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Anticandidal Efficacy of Cinnamon Oil Against Planktonic and Biofilm Cultures of *Candida parapsilosis* and *Candida orthopsilosis*

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Abstract *Candida parapsilosis* is yeast capable of forming biofilms on medical devices. Novel approaches for the prevention and eradication of the biofilms are desired. This study investigated the anticandidal activity of sixteen essential oils on planktonic and biofilm cultures of *C. parapsilosis* complex. We used molecular tools, enumeration of colony-forming units, the colourimetric MTT assay, scanning electron microscopy (SEM) and a chequerboard assay coupled with software analyses to evaluate the growth kinetics, architecture, inhibition and reduction in biofilms formed from environmental isolates of the *Candida parapsilosis* complex; further, we also evaluated whether essential oils would interact synergistically with amphotericin B to increase their anticandidal activities. Of the environmental *C. parapsilosis* isolates examined, *C. parapsilosis* and *C. orthopsilosis* were identified. Biofilm growth on polystyrene substrates peaked within 48 h, after which growth remained relatively stable up to 72 h, when it began to decline. Details of the architectural analysis assessed by SEM showed that *C. parapsilosis* complex formed less complex biofilms compared with *C. albicans* biofilms. The most active essential oil was cinnamon oil (CO), which showed anticandidal activity against *C. orthopsilosis* and *C. parapsilosis* in both suspension (minimum inhibitory concentration—MIC—250 and 500 μg/ml) and biofilm (minimum biofilm reduction concentration—MBRC—1,000 and 2,000 μg/ml) cultures. CO also inhibited biofilm formation (MBIC) at concentrations above 250 μg/ml for both species tested. However, synergism with amphotericin B was not observed. Thus, CO is a natural anticandidal agent that can be effectively utilised for the control of the yeasts tested.

Keywords *C. parapsilosis* · Biofilms · Essential oils · Cinnamon oil · Anticandidal agents

Introduction

Fungi are increasingly important causes of acute and chronic deep-seated human infections, especially recurrent mucosal, cutaneous or nail infections that may be severe in debilitated or immunocompromised individuals. *Candida* spp. are commensal fungi that inhabit various niches of the human body; they are

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also considered the most common fungal pathogens isolated from clinical sites [1].

During the last decade, *Candida parapsilosis* was the second most common species of *Candida* isolated from blood cultures [1–3]. In South American hospitals, the incidence of *C. parapsilosis* is greater than *C. albicans* [3, 4]. *C. parapsilosis* has been renamed as following three distinct species: *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis* [5].

Little information is available regarding the ability of the reclassified species of the *C. parapsilosis* to form biofilms. It is well established that microorganisms contained within biofilms exhibit increased resistance to anticandidal treatments compared to individual cells grown in suspension [6–9]; probably, this phenomenon is most likely caused due to a combination of poor anticandidal agent penetration, nutrient limitation, adaptive stress responses, induction of phenotypic variability and the formation of persistent cells [7, 9].

The small number of drugs available for fungi treatment, most of which are fungistatic, and the emerging resistance to antifungal agents encourage the search for alternatives [10, 11]. Secondary plant metabolites such as essential oils, which are mixtures of multiple heterogeneous compounds, can exhibit anticandidal activity because many essential oils are thought to disrupt cell membranes with their lipophilic components [12, 13].

The potential use of essential oils for the prevention and treatment of *Candida* biofilms has been researched in several studies [11, 14–21]. Therefore, we chose the following sixteen species of medicinal plants that produce essential oils to determine their anticandidal activity on *C. parapsilosis* complex biofilms: *Boswellia thurifera*, *Cinnamomum zeylanicum*, *Citrus bergamia*, *Citrus aurantium amara*, *C. bigaradia*, *Citrus limonum*, *Citrus maxima*, *Commiphora myrrha*, *Copaıfera officinalis*, *Eucalyptus globulus*, *Juniperus virginiana*, *Pogostemon patchouli*, *Salvia officinalis* L., *Santalum album*, *Thymus vulgaris* and *Zingiber officinale*. We sought a possible source of new antibiotics for the treatment of biofilms from pathogenic fungi.

The purpose of this study was threefold. First, we aimed to compare the activities of the different essential oils against planktonic cells of *Candida parapsilosis* complex. Second, we tested the oils that demonstrated anticandidal activity against planktonic cells for their ability to prevent and treat *Candida parapsilosis* complex biofilms. Lastly, we determined the anticandidal activity of amphotericin B in combination with the oils actives against *C. parapsilosis* complex biofilms.

Materials and Methods

Yeast Strains

Seventeen environmental isolates of *C. parapsilosis* that were isolated from a hydraulic circuit of Hemodialysis Center in São Paulo State, Brazil, were tested in this study. They were phenotypically identified as *C. parapsilosis* by the use of API 20 C AUX (Biomerieux, Paris, France). Type strains ATCC 90018 (*C. parapsilosis*), ATCC 96141 (*C. orthopsilosis*), ATCC 96143 (*C. metapsilosis*), ATCC 6258 (*C. krusei*), ATCC 22019 (*C. parapsilosis*) and SC 5314 (*C. albicans* biofilm reference strain) were used as controls.

Essential Oils

The essential oils from the following plants were tested: *Boswellia thurifera*, *Cinnamomum zeylanicum*, *Citrus Bergamia*, *Citrus aurantium amara*, *C. bigaradia*, *Citrus limonum*, *Citrus maxima*, *Commiphora myrrha*, *Copaıfera officinalis*, *Eucalyptus globulus*, *Juniperus virginiana Pogostemon patchouli*, *Salvia officinalis* L., *Santalum album*, *Thymus vulgaris* and *Zingiber officinale*. Plant essential oils were diluted in 5% (vol/vol) in dimethyl sulfoxide (DMSO—*Sigma Chemical Co., St. Louis, MO*) that had been filter sterilised through a 0.22-μm-pore-size filter (Millipore, Billerica, A, USA). All plant essential oils were obtained from a local *Body and Mind Beautiful Com. de Cosméticos Ltda*., Franca, SP, Brazil. The oils that exhibited anticandidal activity were related by the manufacturer as the major components: α-pinene and myrcene in juniper and cinnamaldehyde and caryophyllene in cinnamon oil.

Molecular Identification of *C. parapsilosis* Complex

Yeast genomic DNA was extracted according to the protocol described by Moller et al. [22]. The two-step DNA-based identification test described by Tavanti

et al. [5] was used to screen *C. parapsilosis* isolates. Briefly, a 716-bp fragment of the secondary alcohol dehydrogenase (SADH) gene was amplified and digested with *Ban*I (New England Biolabs, Hitchin, UK). The PCR amplification conditions were as follows: 2 min at 94°C; 35 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 30 s; and a final step of 10 min at 72°C. The PCR products were then digested with the *Ban*I enzyme in a 40 μl volume containing 20 μl of the PCR product and 40 U of *Ban*I. The digestion products were separated on 2% agarose gels. The isolates were discriminated as *C. parapsilosis*, *C. orthopsilosis* or *C. metapsilosis* according to their SADH gene restriction profiles.

The Effect of Essential Oils on *C. orthopsilosis* and *C. parapsilosis* Planktonic Growth

Antifungal testing to determine the MIC for planktonic cells was performed according to the Clinical and Laboratory Standard Institute (formerly the National committee for Clinical Laboratory Standards) M-27A2 broth dilution assay [23]. RPMI 1640 medium containing L-glutamine without sodium bicarbonate (pH 7.0) supplemented with 0.165 M morpholinepropanesulfonic acid (both from Sigma) was utilised. The spectrophotometric method was used for the preparation of inoculums with a concentration of 0.5×10^3 to 2.5×10^3 cells per ml for each of the isolates prepared in the test medium. Stock solutions of essential oils were prepared in 5% DMSO, and twofold serial dilutions were prepared in RPMI in 96-well microtitre plates (Corning Incorporated, Corning, NY, USA); final concentrations ranged from 15.6 to 8,000 μg/ml. Yeast inocula (100 μl) were added to each well in the microtitre plates. Medium alone added to the inoculum was used as a growth control; the blank control contained only the medium. The microtitre plates were then incubated at 35°C; the endpoints were read visually at 48 h. Amphotericin B (final concentration of 0.0156 to 16 μg/ml) and strain ATCC 90018 (*C. parapsilosis*) were used as quality controls.

The minimum fungicidal concentration (MFC) was determined by plating 10 μl from the wells showing no visible growth on SDA. The plates were incubated at 30°C for 48 h. The minimum concentration of essential oil that showed ≥99.9% reduction in the original inoculums was recorded as the MFC [24].

Preparation of Standard Yeast Cell Suspensions for Biofilm Assays

For biofilm growth, the protocol of Ramage et al. was followed [25]. Briefly, *Candida* were grown in SDA plates at 37°C for 18 h and then inoculated into yeast nitrogen base (YNB) medium (Difco) supplemented with 50 mM glucose. After overnight culture in a rotary shaker at 75 rpm, the yeast was harvested in the late exponential growth phase and washed twice with 20 ml of 0.1 M phosphate-buffered saline (PBS) pH 7.2. The suspensions were then diluted in RPMI as required to generate a final concentration of 0.5×10^6–2.5×10^6 cells/ml (counting with a haematocytometer).

Kinetics of Biofilm Formation on Microtitre Plates

Biofilms were formed in microtitre plates (Corning) over a series of time intervals (4, 6, 8, 12, 18, 24, 36, 48, 72, 84 or 96 h) at 37°C. At each time interval, biofilm production was quantified using an MTT reduction assay. For each time interval, four biofilm replicates were evaluated.

At overall time points, viable sessile cell counts were assessed. First, wells were extensively washed, and serial tenfold dilutions in sterile PBS were performed. The inoculum size for each cell suspension was confirmed by plating aliquots of 1:10, 1:100 and 1:1,000 dilutions on SDA (Difco) plates; CFU was obtained after a 48-h incubation period at 37°C. Results are expressed as $\log_{10}$ CFU/ml [17].

MTT analysis of replicate biofilms was performed in parallel with the total viable counts assay to demonstrate a correlation between these two techniques.

MTT Reduction Assay

Fungal viability was analysed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT—Sigma) as described previously [16, 26]. Briefly, 100 μl of prewarmed MTT solution (0.5 mg/ml) in PBS containing 0.1% glucose was added to each well. The plates were incubated at 37°C for 6 h, and the MTT solution was then removed. Biofilms were washed once with PBS, and the formazan product was resuspended in acid isopropanol [5% (vol/vol) 1 M HCl in isopropanol]. Finally, the absorbance was measured

(A_{540nm}). The minimum biofilm reduction concentration (MBRC) was defined as the concentration of essential oil showing A_{540nm} values equal to or lower than those of the control well that contained a biofilm treated with saline; the minimum biofilm fungicidal concentration (MBFC) was defined as the concentration with A_{540nm} values below or equal to the background level (acid isopropanol) [12, 27].

Scanning Electron Microscopy

For SEM, biofilms were formed on sterile PVC discs within 24-well microtitre plates (*Corning*) by dispensing 300 μl of standardised cell suspensions containing 2.5×10^6 cells/ml in RPMI 1640 onto the appropriate discs at 37°C [25]. Following biofilm formation, different CO concentrations (serial twofold dilutions ranging from 500 to 4,000 μg/ml) were added to selected wells, and the plates were incubated for 24 h at 37°C. For SEM observations, the biofilms were assayed as previously described by Priester et al. [28]. Briefly, the discs were removed, washed with PBS and placed in a fixative [4% formaldehyde (vol/vol), 1% glutaraldehyde (vol/vol) in PBS] solution overnight. The samples were rinsed 2 times for 3 min each in 0.1 M phosphate buffer, postfixed with 1% (w/v) OsO_4 for 1 h, dehydrated using an ethanol gradient, critical point dried in CO_2 (Critical Point Dryer, model CPD-030, Balzers, Oberkochen, Germany) and gold coated by sputtering (Denton Vacum, model Desk II, Freehold, NJ, USA). After processing, samples were observed with a scanning electron microscope (JEOL, model JSM 5410, Japan) in high vacuum mode at 15 kV. The images were processed for display using Photoshop software (Adobe Systems Inc., Mountain View, CA.).

Minimum Biofilm Inhibitory Concentrations

The effect of essential oils on *C. parapsilosis* complex biofilm formation was examined by the microbroth dilution method, similar to the MIC assay described above for planktonic cells (M27-A2). Essential oils (100 μl) diluted in RPMI to concentrations that ranged from 15.6 to 8,000 μg/ml were added to 100 μl of the inoculums suspension in 96-well polystyrene microtitre plates (Corning) and incubated for 48 h at 35°C [13, 29]. Controls containing inocula in RPMI and broth alone were included. The reference biofilm strain *C. albicans* SC 5314 was used as a control [25]. Following incubation, the culture supernatants from each well were aspirated, and the non-adherent cells were removed by washing the wells with sterile PBS. A semi-quantitative measure of biofilm formation was calculated using the MTT reduction assay.

Minimum Biofilm Reduction Concentrations and Minimum Biofilm Fungicidal Concentrations

Biofilms were grown as described above but without the presence of essential oils. After 24 h incubation at 37°C, the biofilms were washed three times with sterile PBS and exposed to 100 μl of essential oils, with oil concentrations ranging from 8,000 to 15.6 μg/ml in RPMI. The plates were then incubated for 48 h at 37°C, and the wells were washed once with sterile PBS and the fungal viability was determined using the MTT reduction assay [17, 29]. This assay was performed in duplicate microtitre plates. The reference strain *C. albicans* SC 5314 was used as a biofilm control [25].

Chequerboard Assays to Assess the Anticandidal Activity of *Cinnamomum zeylanicum* Essential Oil in Combination with Amphotericin B Against *C. parapsilosis* Complex Biofilms

The anticandidal activity of CO in combination with AMB was assessed by the chequerboard method [27, 30]. Microtitre plates containing *C. parapsilosis* complex biofilms were washed once with sterile PBS to remove any unbound cells. Serial double dilutions of the anticandidal compounds were prepared (16,000 μg/ml to 62.5 μg/ml for CO and 32 to 0.25 μg/ml for AMB) in RPMI. Next, 50 μl of the essential oil solutions was added to the rows of a 96-well microtitre plates in diminishing concentrations, and 50 μl of AMB was added to the columns in diminishing concentrations. Columns 10 and 11 contained biofilms and anticandidal compounds alone at various concentrations. Column 12 contained the biofilm and saline, and the saline alone controls. The microtitre plates were incubated at 37°C for 48 h. Fractional inhibitory concentrations (FICs) were calculated as the MIC of the combination of amphotericin B and CO divided by the MIC of CO or AMB alone. The FIC index (FICI) was calculated by adding both FICs and was interpreted in the following

manner. A synergistic effect was reported when the FIC value was ≤0.5, no interaction was reported when the FIC value fell between 0.5 and 4.0 ranges, and an antagonistic effect was reported when the FIC value was >4.0 [27, 29]. Assays were performed in duplicate microtitre plates.

Data Analysis

Data analysis was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). Descriptive statistics, such as the mean, standard deviation, median, upper and lower quartile, and highest and lowest value and the Pearson correlation coefficient, were used for statistical analysis. Comparisons of two groups were done using Mann–Whitney test, and a P value <0.05 was considered significant.

Results

C. parapsilosis Complex Identification

To identify the *C. parapsilosis* complex isolates, a fragment of the SADH gene (716 bp) was amplified by PCR (Fig. 1a) from genomic DNA samples. *Ban*I restriction analysis of the SADH amplicons was used to identify *C. orthopsilosis, C. metapsilosis* and *C. parapsilosis*; *C. orthopsilosis* SADH amplicons do not contain a *Ban*I restriction site, while *C. metapsilosis* amplicons contain three *Ban*I restriction sites (at positions 96, 469 and 529), and *C. parapsilosis* amplicons contain only one *Ban*I restriction site (at position 196). Using this technique, 53% of isolates were assigned to the most commonly encountered subtype, *C. parapsilosis*, and 47% of isolates were identified as *C. orthopsilosis* (Fig. 1-b). No *C. metapsilosis* isolates were identified in this study.

Quantification and Visualisation of Biofilms

The ability of *Candida* species to form biofilms was quantified by determining biofilm metabolic activity using the MTT assay and total viable cell counts. The kinetics of biofilm formation for both *C. parapsilosis* species and the *C. albicans* SC 5314 reference strain were similar; the MTT readings peaked at 48 h and declined thereafter (Fig. 2). When the Pearson correlation test was used to compare the spectrometric profile with the CFU counts, a significant correlation was found for *C. parapsilosis*, *C. orthopsilosis* and *C. albicans* ($r = 0.940$, $r = 0.951$, $r = 0.958$, respectively). Maximum cell density was observed at 48 h; decreased OD readings at 72 h suggest that the biofilms entered a declining growth phase at this time point (Fig. 2). SEM microscopy was used in conjunction with these semi-quantitative methods to monitor 24-h biofilm growth. Examination of single species biofilms showed the presence of different cellular morphologies in the biofilm structure. *C. albicans* SC 5314 produced a more profuse biofilm compared to the two *C. parapsilosis* species. *C. orthopsilosis* biofilms presented short and filamentous forms, while *C. parapsilosis* biofilms exhibited a blastospore aggregate layer with irregular clusters. In contrast, *C. albicans* developed a multilayer biofilm structure with a basal yeast layer that was covered by a relatively open network of hyphae penetrating the matrix (Fig. 3). SEM imaging of CO-treated *Candida* biofilms found that not only sessile *Candida* cells effectively are damaged by CO (Fig. 4) but that CO

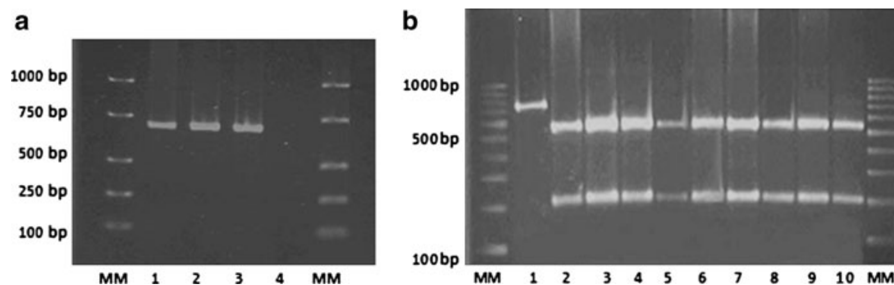

Fig. 1 Discrimination of *C. parapsilosis* complex according to their SADH gene restriction profiles. (**a**) Amplification of a 716-bp SADH gene fragment from the genomic DNA from *C. parapsilosis* complex isolates to provide genetic confirmation. (**b**) Representative SADH gene restriction profile for *C. orthopsilosis* (*lane 1*) and *C. parapsilosis* (*lanes 2–10*) isolates. *Lanes MM*, 100-bp ladder

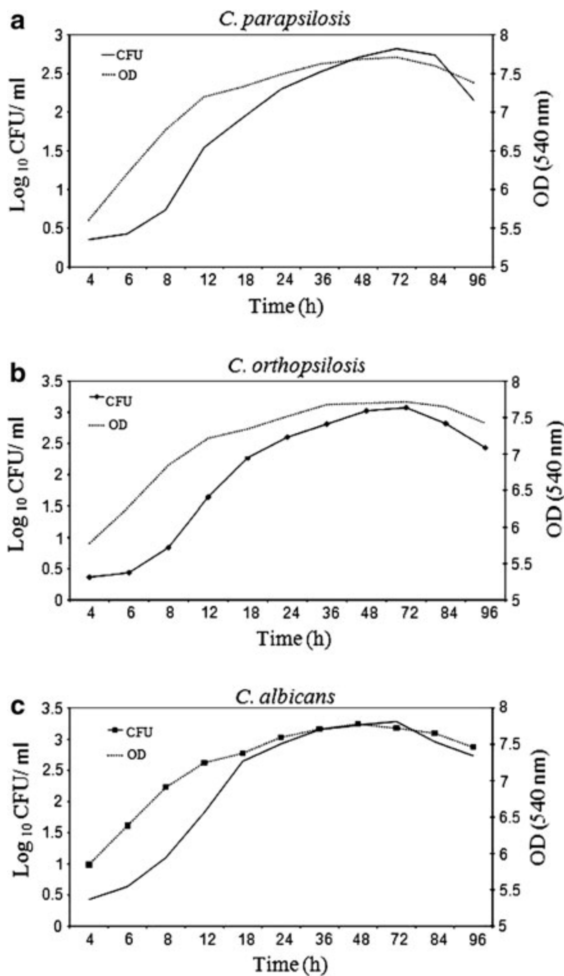

Fig. 2 Biofilm formation by *C. parapsilosis* (**a**), *C. orthopsilosis* (**b**) and *C. albicans* SC 5314 (**c**) growing on microtitre plates. Assays consisted of measurements of MTT formazan formation (*dashed with dotted line*) and enumeration of viable cells (*solid line*). Data represent the means ± standard errors of three independent experiments performed in duplicate. *Error bars* are omitted because the standard errors of the mean were significantly lower

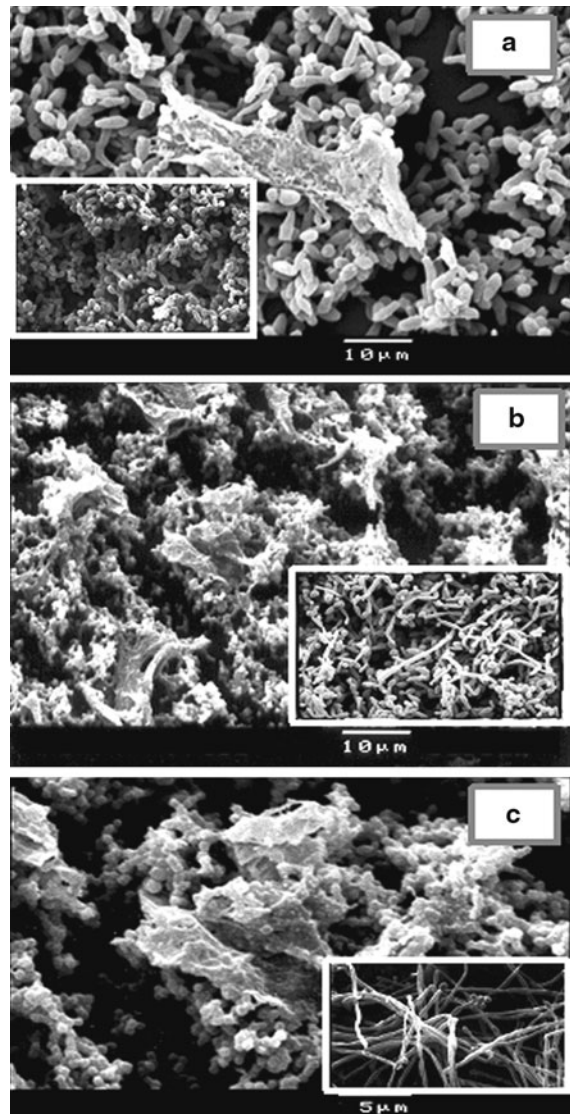

Fig. 3 Scanning electron micrographs (SEM) showing 24-h biofilms formed on PVC discs by different *Candida* species: (**a**) *C. orthopsilosis*, (**b**) *C. parapsilosis* and (**c**) *C. albicans* SC 5314. Samples were fixed prior to processing for SEM

also detached biofilms (Fig. 4). These results indicate that CO has a dual mode of action against *C. parapsilosis* complex biofilms; it is able to detach adhering yeast from a substratum surface while also killing sessile cells.

Effects of Essential Oils on *C. parapsilosis* Complex Planktonic Growth

The MIC and MFC of sixteen essential oils were evaluated in vitro against nine *C. parapsilosis* strains and eight *C. orthopsilosis* strains; all values are listed in Table 1. The MIC and MFC tests showed similar trends for the yeast isolates tested, where the MFC values were similar to or more than twofold greater than the MIC values. The *C. parapsilosis* complex was more susceptible to *Cinnamomum zeylanicum* oil, with MIC values between 250 and 500 μg/ml and MFC values between 500 and 1,000 μg/ml for *C. orthopsilosis* and *C. parapsilosis*, respectively.

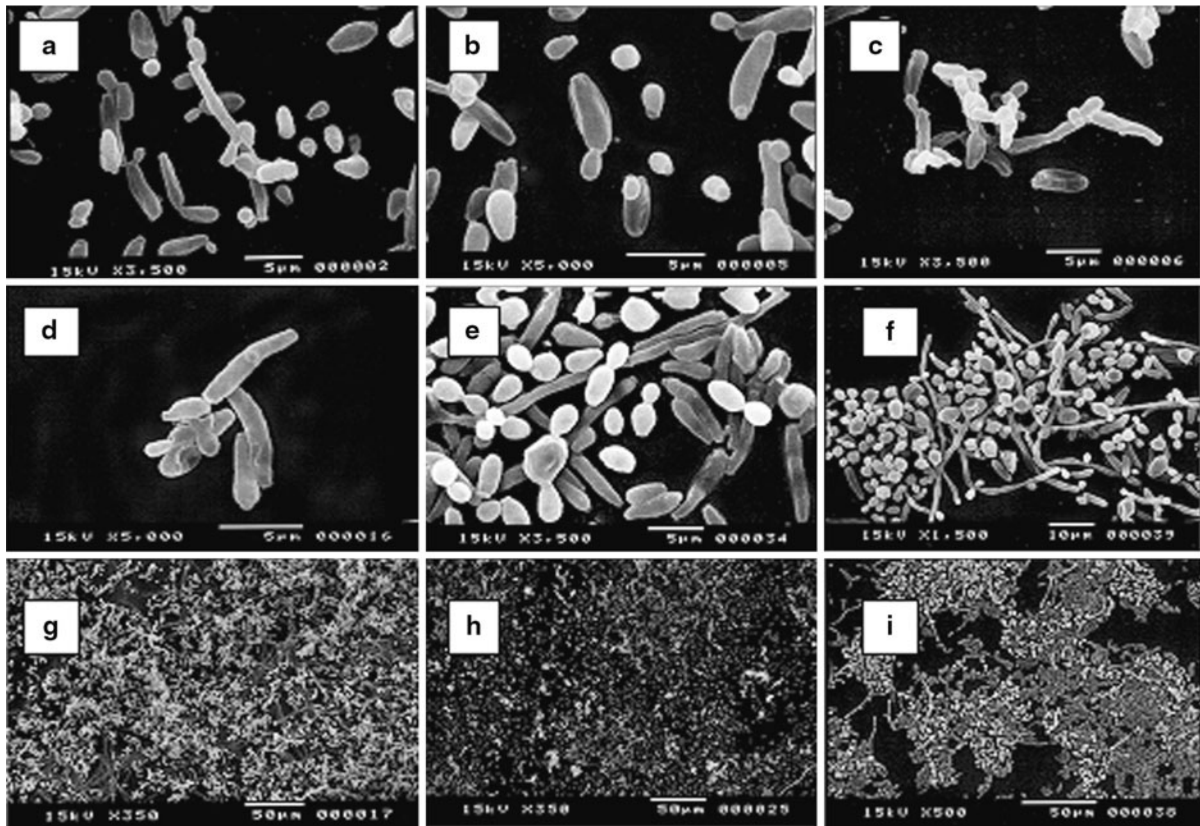

Fig. 4 Representative micrographs showing biofilms (24 h) formed by *Candida orthopsilosis* (**a**, **d**, **e**), *C. parapsilosis* (**b**, **e**, **h**) and reference strain *C. albicans* SC 5314 (**c**, **f**, **i**) after 48-h *Cinnamomum zeylanicum* oil treatment in different magnifications. Sessile *Candida* cells exhibited notable alterations in the membrane and the cell wall, besides a disordered profile of germination (**a–f**). Visualisation of the ultra structure in general revealed a marked reduction in biofilm constituents in all *Candida* species (**g–i**). *Bars*, **a–e** 5 μm; **f** 10 μm; **g–i** 50 μm

Biofilm Susceptibility Assays

Essential oils from two plants, *Juniperus virginiana* and *Cinnamomum zeylanicum*, were selected for the evaluation of their antifungal activity on *C. parapsilosis* complex biofilm growth. The inhibitory effect on the biofilm formation by CO is depicted in Fig. 5. CO concentrations above 250 μg/ml effectively abolished *C. parapsilosis* complex biofilm formation, and in the reference strain, *C. albicans* SC 5314 was abolished at a CO concentration of 125 μg/ml. For the *J. virginiana* essential oil, higher concentrations (4,000 μg/ml) were required to inhibit biofilm formation (data not shown).

The MBRC and MBFC values after treatment with CO were similar between *C. orthopsilosis*, *C. parapsilosis* and the *C. albicans* SC 5314 reference strain (1,000, 2,000 and 2,000 μg/ml, respectively) (Table 2). For *C. orthopsilosis,* the concentration necessary for the reduction of preformed biofilms (1,000 μg/ml, Table 2) was fourfold greater than the concentration required to inhibit biofilm formation (250 μg/ml, Fig. 5). Whereas for *C. parapsilosis*, the concentration necessary for the reduction of preformed biofilms (2000 μg/ml, Table 2) was eightfold greater than the concentration required to inhibit biofilm formation (250 μg/ml, Fig. 5). *J. virginiana* essential oil reduced *C. parapsilosis* complex or *C. albicans* SC 5314 biofilm growth at high concentrations (Table 2). Five per cent (v/v) DMSO, which was used as a cosolvent in the oil suspensions, did not show anticandidal activity against *C.*

Table 1 MICs and MFCs of essential oils for suspensions cultures of *C. parapsilosis* complex determined by the broth microdilution method and agar plating, respectively

Essential oil	Antifungal activity in μg/ml			
	C. orthopsilosis		*C. parapsilosis*	
	MIC	MFC	MIC	MFC
Boswellia thurifera	>4,000	>4,000	>4,000	>4,000
Cinnamomum zeylanicum	250	500	500	1,000
Citrus bergamia	>4,000	>4,000	>4,000	>4,000
Citrus aurantium amara	2,000	4,000	2,000	4,000
Citrus bigaradia	>4,000	>4,000	>4,000	>4,000
Citrus limonum	2,000	4,000	2,000	4,000
Citrus maxima	4,000	4,000	4,000	4,000
Commiphora myrrha	>4,000	>4,000	>4,000	>4,000
Copaifera officinalis L.	2,000	4,000	2,000	4,000
Eucalyptus globulus	2,000	2,000	2,000	2,000
Juniperus virginiana	1,000	1,000	1,000	1,000
Pogostemon patchouli	4,000	4,000	4,000	4,000
Salvia officinalis L.	>4,000	>4,000	>4,000	>4,000
Santalum album	>4,000	>4,000	>4,000	>4,000
Thymus vulgaris	1,000	2,000	1,000	2,000
Zingiber officinale	>4,000	>4,000	>4,000	>4,000

MIC minimum inhibitory concentration, *MFC* minimum fungicidal concentration

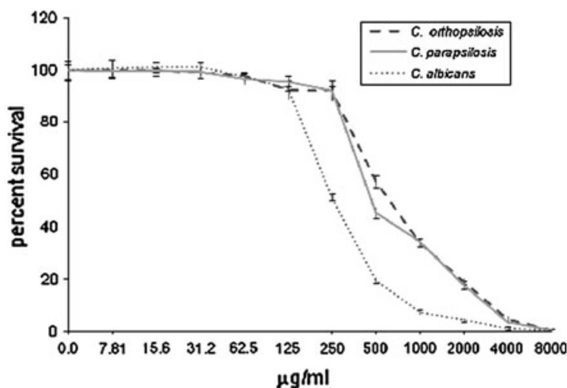

Fig. 5 Effect of cinnamon oil on *Candida* biofilm formation (MBICs). Different cinnamon oil concentrations (7.81–8,000 μg ml) were added to *Candida* cells and incubated under biofilm growing conditions. The *lines* depict the per cent survival of *Candida* cells exposed to cinnamon oil. No statistically significant differences were found among the *C. parapsilosis* complex (Mann–Whitney test, $P = 0.938$). Assays consisted of measurements of MTT formazan formation; the metabolic activity was normalised to the control without oil, which was taken as 100%. All values are means and standard deviations of three independent experiments done in quadruplicate

parapsilosis complex when grown in suspension or as a biofilm.

Chequerboard Assays to Assess the Anticandidal Activity of Cinnamon Oil in Combination with AMB Against *C. parapsilosis* Complex Biofilm Growth

The combination of CO with AMB demonstrated indifferent activity against *C. parapsilosis* and *C. orthopsilosis*, with an FICI value of 1.5 for both (Table 3). Indifference was also demonstrated with the combination against *C. albicans* SC 5314, with an FICI value of 1.5.

Discussion

The increasing incidence of hospital-acquired infections caused by drug-resistant fungal pathogens, host toxicity, the poor efficacy of drugs and high treatment costs has drawn attention to the potential of natural products as antifungals. Several studies have investigated the

Table 2 MBRCs and MBFCs of two essential oils for biofilm cultures of *C. parapsilosis* complex and reference strain *C. albicans* SC 5314

Strains	Antifungal activity in µg/ml			
	Cinnamomum zeylanicum		*Juniperus virginiana*	
	MBRC	MBFC	MBRC	MBFC
C. parapsilosis	2,000	2,000	4,000	8,000
C. orthopsilosis	1,000	1,000	4,000	4,000
C. albicans SC 5314	2,000	2,000	4,000	>8,000

MBRC minimum biofilm reduction concentration, *MBFC* minimum biofilm fungicidal concentration

Table 3 Antimicrobial activities of cinnamon oil and amphotericin B against biofilm cultures of *C. parapsilosis* complex environmental isolates

Strain	Combination	MIC of oil (µg/ml) in combination/alone	FIC of oil	MIC of AMB (µg/ml) in combination/alone	FIC of AMB	FICI	Result
C. parapsilosis	CO + AMB	1,000/2,000	0.5	2/2	1	1.5	Indifference
C. orthopsilosis	CO + AMB	500/1,000	0.5	0.5/0.5	1	1.5	Indifference
C. albicans SC 5314	CO + AMB	1,000/2,000	0.5	2/2	1	1.5	Indifference

MIC minimum inhibitory concentration, *FIC* fractional inhibitory concentrations, *AMB* amphotericin B, *FICI* FIC index, *CO* cinnamon oil

anticandidal effects of essential oils on *Candida* spp. [11, 31–35]. However, there is very limited information regarding the effect of essential oils on *C. parapsilosis*, either in planktonic or biofilm cultures.

The yeast species *Candida parapsilosis*, a nosocomial pathogen, exhibits both genomic and genetic heterogeneity. Recently, based on restriction polymorphism analysis of the SADH gene, which encodes a secondary alcohol dehydrogenase that is common to all species, *C. parapsilosis* complex isolates were distinguished into three species [5]. In this study, molecular analysis found that 53% of the isolates were *C. parapsilosis,* while 47% corresponded to *C. orthopsilosis*. Although substantial regional variation has been noted, most studies [36–39] found that *C. orthopsilosis* occurs in less than 10% of *C. parapsilosis* complex infections. However, a previous study [40] reported a *C. parapsilosis* complex isolate with 23.8% of *C. orthopsilosis.* This study reinforces a hypothesis that *C. parapsilosis* may be related to its ubiquitous nature because it is commonly isolated from different environmental sources.

When grown on a surface, *C. parapsilosis* adapts to a biofilm lifestyle [16]. Sessile, biofilm-associated cells can be distinguished from their planktonic, free-floating counterparts by their generation of an extracellular polymeric matrix, reduced growth rates and the up- and down-regulation of specific genes. In this study, the ability of *C. parapsilosis* complex to adhere to and form biofilms on polystyrene and PVC surfaces under static conditions was demonstrated. Biofilm growth was monitored by a colourimetric assay and total viable cell counts. Our quantitative results are in accordance with a previous study, which reported that *C. parapsilosis* complex exhibited an increased ability to form biofilms [41, 42].

Here, microscopy findings confirmed that *C. albicans* produces larger biofilms compared to *C. parapsilosis* species. Studies of non-albicans *Candida* species such as *C. parapsilosis*, which do not naturally form true hypha, suggest that hyphal morphology is not a prerequisite for biofilm formation [43–45]. Although the current data on *C. parapsilosis* complex biofilm corroborate these

observations, a previous study [17] suggested that *Candida* hyphae contribute to biofilm architecture by forming a scaffold that provides robust support for exopolymeric substances and the blastospores to develop a three-dimensional, spatially organised community with several layers.

Selected natural products of plant origin can influence microbial biofilm formation through different mechanisms. Many plant essential oils, which are mixtures of numerous organic chemicals, contain compounds that inhibit microbial growth. In our study, the responses of *C. parapsilosis* complex biofilms to juniper oil and CO were measured by the MBRC and MBIC assays. The *J. virginiana* essential oil weakly inhibited the formation of biofilms and reduced preformed yeast biofilms. Alpha-pinene and myrcene are the predominate components (61.56%) of *J. virginiana* essential oil. It has been reported that, in yeast cells and isolated mitochondria, α-pinene and *β*-pinene destroy cellular integrity, inhibit respiration and ion transport and increase membrane permeability [46].

In assessing the anticandidal activity of essential oils, the difficulties in comparing results obtained experimentally with the existing data should be taken into account. The chemical composition of essential oils can vary within species for a variety of reasons, including the presence of chemotypes, the time of harvest and the use of different extraction methods [47]. Other factors that may explain our results include the absence of standardised anticandidal assays. Differences regarding experimental conditions, such as broth dilution or agar diffusion, culture medium composition, inoculum concentration, incubation time and temperature, prohibit strict comparisons [34].

In the present study, it was shown that CO exhibited anticandidal activity against both planktonic and biofilm cultures of environmental *C. orthopsilosis* and *C. parapsilosis* isolates; these results are consistent with other accounts where CO has shown effectiveness against other important pathogens [12, 34, 48]. *Cinnamomum zeylanicum* oil, which was predominately composed of cinnamaldehyde (54%), inhibited the growth of *C. parapsilosis* complex biofilms with low MBICs (250 μg/ml). A possible explanation for CO fungicidal activity is that the hydrophobicity of their volatile components allows for their insertion into the lipid bilayer of the cell membrane, disturbing it and increasing its permeability to protons. Subsequently, extensive leakage or the removal of critical molecules and ions eventually leads to microbial cell death [49]. This hypothesis is supported by the SEM results after CO treatment. Obtained images showed a marked decrease in cell aggregates and the complete absence of an extracellular matrix in *Candida* biofilms. Additionally, disruption resulting in the subsidence of the external portion of the cells was observed, indicating sessile cell damage. However, the susceptibility of *C. parapsilosis* complex to an essential oil derived from *Cinnamomum zeylanicum* has not been published for either planktonic organisms or for sessile cells.

Additionally, the MIC of CO for planktonic *C. orthopsilosis* and *C. parapsilosis* was similar to their MBIC values (250 and 500 μg/ml, 250 and 250 μg/ml, respectively). This suggests that CO has similar anticandidal activity against both planktonic and biofilm growth, which could be due to its action on cell membranes. CO and one of its main components, cinnamaldehyde, inhibit the proton motive force, respiratory chain, electron transfer and substrate oxidation in cells, resulting in the uncoupling of oxidative phosphorylation, inhibition of active transport, loss of pool metabolites and the disruption of DNA, RNA, protein, lipid and polysaccharide synthesis [12, 31, 50–52]. The main limitation of this study was the lack of evaluation in vivo anticandidal activities imposed by the complexity of the assays. Although CO and AMB are associated with activity in the plasma membrane and cell wall, suggesting the action potential, synergism was surprisingly not observed when CO and AMB were combined. It is possible that the synergistic interaction does not involve a single target or a single component [12]. It has been suggested that the diffusion of cationic compounds in biofilms is hindered by the negative charge of the extracellular matrix, which changes the physicochemical and tertiary structure of the biofilm. Because essential oils are composed of heterogeneous compounds and AMB is an amphoteric drug, it is possible that AMB treatment alters both the ionic interactions in the biofilm extracellular matrix and its mechanism of action against the extracellular matrix. However, further studies are needed to establish the mode of action of amphotericin B and CO in combination.

In conclusion, this study demonstrated that CO has excellent antifungal activity against biofilm or planktonic cultures of environmental *C. orthopsilosis* and *C. parapsilosis* isolates. CO was able to inhibit biofilm formation and kill yeast cells in *C. parapsilosis* complex biofilms. Importantly, biofilms were equally as sensitive to CO as their planktonic counterparts for *C. orthopsilosis*, mostly likely due to the similar activity of CO on yeast cell membranes. Further studies are required to elucidate the complex mechanism of action of CO and its components against biofilms formed by *C. parapsilosis* complex.

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