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Effect of a casbane diterpene isolated from *Croton nepetaefolius* on the prevention and control of biofilms formed by bacteria and *Candida* species

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ABSTRACT

This study aimed to evaluate the effect of the compound 1,4-dihydroxy-2E,6E,12E-trien-5-one-casbane (CD1CN), a casbane diterpene isolated from *Croton nepetaefolius* stalks, on the biofilm formation or preformed biofilms of bacteria and yeasts. Minimum inhibitory concentration results showed that CD1CN inhibited the growth of single cultures of *Staphylococcus aureus* and *Candida albicans* at 125 and 500 μg/mL, respectively, as well as dual cultures of *S. aureus* with *C. albicans* or *Candida glabrata* at 500 and 250 μg/mL, respectively. In general, CD1CN reduced biofilm biomass when applied to preformed biofilms or when applied during the biofilm formation of single and dual cultures in concentrations ranging from 31.25 to 250 μg/mL, depending on the culture. CD1CN was more effective in reducing the cfu of *S. aureus* in single and dual biofilms (62.5–250 μg/mL) than that of *Pseudomonas aeruginosa*, although this reduction was also significant. For yeasts, CD1CN was generally more effective in reducing *C. glabrata* cfu in single or dual cultures when compared to *C. albicans*. SEM images of the dual-species biofilms confirmed these results. In conclusion, CD1CN could be an effective alternative to conventional antimicrobial agents against infectious biofilms, in particular those attributed to mixed cultures.

1. Introduction

It is well known that a significant proportion of human nosocomial infections are associated with biofilms (Høiby, 2011; Murphy and Clegg, 2013). Biofilms exhibit elevated resistance to host defenses and administered antimicrobial agents (Høiby et al., 2010). These communities are frequently composed of multiple species, including bacteria and yeasts, with several studies describing the association of a range of clinical specimens (Peters et al., 2010; Elias and Banin, 2012).

Candida albicans is the fourth leading cause of nosocomial bloodstream infections in patients in intensive care units and in neutropenic patients (Kett et al., 2011; Bonnin, 2012). *C. albicans* is often found with bacterial species in polymicrobial biofilms where extensive interspecies interactions are likely to occur (Douglas, 2002). For instance, *Candida* and *Pseudomonas* have been coisolated from the cystic fibrosis lung or serious burn wounds (Gupta et al., 2005; Valenza et al., 2008); moreover, their interaction is characterized as an antagonistic fungal–bacterial interaction (McAlester et al., 2008; Williams and Cámara, 2009). *C. albicans* and *Staphylococcus aureus* have also been coisolated from various mucosal surfaces, including vaginal and oral mucosa in a biofilm mode of growth (Klotz et al., 2007). Interestingly, the combined effect of *C. albicans* and *S. aureus* results in a synergistic interaction (Carlson and Johnson, 1985; Carlson, 1988).

Most previous studies examining the interactions between *Candida* and bacteria in mixed biofilms have focused on *C. albicans*, and a few studies have reported on biofilms not containing *C. albicans* in a mixed species environment (Bandara et al., 2010). Previously, *Candida glabrata* was considered a relatively nonpathogenic

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saprophyte of the normal flora of healthy humans, and, as such, it was not readily associated with serious infection. In fact, this species can rapidly disseminate throughout the body, and currently, *C. glabrata* infection is associated with a high mortality rate (Silva et al., 2011).

Analyses of mixed-species infections are limited, and observational studies are confounded by the fact that patients with polymicrobial infections may have other risk factors that correlate with a poor clinical outcome, such as greater severity of illness or inadequate therapy against either or both infecting organisms (Peleg et al., 2010; Morales and Hogan, 2010). In this context, steps should be taken to discover new compounds that are able to prevent or eradicate these biofilms. Natural plant compounds have emerged as potential biotechnological tools in the search for new antimicrobial drugs (Schachter, 2003). Moreover, some studies show that diterpenoids are often associated with the antimicrobial activity of natural extracts (Habibi et al., 2000; Velikova et al., 2000). In particular, the compound 1,4-dihydroxy-2E,6E,12E-trien-5-one-casbane diterpene was isolated previously from *Croton nepetaefolius* (Santos et al., 2008) and was recently shown to have antimicrobial activity against bacteria and yeast pathogenicity (Carneiro et al., 2011; Sá et al., 2012). The aim of the present study was to evaluate the effect of 1,4-dihydroxy-2E,6E,12E-trien-5-one-casbane diterpene on planktonic growth, biofilm formation and preformed biofilms of *C. albicans*, *C. glabrata*, *S. aureus* and *Pseudomonas aeruginosa*, as single species or in yeast–bacteria combinations.

2. Materials and methods

2.1. Plant material

Stalks from *C. nepetaefolius* were collected in Caucaia–Ceará (Brazil) in May 2004. The material was identified by Dr. Edson Paula Nunes at the Herbário Prisco Bezerra (EAC), Departamento de Biologia, Universidade Federal do Ceará, Fortaleza, CE, Brazil, where the voucher specimens (No. 33.582) were deposited.

2.2. Isolation of casbane diterpene

Stalks (5.0 kg) of *C. nepetaefolius* were ground to powder and extracted with ethanol (EtOH) at room temperature. The solvent was removed under reduced pressure to obtain the EtOH extract. Such extract (58.6 g) was fractionated by silica gel chromatography followed by elution with hexane (fractions 1–15), hexane/ethylacetate (EtOAc) 1:1 v/v (fractions 16–25), EtOAc (fractions 26–40) and EtOH (fractions 41–48), resulting in 48 fractions of 100 mL each. The hexane fractions (fractions 1–15; 22.5 g) obtained in the previous step were pooled and re-chromatographed in a silica gel column resulting in new fractions (F′), which were eluted with hexane (F′ 1–10), hexane/EtOAc 1:1 v/v (F′ 11–16), EtOAc (F′ 17–21) and EtOH (F′ 22–25). The hexane/EtOAc fractions (F′ 11–16; 14 g) obtained in the previous step were pooled and re-chromatographed in a silica gel column resulting in new fractions (F″), which were eluted with hexane (F″ 1), hexane/EtOAc 9:1 v/v (F″ 2–5), hexane/EtOAc 8:2 v/v (F″ 6–15), hexane/EtOAc 7:3 v/v (F″ 16–32) and EtOAc (F″ 33). Fractions F″ 10–13, obtained with hexane/EtOAc 8:2 v/v, yielded a diterpene named 1,4-dihydroxy-2E,6E,12E-trien-5-one-casbane (CD1CN) (Fig. 1).

2.3. Microorganisms

The microorganisms used in this study included *S. aureus* JKD 6008, *P. aeruginosa* ATCC10145, *C. albicans* ATCC90028 and *C. glabrata* ATCC2001.

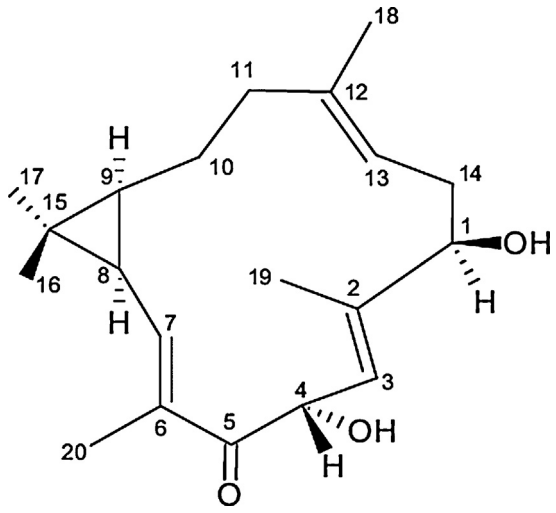

Fig. 1. Structure of 1,4-dihydroxy-2E,6E,12E-trien-5-one-casbane diterpene (CD1CN) isolated from the stalks of *C. nepetaefolius*.

2.4. Culture conditions

Bacteria and yeasts were grown in Trypticase Soy Agar (TSA) and Sabouraud Dextrose Agar (SDA) (both from Liofilchem, Italy), respectively, and incubated at 37 °C for 24 h. An isolated colony was then removed and inoculated into 10 mL of Trypticase Soy Broth (TSB) and Sabouraud Dextrose Broth (SDB) (both from Liofilchem, Italy) and incubated for 18 h at 37 °C under constant agitation. Prior to use, cells were harvested by centrifugation at 5000 × *g* for 10 min at 4 °C and washed using phosphate buffered saline (PBS; pH 7; 100 mM). Thereafter, the final concentration of bacterial inoculum was adjusted to 10^6 cells/mL in Nutrient Broth (NB) (Liofilchem, Italy) using a spectrophotometer (620 nm) and calibration curves previously determined for each bacterium. For yeasts, culture conditions were the same as those described above; however, the concentration of each yeast inoculum was adjusted to 10^6 cells/mL using a Neubauer chamber.

2.5. Minimum inhibitory concentration determination

The antimicrobial potential of CD1CN was assessed through a microdilution test in 96-well "round-bottom" polystyrene plates. CD1CN was diluted in NB with 4% Dimethyl sulfoxide (DMSO) v/v at concentrations ranging from 31.25 to 500 μg/mL and was incubated with 10^6 cells/mL (final concentration of CD1CN and cells). The plates were then incubated at 37 °C for 24 h under constant agitation. The optical density of the contents of each well was recorded at 640 nm (OD_{640}) using an automated Elisa Reader (Synergy TM HT Multi-Detection Microtiter Reader), and the minimum inhibitory concentration (MIC) was determined as the lowest concentration of CD1CN at which there was complete inhibition of the visible growth of the organism. The assays were performed with single- and dual-species (bacteria with yeasts).

2.6. Effect of CD1CN on biofilms

CD1CN activity was tested during biofilm formation, but it was also applied to preformed, mature biofilms. The methodology used to grow biofilms was based on the microtiter plate test developed by Stepanovic et al. (2000) with some modifications. The assays were performed with single and dual-species biofilms (bacteria–yeasts).

Assays to evaluate the formation of biofilms in the presence of CD1CN were performed in sterile 96-well polypropylene plates

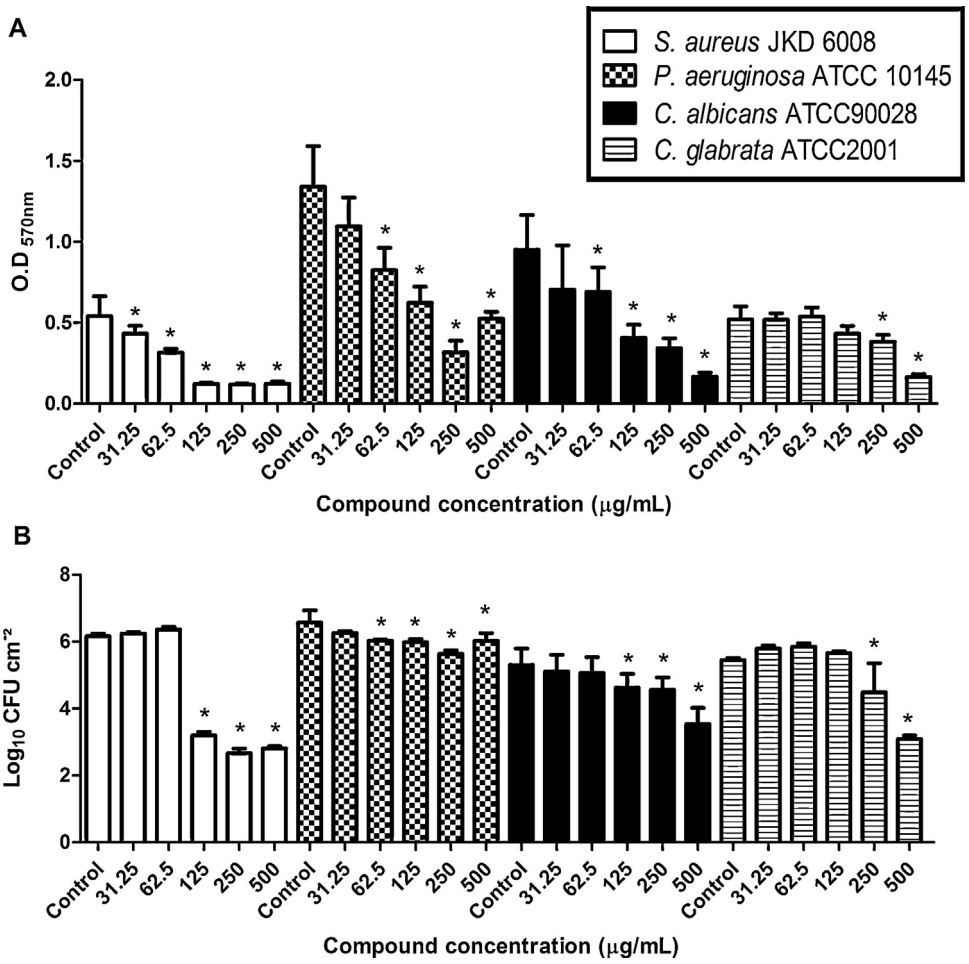

Fig. 2. Quantitative evaluation of single culture biofilms grown in the presence of different concentrations of CD1CN. Mono-species biofilms contain *S. aureus* JKD 6008, *P. aeruginosa* ATCC10145, *C. albicans* ATCC90028 and *C. glabrata* ATCC2001. (A) Biomass quantification measuring the intensity of CV stain at OD_{570}; (B) number of viable cells present in the biofilms. Error bars indicate the standard deviations of the means. *Significantly different ($p < 0.05$) compared to the control group.

prepared using a procedure similar to the one described above for planktonic growth assays including the same initial concentration of cells and CD1CN. All plates were incubated on a horizontal shaker at 37 °C for 24 h for biofilm development. After biofilm growth in the presence or absence of CD1CN, the contents of each well was removed, and the biofilms were washed twice with 200 μL of PBS to remove weakly adherent cells. Then, each well was scraped vigorously with a pipette. For the single-species biofilms, serial decimal dilutions from the obtained suspension were plated on TSA or SDA for bacteria and yeasts, respectively. For dual-species biofilms, the microorganisms were plated in specific media as follows: *S. aureus* was plated on Mannitol Salt Agar (MSA, Liofilchem, Italy), and *P. aeruginosa* was plated on Pseudomonas Isolation Agar (PIA, Fluka Analytical, Switzerland), both inhibiting the growth of yeast. The yeasts were plated on SDA supplemented with 50 μg/mL of ampicillin (Sigma) to inhibit the growth of *S. aureus* and with 50 μg/mL of tetracycline (Sigma) to inhibit the growth of *P. aeruginosa*. Then, the agar plates were incubated for 24 h at 37 °C, and the total number of colony forming units (cfu) was enumerated and represented per unit area ($\log_{10}$ cfu/cm^2) of the microtiter plate well.

Biofilm biomass was determined using crystal violet staining. For fixation of biofilms, 200 μL of 99% methanol (Romil, UK) was added to each well. After 15 min, the methanol was removed, and the plates were allowed to dry at room temperature. Then, 200 μL of crystal violet stain (Merck, Germany) was added to each well. After 5 min, excess crystal violet was removed, and the plates were washed in water. Finally, 200 μL of acetic acid (33%, v/v) (Pronalab, Portugal) was added to all wells to dissolve the crystal violet stain, and the absorbance was measured at 570 nm (OD_{570}).

For the application of CD1CN to preformed biofilms (single- and dual-species), the assays were also performed in 96-well plates. Cell suspensions (200 μL of 10^6 cells/mL in NB) were added to each well and incubated for 24 h at 37 °C under constant agitation. After 24 h, 200 μL of medium was removed, and an equal volume of CD1CN in NB (at concentrations ranging from 31.25 to 500 μg/mL) or NB alone was added. After allowing biofilms to form for 48 h, the medium was aspirated, and each well was rinsed once with 200 μL of PBS to remove nonadherent cells. As described above, samples were removed from the wells and plated for cell enumeration, and crystal violet staining was used for biofilm biomass quantification.

2.7. Scanning electron microscopic observation of biofilms

Dual biofilms (24 h and preformed) were analyzed by Scanning Electron Microscopy (SEM) (S-360, Leo, Cambridge, USA) following standard preparative techniques using CD1CN at 250 μg/mL. Briefly, biofilms were formed in 24-well microplates. After biofilm formation, the plates were washed with sterilized water, dehydrated with alcohol (70% ethanol for 10 min, 95% ethanol for 10 min, and 100% ethanol for 20 min), and allowed to dry prior to gold coating.

Table 1
Minimum inhibitory concentrations (MIC) of casbane diterpene against the microorganisms single and dual species combinations.

Microorganisms	MIC (μg/mL)
S. aureus JKD6008	125
P. aeruginosa ATCC10145	ND
C. albicans ATCC902008	500
C. glabrata ATCC2001	ND
S. aureus JKD6008/*C. albicans* ATCC902008	500
S. aureus JKD6008/*C. glabrata* ATCC2001	250
P. aeruginosa ATCC10145/*C. albicans* ATCC902008	ND
P. aeruginosa ATCC10145/*C. glabrata* ATCC2001	ND

ND – not determined.

2.8. Statistical analysis

Statistical analyses were performed using GraphPad Prism® version 5.0 from Microsoft Windows®. The data from all assays were compared using one-way analysis of variance (ANOVA) with Bonferroni post hoc test. $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Minimum inhibitory concentration

The results of these experiments showed that CD1CN reduced the planktonic growth of all microorganisms tested (data not shown). However, growth inhibition was not complete for *P. aeruginosa* and *C. glabrata* in single-species cultures or when mixed together in dual cultures. Therefore, the MIC was not determined (Table 1). In single-species culture, growth inhibition of 73% and 50% for *P. aeruginosa* and 64% and 45% for *C. glabrata* was obtained with concentrations of 500 and 250 μg/mL, respectively. For dual-species cultures, CD1CN inhibited 78% and 48% of *P. aeruginosa*/*C. albicans* and 87% and 45% of *P. aeruginosa*/*C. glabrata* at the highest concentration (data not shown). As expected (Table 1), the MIC of CD1CN against *S. aureus* (125 μg/mL) was smaller than the MIC found for *C. albicans* (500 μg/mL). For the MIC of dual-species, the compound showed MICs of 500 μg/mL for *S. aureus*/*C. albicans* and 250 μg/mL for *S. aureus*/*C. glabrata*.

3.2. Effect of CD1CD on single- and dual-species biofilm formation

Similar to planktonic growth results, CD1CN impaired the formation of single- and dual-species biofilms (Figs. 2–4).

S. aureus, *P. aeruginosa* and *C. albicans* were sensitive to almost all concentrations of CD1CN tested (Fig. 2A); however, the biomass of *C. glabrata* was only reduced at the two highest concentrations. Similar to the single-species biofilms, CD1CN greatly reduced the biomass of *S. aureus* with the two yeasts (Fig. 3A and C) and also reduced the biomass of *P. aeruginosa* with *C. albicans* (Fig. 4A). Interestingly, CD1CN showed low reduction of *P. aeruginosa* with *C. glabrata* (Fig. 4C).

CD1CN reduced the number of viable *S. aureus* cells in single culture biofilms by approximately 3.0 $\log_{10}$ at 500, 250 and 125 μg/mL, but only reduced *P. aeruginosa* levels by 1.0 $\log_{10}$ (Fig. 2B). For yeasts, CD1CN at 500, 250 and 125 μg/mL reduced *C. albicans* by 1.8, 0.8 and 0.7 $\log_{10}$, respectively, and reduced *C. glabrata* by 2.4 and 0.9 $\log_{10}$ at the highest concentrations (Fig. 2B). The activity of CD1CN was also assessed for mixed biofilms (Figs. 3 and 4). For biofilms containing *S. aureus* with *C. albicans* or *C. glabrata*, the effect of CD1CN was identical to that observed with *S. aureus* in single

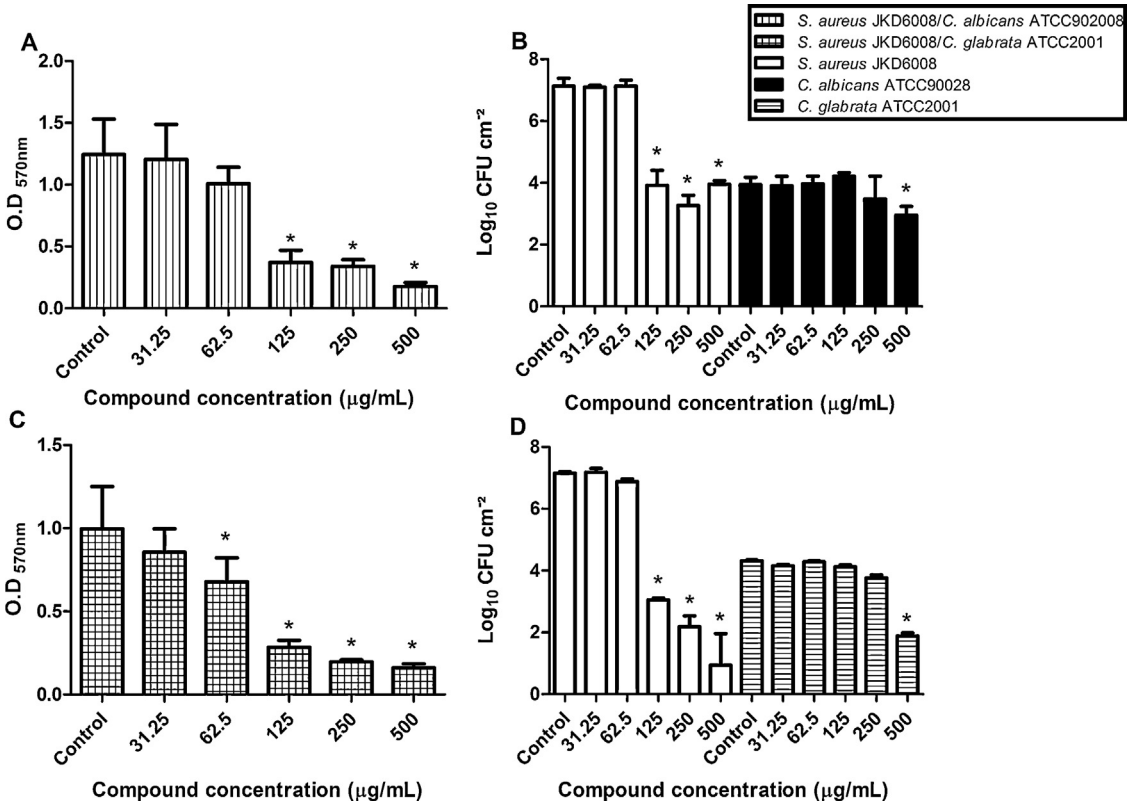

Fig. 3. Quantitative evaluation of dual culture biofilms grown in the presence of different concentrations of CD1CN. Dual-species biofilms are composed of *S. aureus* JKD 6008 with *C. albicans* ATCC90028 or *S. aureus* JKD 6008 with *C. glabrata* ATCC2001. Biomass quantification measuring the intensity of CV stain at OD_{570} for *S. aureus* with *C. albicans* (A) or *C. glabrata* (B); number of viable cells present in biofilms of *S. aureus* with *C. albicans* (C) or *C. glabrata* (D). Error bars indicate the standard deviations of the means. *Significantly different ($p < 0.05$) compared to the control group.

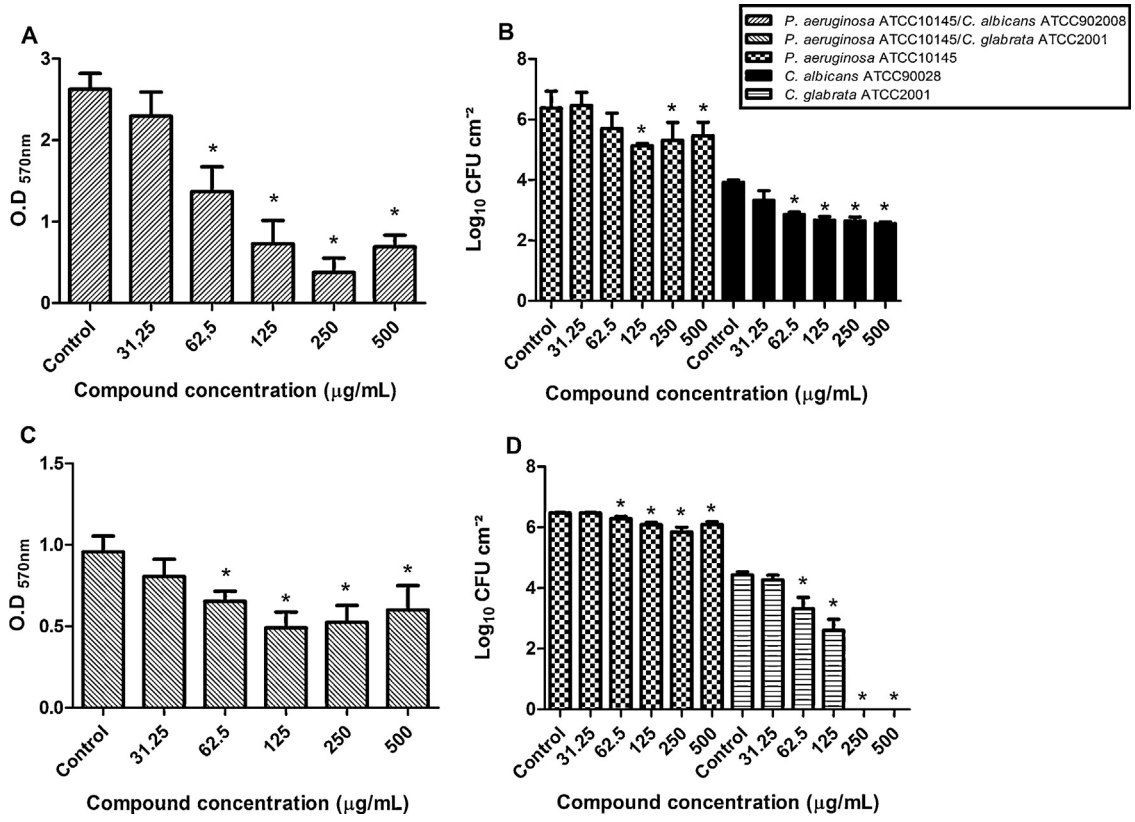

Fig. 4. Quantitative evaluation of dual culture biofilms grown in the presence of different concentrations of CD1CN. Dual-species biofilms are composed of *P. aeruginosa* ATCC10145 with *C. albicans* ATCC90028 or *P. aeruginosa* ATCC10145 with *C. glabrata* ATCC2001. Biomass quantification measuring the intensity of CV stain at OD_{570} for *P. aeruginosa* with *C. albicans* (A) or *C. glabrata* (B); number of viable cells present in biofilms of *P. aeruginosa* with *C. albicans* (C) or *C. glabrata* (D). Error bars indicate the standard deviations of the means. *Significantly different ($p < 0.05$) compared to the control group.

biofilms (Fig. 3B and D). However, the yeasts were less sensitive to CD1CN in the presence of *S. aureus*. CD1CN reduced *C. albicans* and *C. glabrata* by 1.0 and 2.0 $\log_{10}$, respectively, but only at 500 μg/mL (Fig. 3B and D). Interestingly, when yeasts were associated with *P. aeruginosa*, the reduction of the bacteria was similar to that noted in single-species biofilms (Fig. 4B and D), but inhibition of *Candida* species was also observed, especially for *C. glabrata*. For *C. glabrata*, the presence of *P. aeruginosa* appeared to potentiate the activity of CD1CN, decreasing viable *C. glabrata* levels to almost zero at the two higher concentrations (Fig. 4D).

Interestingly, the number of *C. albicans* and *C. glabrata* cells in biofilms formed in the presence of bacteria was approximately 1.0 $\log_{10}$ lower than the number obtained in single-species biofilms. Moreover, in the presence of the yeasts, the number of *S. aureus* cells increased approximately 1.0 $\log_{10}$, whereas the number of *P. aeruginosa* cells remained the same.

3.3. Effect of CD1CD on single- and dual-species preformed biofilms

In addition to assessing biofilm formation in the presence of CD1CN, the ability of this compound to disrupt single- and dual-species preformed biofilms was analyzed by allowing biofilms to form for 24 h and then incubating them with CD1CN for another 24 h.

The results of these experiments showed that CD1CN reduced the biomass of all single-species biofilms tested (Fig. 5A). In contrast to bacteria and the other *Candida* species, *C. glabrata* biomass decreased only at the two highest CD1CN concentrations. For dual-species biofilms, CD1CN generally promoted biomass reduction (Figs. 6 and 7). For the biofilms of *S. aureus* and yeasts, CD1CN showed biomass reduction at all concentrations tested (Fig. 6A and B). For the combination of *P. aeruginosa* and *C. albicans*, the effect of CD1CN was similar to that observed in the single-species biofilms in that the compound showed biomass reduction of the biofilm at 500, 250, 125 and 62.5 μg/mL (Fig. 7A). Interestingly, CD1CN reduced the biomass of the *P. aeruginosa* and *C. glabrata* combination at 500, 250 and 31.25 μg/mL, but it did not show a concentration-dependent behavior (Fig. 7C).

After incubating biofilms with CD1CN for 24 h, the effect of CD1CN on the number of viable cells was also observed (Fig. 5B). The application of CD1CN to the mature biofilm of *S. aureus* reduced cell viability approximately 3.5 $\log_{10}$ at concentrations of 500 and 250 μg/mL and approximately 2.0 $\log_{10}$ at 62.5 and 31.25 μg/mL. However, for *P. aeruginosa*, CD1CN reduced cell viability by less than 1.0 $\log_{10}$ at the higher concentrations. Similarly, CD1CN treatment showed low reduction of *C. albicans* biofilms with an approximately 0.5 $\log_{10}$ decrease at the higher concentrations. On the other hand, CD1CN showed efficacy in the treatment of *C. glabrata* biofilms with $\log_{10}$ reductions of approximately 3 and 2 at 500 and 250 μg/mL, respectively.

For dual-species biofilms containing *S. aureus* and yeasts, treatment with CD1CN showed results for *S. aureus* that were similar to those observed with single-species biofilms (Fig. 6B and D). However, similar to its effect on yeast single-species biofilms, CD1CN was less effective in decreasing the number of viable *C. albicans* cells, with $\log_{10}$ reductions of 0.7 and 0.4 at the two higher concentrations. For *C. glabrata*, CD1CN reduced viable cell numbers at 500, 250 and 125 μg/mL by 2.0, 0.4 and 0.3 $\log_{10}$, respectively (Fig. 6B and D), but this result was also less effective when compared to single-species biofilm. Curiously, when *P. aeruginosa* was grown with *Candida* species, no significant effect was observed on

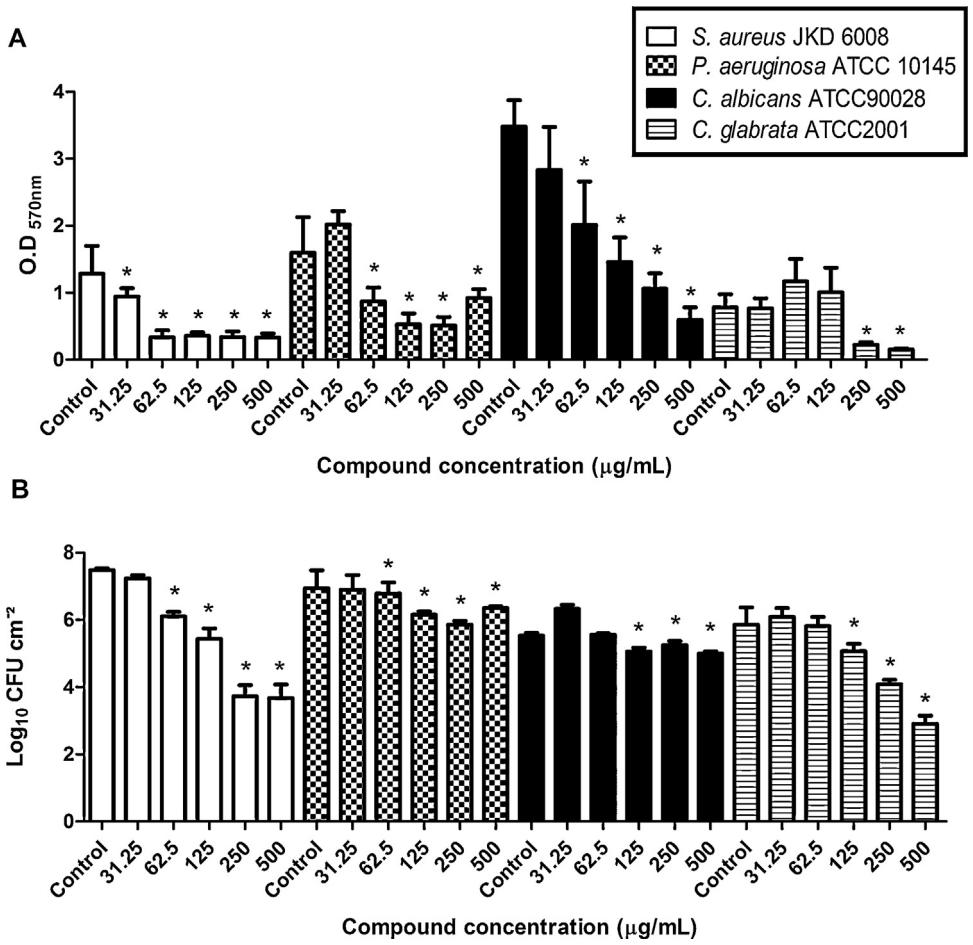

Fig. 5. Quantitative evaluation of preformed single culture biofilms treated with different concentrations of CD1CN. Mono-species biofilms are composed of *S. aureus* JKD 6008, *P. aeruginosa* ATCC10145, *C. albicans* ATCC90028 and *C. glabrata* ATCC2001. (A) Biomass quantification measuring the intensity of CV stain at OD_{570}; (B) number of viable cells present in the biofilms. Error bars indicate the standard deviations of the means. *Significantly different ($p < 0.05$) compared to the control group.

the number of viable *P. aeruginosa* cells in comparison to single-species biofilms (Fig. 7B and D), but CD1CN did have an abrupt effect on *Candida* cells. Specifically, for *C. albicans*, CD1CN showed a $\log_{10}$ reduction of approximately 3.5 for all of the higher concentrations and up to a 4 $\log_{10}$ reduction for *C. glabrata* (Fig. 7B and D).

The presence of *S. aureus* did not change the number of *C. albicans* cells; however, the number of *C. glabrata* cells was decreased by approximately 1.5 $\log_{10}$, and the presence of *P. aeruginosa* reduced the number of cells by approximately 1.5 $\log_{10}$ for both yeasts. As shown previously, the presence of the yeasts increased the number of *S. aureus* cells by approximately 1.0 $\log_{10}$, while, for *P. aeruginosa*, the number of cells was decreased by less than 1.0 $\log_{10}$ with *C. albicans*.

3.4. Analysis of the dual-species biofilms by SEM

The influence of CD1CN on dual-species biofilms during formation and in preformed biofilms was assessed by SEM (Figs. 8 and 9). The interaction between bacteria and yeasts was clearly visible at the periphery of the biofilm, with *S. aureus* and *P. aeruginosa* located firmly on hyphal elements of *C. albicans* (Figs. 8 and 9A, B, E, F) and on *C. glabrata* cells (Figs. 8 and 9C, D, G, H). The images show that CD1CN induced a reduction in cell number, as well as changes in biofilm structure.

During biofilm formation, it is noteworthy that CD1CN in the presence of *S. aureus* inhibited hypha formation in *C. albicans* and substantially reduced the number of *S. aureus* cells (Fig. 8B). However, a similar effect was not observed for *C. albicans* in dual-species biofilms with *P. aeruginosa* (Fig. 8F). A clear decrease in the number of cells of bacteria and yeast could be observed in dual-species biofilms with bacteria and *C. glabrata* (Fig. 8D and H).

For the preformed, mature biofilms, the presence of CD1CN in biofilms containing *S. aureus* and *C. albicans* resulted in a decrease in *S. aureus* cell number but no visible decrease in the number of *C. albicans* cells or inhibition of hypha formation (Fig. 9B). On the other hand, CD1CN in the presence of *P. aeruginosa* showed a strong decrease in the number of *C. albicans* but little change in the number of bacterial cells (Fig. 9F). In preformed, mature biofilms of *S. aureus* and *C. glabrata* and during formation of these biofilms, a clear decrease in the number of bacteria and yeast cells was observed (Fig. 9D). In the biofilms of *P. aeruginosa* and *C. glabrata*, only yeasts showed a strong decrease in cell number (Fig. 9H).

4. Discussion

This work evaluated the effect of a casbane diterpene on planktonic growth and biofilms of *S. aureus*, *P. aeruginosa*, *C. albicans* and *C. glabrata* through quantification of their biomass and number of viable cells. The activity of this compound was also evaluated against dual-species cultures, as indicated in the results. The results showed that CD1CN activity against the tested microorganisms was concentration-dependent. The antimicrobial result corroborates the work of other studies reporting on the antimicrobial activity

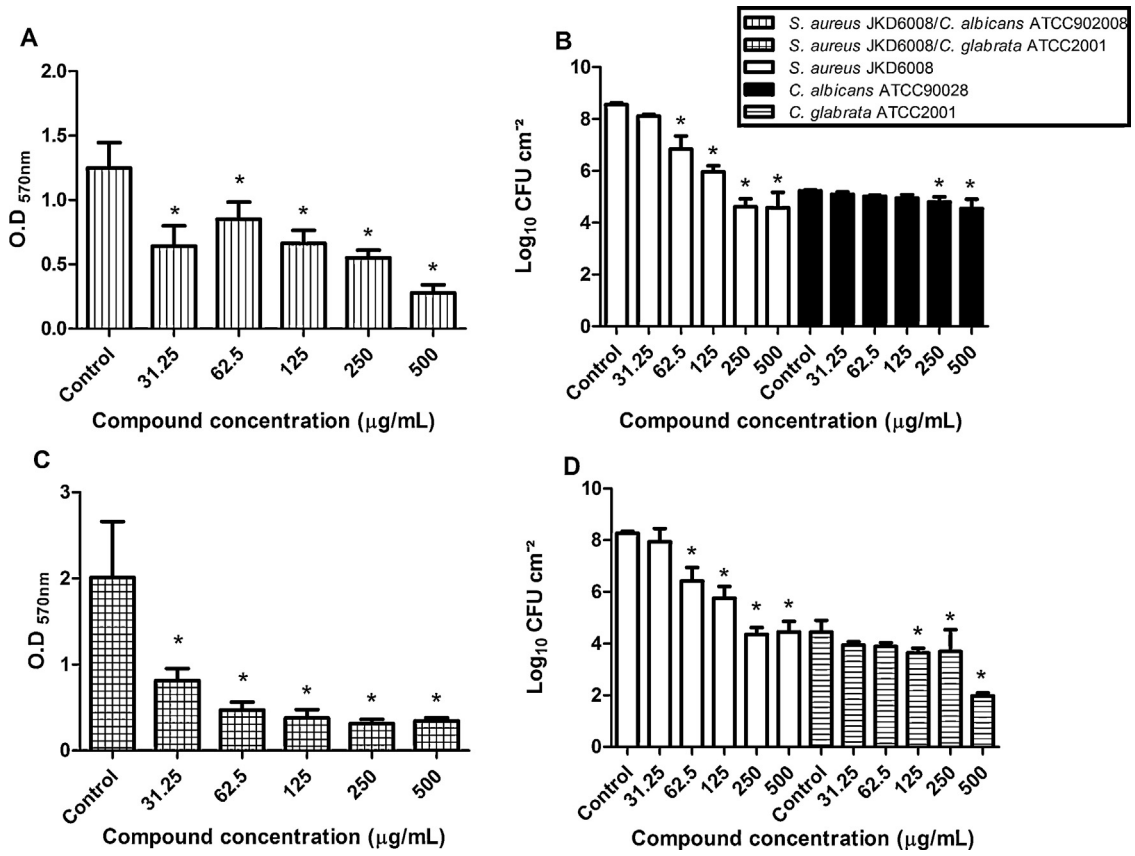

Fig. 6. Quantitative evaluation of preformed dual culture biofilms treated with different concentrations of CD1CN. Dual-species biofilms are composed of *S. aureus* JKD 6008 with *C. albicans* ATCC90028 or *S. aureus* JKD 6008 with *C. glabrata* ATCC2001. Biomass quantification measuring the intensity of CV stain at OD_{570} for *S. aureus* with *C. albicans* (A) or *C. glabrata* (B); number of viable cells present in biofilms of *S. aureus* with *C. albicans* (C) or *C. glabrata* (D). Error bars indicate the standard deviations of the means. *Significantly different ($p < 0.05$) compared to the control group.

of diterpenes (Habibi et al., 2000; Velikova et al., 2000; Mendoza et al., 2002; Urzúa et al., 2006).

The MIC test showed that both bacteria and yeasts, even in dual-species cultures, were susceptible to CD1CN (Table 1). CD1CN MICs were found to be between 125 and 500 μg/mL for *S. aureus* and *C. albicans* in single cultures and in dual cultures of *S. aureus* with *C. albicans* or *C. glabrata*. In contrast, although CD1CN treatment did not lead to a 100% reduction in *P. aeruginosa*/*C. glabrata* dual culture, it did significantly reduce planktonic growth when compared with controls (data not shown). These MIC results are similar to those found for CD1CN against oral *Streptococcus* (Sá et al., 2012). Moreover, Carneiro et al. (2011) tested CD1CN against bacteria and yeasts, including those tested in the present study, but their results showed an MIC of only 250 μg/mL for *S. aureus* while our results showed an MIC of 125 μg/mL against that species. In addition, their study showed lower reductions in planktonic growth of *P. aeruginosa* and *C. albicans* when compared with the results presented in this study. Although the same species and CD1CN concentration were used, these activity differences could result from small differences in assay protocols, including different media.

The mechanism of action underlying the antimicrobial activity of diterpenes remains unclear, but it is believed that these compounds can interact with lipid bilayer fatty acids that destabilize membrane phospholipids (Urzúa et al., 2008; Carneiro et al., 2011). Using simulation, Urzúa et al. (2008) explained that the antimicrobial activity of diterpenes results from the presence of a hydrophobic moiety and a hydrophilic region possessing two hydrogen-bond donor groups. These two structural requirements may be responsible for the optimal insertion of this compound into cells. In the present study, CD1CN was shown to be more effective against the Gram-positive bacterium *S. aureus* compared to activity against the Gram-negative bacterium *P. aeruginosa* (Table 1). Other studies show that some diterpenes exhibit antibacterial activity against Gram-positive bacteria but not Gram-negative bacteria (Urzúa et al., 1998, 2006; Carneiro et al., 2011). The outer membrane present in Gram-negative bacteria can constitute a barrier against CD1CN permeability, thus inhibiting the antimicrobial effect of the compound.

Although some studies have reported on antimicrobial diterpenes, few have described the activity of these compounds on biofilms. In this context, the present study shows that CD1CN reduces the biomass and number of viable cells of *S. aureus*, *P. aeruginosa*, *C. albicans* and *C. glabrata* during biofilm formation and upon application to preformed biofilm. As shown previously, CD1CN reduces planktonic growth of the species tested (Table 1). These results may explain why CD1CN decreased the biomass and number of viable cells when applied at the beginning of biofilm formation. The application of CD1CN in preformed biofilms also resulted in a reduction of the biomass and cfu. In fact, CD1CN is a small molecule (Santos et al., 2008) that most likely penetrates into the biofilm matrix and directly diffuses inside the matrix layer through the pores, as water channels, to impart its antimicrobial activity. Although CD1CN shows low efficiency in reducing the number of viable *P. aeruginosa* cells, it is noteworthy that it significantly reduced biofilm biomass, most likely as a result of an interaction with their matrix. It has already been reported that the *P. aeruginosa* matrix plays a role in the bacterium's resistance to antibiotics and its evasion of host defense mechanisms (Evans et al.,

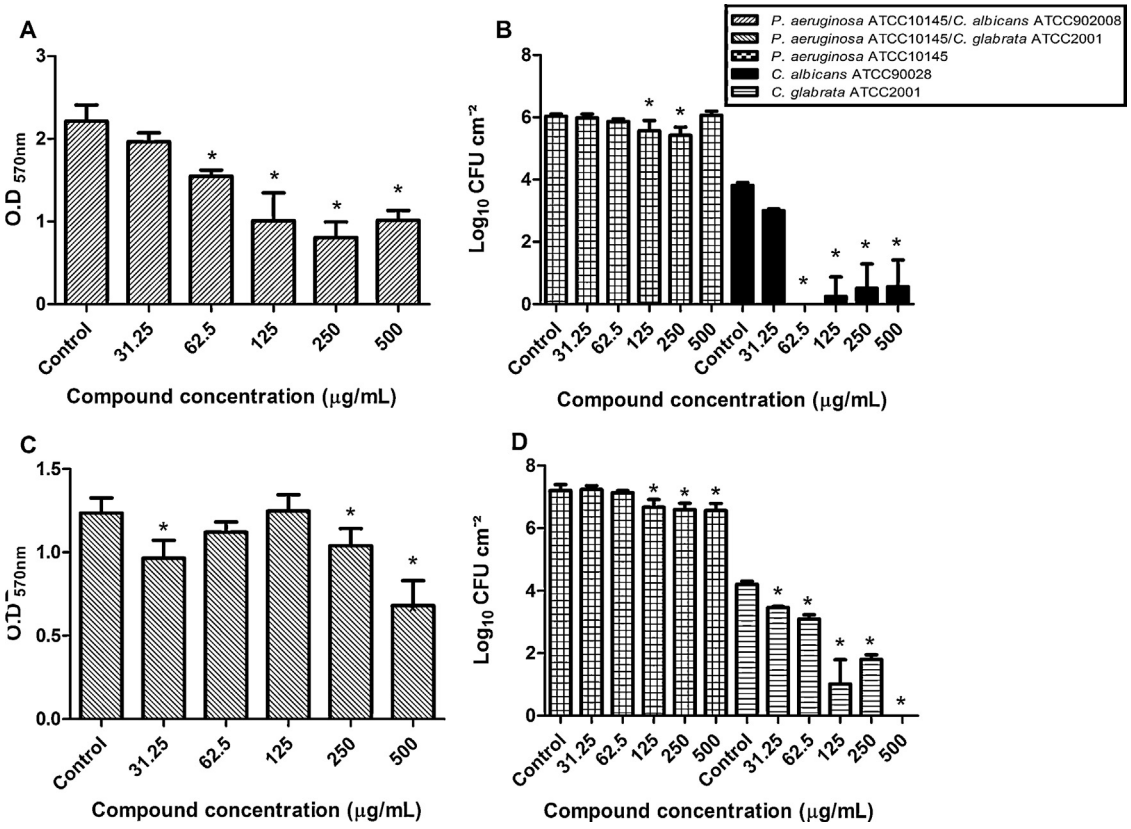

Fig. 7. Quantitative evaluation of preformed dual culture biofilms treated with different concentrations of CD1CN. Dual-species biofilms are composed of *P. aeruginosa* ATCC10145 with *C. albicans* ATCC90028 or *P. aeruginosa* ATCC10145 with *C. glabrata* ATCC2001. Biomass quantification measuring the intensity of CV stain at OD_{570} for *P. aeruginosa* with *C. albicans* (A) or *C. glabrata* (B); number of viable cells present in biofilms of *P. aeruginosa* with *C. albicans* (C) or *C. glabrata* (D). Error bars indicate the standard deviations of the means. *Significantly different ($p < 0.05$) compared to the control group.

1996). Thus, according to the results obtained herein, it can be noted that CD1CN can facilitate the action of other drugs in the eradication *P. aeruginosa* cells from biofilms.

This study has shown that *S. aureus* and *P. aeruginosa* form biofilms with *Candida* species and that the bacteria–yeast interaction induces changes in the biofilm of both species. Interestingly, after 24 and 48 h of biofilm formation, the number of viable *C. albicans* and *C. glabrata* cells was significantly reduced in the presence of the bacteria (*S. aureus* and *P. aeruginosa*), compared with single-species biofilms. Several works suggest the possible negative influence of *P. aeruginosa* on the growth of *Candida* species (Hogan and Kolter, 2002; Williams and Cámara, 2009; Gibson et al., 2009; Holcombe et al., 2010; Bandara et al., 2010). Hogan and Kolter (2002) demonstrated that several *P. aeruginosa* virulence factors, including pili and secreted molecules, act in concert to kill *Candida* hyphae. Moreover, *C. albicans* cells were capable of suppressing filamentation upon exposure to a *P. aeruginosa* QS molecule (3-oxo-C12 homoserine lactone) (Williams and Cámara, 2009). Gibson et al. (2009) showed that a *P. aeruginosa*-produced precursor to pyocyanin, proposed to be 5-methyl-phenazinium-1-carboxylate (5MPCA), induces fungal death of *C. albicans*. However, in the present study, no evidence of such an abrupt decrease in the number of yeast cells was noted, and the presence of *P. aeruginosa* did not suppress the development of hyphae (Figs. 8E and 9E). Other studies have shown that *Staphylococcus* species and *Candida* were coisolated and that an infectious interaction seems to be synergistic (Costerton et al., 1999; Timsit et al., 2001; Ramage et al., 2004). The present study showed that the number of viable *S. aureus* cells was increased in the presence of *Candida*. Corroborating these results, Carlson (1983) showed that *C. albicans* directly stimulates the growth of *S. aureus* and increases its virulence.

Based on the present study, it is also worth noting that CD1CN shows a potentially synergistic effect with a factor secreted from *P. aeruginosa* that acts against *C. albicans* and *C. glabrata*, strongly decreasing the number of viable yeast cells when compared with the activity of CD1CN alone (Figs. 4D and 7B and D). Interestingly, the presence of CD1CN together with *S. aureus* inhibited the hyphal development of *C. albicans*, when compared to treatment with no CD1CN (Fig. 8A and B). This phenomenon was, however, not observed with *P. aeruginosa* (Fig. 9E and F). The ability to undergo morphological transformations has been suggested as an important virulence factor in *C. albicans* (Hammer et al., 2000). Thus, although CD1CN and *S. aureus* together did not decrease the number of viable *C. albicans* cells when compared to single-species biofilms, a possible synergistic effect appears to inhibit an important virulence factor of the yeasts. Shirtliff et al. (2009) state that the initial observed interaction between *C. albicans* and *S. aureus* seems to be synergistic; however, at some point during biofilm development, it is possible that the relationship becomes competitive or antagonistic. In a study of biofilms involving bacteria and *C. glabrata*, Bandara et al. (2010) showed a significant reduction in *C. glabrata* counts (58%) after 24 h in dual-species biofilms with *P. aeruginosa* and a nearly complete suppression of *C. glabrata* in the presence of *P. aeruginosa* after 48 h growth. Moreover, Grillot et al. (1994) observed complete or partial inhibition of *Candida* species, including *C. glabrata*, by *P. aeruginosa* in pure and mixed blood cultures using *in vitro* yeast inhibition assays, and they suggested that preclusion of yeast recovery from blood cultures in mixed infections could result from the suppression of yeast by *P. aeruginosa*.

It is also noteworthy that CD1CN, in general, was found to be more effective in *C. glabrata* biofilms compared to *C. albicans* in both single and dual biofilms. According to Samaranayake et al. (2005),

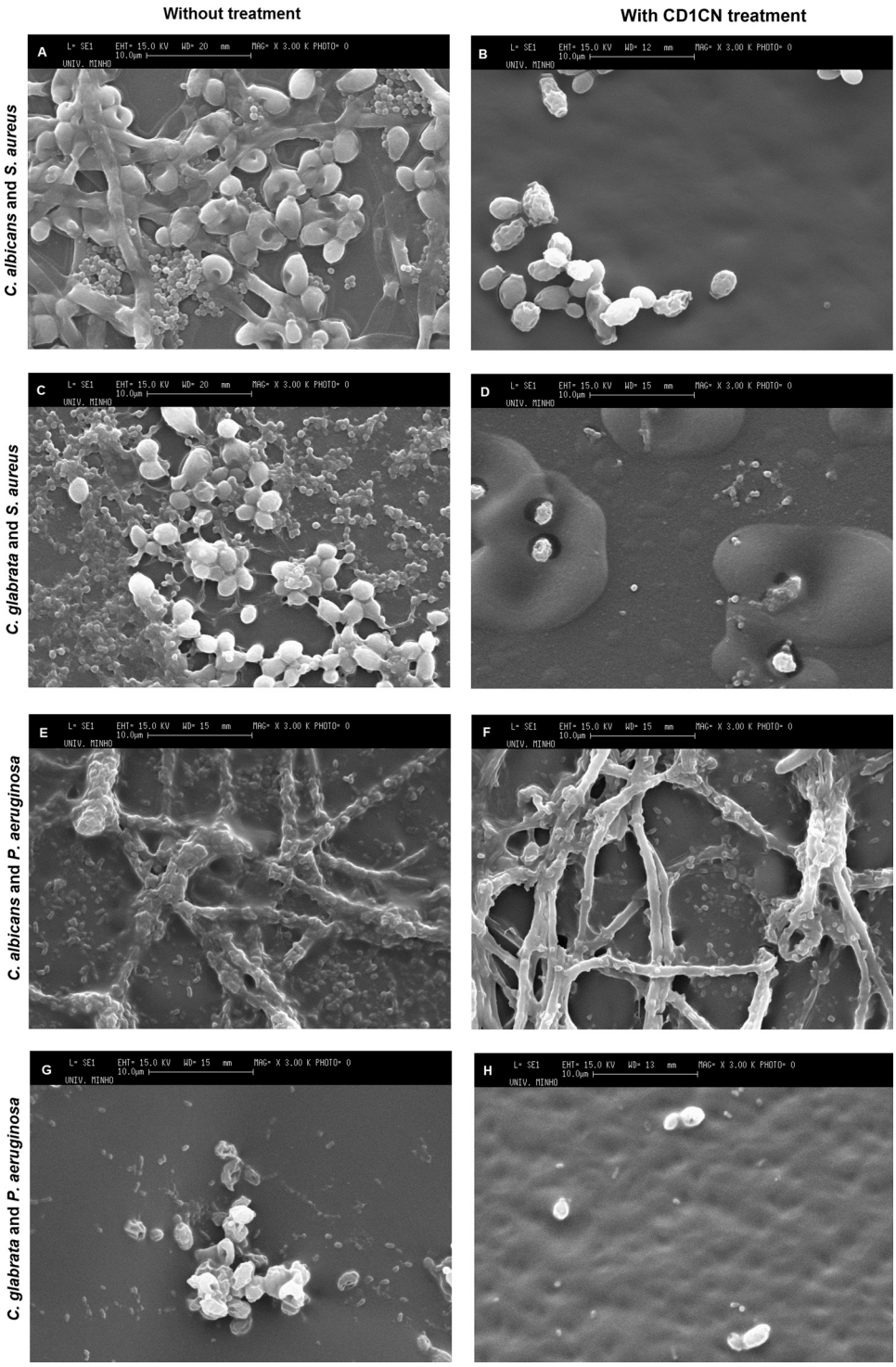

Fig. 8. SEM images of dual-species biofilm formation in the presence and absence of CD1CN. Treatment with 250 μg/mL of CD1CN. 3000× magnification. Bar = 10 μm.

C. albicans forms larger and more complex biofilms than *C. glabrata*. Furthermore, *C. albicans* is a polymorphic organism with biofilms composed of hyphae, pseudo-hyphae and blastospores, while *C. glabrata* biofilms are exclusively composed of blastospores. Thus, hyphal elements of *C. albicans* facilitate the architecture of biofilms, making them more difficult to eliminate (Seneviratne et al., 2009; Silva et al., 2011). The different characteristics between *C. albicans* and *C. glabrata* biofilms may explain why CD1CN is effective on *C. glabrata* biofilms.

In nature, biofilms associated with infections are commonly formed by multiple microbial species. In mixed-species biofilms containing bacteria and fungi, it has been demonstrated that a range of different interactions can occur, including increased resistance to antimicrobials, enhanced surface colonization and interspecies antagonism (Shirtliff et al., 2009; Morales and Hogan, 2010; Peleg et al., 2010). The present study shows that CD1CN is able to inhibit biofilm formation and disrupt the mature biofilm of single- and dual-species biofilms composed of bacteria and yeasts. Thus,

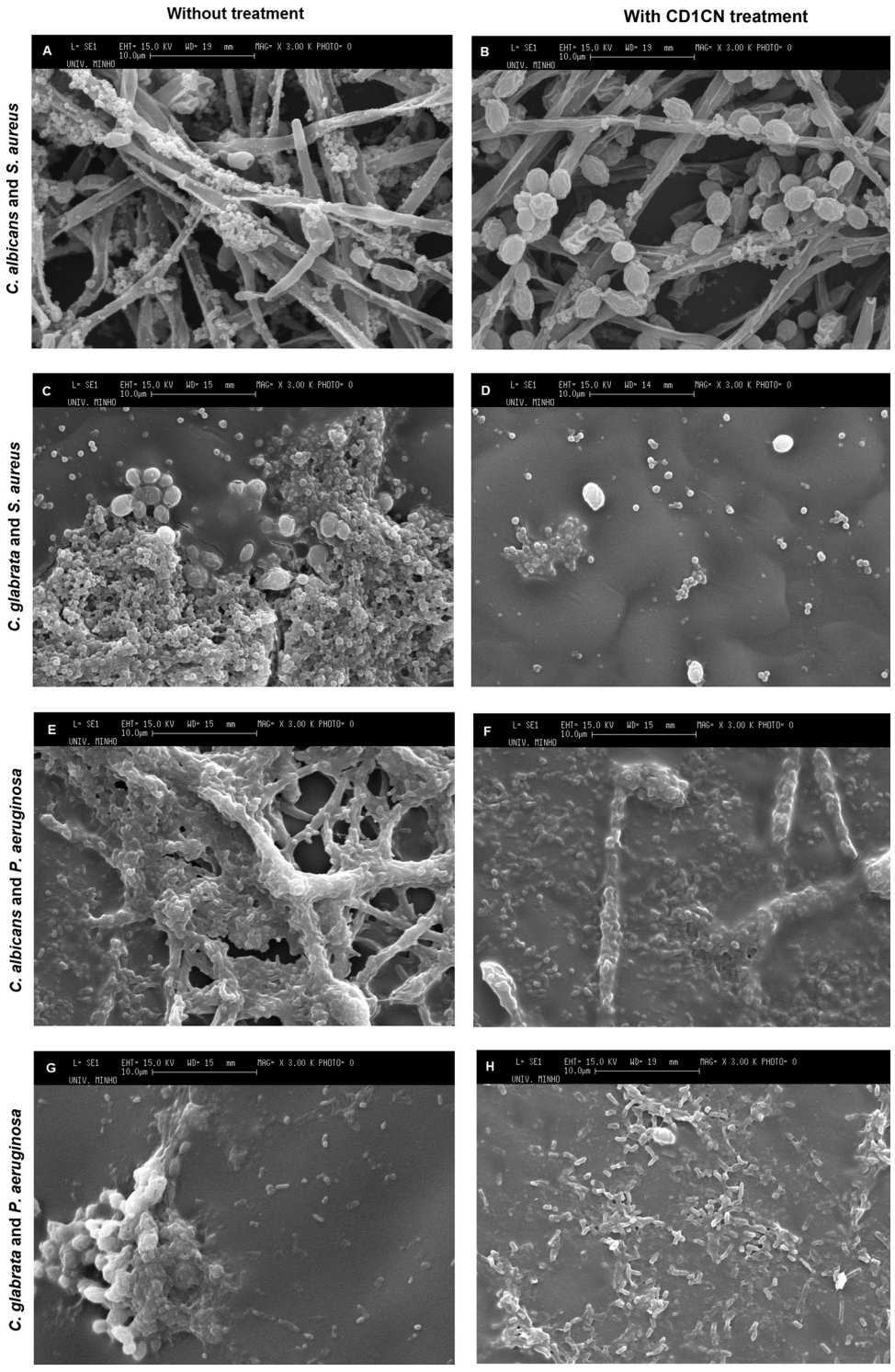

Fig. 9. SEM images of preformed dual-species biofilms treated with 250 μg/mL of CD1CN. 3000× magnification. Bar = 10 μm.

CD1CN appears to be a potentially promising drug in the treatment of infections caused by bacteria and *Candida* together, as well as an alternative to using antibacterial and antifungal agents together.

Conflicts of interest

None.

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