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In vitro activities of carvacrol, cinnamaldehyde and thymol against *Candida* biofilms

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ABSTRACT

Oral candidiasis is frequently associated with *Candida* biofilms. Biofilms are microbial communities related to persistent, recalcitrant and difficult to-treat infections. Conventional treatments are not sufficient to overcome biofilm-associated candidiasis; thus, the search of new antifungal compounds is necessary. In the current study, we have evaluated the effect of three phytocompounds, carvacrol, cinnamaldehyde and thymol, against *Candida* planktonic and sessile cells. Reduction in biofilm biomass and metabolic activity was assessed during adhesion and mature biofilm phases. *Candida albicans* was the most biofilm-producing *Candida* species. All phytocompounds tested were fungicidal against *Candida* planktonic cells. Cinnamaldehyde was the most active in inhibiting biofilm adhesion, but carvacrol and thymol significantly reduced both mature biofilm biomass and metabolic activity. These results highlight the role of cinnamaldehyde, carvacrol and thymol as promising alternatives for the treatment of candidiasis due to their antibiofilm capacities, and stress the necessity to continue studies on their safety, toxicity and pharmacodynamics and pharmacokinetics.

1. Introduction

Oral candidiasis is one of the most prevalent opportunistic infections that causes oral discomfort, pain and dysgeusia and it is often associated with a poor immune status that can lead to complications, such as esophageal or systemic candidiasis [1–3]. Risk for systemic infection increases in immunocompromised patients colonized by *Candida*. Although most oral infections are easily treatable, they often follow a protracted course in those patients carrying dentures, HIV-infected or under chemotherapy [2]. Treatment of oral candidiasis is based on the correction of underlying diseases, the maintenance of good oral hygiene and the use of antifungal drugs. *Candida albicans* is the most frequent etiology, followed by *Candida glabrata* [1,4]. *C. albicans* outstands in the oral cavity due in part to its biofilm forming ability that challenges the efficacy of treatment. Extracellular polymeric substances, such as carbohydrate and extracellular DNA, often hamper antifungal penetration through biofilms extracellular matrix (ECM). This clinical concern is magnified by the emergence of azole resistant isolates and by the selection of species of *Candida* with reduced antifungal susceptibility [3]. In recent years, essential oils and their components have gathered significant attention as potential antimicrobial agents due to its relative safety, low long-term genotoxicity and scarcity of side effects [5]. Main strategies focus on studying the effectiveness of key phytocompounds against *Candida* biofilms as monotherapy or in combination with current antifungal drugs, thus limiting development of resistance or decreasing antifungal selective pressure. Some phytocompounds have been included in rinses or mouthwashes for preventing oral infections [6]. However, most studies evaluate the activity of essential oils as a whole, rather than studying key components, which could be more advantageous in safety and reproducibility [7]. Therefore, the aim of the present

Abbreviations: IC, Inhibitory concentration; SICs, Sessile inhibitory concentrations; PSICs, Pre-sessile inhibitory concentrations; SMIC, Sessile minimum inhibitory concentration; PSMIC, Pre-sessile minimum inhibitory concentration; CV, crystal violet assay; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; HBB, High biomass biofilm producer; MBB, Moderate biomass biofilm producer; LBB, Low biomass biofilm producer; HMA, High metabolic activity biofilm producer; MMA, Moderate metabolic activity biofilm producer; LMA, Low metabolic activity biofilm producer.

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study was to assess the *in vitro* activity of carvacrol, cinnamaldehyde and thymol, compared to anidulafungin, fluconazole and isavuconazole, against planktonic and sessile *Candida* cells.

2. Materials and methods

2.1. Microorganisms

Thirty-five oral isolates from patients suffering oral candidiasis attending the Dental Clinic Service at the Universidad del País Vasco/Euskal Herriko Unibertsitatea (UPV/EHU), Bilbao (Spain) were analyzed. Isolates were identified by conventional techniques, such as colony morphology on *Candida* Chromogenic agar (Laboratorios Conda, Spain) and ChromID *Candida* (BioMérieux, France), carbon source assimilation kit API ID 32C system (BioMérieux), and molecular methods (Multiplex PCR and PCR-RFLP to *C. glabrata* complex and *C. parapsilosis* complex, respectively) [4]. These isolates included 10 *C. albicans*, 10 *C. glabrata*, three isolates each of *Candida dubliniensis* and *Candida krusei*, two isolates each of *Candida guilliermondii*, *Candida orthopsilosis*, *Candida parapsilosis* and *Candida tropicalis*, and one *Candida metapsilosis*. Moreover, seven reference strains from the American Type Culture Collection (ATCC) and the National Collection of Pathogenic Fungi (NCPF), and the hypha-defective mutant *C. albicans* Ca2 (kindly donated by Professor Antonio Cassone, Istituto Superiore di Sanità, Rome, Italy) were studied. Isolates and reference strains were cultured on Sabouraud dextrose agar (Difco, Becton Dickinson, USA) at 37 °C for 24 h before testing.

2.2. Biofilm production

Biofilm production by oral isolates and reference strains was assessed. Prior to each experiment, they were cultured overnight at 30 °C in an orbital shaker on yeast peptone dextrose (YPD) medium containing 1% weight/volume (w/v) yeast extract, 2% w/v peptone and 2% w/v dextrose. Cells were washed thrice in sterile phosphate buffered saline solution (PBS), and adjusted to a cellular density of 1.0×10^6 cells/ml in RPMI-1640 supplemented with L-glutamine and buffered at pH 7 with 0.165 M 3-(N-morpholino)propanesulfonic acid, MOPS (Sigma-Aldrich). *Candida* biofilms were developed in sterilized, flat-bottomed honeycomb 100-well polystyrene microtiter plates (Labsystems, Finland) by adding 100 μl of the adjusted standard cell suspension into each well. Two identical microtiter plates were prepared, one to determine metabolic activity and the other to quantify biomass. Microtiter plates were incubated at 37 °C in a computer-controlled incubator (BioScreen C MBR, Growth Curves Ltd, Finland). After 24 and 48 h non-adherent and loosely adherent cells were removed by washing three times with sterile PBS.

2.2.1. Metabolic activity determination assay

Metabolic activity of the biofilm was measured following the colorimetric method described by Ramage et al. [8]. The 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT, Sigma-Aldrich) reagent was prepared as a saturated solution at 0.5 g/l in Ringer's lactate and the solution was sterilized by filtration, aliquoted and stored at −70 °C until required. Before each assay, an aliquot of stock XTT was thawed and menadione was added to a final concentration of 1 μM. Then, 100 μl of XTT-menadione was added to each prewashed well and incubated in dark for 2 h at 37 °C. The colorimetric changes showing metabolic activity of the biofilm were measured on the microtiter plate reader BioScreen C MBR at 490 nm wavelengths.

2.2.2. Biomass quantification assay

Biomass quantification was performed following the method described by Peeters et al. [9] using crystal violet. After removing non-adherent cells, microplates were air-dried for 30 min and then, 100 μl of 0.4% crystal violet solution was added to each well and incubated for a further 20 min at room temperature. Microplates were washed twice using 250 μl of sterile distilled water and 150 μl of 33% acetic acid were afterwards added to each well. Absorbance of the biomass was measured at 600 nm wavelengths.

2.3. Phytocompounds and antifungal agents

Carvacrol, cinnamaldehyde and thymol at 98%, 95% and 99% purity, respectively, were purchased from Sigma-Aldrich. Anidulafungin (Pfizer, Spain), fluconazole (Sigma-Aldrich) and isavuconazole (Basilea Pharmaceutica, Switzerland) were also used.

Stock solutions were prepared in dimethylsulfoxide (DMSO, Sigma-Aldrich). Antifungal drugs were stored at −70 ºC and phytocompounds were prepared on the same day of the susceptibility test. The tested compounds contained 0.5% DMSO, therefore all drug-free controls also included this concentration of DMSO.

2.4. In vitro antifungal activity against planktonic cells

In vitro antifungal susceptibility was assessed according to the methodology proposed by the European Committee for Antimicrobial Susceptibility Testing [10,11]. Final concentrations of anidulafungin ranging from 0.016 to 8 mg/l, of fluconazole from 0.12 to 64 mg/l and of isavuconazole from 0.016 to 8 mg/l were used. Susceptibility to carvacrol, cinnamaldehyde and thymol was assayed at concentrations ranging from 2 to 1024 mg/l. *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were used as quality controls. Absorbance of each microplate was measured at 450 nm wavelengths by the iMark reader (BioRad, USA) after 24 and 48 h of incubation at 37 °C. Absorbance values equal to or less than 0.2 after 48 h were considered a failed test. Antifungal activities were studied in triplicate and in at least three separate experiments. Anidulafungin, fluconazole and isavuconazole minimum inhibitory concentrations (MIC) were calculated at 24 h as the lowest drug concentration inhibiting ≥50% of growth in comparison to controls without antifungal drugs [10,11]. Carvacrol, cinnamaldehyde and thymol inhibitory concentrations (IC) were calculated at 24 h as the lowest drug concentration inhibiting ≥50% of growth in comparison to controls without phytocompounds [12]. Clinical isolates were classified as susceptible, susceptible-dose dependent and resistant using the species-specific MIC breakpoints defined by EUCAST for anidulafungin and fluconazole [13]. However, EUCAST breakpoints for isavuconazole have not been established.

Minimum fungicidal concentration (MFC) was calculated by seeding 100 μl of each well without growth onto Sabouraud dextrose agar plates [14]. Fungicidal activity was defined as the lowest concentration of antifungal agent resulting in the death of 99.9% of the inoculum.

2.5. Effect of phytocompounds against adhesion and mature Candida biofilms

Ten clinical isolates were selected according to their biofilm production in the previous assay: eight *C. albicans* and one isolate each of *C. dubliniensis* and *C. tropicalis*. *C. albicans* SC5314 and the hypha-deficient *C. albicans* Ca2 were included as controls. Activities of carvacrol, cinnamaldehyde and thymol to prevent *Candida* biofilm formation were assessed according to the method described by Van Dijck et al. [12]. Briefly, 100 μl of the adjusted standard cell suspension of each isolate were inoculated into the 100-well polystyrene microtiter plates plus 100 μl of the phytocompound at final concentrations ranging from 8 to 1024 mg/l. Pre-sessile ICs (PSICs) were those concentrations causing 50% metabolic inhibition and 50% biomass reduction with respect to controls without compound.

For the susceptibility assay against the sessile cells of the mature biofilm, concentrations of phytocompound ranging from 16 to 2048 mg/l were used. Briefly, biofilms were developed into 100-well polystyrene microtiter plates by adding 100 μl of the adjusted *Candida* inoculum into each well. After an incubation of 24 h at 37 °C, non-adherent and loosely adherent cells were removed from the mature biofilm by washing twice with sterile PBS. Then, 100 μl of the final concentrations of phytocompounds in RPMI medium were added into microtiter plates for a

further incubation of 24 h at 37 °C. Compound-free wells and biofilm-free wells were also included as positive and negative controls, respectively. Sessile ICs (SICs) were those concentrations causing 50% metabolic inhibition and 50% biomass reduction with respect to controls.

2.6. Morphology and architecture of the biofilm

Morphology and architecture of the mature 24 h biofilms of *C. albicans* SC5314 strain developed on nitrocellulose filters of 13 mm (Merck Millipore, Germany) or 8-well tissue culture chambers (Sarstedt, Germany) were studied by scanning electron microscopy (SEM) and confocal microscopy (CLSM), respectively. Mature biofilms were also treated with 2048 mg/l of carvacrol, cinnamaldehyde and thymol to observe the effect on *Candida* biofilms by SEM and CLSM.

Samples analyzed by SEM were fixed in 2% glutaraldehyde phosphate buffer solution. After three washes with 6% sucrose in Sorenson's buffer, biofilms were dehydrated using graded ethanol solutions (50% and 70% in distilled water, and 100%) for 5 min each, and washed three times in hexamethyldisilazane (Electron Microscopy Sciences, USA) before air drying. Afterwards, samples were mounted on SEM stubs and gold coated using an Emitech k550x ion sputter. Finally, images were acquired using a Hitachi S-4800 scanning electron microscope.

Biofilms analyzed by CLSM were stained with the LIVE/DEAD Yeast Viability Kit (Thermo Fisher Scientific S.L., USA) using FUN-1 and calcofluor white M2R following the instructions of manufacturers. Briefly, biofilms formed on the 8-well tissue culture chambers slides were washed with buffer HEPES (10 mM Na-HEPES; pH: 7.2, with 2% glucose). Subsequently 500 µl of FUN-1 and M2R white calcofluor solution (1:5) in HEPES buffer were dispensed into each well. Samples were incubated in dark for 30 min and afterwards, they were observed with standard filter set of FICT and DAPI by an Olympus Fluoview FV500 confocal microscope. Live cells show green color and dead cells yellow or orange.

2.7. Statistical analysis

Statistics were analyzed using Graphpad Prism 5.0 version (GraphPad Software, USA). Comparisons between quantitative results were analyzed by t Student's test when data showed a normal distribution and non-parametric Kruskal Wallis test with Dunn's multiple comparison test and Mann Whitney non-parametrical test when data did not show a normal distribution. In all the cases, $p < 0.05$ was considered statistically significant.

3. Results

3.1. Biofilm production of oral Candida isolates

Biofilm production of 35 isolates and reference strains was divided into terciles for CV and XXT assays. It allowed the classification of isolates as high biomass (HBB) or high metabolically active (HMA) biofilm producers when the mean absorbance (OD) was greater than 0.500; moderate biomass (MBB) or with moderate metabolic activity (MMA) biofilm producers when absorbance ranged between 0.300 and 0.499, and low biomass (LBB) or low metabolically active (LMA) biofilm producers when the mean values of the absorbance were less than 0.300 for each method (Fig. 1, Table S1). *C. albicans* isolates developed the most metabolically active biofilm and showed the highest biomass production, including the isolate resistant to fluconazole and with reduced susceptibility to isavuconazole *C. albicans* UPV 15–157, classified as HBB and HMA biofilm producer. Highest biofilm producers among *C. glabrata* isolates exhibited moderate biofilm production (MBB and MMA). *C. dubliniensis* UPV 11–366, a fluconazole resistant isolate and with reduced susceptibility to isavuconazole was HMA and HBB, while two *C. tropicalis* isolates (UPV 06–115 and UPV 05–016) and *C. parapsilosis* UPV 12–296 were HBB but their biofilms showed moderate or low metabolic activities. All isolates of *C. guilliermondii*, *C. krusei* and the rest of species inside the *C. parapsilosis* complex produced biofilms with low or moderate metabolic activity and biomass.

C. albicans produced more biofilm than *C. krusei*, *C. glabrata* and *C. parapsilosis* complex ($p < 0.0005$). Nevertheless, no significant differences were found in biofilm production among *C. albicans*, *C. tropicalis*, *C. dubliniensis* and *C. guilliermondii*.

3.2. In vitro antifungal susceptibility testing against Candida planktonic cells

Table 1 shows the MIC and IC at 24 h of all oral isolates and reference strains. Carvacrol, cinnamaldehyde and thymol showed antifungal activity against planktonic cells of all isolates (geometric mean -GM- of IC were 105 mg/l; 61.5 mg/l and 93.2 mg/l, respectively), including those resistant to fluconazole isolates (*C. albicans* UPV 15–157 and *C. dubliniensis* UPV 11–366), and susceptible-dose dependent and fluconazole resistant isolates of *C. glabrata* and *C. krusei*. Cinnamaldehyde was active against the 90% of isolates studied at 64 mg/l (IC90), followed by thymol and carvacrol (IC90 128 mg/l for both compounds); therefore, cinnamaldehyde was the most active against all *Candida*

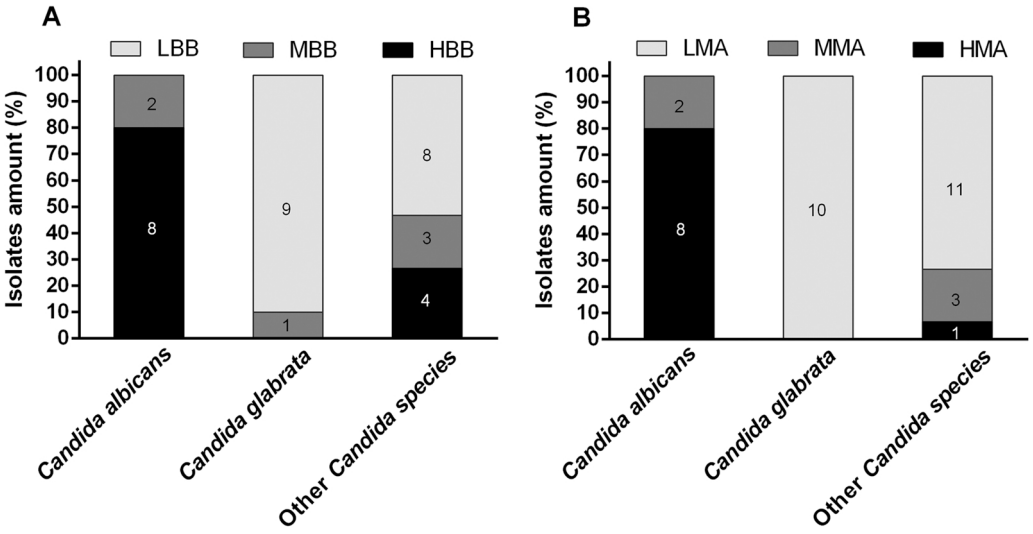

Fig. 1. Biomass (A) and metabolic activity (B) of *Candida* biofilms. Percentages and number de isolates of each *Candida* species. Isolates were classified as high (HBB), moderate (MBB) and low (LBB) biomass biofilm producers; and with high (HMA), moderate (MMA) and low (LMA) metabolic activity.

Table 1
In vitro activity of carvacrol, cinnamaldehyde, thymol and current antifungal drugs against 35 isolates of *Candida*.

***Candida* species (n)**		**Carvacrol [2–1024 mg/l]**	**Cinnamaldehyde [2–1024 mg/l]**	**Thymol [2–1024 mg/l]**		**Anidulafungin [0.016–8 mg/l]**	**Isavuconazole [0.016–8 mg/l]**	**Fluconazole [0.12–64 mg/l]**
***C. albicans* (10)**	**IC GM**	128	59.7	104.0	**MIC GM**	0.02	0.03	0.35
	Mode	128	64	128	**Mode**	0.016	0.016	0.25
	Range IC	128	32–64	32–256	**Range MIC**	0.016–0.03	0.016–8	0.12 – >64
***C. glabrata* (10)**	**IC GM**	119.4	68.6	111.4	**MIC GM**	0.04	0.09	12.1
	Mode	128	64	128	**Mode**	0.06	0.125	16
	Range IC	16–512	64–128	32–128	**Range MIC**	0.03–0.06	0.03–0.5	4 – >64
***C. krusei* (3)**	**IC GM**	80.6	50.8	64	**MIC GM**	0.04	0.1	50.8
	Mode	128	64	–	**Mode**	0.03	0.125	64
	Range IC	32–128	32–128	32–128	**Range MIC**	0.03–0.06	0.06–0.12	32 – >64
***C. dubliniensis* (3)**	**IC GM**	128	64	64	**MIC GM**	0.02	0.13	1
	Mode	128	64	64	**Mode**	0.03	0.016	0.125
	Range IC	128	64	64	**Range MIC**	0.016–0.03	0.016–8	0.12 – >64
***C. parapsilosis* (2)**	**IC GM**	64	90.5	90.5	**MIC GM**	2	0.02	0.5
	Range IC	64	64–128	64–256	**Range MIC**	0.03–2	0.016–0.5	0.5–2
***C. guilliermondii* (2)**	**IC GM**	64	64	90.5	**MIC GM**	0.25	0.24	4
	Range IC	64	64	64–128	**Range MIC**	0.25	0.12–0.5	2–8
***C. orthopsilosis* (2)**	**IC GM**	64	32	64	**MIC GM**	0.5	0.016	0.4
	Range IC	64	32	64	**Range MIC**	0.5	0.016	0.25–0.5
***C. tropicalis* (2)**	**IC GM**	128	64	128	**MIC GM**	0.02	0.016	5.7
	Range IC	128	64	128	**Range MIC**	0.016–0.03	0.016	0.5 – >64
***C. metapsilosis* (1)**	**IC**	64	256	64	**MIC**	0.25	0.016	2
Total	**IC GM**	105	61.5	93.2	**MIC GM**	0.05	0.05	2.4
	Mode	128	64	128	**Mode**	0.016	0.016	0.125
	Range IC	16–512	32–128	32–256	**Range MIC**	0.016–2	0.016–8	0.12 – >64

IC: inhibitory concentration, GM: geometric mean, MIC: minimum inhibitory concentration.

species except *C. metapsilosis* (IC 256 mg/l). The activity of all phytocompounds was fungicidal against *Candida* planktonic cells (Table 2). Cinnamaldehyde was the most effective agent, followed by thymol and carvacrol (GM MFC: 99.0 mg/l, 241.2 mg/l and 251.0 mg/l, respectively).

3.3. In vitro antifungal susceptibility testing against Candida sessile cells

Ten clinical isolates, including eight *C. albicans* (one fluconazole resistant), one fluconazole resistant *C. dubliniensis* and one *C. tropicalis*, were selected for their high biofilm production to study the effect of phytocompounds and antifungal agents against adhesion and preformed biofilms (Table 3). Anidulafungin was active reducing both the biomass and the metabolic activity in the adhesion phase of *Candida* biofilm formation of all isolates (GM PSMIC 0.134 and 0.125 mg/l, respectively). Carvacrol, cinnamaldehyde and thymol were active inhibiting the metabolic activity of the adhesion phase (GM PSIC 104 mg/l, 97 mg/l and 157.6 mg/l); as well as reducing the biomass, in which the cinnamaldehyde was the most effective (GM PSIC 111.4 mg/l), although thymol and carvacrol also showed good activity (GM PSIC 147 mg/l and 157.6 mg/l, respectively).

Fig. 2 shows mature biofilm growth inhibition of ten *Candida* isolates analyzed by biomass and metabolic activity determination assays. Anidulafungin and cinnamaldehyde showed activity inhibiting the metabolic activity but were less effective reducing biomass (GM SMIC 0.9 mg/l with XTT and 29.6 mg/l ml with CV; GM SIC 118.5 mg/l with XTT and 1896.2 mg/l with CV assays, respectively). Conversely, carvacrol and thymol reduced the biomass of most of the mature biofilms, and also showed activity inhibiting metabolic activity (GM SIC 188.1 mg/l with XTT and 812.7 mg/l with CV; 128 mg/l with XTT and 1106 mg/l with CV assays, respectively); while isavuconazole was less active reducing biomass and metabolic activity of mature biofilms.

All phytocompounds were effective against the azole-resistant *Candida* isolates inhibiting significantly the metabolic activity of mature biofilm, with a reduction of 80% or more with the highest concentration assayed (2048 mg/l, Fig. 2). Moreover, a significant reduction was observed with 64 mg/l of the three phytocompounds against *C. albicans* UPV 15–157 isolate, and from the lowest concentration (16 mg/l) against *C. dubliniensis* UPV 11–366 isolate and against the susceptible isolates.

3.4. Morphology and architecture of the biofilm after treatment with phytocompounds

Fig. 3 shows the cell morphology and the changes in the ultrastructure of the mature *C. albicans* SC5314 biofilm treated with the respective SIC of carvacrol, cinnamaldehyde and thymol (2048 mg/l) observed by SEM. Untreated *Candida* biofilm presented filamentation, abundant hyphae with well-defined shapes and oval shaped yeast cells with smooth surfaces and polar bud scars. In biofilms treated with all phytocompounds, damage was evident presenting deformed hyphae, irregular surfaces in some sites with deposit of lytic material, reduction of number of cells and absence of hypha production. Untreated and treated biofilm showed absence of ECM due to dehydration during SEM procedures.

The mature biofilm of *C. albicans* SC5314 treated with 2048 mg/l of each phytocompound in the same conditions described above but monitored by CLSM is shown in Fig. 4. Untreated *Candida* biofilm exhibited high cell viability, while on the treated biofilms, the viability decreased significantly, and the amount of dead *Candida* cells increased. CLSM allowed to visualize cell viability of treated and untreated biofilms and confirmed the results of metabolic activity assay.

4. Discussion

Oral candidiasis is a common superficial infection in immunocompromised patients, denture wearers, and in the elderly and new-borns [1]. Pseudomembranous candidiasis, erythematous candidiasis, hyperplastic candidiasis, denture stomatitis and angular cheilitis are clinical manifestations of this *Candida* infection. Classical treatment of oral candidiasis has some important issues mainly related to the development of antifungal resistances, the limited availability of antifungal agents and their potential toxicity, so new therapeutic alternatives should be implemented [15]. Essential oils stand out in the management of oral infections because of their antimicrobial activity and have been included in oral rinses [6,16]. However, reliable information on their potential use in antifungal therapy is scarce. These oils are complex mixtures of chemically heterogeneous components obtained from aromatic plants that have numerous biological properties including antioxidant, antimicrobial, antitumor and analgesic activities [17–21].

While the study of antimicrobial properties of essential oils as a

Table 2
Minimum fungicidal concentration (MFC) of carvacrol, cinnamaldehyde and thymol against 35 isolates of *Candida*.

Candida species (n)		Carvacrol [2–1024 mg/l]	Cinnamaldehyde [2–1024 mg/l]	Thymol [2–1024 mg/l]
***C. albicans* (10)**	**MFC GM**	256	119.4	256
	Mode	256	128	256
	Range MFC	256	64–128	256
***C. glabrata* (10)**	**MFC GM**	238.9	119.4	238.9
	Mode	256	128	256
	Range MFC	128–512	64–128	128–256
***C. krusei* (3)**	**MFC GM**	256	64	256
	Mode	256	64	256
	Range MFC	256	64	256
***C. dubliniensis* (3)**	**MFC GM**	161.3	64	161.3
	Mode	128	64	128
	Range MFC	128–256	64	128–256
***C. parapsilosis* (2)**	**MFC GM**	256	128	256
	Range MFC	256	128	256
***C. guilliermondii* (2)**	**MFC GM**	362	64	256
	Range MFC	256–512	64	256
***C. orthopsilosis* (2)**	**MFC GM**	256	64	256
	Range MFC	256	64	256
***C. tropicalis* (2)**	**MFC GM**	362	91	256
	Range MFC	256–512	64–128	256
***C. metapsilosis* (1)**	**MFC**	256	128	256
Total	**MFC GM**	251.0	99.0	241.2
	Mode	256	128	256
	Range MFC	128–512	64–128	128–256

GM: geometric mean, MFC: minimum fungicidal concentration.

Table 3
In vitro activity of carvacrol, cinnamaldehyde, thymol and current antifungal drugs against pre-sessile and sessile *Candida* cells.

Candida isolates	Carvacrol				Cinnamaldehyde				Thymol				Anidulafungin				Isavuconazole			
	Range analyzed [mg/l]				Range analyzed [mg/l]				Range analyzed [mg/l]				Range analyzed [mg/l]				Range analyzed [mg/l]			
	[8–1024]		[16–2048]		[8–1024]		[16–2048]		[8–1024]		[16–2048]		[0.125–16]		[0.25–32]		[0.125–16]		[0.25–32]	
	PSIC		SIC		PSIC		SIC		PSIC		SIC		PSMIC		SMIC		PSMIC		SMIC	
	CV	XTT	CV	XTT	CV	XTT	CV	XTT	CV	XTT	CV	XTT	CV	XTT	CV	XTT	CV	XTT	CV	XTT
***C. albicans* SC5314**	128	64	≥2048	128	64	64	≥2048	128	256	64	≥2048	128	0.125	0.125	32	2	8	0.125	≥32	≥32
C. albicans Ca2	128	256	–	–	64	64	–	–	128	128	–	–	0.125	0.125	–	–	0.25	≥16	–	–
***C. albicans* UPV 05–013**	128	64	≥2048	128	128	64	≥2048	128	128	32	≥2048	128	0.125	0.125	≥32	0.25	2	0.125	≥32	32
***C. albicans* UPV 05–007**	256	128	512	128	128	128	≥2048	64	256	256	≥2048	128	0.125	0.125	≥32	0.25	8	≥16	≥32	≥32
***C. albicans* UPV 11–342**	256	64	512	512	128	128	≥2048	256	256	128	1024	256	0.125	0.125	≥32	≥32	4	≥16	≥32	≥32
***C. albicans* UPV 11–345**	256	64	1024	512	128	128	≥2048	64	256	128	≥2048	512	0.25	0.125	≥32	0.25	8	0.125	≥32	≥32
***C. albicans* UPV 12–298**	128	128	512	128	64	64	≥2048	128	32	64	512	256	0.125	0.125	≥32	0.25	≥16	≥16	≥32	≥32
***C. albicans* UPV 15–101**	128	128	512	128	256	128	≥2048	64	128	256	512	16	0.125	0.125	16	≥32	2	0.125	1	4
***C. albicans* UPV 15–106**	128	256	512	128	128	128	1024	128	128	256	256	32	0.125	0.125	≥32	0.25	≥16	≥16	32	0.25
***C. albicans* UPV 15–157**	128	64	1024	256	128	128	≥2048	256	128	64	≥2048	256	0.125	0.125	≥32	0.25	≥16	1	≥32	≥32
GM	157.6	1040.5	812.7	188.1	111	97	1896.2	118.5	147	157.6	1106	128	0.134	0.125	29.6	0.9	4.9	1.7	21.8	14.8
Mode	128	64	512	128	128	128	≥2048	128	128	64	≥2048	128	0.125	0.125	≥32	0.25	≥16	≥16	≥32	≥32
***C. dubliniensis* UPV 11–366**	128	32	512	128	128	32	≥2048	128	64	64	≥2048	128	0.125	0.125	≥32	16	≥16	≥16	≥32	≥32
***C. tropicalis* UPV 06–115**	128	64	512	128	128	128	≥2048	256	64	64	512	128	0.125	0.125	≥32	≥32	≥16	0.125	≥32	≥32

Pre-sessile ICs (PSICs) were those concentrations causing 50% metabolic inhibition and 50% biomass reduction with respect to controls without compound. Sessile ICs (SICs) were those concentrations causing 50% metabolic inhibition and 50% biomass reduction with respect to controls. CV: crystal violet assay - biomass quantification, XTT: XTT assay - metabolic activity determination.

whole has gained considerable interest, comparisons of published results is often difficult and has limitations that hinder its clinical application. These limitations include the variability among studies due to different composition of chemical constituents, the influence of the time of harvesting, the part and lot of the plant used or the methodology of extraction, and the hypersensitivity reactions associated to their use [22]. On the other hand, the study of pure phytochemical compounds can present important advantages, as it facilitates the comparison of results among research and toxic side effects related to other components present in the essential oils are avoided. Moreover, previous studies have indicated a significantly superior and sustained inhibitory effect of components compared to essential oils [7].

Carvacrol and its isomer thymol are monoterpenoid phenols present in major proportion in the essential oils extracted from plants of *Lamiaceae*, *Verbenaceae*, *Scrophulariaceae*, *Ranunculaceae*, and *Apiaceae* families [20]. Cinnamaldehyde is a phenylpropanoid present in essential oils from several trees from genus *Cinnamomum* of *Lauraceae* family [21]. In the current study, carvacrol, cinnamaldehyde and thymol showed activity against planktonic and sessile *Candida* cells. In the case of planktonic cells, cinnamaldehyde presented the strongest antifungal fungicidal activity from low concentrations as reported by Rajput and Karuppayil [23], followed by thymol and carvacrol. The latter phytocompounds were also very active against all *Candida* species studied, even against isolates with reduced susceptibility or resistance to fluconazole, as previously described [24,25]. Although the mode of action seems to be directed towards ergosterol synthesis, differences and similarities between these phytocompounds could be explained by their chemical structure. Carvacrol and thymol both have hydroxyl groups and belong to the same chemical class, while cinnamaldehyde would exert its greater activity due to its aldehyde group, as hypothesized in a study of the inhibitory activity of these compounds via vapor phase mediated susceptibility assay [26].

Biofilms are communities of adherent sessile cells with different properties from those of planktonic cells [27]. Biofilm formation is an important virulence factor because the presence of these microbial communities is related to increased resistance to antimicrobial agents and recalcitrant infections such as denture stomatitis, chronic mucocutaneous candidiasis and chronic multifocal candidiasis [1]. In the current study, *C. albicans* was the most biofilm producing species, but also *C. tropicalis* isolates were high biofilm producers. *C. tropicalis* and *C. glabrata* are frequently co-isolated with *C. albicans* from patients with oral infections or colonization [4,28]. Moreover, co-infection or prior infection with *C. albicans* may facilitate *C. glabrata* infection [29]. These fungal associations could change antifungal susceptibility patterns and reduce the effectiveness of conventional treatments for candidiasis [15]. In the current study, the three phytochemicals tested and anidulafungin have shown anti-*Candida* biofilm activity, in contrast with the low antibiofilm activity of isavuconazole. The last one, a second-generation triazole, although active against planktonic fungal cells, was not active against *Candida* biofilms as reported in studies with antifungal agents of the same family [30]. Anidulafungin showed activity against almost all *C. albicans* biofilms, although it was not active against *C. tropicalis* biofilms, as described in previous reports showing that echinocandins do not eradicate or kill *C. tropicalis* biofilms [31,32]. Comparisons on biofilm production capacity among different species are subject to a number of limitations inherent to the methodologies employed due to both the methodologies employed and the specific biofilm production patterns of each species. Isolate classification according to biofilm production capacity contemplated both metabolic activity and biomass results, as recommended by other authors [33]. These procedures are broadly used in the literature, despite the lack of standardized methodologies. However, other media did not used in the present work could be tested to assess the biofilm production in order to resemble the normal conditions in the oral cavity.

Carvacrol and thymol may be efficient alternatives for eradicating biofilm-associated recalcitrant infections, considering that in our study

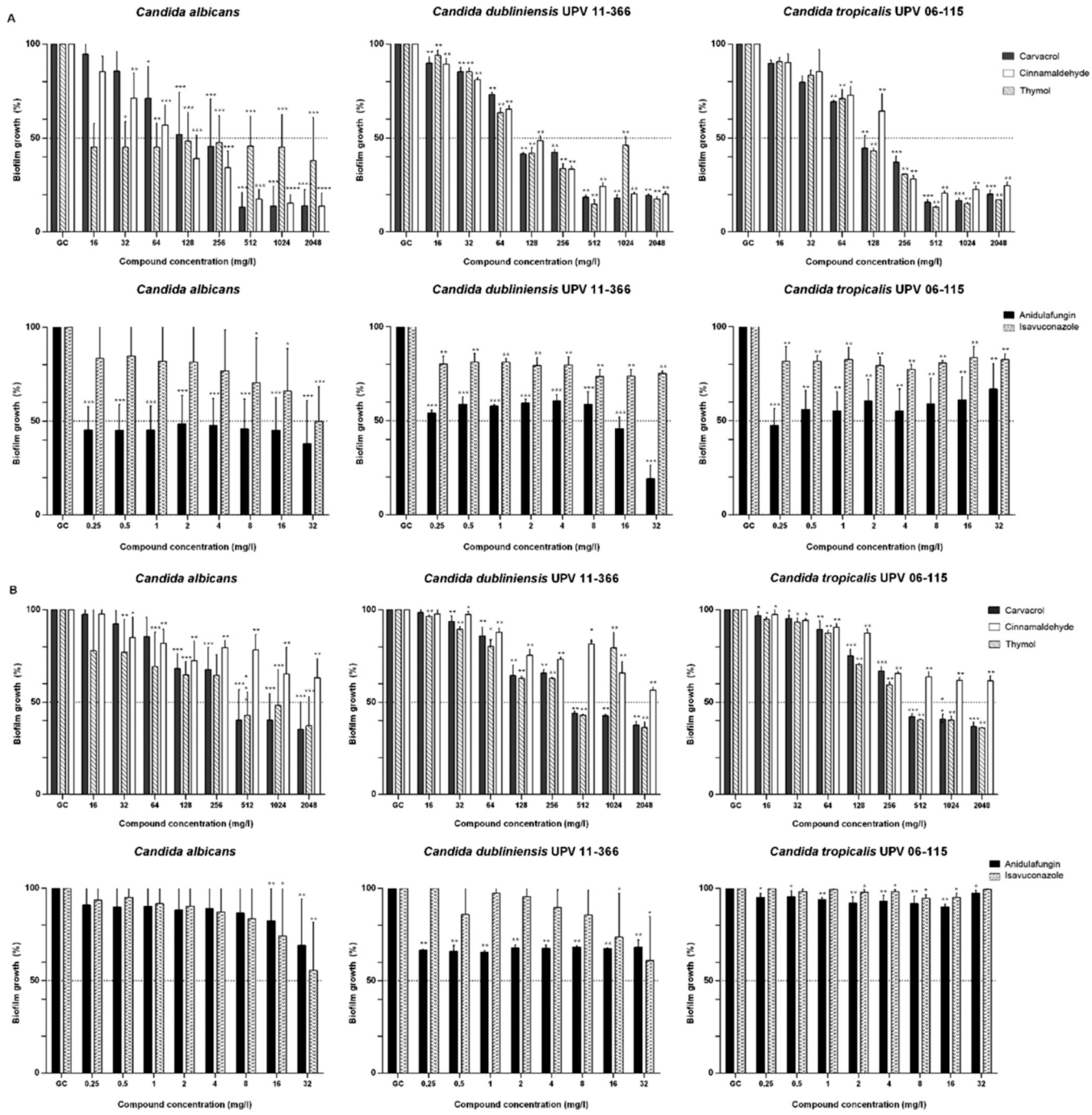

Fig. 2. Biofilm growth inhibition of mature biofilms treated with carvacrol, cinnamaldehyde, thymol, anidulafungin and isavuconazole. Metabolic activity (A) and biomass (B). *Candida albicans*: mean of eight isolates including a fluconazole resistant isolate. GC: growth control. * : $p < 0.05$, * *: $p < 0.01$ respect to the growth control without antifungal agents.

they were more active than cinnamaldehyde in reducing metabolic activity and biomass of mature biofilm. Biomass reduction could play a crucial role in the management of recalcitrant infections, as biofilms are a source for dispersal of cells with advantageous characteristics, such as the ability to form new biofilms more efficiently, enhanced adhesion and virulence, as reported in mice models of candidiasis [27,34]. Carvacrol and thymol reduced at 24 h more than 85% of *Candida* biofilms metabolic activity of azole-susceptible isolates and until 80% of mature biofilms of azole-resistant isolates. These results are supported by the CLSM observation of *Candida* biofilms treated with these phytocompounds in which loss of cell viability was evident. Moreover, these results are in line with those described by Braga et al. [35], who observed a reduction of 45.1% in metabolic activity of *C. albicans* biofilm at 6 h of incubation with thymol, 68% inhibition at 12 h and 88.3% at 24 h. Similarly, Doke et al. [17] stated that carvacrol eugenol and thymol were very effective in reducing >80% of metabolic activity of *C. albicans* biofilm. Dalleau et al. [24] showed that mature biofilms of *C. albicans*, *C. glabrata* and *C. parapsilosis* isolates were susceptible to carvacrol, geraniol and thymol, even the fluconazole-resistant isolates. Carvacrol and thymol are moderately water-soluble and their effectiveness against *Candida* is associated to cell membrane rupture and solubilization, together with ergosterol biosynthesis inhibition [20,36].

Cinnamaldehyde, in our study, reduced biofilm metabolic activity but its effect on biomass reduction was lower. Moreover, the highest

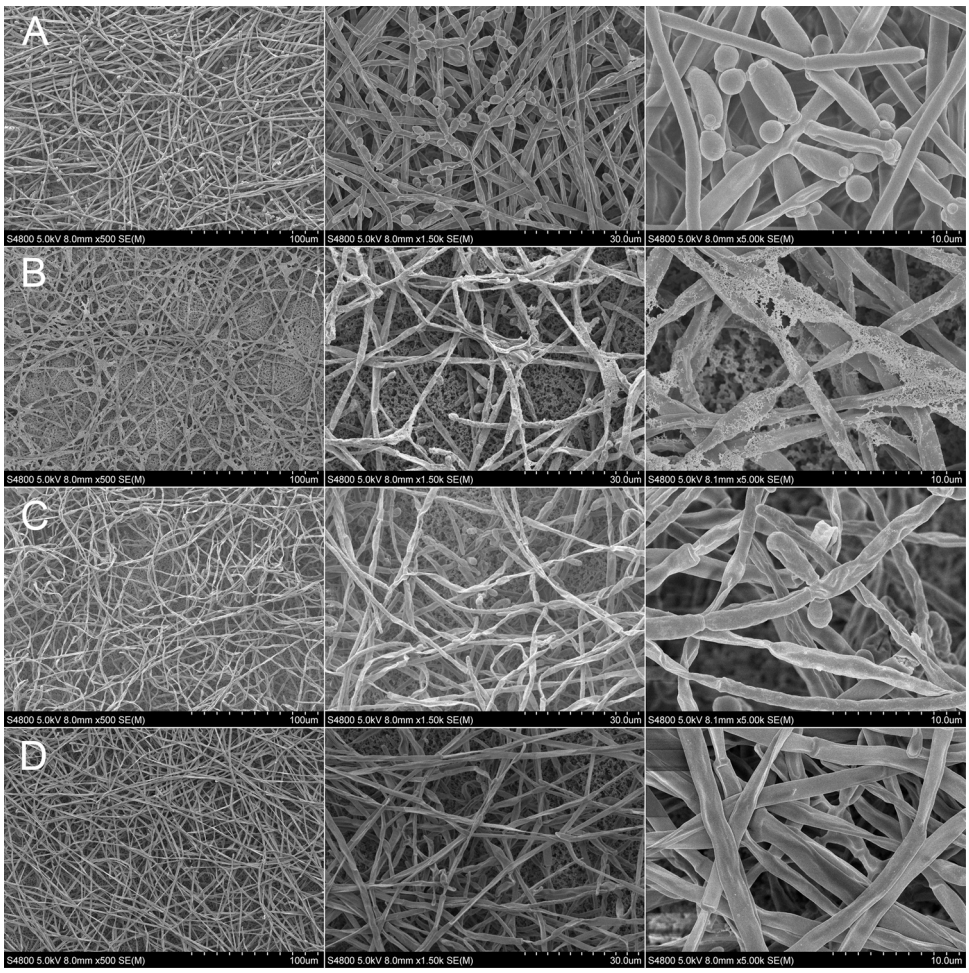

Fig. 3. Images of *C. albicans* SC5314 biofilm by scanning electron microscopic at 500×, 1000× and 5000×. Untreated biofilm (A), biofilm treated with 2048 mg/l of carvacrol (B), 2048 mg/l of cinnamaldehyde (C) and 2048 mg/l of thymol (D).

activity of this phytocompound was obtained in the early phase, preventing the formation of biofilm. Almeida et al. [16] reported anti--*Candida* biofilm activity of cinnamon and its components, among which is cinnamaldehyde. The mode of action of cinnamaldehyde against biofilm could be related to its capacity to reduce adhesion on biotic and abiotic surfaces by downregulating *HWP1* gene [18]. This gene is involved in hyphal formation on early stages of biofilm development. In addition, cinnamaldehyde could cause a loss in cell wall integrity by ergosterol depletion [21,23] and an apoptotic effect [18].

Oral and denture cleansers that include phytocompounds in their formula have been extensively used. However, there are interesting reports that indicate increased *C. albicans* cell counts from mixed oral biofilms exposed to denture cleanser [37]. Therefore, given the importance of *C. albicans* in the pathobiology of oral infections such as denture stomatitis, dental caries and other biofilm-related clinical presentations [38,39], a careful design of the formula of these cleansers should be advisable.

Unlike to the findings of previous studies, our study evaluated biofilm biomass as an important parameter related to the persistence of the biofilm after treatment, and not only with its metabolic activity. Hence, the present work provides consistent data on the effect of pure compounds against biofilm formation of different *Candida* species and relevant knowledge about the capacity to remove biomass of mature biofilm, thus limiting recurrence. These properties could be promising for its application in biomedical fields, such as the design of antifungal coatings for biomedical devices from pure phytocompounds avoiding the disadvantages of the essential oils described above.

Nonetheless, limitations of the use of phytocompounds from essential oils lie in its poor solubility in aqueous solution, volatility and instability, and possible hypersensitivity reactions. Furthermore, the typical dosage of oral rinses enables only short exposure to phytocompounds and may not be suitable to treat active infections. Indeed, the clinical applications of pure phytochemical compound may be attainable using mainly prolonged release formulations instead of cleanser solutions. Nanotechnology can help developing nanoparticles with less adverse effects, better bioavailability, less phytocompound concentration and site-specific delivery. Cinnamaldehyde could be also included in antibiofilm material coatings, while carvacrol and thymol can be used to design curative antibiofilm therapy, since carvacrol and thymol at concentrations $\leq$32 mg/l show less cytotoxicity than cinnamaldehyde in a murine cell line, RAW 264.7 [40]. In addition, likewise it has been observed synergistic effect against planktonic cells (Table S2), the combination of phytocompounds and antifungal drug in sessile cells can improve their antifungal activity. Thus, use of drug combinations, in which the phytocompounds would degrade the matrix and other drugs exert antifungal actions, could be beneficial to improve the clinical response to therapy in candidiasis associated to biofilms, where azoles have proven little or no effect [41].

5. Conclusion

The use of phytocompounds from essential oils has potential in the improvement of established infections without developing further resistance associated to treatment. Carvacrol, cinnamaldehyde and

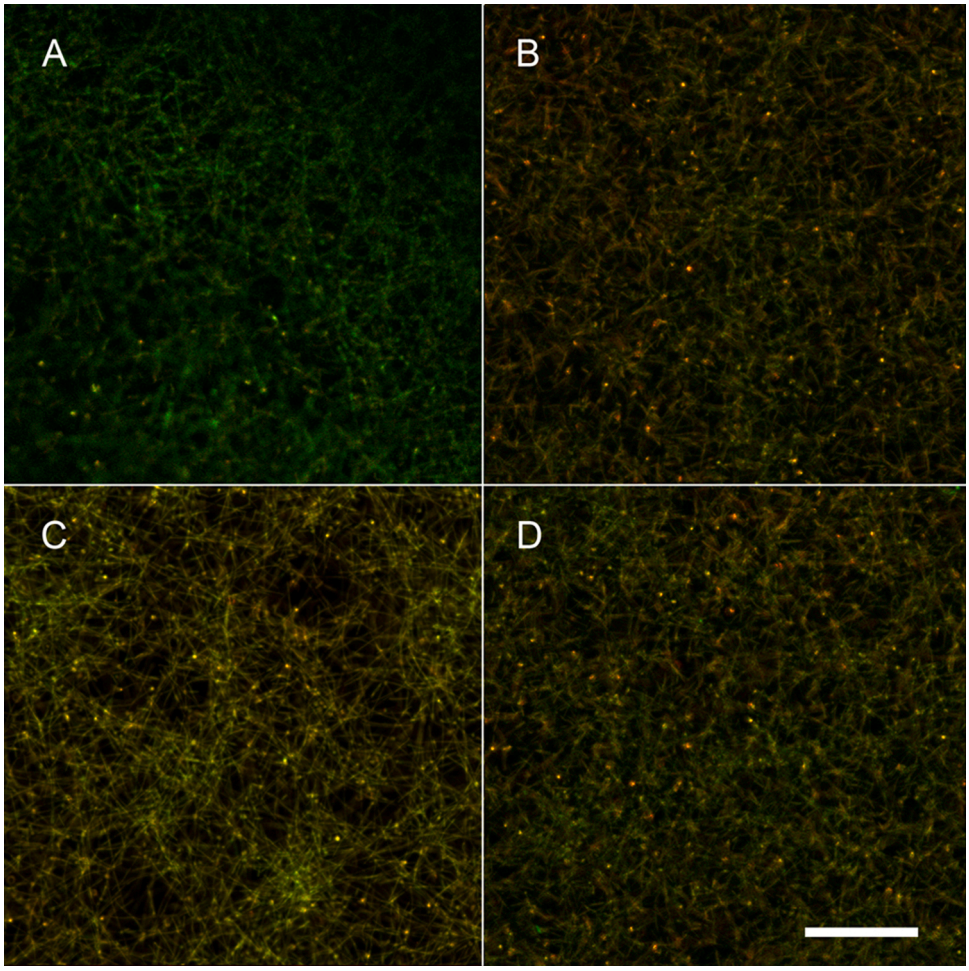

Fig. 4. CLSM analysis of *C. albicans* SC5314 biofilm stained by LIVE/DEAD Yeast kit. Untreated biofilm (A), biofilm treated with 2048 mg/l of carvacrol (B), 2048 mg/l of cinnamaldehyde (C) and 2048 mg/l of thymol (D). Green staining indicates live cells and yellow/orange staining indicates dead cells. Bar scale of 100 μm.

thymol are promising alternatives for candidiasis treatment: cinnamaldehyde by preventing biofilm formation and carvacrol and thymol against established biofilms. However, toxicity and viability studies must be done in animal models and cellular cultures to establish the optimal use of these phytocompounds as antifungal agents in clinical assays.

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CRediT authorship contribution statement

Katherine Miranda-Cadena: Conceptualization, Methodology, Formal analysis, Writing – original draft. **Cristina Marcos-Arias:** Conceptualization, Formal analysis, Writing – original draft. **Estibaliz Mateo:** Formal analysis, Writing – review & editing. **José Manuel Aguirre-Urizar:** Resources, Writing – review & editing. **Guillermo Quindós:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **Elena Eraso:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. All authors have read and agreed to the published version of the manuscript.

Conflict of interest statement

The authors declare that there are no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2021.112218.

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