ISHAM INTERNATIONAL SOCIETY FOR HUMAN AND ANIMAL MYCOLOGY

Medical Mycology, 2016, 54, 515–523
doi: 10.1093/mmy/myw003
Advance Access Publication Date: 11 February 2016
Original Article

Original Article

Antifungal activity of plant-derived essential oils on *Candida tropicalis* planktonic and biofilms cells

Caio Marcelo Cury Souza[1], Silvio Alves Pereira Junior[1], Thaís da Silva Moraes[1], Jaqueline Lopes Damasceno[1], Suzana Amorim Mendes[1], Herbert Júnior Dias[2], Ricardo Stefani[3], Denise Crispim Tavares[1], Carlos Henrique Gomes Martins[1], Antônio Eduardo Miller Crotti[2], Maria José Soares Mendes-Giannini[4] and Regina Helena Pires[4,*]

[1]Laboratório de Pesquisa em Microbiologia Aplicada, Universidade de Franca, Franca, SP, Brazil, [2]Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, São Paulo, Brazil, [3]Instituto de Ciências Exatas e da Terra, Universidade Federal do Mato Grosso, Pontal do Araguaia, MT, Brazil and [4]Faculdade de Ciências Farmacêuticas, Universidade Estadual Paulista Júlio de Mesquita Filho, Araraquara, São Paulo, Brazil

*To whom correspondence should be addressed. Regina Helena Pires, Faculdade de Ciências Farmacêuticas, Núcleo de Proteômica, Rodovia Araraquara-Jaú, km1, 14.902-801- Araraquara, SP, Brazil. Tel: +55 -16- 3711-8728; E-mail: rehepi@gmail.com

Received 13 July 2015; Revised 23 December 2015; Accepted 5 January 2016

Abstract

Dental prosthesis supports *Candida* species growth and may predispose the oral cavity to lesions. *C. tropicalis* has emerged as a colonizer of prosthesis and has shown resistance to clinically used antifungal agents, which has increased the search for new antifungals. This work describes the effectiveness of fifteen essential oils (EOs) against *C. tropicalis*. The EOs were obtained by hydrodistillation and were chemically characterized by gas chromatography-mass spectrometry. The antifungal activities of the EOs were evaluated by the microdilution method and showed that *Pelargonium graveolens* (Geraniaceae) (PG-EO) was the most effective oil. Geraniol and linalool were the major constituents of PG-EO. The 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT) assay showed that all the clinical *C. tropicalis* strains formed viable biofilms. Scanning electron microscopy examination of the biofilms revealed a complex architecture with basal layer of yeast cells and an upper layer of filamentous cells. Treatments with PG-EO, linalool, and geraniol significantly reduced the number of viable biofilm cells and inhibited biofilm formation after exposure for 48 h. PG-EO, geraniol, and linalool were not toxic to normal human lung fibroblasts (GM07492A) at the concentrations they were active against *C. tropicalis*. Together, our results indicated that

C. tropicalis is susceptible to treatment with PG-EO, geraniol, and linalool, which could become options to prevent or treat this infection.

Key words: *C. tropicalis*, essential oils, *P. graveolens*, biofilms.

Introduction

Denture stomatitis is a common disorder affecting denture wearers. *C. albicans* is usually isolated from both the fitting surface of the denture and the denture-bearing mucosa of the affected patients.[1,2] Other *Candida* species are often recoverable from dentures and underlying mucosal tissues.[3,4]

Recently, accurate fungal identification by advanced technologies has revealed that *C. tropicalis* is a potential opportunistic fungus that affects an increasing number of patients susceptible to opportunistic parasites, such as immunosuppressed patients.[5,6] In addition, *C. tropicalis* has been isolated from denture stomatitis.[2,7]

Currently, infections caused by *Candida* spp. are associated with biofilm formation on the surface of medical devices or the host's epithelium. By definition, biofilms consist of a microbial community characterized by cells that form microcolonies. These microcolonies are irreversibly adhered to a substrate or to each other and are embedded in a matrix of extracellular substances. Consequently, they exhibit an altered phenotype with respect to growth rate and gene transcription.[8] Previous reports of *C. tropicalis* biofilms isolated from denture stomatitis[7] and of *C. tropicalis* mixed bacterial biofilms[9] have demonstrated that this species can adhere to dentures. These findings have made investigation into the biofilm-forming properties of this organism a matter of utmost importance.

From a medical viewpoint, the most critical feature of biofilm growth is the development of antimicrobial resistance by the microorganisms that constitute the biofilm.[10] Studies have demonstrated that biofilms formed by *Candida* species are resistant to the antifungal agents used in routine clinical practice; for example, amphotericin B, fluconazole, itraconazole, and ketoconazole.[10] Additionally, some patients infected by *Candida* species have experienced microorganism resistance to many existing antifungals and have had to cope with undesirable side effects like hemolysis and nephrotoxicity.[11] All these issues have created a pressing need for novel efficient and pathogen-specific antifungal agents.

Natural products of plant origin and their analogs are an important source of antimicrobial agents. Among these products, essential oils (EOs), which are a mixture of volatile and low-polarity secondary metabolites, are noteworthy. Although EOs have been tested against *C. albicans* biofilms[12,13] very few studies have been conducted on the activity of EOs against *Candida* non-*albicans* biofilms, especially *C. tropicalis*.

This study describes the susceptibility of *C. tropicalis* to 15 different EOs, the ability of this *Candida* species to form biofilms on polystyrene microtiter plates *in vitro*, and the susceptibility of candidal biofilms to the most active of the 15 investigated EOs and its major components.

Materials and methods

Plant material and essential oil (EO) extraction

The EOs of 15 plant species were investigated. The plant species codes and voucher specimen numbers were as follows: *Artemisia absinthium* L. (Asteraceae) (AA, SPFR 12417); *Artemisia camphorata* L. (Asteraceae) (ACa, SPFR 10006); *Ageratum conyzoides* L. (Asteraceae) (ACo, SPFR 12324); *Bidens sulphurea* (Cav.) Sch. Bip (Asteraceae) (BS, SPFR 12020); *Chenopodium ambrosioides* L. (Chenopodiaceae) (CA, SPFR 13163); *Coreopsis lanceolata* L. (Asteraceae) (CL, SPFR 10007); *Citrus reticulata* Blanco (Rutaceae) (CR, UFMT 4562); *Foeniculum vulgare* Mill. (Apiaceae) (FV, SPFR 12024); *Lavandula officinalis* L. (Lamiaceae) (LO, SPFR 13163); *Ocimum gratissimum* L. (Lamiaceae) (OG, SPFR 12420); *Pelargonium graveolens* Schl. (Geraniaceae) (PG, SPFR 12023); *Syzigium aromaticum* (L.) Merr. & L.M.Perry (SA, SPFR 12418); *Tagetes erecta* L. (Asteraceae) (TE, SPFR 10009); and *Tetradenia riparia* (Hochst.) Codd (TR, SPFR 12421). *C. reticulata Blanco* (*Rutaceae*) was collected near the city of Catalão (18°10′47″S 46°56′36″W G), State of Goiás, Brazil; it was identified by Prof. Dr. José Realino de Paula (Departamento de Tecnologia Farmacêutica, Faculdade de Farmácia, UFMT), and a voucher specimen was deposited at the Herbarium of Universidade Federal do Mato Grosso (UFMT). The other plant species were collected at "May 13th Farm" (20°26′S 47°27′W 977 m) near Franca, state of São Paulo, Brazil; they were identified by Prof. Dr. Milton Groppo (Departamento de Botânica, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo), and one voucher of each specimen was deposited at the Herbarium of the Department of Biology (Herbarium SPFR).

Fresh leaves of each plant were subjected to hydrodistillation in a Clevenger-type apparatus for 4 h. Briefly, 450 g of the plant material was divided into three samples of 150 g

each, and 500 ml of distilled water was added to each sample. After manual collection of the EOs, anhydrous sodium sulfate was used to remove traces of water, and the EOs were filtered. Then, the EOs were stored in an amber bottle and kept in the refrigerator at 4°C until analysis. The EO yield was calculated from the weight of fresh leaves and expressed as the average of triplicate analysis. The EO that showed the best antimicrobial activity (MIC < 250 μg/ml) was used for further analysis. The major components of the most active EO were also submitted to the antimicrobial assay.

Gas chromatography (GC) and gas chromatography/mass spectrometry (GC-MS)

The essential oil of *P. graveolens* (PG-EO) was analyzed by gas chromatography (GC) on a Hewlett-Packard G1530A 6890 gas chromatograph fitted with a flame ionization detector (FID) and a data-handling processor. An HP-5 (Hewlett-Packard, Palo Alto, CA, USA) fused-silica capillary column (length = 30 m, i.d. = 0.25 mm, film thickness = 0.33 μm) was used during the analysis. The operation conditions were as follows: column temperature = from 60 to 240°C at 3°C/min, followed by 5 min at 240°C; carrier gas = N_2 at a flow rate of 1.0 ml/min; chromatograph operation mode = injection mode; injection volume = 0.1 μl (split ratio of 1:10); injector and detector temperatures = 240 and 280°C, respectively. The concentrations of the EO components were obtained by relative peak area normalization (%); the relative areas were the average of triplicate GC-FID analyses. GC-MS analyses were carried out on a Shimadzu QP2010 Plus (Shimadzu Corporation, Kyoto, Japan) system equipped with an AOC-20i autosampler. The column consisted of Rtx-5MS (Restek Co., Bellefonte, PA, USA) fused silica capillary (length = 30 m, i.d. = 0.25 mm, film thickness = 0.25 μm. The equipment was set to operate in the electron ionization mode at 70 eV. Helium (99.999%) at a constant flow of 1.0 ml/min was the carrier gas. The injection volume was 0.1 μl (split ratio of 1:10). The injector and the ion-source temperatures were set at 240 and 280°C, respectively. The oven temperature program was the same as the one used for GC. The mass spectra were taken with a scan interval of 0.5 s, from 40 to 600 Da. The PG-EO components were identified based on their retention indices on an Rtx-5MS capillary column under the same operating conditions as in the case of GC; peaks were identified relative to a homologous series of *n*-alkanes (C8-C24). Structures were computer-matched with the Wiley 7, NIST 08, and FFNSC 1.2 spectra libraries, and their fragmentation patterns were compared with literature data.[14] The standard compounds geraniol and linalool were purchased from Sigma (Sigma Chemical Co., St. Louis, MO) and co-eluted with PG-EO to confirm their identity as major components of this essential oil.

Yeast strain and growth conditions

In this study, eight clinical isolates of *C. tropicalis* belonging to the mycology collection of the University of Franca, state of São Paulo, Brazil, were used. The isolates (9b; 20a; 26b; 29; 30; 31; 38; 39a) had been previously recovered from the fitting surface of complete or partial dental prostheses of outpatients assisted at the clinics of the Dentistry School of the University of Franca. The strain *C. tropicalis* ATCC 13803 was also included. All the subjects provided an informed consent according to the protocols (84/10) approved by the Human Subjects Committee of the University of Franca. All the *Candida* strains were subcultured from thawed suspensions of pure clinical isolates and ATCC stock cultures. Before each experiment, yeasts were inoculated into Sabouraud dextrose agar (SDA; Difco, Detroit, MI, USA) and incubated at 30°C for 48 h, and a single colony was isolated. Use of API ID 32 C™ (bio-Merieux, Marcy-l'Etoile, France) and Vitek Yeast Biochemical Card™ (bio-Merieux), following the manufacturer's instruction, confirmed that the *Candida* strains were *C. tropicalis*.

Antifungal effect of compounds against planktonic cells

The individual susceptibility profile of planktonic cells was obtained by the broth microdilution[15] method conducted in 96-well microtiter plates (TPP, Biogen, Europe). Two percent (v/v) DMSO (Sigma Chemical Co., St. Louis, MO) was added to the EO stock suspensions, and dilutions of each EO (ranging from 15.6 to 8000 μg/ml) were prepared in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid buffer (MOPS - Sigma). Yeasts from the Sabouraud Dextrose Agar (SDA - Difco, Detroit, MI, USA) plates were incubated at 30°C overnight, resuspended in RPMI, and adjusted to 0.5–1.5 × 10^3 cells/ ml. A total of 100 μl of each EO dilution was added to the wells containing 100 μl of *Candida* suspension, and incubated at 35°C 24 h. Controls containing antimicrobial agent in broth and broth with inoculum were also included. The Minimal Inhibitory Concentration (MIC) was determined visually and defined as the lowest concentration of EO that caused 50% inhibition of fungal growth relative to the growth detected in control well. The antimicrobial activity of 2% (v/v) DMSO (Sigma) was also studied on a

separate microtiter plate alongside the assay. Tests on the CLSI-recommended strains *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 ensured quality control. Fluconazole (Pfizer, Inc., New York, NY, at concentrations ranging from 0.12 to 128 μg/ml) was used as standard antifungal drug. Because there is no consensus regarding the acceptable inhibition level for natural products, this study considered the definition proposed by Duarte et al.,[16] which considers MIC up to 500 μg/ml, between 510 and 1000 μg/ml, and above 1100 μg/ml as denoting strong, moderate, and weak activity, respectively. Each compound was tested in duplicate at least twice, on two separate dates.

Biofilm formation

Candida biofilms were grown according to a previously published protocol.[17] The biofilms were induced in 96-well microtiter plates (TPP) and incubated at 37°C for 48 h. Controls for medium sterility were also included in each plate. The viability of the biofilms was assessed by the XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, Sigma] assay at 492 nm (Asys, Eugendorf, Salzburg, Austria). *C. albicans* SC5314 was used as the control strain. Strains with maximal OD lower than or equal to 0.120 (three standard deviations (0.023) above the mean OD (0.050) of a clean tissue culture plate) were not considered to form a biofilm. Experiments were performed in duplicate on two separate occasions for each strain with inoculated controls.

Effects of compounds on pre-formed biofilms

The essential oil of *P. graveolens* (PG-EO) and its major constituents (geraniol and linalool) were evaluated against preformed *C. tropicalis* biofilms (24 h). The compounds were serially diluted in RPMI medium (Sigma) at concentrations ranging from 8000 μg/ml to 15.6 μg/ml. The best *C. tropicalis* biofilm-forming strains were used in these assays. Biofilms were formed as described above and exposed to the target compounds at 35°C for 48 h. A negative control (well with RPMI medium) and 100% growth control (well with standard cell suspension without compounds) were prepared for each plate. After treatment, the *C. tropicalis* biofilms were scraped from the wells and serially diluted. Then, 100 μl of the diluted suspension was plated on SDA (Difco) plates. The percentage of survival was determined by the formula: $S = n/N \times 100$, where S = survival (%), n = number of colonies in one experimental plate, and N = number of colonies in the growth control plate. Fluconazole (Pfizer) at concentrations ranging from 2 to 1024 μg/ml was used as standard antifungal drug. *C. albicans* SC5314 was the reference strain. Experiments were performed in duplicate on two separate occasions. The mean of the replicates were calculated, and results are expressed as a percentage of survival.

Effect of antifungal drugs on *C. tropicalis* biofilm growth

For the assays on biofilm growth inhibition, the best *C. tropicalis* biofilm-forming strains were used. The wells of the microtiter plate (TPP, 96 wells) were filled with 100 μl of fungal standardized inoculum. Then, 100 μl of the samples (PG-EO or pure compounds) diluted in RPMI medium (Sigma), at concentrations ranging from 0 μg/ml (untreated control) to 8000 μg/ml, was added to the wells. Plates were incubated at 37°C for 48 h, and biofilm growth was quantified by using the XTT (Sigma) reduction assay at 492 nm (Asys). The mean of the replicates was calculated, and results are expressed as a percentage of optical density (OD) reduction in relation to the OD of the untreated control. Each assay was carried out twice in duplicate.

Scanning electron microscopy analysis

Biofilms were formed on 24-well cell culture plates by dispensing cell suspensions containing 5.0×10^6 cells/ml in RPMI 1640 (Sigma) at 37°C for 48 h; the supernatant was aspirated. The biofilms were fixed [4% formaldehyde (Sigma, v/v), 1% glutaraldehyde (Sigma - v/v)], buffered with phosphate potassium (1 M, Sigma), added with 1% (w/v) osmium tetroxide (Sigma)], and dehydrated with a series of ethanol (Sigma) solutions (30% (v/v) to 100%). Samples were cut from the microtiter plates, dried to critical point by using a critical-point drier (Balzers, Oberkochen, Germany), coated with gold (Denton Vacum, model Desk II, Freehold, NJ, USA), and viewed under a JEOL scanning electron microscope (model JSM 5410, Japan).

XTT-based cytotoxicity assay

Cytotoxicity was measured with the in vitro Toxicology Colorimetric Assay Kit (XTT; Roche Diagnóstica Brasil Ltda., São Paulo, SP) according to the manufacturer´s instructions. To evaluate cytotoxicity, normal human lung fibroblasts (GM07492A cells, Banco de Células do Rio de Janeiro (BCRJ), Rio de Janeiro, RJ) were used. The cell line was cultured in HAM-F10 (Sigma) and DMEM (Sigma) (1:1) culture medium supplemented with 10% fetal bovine serum (Nutricell, Campinas, SP), antibiotics (0.01 mg/ml streptomycin and 0.005 mg/ml penicillin, Sigma), and 2.38 mg/ml Hepes (Sigma) at 37°C with 5%

Table 1. *In vitro* antifungal activity of the fifteen essential oils against *Candida tropicalis* planktonic cells.

Essential oils	MIC of *C. tropicalis* strains (μg/ml)								
	DA9b	DA20a	DA26b	DA29	DA30	DA31	DA38	DA39a	ATCC 13803
AA-EO	500	500	500	500	500	500	1000	1000	500
Aco-EO	1000	1000	1000	1000	1000	1000	1000	1000	1000
ACa-EO	1000	1000	1000	1000	1000	1000	1000	1000	1000
BS-EO	500	500	500	500	500	500	500	500	500
CA-EO	500	500	500	500	500	500	500	500	500
CL-EO	1000	1000	1000	1000	1000	1000	1000	500	500
CR-EO	500	500	1000	500	500	500	1000	1000	500
FV-EO	500	500	500	500	500	500	500	500	500
LO-EO	500	500	500	500	500	500	500	500	500
OG-EO	500	500	500	500	500	500	500	500	500
PG-EO	**125**	**125**	**125**	**125**	**125**	**125**	**125**	**125**	**125**
PN-EO	1000	1000	1000	1000	1000	1000	1000	1000	1000
SA-EO	500	500	500	500	500	500	500	500	500
TE-EO	500	500	500	500	500	500	500	500	500
TR-EO	250	250	500	250	250	250	500	250	250

Notes: MIC: Minimum Inhibitory concentration; AA-EO: *Artemisia absinthum* L.; ACo-EO *Artemisia camphorata* L.; ACa-EO: *Ageratum conyzoides* L.; BS-EO: *Bidens sulphurea* (Cav.) Sch.Bip.; CA-EO: *Chenopodium ambrosioides* L.; CL-EO: *Coreopsis lanceolata* L.; CR-EO: *Citrus reticulata* Blanco; FV-EO: *Foeniculum vulgare* Mill.; LO-EO: *Lavandula officinalis* L.; OG-EO: *Ocimum gratissimum* L.; PG-EO: *Pelargonium graveolens* L´Hér.; PN-EO: *Plectranthus neochilus* Schl.; SA-EO: *Syzigium aromaticum;* TE-EO: *Tagetes erecta* L.; TR-EO: *Tetradenia riparia* (Hochst.) Codd.

CO_2. For these experiments, cells (10^3 cells/well) were plated in 96-well microplates; each well received 100 μl of culture medium. Twenty-four hours after seeding, culture medium containing the desired concentrations of PG-EO, geraniol, or linalool dissolved in 0.02% DMSO (Sigma) was added to the cells. Concentrations ranging from 2.5 to 5000 μg/ml were tested. The negative (without treatment), solvent (0.02% DMSO), and positive (25% DMSO) controls were included. After incubation at 37°C for 24 h, the medium was removed; the cells were washed with 100 μl of phosphate buffered-saline (PBS) and exposed to 100 μl of HAM-F10 medium without phenol red. Then, 20 μl of XTT was added to each well. The microplates were covered and incubated at 37°C for 17 h. The absorbance of the sample was determined by a multiplate reader (Asys – UVM 340 / Microwin 2000) at a test wavelength of 450 nm and at a reference wavelength of 620 nm. Cell viability was expressed as a percentage of untreated cells, which served as the negative control group and was designated as 100%. Assessment of the antiproliferative activity was based on the parameter 50% inhibition of cell line (IC_{50}) growth.[18] The experiments were performed in triplicate.

Statistical analysis

All the statistical analyses were performed with Graph Pad Prism 5.0 for Windows (GraphPad Software, San Diego, CA, USA). Significance of the difference ($P < .05$) was assessed by means of one-way ANOVA. The Bonferroni test was carried out for post-hoc multiple comparisons.

Results

The minimal inhibitory concentration (MIC) of the essential oils (EOs) against *C. tropicalis* planktonic cells is shown in Table 1. In general, the EOs were active against planktonic *C. tropicalis* ATCC 13803 and clinical isolates; however, the essential oil of *P. graveolens* (PG-EO) inhibited all the tested strains at MIC values of 125 μg/ml. The MIC values of fluconazole for the control strains *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were within the CLSI-acceptable range.

PG-EO was obtained in 0.45 ± 0.12% yield (w/w). Table 2 shows the chemical composition of PG-EO as determined by GC and GC-MS. A total of thirteen compounds were identified in PG-EO; monoterpenes (90.2%) predominated in this oil. The major constituents of PG-EO (Fig. 1) were the oxygenated monoterpenes geraniol (**1**, 42.3%), linalool (**2**, 20.1%), citronellol (**3**, 11.1%), and menthone (**4**, 8.0%).

Biofilm formation is an additional tool to measure microorganism pathogenicity. All the strains investigated in this study were evaluated for their ability to form biofilms. All the clinical isolates (100%) formed a biofilm after

Table 2. Chemical composition of the essential oil of *P. graveolens.*

Compound	RT[a]	$R_{I\ exp}$[b]	$R_{I\ lit}$[c]	% RA[d]	Identification[e]
Limonene	8.76	1028	1031	1.0	R_I, MS
Linalool	11.25	1110	1098	20.1	R_I, MS
Menthone	13.31	1161	1154	8.0	R_I, MS
α-terpineol	14.78	1195	1189	1.3	R_I, MS
Citronellol	16.26	1229	1228	11.1	R_I, MS
Geraniol	17.34	1253	1255	42.3	R_I, MS
Aristolene	24.61	1437	1428	4.2	R_I, MS
Neryl propanoate	25.79	1464	1454	2.6	R_I, MS
Germacrene D	26.03	1477	1480	2.4	R_I, MS
α-Calacorene	29.25	1553	1548	2.0	R_I, MS
Phenylethyl tiglate	29.87	1583	1590[b]	0.7	R_I, MS
Unknown	32.49	1617	—	0.5	
Torreyol	33.35	1638	1640[c]	0.8	R_I, MS
Geranyl tiglate	35.40	1697	1700	3.0	R_I, MS
Total				100.0	

Monoterpenes: 89.4% (hydrocarbons, 1.0%; oxygenated, 88.4%)
Sesquiterpenes; 9.4% (hydrocarbons, 8.6%; oxygenated: 0.8%)
Others: 0.7%; not identified: 0.5%.

[a]**RT**: retention time (min); [b]$\mathbf{R_{I\ exp}}$: retention index determined relative to *n*-alkanes (C_8–C_{20}) on the Rtx-5MS column. [c]$\mathbf{R_{I\ lit}}$: Retention index from the literature.(Adams 2007) [d]Calculated from the peak area relative to the total peak area of the GC-FID chromatogram. [e]Compound identification: R_I, comparison of the R_I with literature values(Adams 2007); MS, comparison of the mass spectra with those of the Wiley 7, NIST 08, and FFNSC 1.2 spectral libraries as well as literature data (Adams 2007).

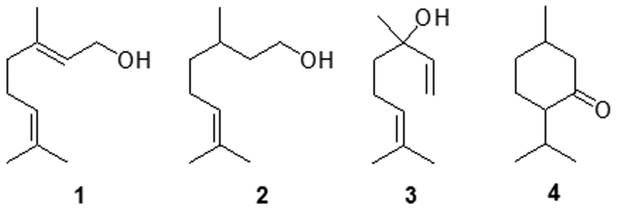

Figure 1. Chemical structures of geraniol (1), linalool (2), citronellol (3), and menthone (4).

48 h at 37°C, except for *C. tropicalis* ATCC 13803. Compared with the reference strain *C. albicans* SC 5314, *C. tropicalis* 38 was the best biofilm-forming strain, followed by *C. tropicalis* 9b, 26b, 38, and 39a strains ($P > .05$).

SEM provided useful information about the different cellular morphologies that were present in the biofilm structure. Images of representative biofilms of *C. tropicalis* 38 strains showed that the biofilms consisted of the yeast form, pseudohyphal cells, and certain amounts of the matrix material (Fig. 2).

Geraniol and linalool, the major chemical constituents of PG-EO, were also tested against preformed *C. tropicalis* biofilms (24 h). The percentage of cell survival in the biofilm was determined by counting the number of CFU in wells treated with the compounds and by comparing this number to the number of colonies obtained from untreated wells. Treatment with PG-EO and monoterpenes **1** and **2** at concentrations of 4000 μg/ml, approximately 500 μg/ml, and 1000 μg/ml, respectively, reduced yeast survival within the biofilm by approximately 50% after 48 h (Fig. 3). Compounds **1** and **2** were significantly more active ($P < .001$) than PG-EO against *C. tropicalis* biofilms (Fig. 3). The biofilm generated by reference strain *C. albicans* SC5314 was not susceptible to fluconazole (99% survival at 1024 μg/ml).

The effect of co-incubation of PG-EO, geraniol (**1**), and linalool (**2**) with yeast cells on *C. tropicalis* biofilm formation was evaluated in vitro by the XTT reduction assay (Fig. 4). At a concentration of 500 μg/ml, both compounds **1** and **2** inhibited 50% biofilm formation by *C. tropicalis*. Although the 50% inhibition values of compounds **1** and **2** were not significantly different, overall compound **1** was more active ($P < .0001$) as compared with PG-EO (Fig. 4).

Cytotoxicity to GM07492 cells was assessed by the XTT assay for 24 h and at concentrations ranging from 2.5 to 5000 μg/ml. The viability of the cultures was determined by establishing a relation between the absorbance obtained in the treated and untreated (control) groups. The IC_{50} values for PG-EO, geraniol, and linalool were 2907.50 ± 4.94 μg/ml, 666.36 ± 28.39 μg/ml, and 1253.00 ± 2.83 μg/ml, respectively.

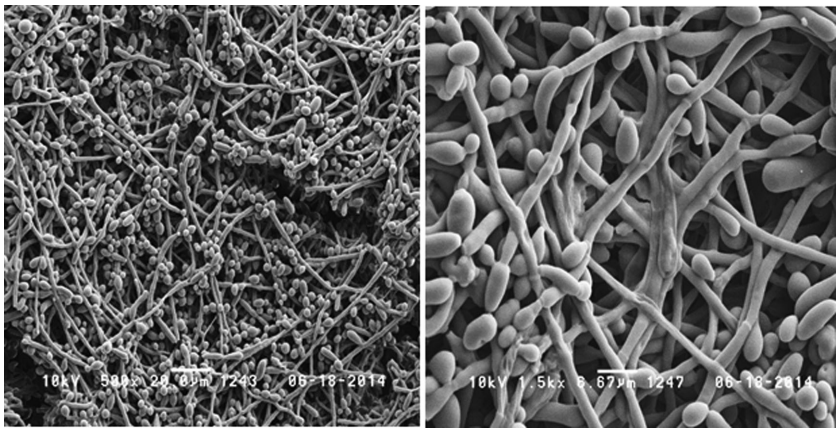

Figure 2. Representative electron micrograph of *C. tropicalis* biofilms (strains 38). The biofilms grown in the microtiter plates showed a dense layer of blastoconidia and pseudohyphae surrounded by an exopolymeric matrix. Bars, A: 20 μm; B: 6.67 μm.

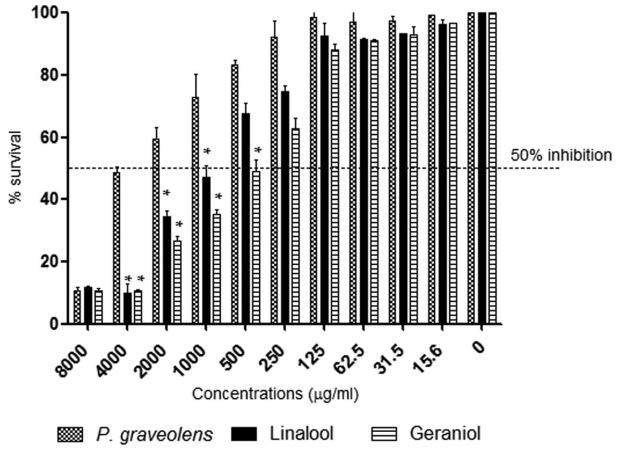

Figure 3. Susceptibility of *C. tropicalis* biofilms to PG-OE, linalool, and geraniol. The biofilms were treated with different concentrations of the tested compounds for 48 h. The number of surviving organisms was compared to the number of surviving organism in biofilms incubated in RPMI alone. Analysis of variance followed by the Bonferroni post-test showed that both geraniol and linalool are significantly [$P < .001$ (*)] more active than PG-EO. The bars are the average of measurements, and brackets denote standard deviations. These experiments were done twice, and similar results were obtained each time.

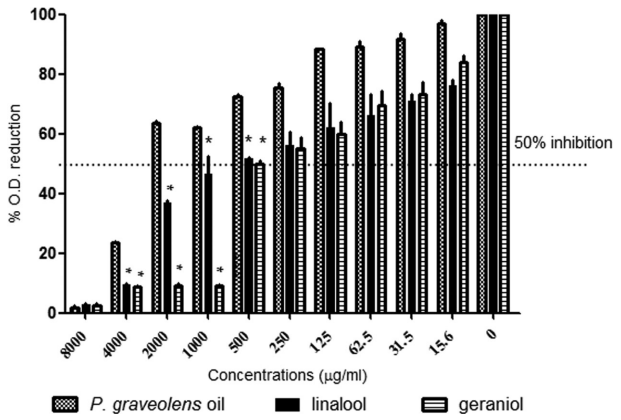

Figure 4. Inhibition of *Candida tropicalis* biofilm formation by PG-EO and its major constituents (linalool and geraniol) on microtiter plates (96 wells). The metabolic activities of *C. tropicalis* were measured by the XTT reduction assay. Percent OD reduction represents the decrease in biofilm formation in the presence of compounds relative to biofilm formation when no compound was present. Bars represent the standard deviation of the mean of duplicate experiments conducted on two different days. Controls with no compound were included in each experiment, on each plate. Asterisks denote $P < .0001$, calculated by analysis of variance and adjusted by use of the Bonferroni correction.

Discussion

Denture stomatitis elicits an inflammatory reaction under maxillary prosthesis, and *Candida* species are the etiological agent of this condition.[7] A wide variety of plant extracts can inhibit *Candida* species proliferation.[12,19] This study tested fifteen essential oils (EOs) against *C. tropicalis* planktonic cells. The essential oil of *Pelargonium graveolens* (*Geraniaceae*) (PG-EO) displays the highest antifungal activity among the assayed EOs with MIC at concentration of 125 μg/ml. In accordance with the criteria adopted in this study, PG-EO has strong antifungal effects against clinically *C. tropicalis* strains. EOs from some *Geraniaceae* species also possess antifungal and immunomodulatory activities.[20,21] Some beneficial therapeutic effects of PG-EO have been reported in the treatment of denture stomatitis.[22]

GC-MS analyses revealed that monoterpenes (90.2%) are the main constituents of PG-EO; geraniol (42.3%), linalool (20.1%), citronellol (11.1%), and menthone (8.0%) are the major constituents of PG-EO, as reported previously,[23–26] but in different proportions. The relative concentrations of the constituents of an EO depend on factors such as the plant growth location, the cultivar, the distillation method, the plant part, the age of the leaves, and seasonal changes.[24,26]

Rosato and coworkers[27] investigated the antifungal activity of the EOs of *Melaleuca alternifolia*, *Origanum*

vulgare, and *P. graveolens* against 11 *Candida* strains, including *C. tropicalis*. These authors verified that EO-PG has anti-*Candida* activity, with inhibitory concentration at 700 μg/ml. The inhibitory concentration of EO-PG reported here is lower than this value. These differences can result from the different methodologies used to test PG-EO. Rosato and coworkers[27] analyzed the activity of PG-EO by the agar diffusion method, whereas we opted to determine the inhibitory concentration of this EO by the broth microdilution because it is more sensitive than other literature methods.[28]

C. tropicalis adheres to and forms biofilm in microtiter microplates. The organization of the various *Candida* species biofilms is similar; however, structural details heavily depend on the conditions of biofilm formation, including growth medium, carbohydrates supplementation, nature of the colonized surface,[29] and *Candida* species.[30] In RPMI supplemented with 2% glucose, the *C. tropicalis* biofilm grows as a three-dimensional structure comprising blastoconidia and pseudohyphae linked by the matrix and permeated by water channels, as demonstrated by Bizerra and coworkers.[31] Compared with *C. albicans* SC5314, the *C. tropicalis* 26b, 38, and 39a strains form biofilms more effectively, and the MFC values of PG-EO against these strains differ only slightly (Table 1). Different expression of genes related to protein synthesis; amino acids, nucleotides, lipids, carbohydrate metabolism, and control of transcription and cellular organization in the biofilm-forming strains could account for these small differences.[30,32–34]

Crude PG-EO at 4000 μg/ml inhibits 50% of *C. tropicalis* biofilm population. This concentration is much higher than the concentration needed to elicit the same effect on planktonic cells (125 μg/ml). This result agrees with data presented in other reports showing that the biofilm phenotype confers resistance to antifungal therapy.[10,30] In the absence of an exopolymeric matrix, PG-EO interacts more freely with the fungal cell wall of planktonic cells, making them more susceptible to penetration and disruption.

The cell wall is the structure that mediates cell interaction with the environment, and it might be important for the adhesion of fungi to solid surfaces such as mucosal surfaces or dentures. The hydrophobicity of EO molecules allow them to pass through the cell wall and penetrate fatty acid chains of the phospholipid bilayer, rendering the cell membrane more permeable and causing intracellular contents to leak. Such loss of cell homeostasis can culminate in lysis and cell death. In agreement with previous studies, the EOs[22,35] are promising alternatives for prophylaxis against oral microorganisms.

According to the present research, linalool and geraniol have activities against *C. tropicalis* biofilms. Geraniol is significantly more active against *C. tropicalis* biofilms than PG-EO ($P < .001$). In line with our observations, other authors found that primary monoterpenic alcohols (e.g., geraniol) display more potent anticandidal activity than tertiary alcohols (e. g., linalool).[36,37] Uribe et al.[38] described that terpenes inhibit *Candida* respiration, thus damaging the mitochondria. To the best of our knowledge, this is the first study that has provided data on the antifungal activity of the PG-EO and its major constituents against *C. tropicalis* biofilms.

In conclusion, our results have pointed out the in vitro potential of PG-EO and its major terpene derivatives as antifungal and antibiofilm agents against *C. tropicalis*. Geraniol is especially interesting: at 500 μg/ml, it inhibits 50% *C. tropicalis* biofilm formation and kills 50% of biofilm population. This concentration is smaller than that which inhibited 50% of viability of human lung fibroblasts (666.36 μg/ml). However, considering that these values are close, further *in vivo* studies will be conducted. Together, our data suggest that the extracts of this aromatic plant can be useful alternative antimicrobial agents in natural medicine for the treatment of oral infectious diseases.

Acknowledgments

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) [2011/12734-3 and 2007/54241-8].

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

References

1. Gasparetto A, Negri MFN, Paula CR et al. Biofilm production by yeasts isolated from bucal cavity of prosthesis. *Acta Sci Health Sci* 2005; **27**: 37–40.
2. Pereira CA, Toledo BC, Santos CT et al. Opportunistic microorganisms in individuals with lesions of denture stomatitis. *Diagn Microbiol Infect Dis* 2013; **76**: 419–424.
3. Pires-Gonçalves RH, Miranda ET, Baeza LC et al. Genetic relatedness of commensal strains of *Candida albicans* carried in the oral cavity of patients' dental prosthesis users in Brazil. *Mycopathologia* 2007; **164**: 255–263.
4. Zomorodian K, Rahimi MJ, Pakshir K et al. Determination of antifungal susceptibility patterns among the clinical isolates of *Candida* species. *J Glob Infect Dis* 2011; **3**: 357–360.
5. Guinea J. Global trends in the distribution of *Candida* species causing candidemia. *Clin Microbiol Infect* 2014; **20**(Suppl 6): 5–10.

6. Nucci M, Queiroz-Telles F, Alvarado-Matute T et al. Epidemiology of candidemia in Latin America: a laboratory-based survey. *PLoS One* 2013; **8**: e59373.
7. Tay LY, Jorge JH, Herrera DR et al. Evaluation of different treatment methods against denture stomatitis: a randomized clinical study. *Oral Surg Oral Med Oral Pathol Oral Radiol* 2014; **118**: 72–77.
8. Costerton JW, Lewandowski Z, Caldwell DE et al. Microbial biofilms. *Annu Rev Microbiol* 2003; **49**: 711–745.
9. Campos MS, Marchini L, Bernardes LA et al. Biofilm microbial communities of denture stomatitis. *Oral Microbiol Immunol* 2008; **23**: 419–424.
10. Al-Fattani MA, Douglas LJ. Penetration of *Candida* biofilms by antifungal agents. *Antimicrob Agents Chemother* 2004; **48**: 3291–3297.
11. Hospenthal DR, Murray CK, Rinaldi MG. The role of antifungal susceptibility testing in the therapy of candidiasis. *Diag Microbiol Infect Dis* 2004; **48**: 1153–1160.
12. Curvelo JA, Marques AM, Barreto AL et al. A novel nerolidol-rich essential oil from *Piper claussenianum* modulates *Candida albicans* biofilm. *J Med Microbiol* 2014; **63**(Pt 5): 697–702.
13. Sudjana AN, Carson CF, Carson KC et al. *Candida albicans* adhesion to human epithelial cells and polystyrene and formation of biofilm is reduced by sub-inhibitory *Melaleuca alternifolia* (tea tree) essential oil. *Med Mycol* 2012; **50**: 863–870.
14. Adams R. *Identification of essential oils components by gas chromatography/mass spectrometry*. Allured Publishing: Carol Stream, 2007.
15. CLSI. *Reference method for broth dilution antifungal susceptibility testing of yeasts: 4th informational supplement*. CLSI document M27–A3. Wayne, PA: CLSI, 2008.
16. Duarte MCT, Figueira GM, Sartoratto A et al. Anti-*Candida* activity of Brazilian medicinal plants. *J Ethnopharmacol* 2005; **97**: 305–311.
17. Ramage G, Vande Walle K, Wickes BL et al. Standardized method for in vitro antifungal susceptibility testing of *Candida albicans* biofilms. *Antimicrob Agents Chemother* 2001; **45**: 2475–2479.
18. Berridge MV, Herst PM, Tan AS. Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. *Biotechnol Ann Rev* 2005; **11**: 127–152.
19. Białoń M, Krzyśko-Łupicka T, Koszałkowska M et al. The influence of chemical composition of commercial lemon essential oils on the growth of *Candida* strains. *Mycopathologia* 2014; **177**: 29–39.
20. Ganapaty S, Beknal AK. Chemical composition and anti-inflammatory activity of *Pelargonium graveolens* oil (geranium). *J Nat Prod* 2004; **20**: 18–20.
21. Guerrini A, Rossi D, Paganetto G et al. Chemical characterization (GC/MS and NMR Fingerprinting) and bioactivities of South-African *Pelargonium capitatum* (L.) L' Her. (Geraniaceae) essential oil. *Chem Biodiv* 2011; **8**: 624–642.
22. Sabzghabaee AM, Shirdare Z, Ebadian B et al. Clinical evaluation of the essential oil of *Pelargonium graveolens* for the treatment of denture stomatitis. *Dental Res J (Isfahan)* 2011; **8**(Suppl 1): S105–108.
23. Baldin ELL, Aguiar GP, Fanela TLM et al. Bioactivity of *Pelargonium graveolens* essential oil and related monoterpenoids against sweet potato whitefly, *Bemisia tabaci* biotype. *J Pest Sci* 2015; **88**: 191–199.
24. Boukhris M, Simmonds MS, Sayadi S et al. Chemical composition and biological activities of polar extracts and essential oil of rose-scented geranium, *Pelargonium graveolens*. *Phytother Res* 2013; **27**: 1206–1213.
25. Ghannadi A, Bagherinejad M, Abedi D et al. Antibacterial activity and composition of essential oils from *Pelargonium graveolens* L'Her and Vitex agnus-castus L. *Iran J Microbiol* 2012; **4**: 171–176.
26. Rao BR, Kaul PN, Syamasundar KV et al. Water soluble fractions of rose-scented geranium (*Pelargonium* species) essential oil. *Bioresour Technol* 2002; **84**: 243–246.
27. Rosato A, Vitali C, Gallo D et al. The inhibition of *Candida* species by selected essential oils and their synergism with amphotericin B. *Phytomedicine* 2008; **15**: 635–638.
28. Ostrosky EA, Mizumoto MK, Lima MEL et al. Methods for evaluation of the antimicrobial activity and determination of Minimum Inhibitory Concentration (MIC) of plant extracts. *Rev Bras Farmacogn* 2008; **18**: 301–307.
29. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* 2002; **15**: 167–193.
30. Sherry L, Rajendran R, Lappin DF et al. Biofilms formed by *Candida albicans* bloodstream isolates display phenotypic and transcriptional heterogeneity that are associated with resistance and pathogenicity. *BMC Microbiol* 2014; **14**: 182.
31. Bizerra FC, Nakamura CV, de-Poersch C et al. Characteristics of biofilm formation by *Candida tropicalis* and antifungal resistance. *FEMS Yeast Res* 2008; **8**: 442–450.
32. Garcia-Sanchez S, Aubert S, Iraqui I et al. *Candida albicans* biofilms: a developmental state associated with specific and stable gene expression patterns. *Eukaryot Cell* 2004; **3**: 536–545.
33. Murillo LA, Newport G, Lan CY et al. Genome-wide transcription profiling of the early phase of biofilm formation by *Candida albicans*. *Eukaryot Cell* 2005; **4**: 1562–1573.
34. Yeater KM, Chandra J, Cheng G et al. Temporal analysis of *Candida albicans* gene expression during biofilm development. *Microbiology* 2007; **153**(Pt 8): 2373–2385.
35. Silva CB, Guterres SS, Weisheimer V et al. Antifungal activity of the lemongrass oil and citral against *Candida* spp. *Braz J Infect Dis* 2008; **12**: 63–66.
36. Carmen G, Hancu G. Antimicrobial and antifungal activity of *Pelargonium roseum* essential oils. *Adv Pharm Bull* 2014; **4**(Suppl 2): 511–514.
37. Tampieri MP, Galuppi R, Macchioni F et al. The inhibition of *Candida albicans* by selected essential oils and their major components. *Mycopathologia* 2005; **159**: 339–345.
38. Leite MC, de Brito Bezerra AP, de Sousa JP et al. Investigating the antifungal activity and mechanism(s) of geraniol against *Candida albicans* strains. *Med Mycol* 2015; **53**: 275–284.
39. Uribe S, Ramirez J, Peña A. Effects of beta-pinene on yeast membrane functions. *J Bacteriol* 1985; **161**: 1195–1200.