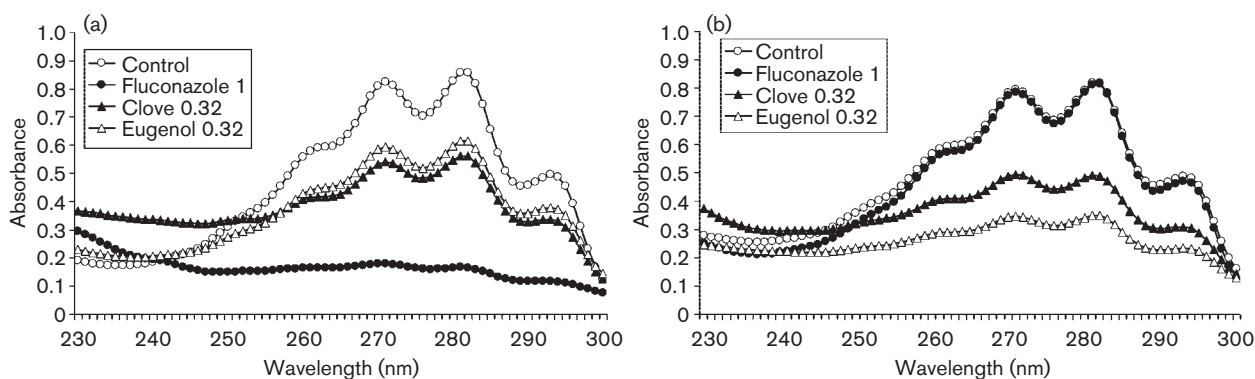
Fig. 4. UV spectrophotometric sterol profiles of C. albicans (a) and C. krusei (b) strains treated with clove oil or eugenol at 0.32 µl ml^{-1} and comparison with those of an untreated control and a control treated with 1 µg fluconazole ml^{-1}.

ACKNOWLEDGEMENTS

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