RESEARCH ARTICLE

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Future MICROBIOLOGY

Propolis: a potential natural product to fight *Candida* species infections

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Aim: To evaluate the effect of propolis against *Candida* species planktonic cells and its counterpart's biofilms. **Materials & methods:** The MIC values, time-kill curves and filamentation form inhibition were determined in *Candida* planktonic cells. The effect of propolis on *Candida* biofilms was assessed through quantification of CFUs. **Results:** MIC values, ranging from 220 to 880 µg/ml, demonstrated higher efficiency on *C. albicans* and *C. parapsilosis* than on *C. tropicalis* cells. In addition, propolis was able to prevent *Candida* species biofilm's formation and eradicate their mature biofilms, coupled with a significant reduction on *C. tropicalis* and *C. albicans* filamentation. **Conclusion:** Propolis is an inhibitor of *Candida* virulence factors and represents an innovative alternative to fight candidiasis.

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KEYWORDS
• biofilm • *Candida* species • propolis extract • virulence factors

Candida species are human commensal microbes that commonly reside on skin, GI tract, genitourinary system, oropharynx and upper respiratory tract without causing harm to healthy individuals [1]. However, when the host immune and defense system are debilitated or under certain favorable conditions, these species, which are opportunistic, can cause infections [2]. These infections can range from superficial, such as vulvovaginal, esophageal or oropharyngeal candidiasis, to life-threatening invasive disorders, including candidemia, which is associated with high mortality among immunocompromised populations [1].

For many years, *Candida albicans* has been reported as the predominant species responsible for the majority (60–80%) of infections caused by the genus *Candida* [3]. However, other non-*C. albicans Candida* (NCAC) species, such as *Candida glabrata*, *Candida tropicalis* and *Candida parapsilosis*, have been frequently isolated mainly due to the indiscriminate prescription of antifungal agents [4–6]. Moreover, the pathogenesis of candidiasis is common to all *Candida* species and is facilitated by a number of virulent factors, including the ability to adhere to medical devices or host cells, biofilm development and filamentous form transition [7]. From a clinical point of view, *Candida* biofilms are associated with treatment failure due to a high level of antifungal resistance [8,9]. This fact triggers serious clinical concerns, not only regarding the treatment of patient infection but also for public health [10–12].

The increasing incidence of drug-resistant pathogens, limited number of therapeutic options and the toxicity of compounds have drawn attention towards the antimicrobial activity of

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natural products encouraging the development of alternative treatments [13–15].

Propolis is a resinous substance that honeybees, especially *Apis mellifera*, collect from branches and flowers. It has a complex chemical composition and is known to be rich in polyphenols (mainly flavonoids), waxes, resins, balsams, amino acids and other oils, thus propolis composition varies according to the plant source [16–18]. Propolis is reported to have a wide range of therapeutic properties, such as antimicrobial, antioxidant, anticancer, antiviral, immunomodulatory, wound healing, and antiseptic effect [19–28].

Thus, knowing the problems associated with *Candida* infections, the development of alternative therapies, able to attenuate microbial virulence, is of utmost importance [29]. A little knowledge is already available regarding inhibition of virulence factors of *C. albicans* by propolis [24,30–31]; nevertheless the knowledge concerning NCAC species is still scarce. Therefore, the main goal of this study was to investigate the effect of propolis on clinical isolates of *C. albicans*, *C. parapsilosis* and *C. tropicalis* planktonic cells and their counterpart's biofilms.

Materials & methods

• Origin of propolis, preparation & characterization of extract

Green Brazilian propolis was purchased from the company Mel Apinor (Wal-Luz apiary, Maringá, Paraná State, Brazil). This material was cooled at -18°C for at least 24 h. Then, *in natura* propolis was crushed in an industrial blender, packaged in plastic bags and stored in a freezer (-18°C).

The propolis extract (PE) was prepared from the previously reduced propolis, 30% (w/w) in ethanol by turbo-extraction technique [32]. Briefly, in a glass of turbo extractor, 30 g of propolis were mixed with 70 g of ethanol (96%, v/v) and this system was kept in the refrigerator for 24 h. After this period, the evaporated alcohol weight was completed and the mixture was subjected to turbo extraction. Subsequently, it was vacuum filtered through filter paper and stored in amber glass bottle.

For the evaluation of the quality control of the PE, the techniques used were approved by official codes and were described by many authors, namely relative density, pH, dryness residue (DR) and total phenol content (TPC) [18]. To determine the DR, an amount of 3.0 g of PE was evaporated in water bath, with slow shaking. Afterwards, the concentrated material was dried on the Ohaus-MB 200 infrared analytical balance (Pine Brook, NJ, USA), at 110°C until constant weight. The DR represents the average of, at least, three determinations. The TPC was measured by the Folin–Ciocalteau method with some modifications [33]. For that, in a 25-ml flask an aliquot of PE (2.0 µl) was mixed with 10 ml of purified water and 1 ml of phosphomolybdotungstic reagent R (Folin–Ciocalteau). Then the volume was completed with an aqueous solution of sodium carbonate 14.06% (w/v). As compensatory solution, purified water was employed. The solutions were allowed to stand, protected from light for 15 min under room temperature and then the absorbance was read in a Shimadzu double beam UV-VIS spectrophotometer (Model 1650, Tokyo, Japan) at wavelength of 760 nm. A calibration curve with different dilutions of gallic acid was used as reference. Thus, the TPC was expressed as a percentage of total phenolic substances in PE. The tests accounted for an average of six evaluations.

• *Candida* strains

Fourteen *C. albicans* (12 isolates from blood and two from urine), 14 *C. parapsilosis* (13 from blood and one from urine) and 14 *C. tropicalis* (four from blood and ten from urine), were used. Three *Candida* reference strains from the American Type Culture Collection (ATCC), namely *C. albicans* ATCC 90028, *C. parapsilosis* ATCC 22019 and *C. tropicalis* ATCC 40042 were included in this work. The clinical isolates from urine and blood were selected due to the high level of resistance to commercial antifungals [34] and were obtained to archive collection of the Laboratory of Medical Mycology, Universidade Estadual de Maringá, Brazil.

In each experiment, the isolates were subcultured on Sabouraud Dextrose Agar (SDA; Merck, Munich, Germany) or on Sabouraud Dextrose Broth (SDB; Merck, Munich, Germany) overnight at 37°C. The cellular density was adjusted using a Neubauer chamber before each assay.

• Effect of propolis on planktonic cells

Antifungal susceptibility testing

The antifungal activity of PE was determined by the broth microdilution method according to CLSI standard M27-A3 [35] with some modifications for natural products [36]. For this test, the serial dilution was performed at a ratio of two, from 1:2 to 1:1024. In this

way, PE's concentrations ranged from 13.9 to 7100 µg/ml of total phenol content expressed in gallic acid. The test was carried out in RPMI 1640 (Roswell Park Memorial Institute, Gibco) with L-glutamine (with sodium bicarbonate) and 0.165 M 3-(N-morpholino)propanesulfonic acid (pH 7.2) as buffer (Sigma), and 2% glucose, in 96-well flat-bottomed microtitration plates (Orange Scientific, Braine-l'Alleud, Belgium). After incubation at 37°C for 72 h, MICs were determined by direct observation. The results of the MIC were considered relative to the TPC and were defined as the concentration of TPC that reduced 100% of the growth compared with the organisms grown in the absence of the drug. The minimum fungicidal concentration (MFC) was determined by seeding, on SDA plates, the suspensions exposed to different PE concentrations. Plates were then incubated at 37°C for 24 h. The MFC was defined as the lowest concentration of the test compound in which no recovery of microorganisms was observed. Fluconazole was used as a control (Pfizer, Brazil), and the tests were also determined according to the M27-A3 guidelines of the CLSI. The MIC of fluconazole was defined, as the lowest concentration of this antifungal that was able to inhibit 50% of growth relative to the positive control without drug. As defined by the CLSI, negative controls (medium only), positive controls (medium and yeast), and the reference strain *C. albicans* ATCC 90028 were used in each test. The cutoff levels of susceptibility to fluconazole were used according to CLSI supplement M27-S3 [37] to identify strains as susceptible (S), dose-dependent susceptible (DDS) and resistant (R): fluconazole (S ≤ 8 µg/ml; DDS = 16–32 µg/ml; R ≥ 64 µg/ml).

Time-kill curve procedures

Time-kill curves were determined for the three *Candida* reference strains, *C. albicans* ATCC 90028, *C. parapsilosis* ATCC 22019 and *C. tropicalis* ATCC 40042 with slight modifications to that previously described [38]. Prior to testing, fungi were subcultured on SDA and the inoculum adjusted to 1- 5 × 10^5 yeasts/ml, in RPMI 1640 medium, using a Neubauer chamber. Then, each *Candida* strain suspension was grown in the presence of PE at concentrations equivalent to 450 and 900 µg/ml of TPC. The RPMI 1640 medium without propolis was used as a positive control. Test suspensions were placed on a shaker and incubated at 37°C. At predetermined time points (0, 1, 2, 3, 4, 6, 8, 12, 24, 28 and 36 h), serial dilutions were performed on SDA for CFUs determination. Following incubation at 37°C for 24 h, the number of CFU was determined.

Effect of propolis on filamentation form transition

To evaluate the effect of propolis against *Candida* species filamentation four *C. albicans,* four *C. parapsilosis* and four *C. tropicalis* clinical isolates, and their respective references strains were tested. The clinical isolates were chosen randomly. *Candida* cells were grown overnight in YPD (1% yeast extract; 2% peptone; 2% dextrose) medium. And then, 1 × 10^6 yeasts/ml were incubated in RPMI 1640 medium with 10% fetal bovine serum (FBS), in the presence or absence of PE (450 µg/ml of TPC, selected in order to use a concordant concentration to all species in accordance with its MICs values), at 37°C for 4 h. Blastospore and filamentous forms were counted by observation under a phase contrast microscope, according to the criteria described by Toenjes *et al.* (2005) [39]. More than 100 cells were counted, in duplicate, for each strain. Additionally, images of cell morphologies were obtained, after staining the microorganisms with calcofluor white (Sigma-Aldrich, St Louis, Missouri, EUA). The cells were visualized with BX51 Olympus epifluorescence microscope coupled with a DP72 digital camera (Olympus Portugal SA, Porto, Portugal). All images were acquired using the Olympus Cell-B software.

• Antibiofilm effect of propolis

As known, biofilms are microorganism's community described as ten a 100-times more resistance than its counterpart's planktonic cells [7]. Thus, the PE concentrations used in this part of the study were based on this concept and on our previous findings of antimicrobial susceptibility.

Influence of propolis on biofilm formation

In order to evaluate the effect of PE on *Candida* species' biofilm formation, PE was added after adhesion phase (2 h). For that, *Candida* cells were grown on SDA for 24 h at 37°C, then inoculated in SDB and incubated for 18 h at 37°C under agitation at 120 rpm. After incubation, cells were harvested by centrifugation at 3000 × *g* for 10 min, at 4°C, and washed twice with 15 ml of phosphate-buffered saline ([PBS]; pH 7; 0.1 M). Cell suspensions of

1×10^5 yeasts/ml were prepared in RPMI 1640 medium, 200 µl of suspensions were inoculated into 96-well polystyrene plates, and incubated at 37°C on a shaker at 120 rpm/min for 2 h, to allow attachment of cells to the abiotic surface. Nonadhered cells were removed by wash with sterile PBS. And then 200 µl of PE (concentrations of 500, 700 and 1400 µg/ml of TPC in RPMI 1640 medium) were added to each well. The plates were incubated at 37°C for 24 h to allow biofilm formation. Negative controls (200 µl of only RPMI 1640 medium) were also included.

Biofilms were analyzed by CFU determination. For that, the total medium was removed and the biofilms washed once with 200 µl of PBS. Then, the biofilms were scraped from the respective wells and the suspensions vigorously vortexed for approximately 2 min to disaggregate cells from the matrix [13]. Serial dilutions were made in PBS, plated onto SDA and incubated for 24 h at 37°C. The results were presented in terms of log of CFUs.

Influence of propolis on preformed biofilms

The effect of PE was evaluated on 24 h preformed biofilms. For that, biofilms were formed during 24 h, as described above, the medium was aspirated and the nonadherent cells were removed by washing the biofilms once with 200 µl of PBS. Then, 200 µl of PE (500, 700 and 1400 µg/ml of TPC in RPMI 1640 medium) were added to each well. The biofilms were incubated for further 24 h, at 37°C on a shaker at 120 rpm/min. The effect of PE on *Candida* biofilms was assessed through quantification of the number of CFU as described above. The results were presented in terms of log of CFU.

Effect of propolis on biofilm structure

Candida biofilm's structure and cell morphology, after growth in the presence and absence of PE (1400 µg/ml of TPC) was characterized by scanning electron microscopy (SEM). Biofilms were prepared as described above, but 24-well microtiter plates (orange Scientific, Braine-l'Alleud, Belgium) were used. The biofilms were dehydrated with increasing concentrations of ethanol (using 70% ethanol for 10 min, 95% ethanol for 10 min and 100% ethanol for 20 min) and then air dried for 20 min. Samples were kept in a desiccator until analysis. Prior to observation, the bottom of the wells was removed and mounted on aluminium stubs, sputter coated with gold and imaged using an S-360 scanning electron microscope (Leo, MA, USA).

• Cytotoxicity assay

Fibroblasts 3T3 (CCL-163) were grown in Dulbecco Modified Eagle Medium (DMEM – Gibco) containing 10% of calf bovine serum (Gibco) and 1% penicillin streptomicin (Gibco). After detachment, a suspension with 10^5 cells/ml was added to a 96-well plate and cells were allowed to grow until attaining 80% of confluence. Prior to the cytotoxicity assays, the wells were washed twice with PBS. PE (concentrations from 220 to 1400 µg/ml of TPC) was added to the cells and incubated for 24 h at 37°C under 5% CO2. Cells treated with the same concentration of ethanol were used as control. Afterwards, cytotoxicity was assessed using the Promega CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay, based on the reduction of MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium) in DMEM without phenol red. After 4 h, the absorbance of the resulting solution was read at 490 nm. The cytotoxicity of the compound is presented as the average of three independent experiments with three replicates [40]. The percentage of cell viability (%CV) was calculated by the following equation: %CV = (Abs sample/ Abs blank) × 100, where blank is the medium with cells and MTS.

• Statistical analysis

Data are expressed as the mean ± standard deviation (SD) of at least three independent experiments. Results were compared using two-way ANOVA followed by Bonferroni multiple comparisons, using GraphPad Prism version 6 (GraphPad Software, CA, USA). The significance level was set at $p < 0.05$.

Results

• Preparation & characterization of the propolis extract

The green propolis sample used in this study was collected from hives located in the North of Paraná state (Brazil). The apiary is surrounded by native forest with a predominance of *Baccaris drancunculifolia* and eucalyptus reserve. Green Brazilian propolis of this region is classified as 'type BRP'. In previous studies, the ethanolic extracts prepared containing this type of propolis were standardized and already chemically characterized [18,41].

The physicochemical evaluation of PE showed that pH was 5.12 ± 0.05 and relative density was 0.8722 ± 0.0009 g/ml^{-1}. The DR value of the PE was determined as 19.33% ± 0.01 (w/w) and the TPC value obtained was 1.42% ± 0.07 (w/w). These values are in accordance to the literature, showing the good physicochemical characteristics of it, and indicating, then, that PE can be used in the present study [18,36,41].

• Effect of propolis on *Candida* planktonic cells

MICs & MFCs

The results of PE's MICs 50% (i.e., the concentration that was able to inhibit 50% of the isolates tested) and 90% (i.e., the concentration that was able to inhibit 90% of the isolates tested) for the different pathogenic yeasts are shown in **Table 1**. PE showed similar and potent inhibitory activity against all clinical isolates of *Candida* species with MICs values ranging from 220 to 880 µg/ml of TPC. In all cases, the MIC value was equivalent to its correspondent MFC value.

Based in these results, PE concentrations among 450–1400 µg/ml of TPC were selected to be used in the following experiments.

Moreover, the percentage of fibroblasts viability, after direct contact with the PE (in these concentrations) was determined in order to allow cytotoxicity evaluation. Results shown that cytotoxicity was below 35% (15–35%), for all the PE's concentrations tested (data not shown).

Time-kill curves determination

The killing activity of PE, plotted from $\log_{10}$ CFU/ml versus time (36 h), is represented in **Figure 1**. Two distinct effects were observed on the growth of the *Candida* species. At 450 µg/ml of TPC, for all species tested, slight inhibitory effect was observed until 12 h, however, after this time the resultant curves were nearly identical to those for the control. At concentration 900 µg/ml of TPC, a substantial time-dependent reduction in the number of viable cells was observed compared with the control group. Additionally, results revealed that the PE effect was more pronounced in *C. albicans* and *C. parapsilosis* species, with a decreased of ≥99.9% (4 and 3 log) at 36 h, comparatively to control group (without PE). In fact even at 36 h the reduction observed by *C. tropicalis* did not exceed approximately 90% (1.5 log) of the reduction. This species had also higher MIC to PE, when compared with *C. albicans* and *C. parapsilosis*.

Propolis effect on *Candida* species filamentous forms formation

The effect of propolis on the transition of yeast to filamentous forms was evaluated **(Figure 2)**. Four clinical isolates of *C. albicans*, *C. tropicalis* and *C. parapsilosis* species and the respective reference strains were analyzed. The results revealed that *C. parapsilosis* was unable to form filamentous forms (data not shown) and that *C. albicans* presented higher number of filamentous forms than *C. tropicalis*. It was also observed that PE (at concentration of 450 µg/ml of TPC), after 4 h of exposition, reduced approximately from 80 to 5% the formation of filamentous forms on all *C. albicans* and *C. tropicalis* strains **(Figure 2)**.

• Propolis antibiofilm activity

The second aim of this work was to evaluate the activity of propolis on *Candida* biofilms formation **(Figure 3)** and against *Candida* preformed biofilms **(Figure 4)**. The results revealed that PE was able to reduce *Candida* biofilms, however, in a species- and strain-dependent manner. Concerning the effect of the PE on biofilm formation the results revealed a significant reduction in the number of cultivable cells for the four clinical isolates of the each species and its

Table 1. *In vitro Candida* species antifungal susceptibility to propolis extract and fluconazole.

Candida species	Antifungal agent	MIC (µg/ml)		
		Range	MIC 50	MIC 90
C. albicans	PE	440	440	440
	FLU	≤0.125–0.25 (S)	0.125	0.25
C. parapsilosis	PE	220–880	220	440
	FLU	0.25–4.0 (S)	0.5	2.0
C. tropicalis	PE	440–880	880	880
	FLU	0.25–16 (S–DDS)	0.5	8.0

MIC 50 and MIC 90: MIC that could inhibit 50 and 90% of the growth of the isolates, respectively.
DDS: Dose-dependent susceptible; FLU: Fluconazole; PE: Propolis extract; S: Susceptible.

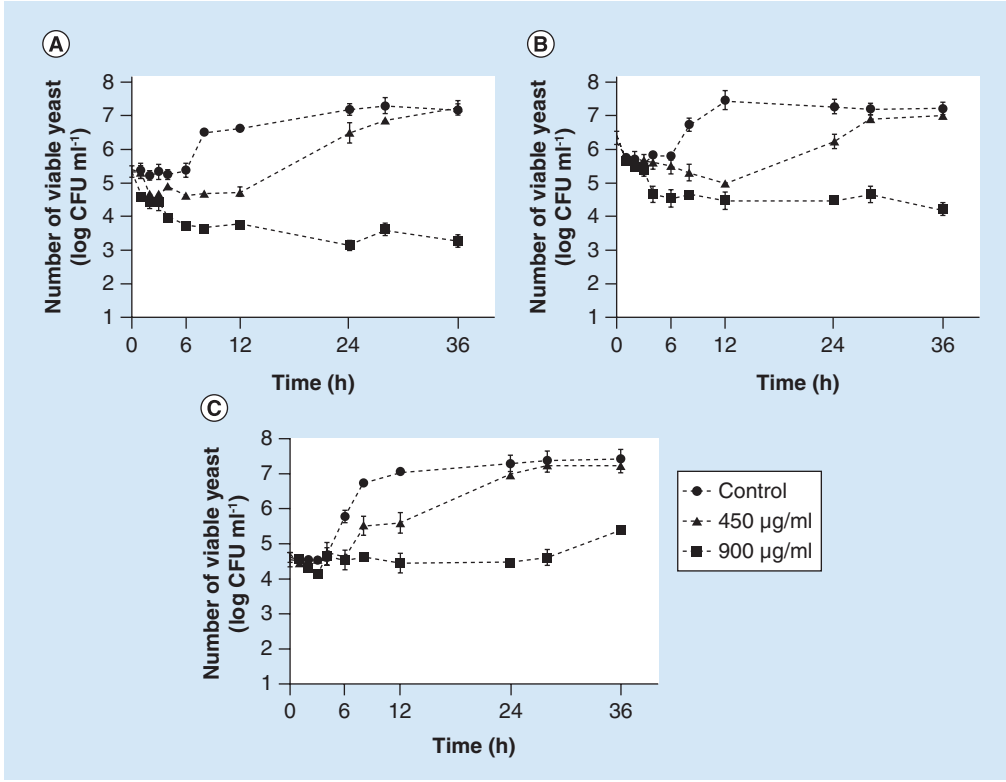

Figure 1. Killing kinetics of propolis extract against *Candida* species. (A) *C. albicans* American Type Culture Collection (ATCC) 90028; **(B)** *C. parapsilosis* ATCC 22019; **(C)** *C. tropicalis* ATCC 40042. Standardized yeast cells suspensions were exposed to 450 and 900 µg/ml of total phenol contents. At determined time intervals, samples were serially diluted and plated for colony counts. Each data point represents mean result ± standard deviation (error bars) from three experiments.

respective reference strains **(Figure 3A)**. No significant differences were observed between the three PE concentrations tested. *Candida albicans* strains presented the highest biofilm reduction (~3.5 log), followed by *C. parapsilosis* and *C. tropicalis*, with a reduction approximately 2.8 and 2 log, respectively, for all PE concentrations tested **(Figure 3A)**.

The propolis effect against preformed *Candida* biofilms **(Figure 4A)** was lower comparatively to the effect on biofilm formation. In fact, at the biofilm maturation stage, no biofilm reduction was observed for 500 µg/ml of TPC for all *Candida* strains under study. Moreover, with PE concentrations ≥700 µg/ml of TPC the preformed biofilm reduction was similar to those observed for the biofilm formation, when compared with the control group. Concerning *C. tropicalis,* PE at 500 and 700 µg/ml of TPC was able to reduce approximately 1.5 and 2.4 log, respectively. This reduction was higher than the observed in the biofilm formation studies **(Figure 3A)**, even to for PE concentrations of the 1400 µg/ml of TPC where it was observed a reduction of approximately 3.5 log in the number of CFUs.

• Effect of propolis on biofilm structure

SEM analysis was performed to examine the effect of the PE on *Candida* species biofilm formation **(Figure 3B)** and against preformed biofilms **(Figure 4B)**. For that, biofilms of one clinical isolate and its respective reference strain were treated with PE at 1400 µg/ml of TPC and compared with untreated biofilms.

Examination of untreated biofilms showed the presence of different cellular morphologies in the *Candida* biofilms. *Candida albicans* and *C. parapsilosis* biofilms exhibited a blastoconidia aggregate layer with irregular clusters, while *C. tropicalis* biofilms developed a more compact and continuous structure with yeast cells more interlinked (**Figures 3B & Figure 4B**: Controls). Interestingly, it was observed that *Candida* species' biofilms when treated with PE (1400 µg/ml of TPC) presented a significant reduction on the

number of cells and a consistent biofilm disruption (**Figures 3B & Figure 4B**: Treated). In addition, yeasts cells on biofilms treated with PE underwent morphological alterations and loss of integrity on their cell wall. Moreover, in the presence of PE, *C. albicans* biofilms presented a reduction in the number of filamentous forms.

Discussion

The incidence of candidiasis in the last two decades had a significant increment and *C. albicans* is still the most prevalent species, however, the frequency of the NCAC species has also been increasing [42–44]. This fact can be due to the lower sensibility of the yeasts to the antifungal agents most commonly used in clinical practice [45]. Moreover, the expression of the virulence factors, such as morphological transition and biofilm formation has been associated to difficulties on their treatment [2,46]. The increasing incidence of drug-resistant pathogens, the limited number of therapeutic options and the toxicity of traditional compounds have drawn attention towards the antimicrobial activity of natural products encouraging the development of alternative treatments [47].

Propolis has been demonstrated important antimicrobial activity and this bioactivity has been investigated in the last years [24,48]. The antimicrobial activity of propolis is complex and has been attributed to the synergistic activity between its various potent biological ingredients, mainly phenolic and flavonoid compounds [49]. The flavonoids constitute a very important class of polyphenols, widely present in propolis [50]. The great part of propolis biological activity is attributed to polyphenols [51]. Green Brazilian propolis type BRP is rich source of phenolic substances; most of them are prenylated phenylpropanoids, and cinnamic acids, chiefly compounds bearing prenyl groups [18,36,41]. Therefore, the physicochemical analysis is fundamental for the

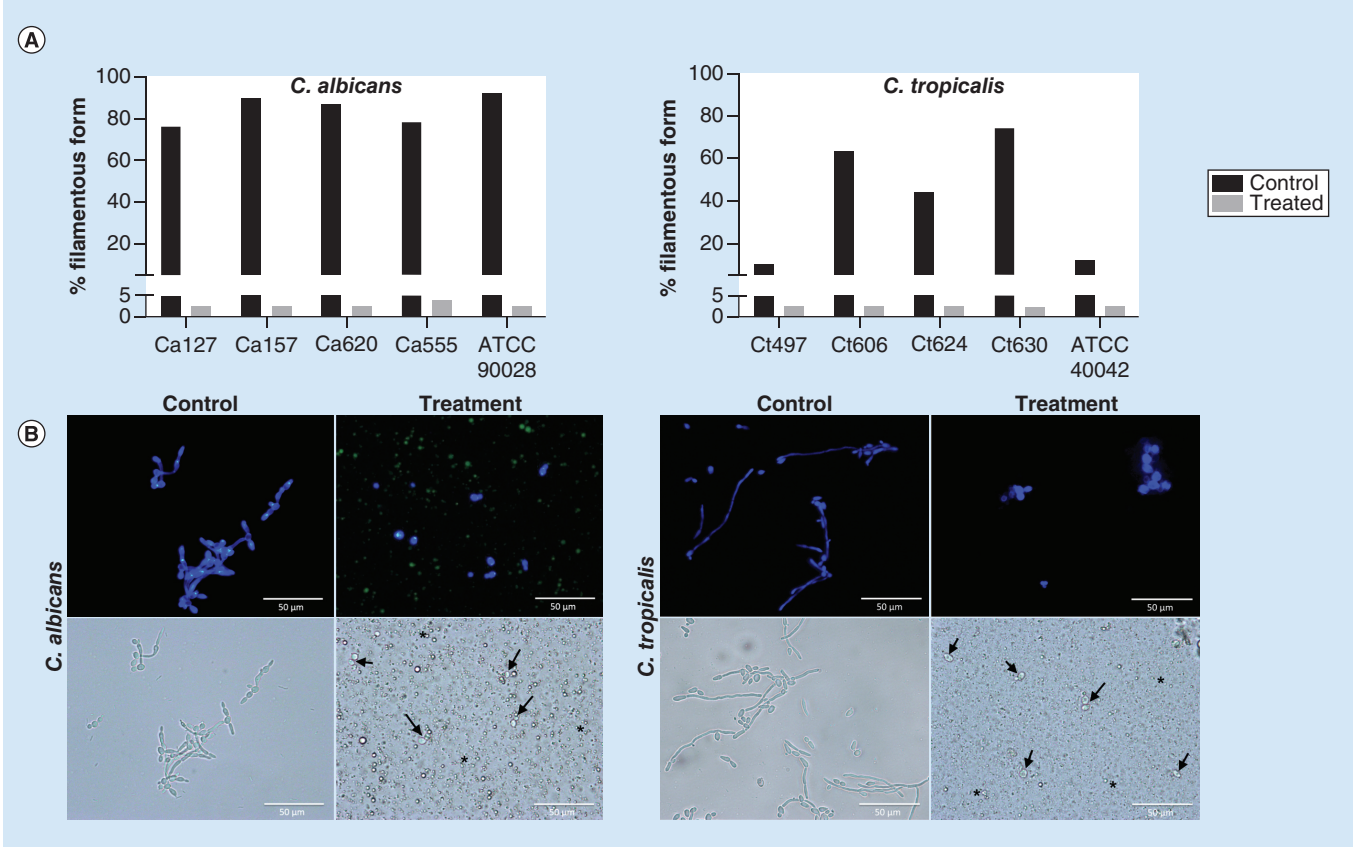

Figure 2. Inhibition of *C. albicans* and *C. tropicalis* filamentation by propolis extract in fetal bovine serum RPMI 1640 medium at 37°C for 4 h. (A) Percentage of the filamentous forms observed after exposing *Candida* planktonic cells to propolis (treated). **(B)** The images are representative of *C. albicans* and *C. tropicalis* cells obtained using a fluorescent microscope stained with calcofluor white. Propolis extract was added at a concentration of 450 µg/ml. The asterisks represent propolis residues.
ATCC: American Type Culture Collection.

evaluation of PE quality. The results showed the good characteristics of PE and the spectrophotometric determination of TPC was useful to characterize the amount of polyphenols. The value obtained was 1.42% ± 0.07 (w/w) of TPC, and this amount is in accordance with other researches [36,41].

Despite some work developed about the effect of propolis against virulence factors of *C. albicans* [24,30–31] scarce are the studies involving NCAC species. Thus, the main goal of this study was to investigate the effect of propolis on the three most important *Candida* species, *C. albicans, C. tropicalis* and *C. parapsilosis*. It was a goal to evaluate the effect on both planktonic cells and biofilms.

Firstly, the planktonic susceptibility of *C. albicans, C. tropicalis* and *C. parapsilosis* strains to PE was determined **(Table 1)**. Our data demonstrated that, all *Candida* species were susceptible to PE with a MIC range of 220 to 880 µg/ml of TPC. Moreover, these work showed that PE was effective even against strains with sensitivity dose dependence to fluconazole (MIC 16 µg/ml), namely in the case of *C. tropicalis*. Therefore these results are in agreement with Dalben-Dota *et al.* (2010) [36] that showed *Candida* species' sensitivity to PE. These authors observed that the MIC of PE ranged from 6.14 to 3145.50 mg/ml of flavonoids content (which are included in polyphenol content), evidencing an efficacy of

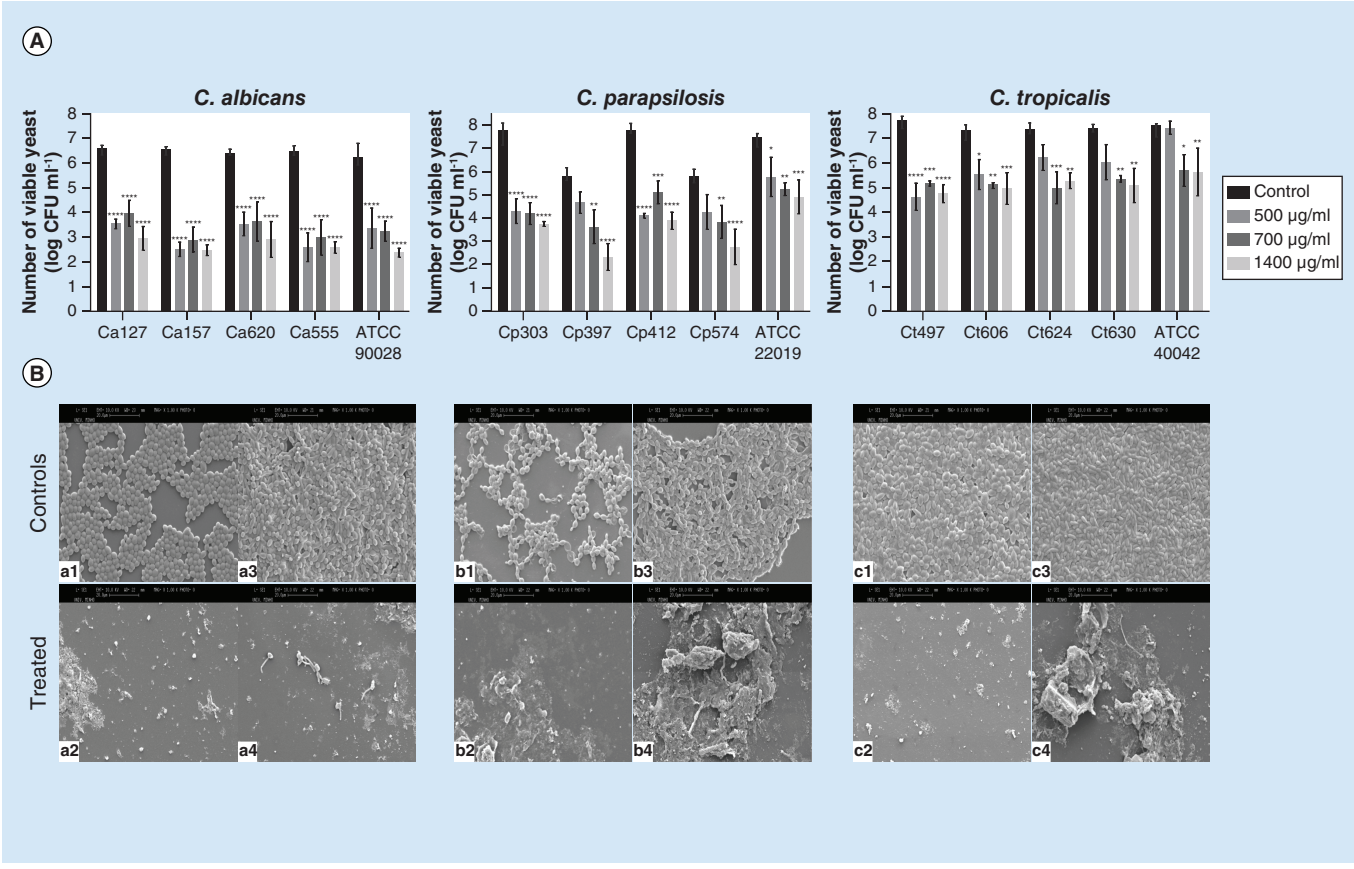

Figure 3. Logarithm of number of *Candida* cells biofilms (A) and scanning electron microscopy images (B) of propolis extract effect during biofilm formation by *Candida* species. *Candida* cells, were allowed to adhere for 2 h, then propolis was added and incubated further for 24 h at 37°C. Error bars represented in graphics indicate the standard deviation. In the images **(a1 & a2)** represent isolate clinical *C. albicans* **(a3 & a4)** ATCC *C. albicans* 90028 **(b1 & b2)** isolate clinical *C. parapsilosis* **(b3 & b4)** ATCC *C. parapsilosis* 22019 **(c1 & c2)** isolate clinical *C. tropicalis* **(c3 & c4)** ATCC *C. tropicalis* 40042.

*, ** and **** correspond to $p < 0.05$, $p < 0.01$ and $p < 0.0001$, respectively.

Controls: biofilms grown in RPMI medium in the absence of propolis extract; Treated: biofilms grown in RPMI medium in the presence of 1400 µg/ml of total phenol content. The bar in the images corresponds to 20 µm. Magnification × 1000.

ATCC: American Type Culture Collection.

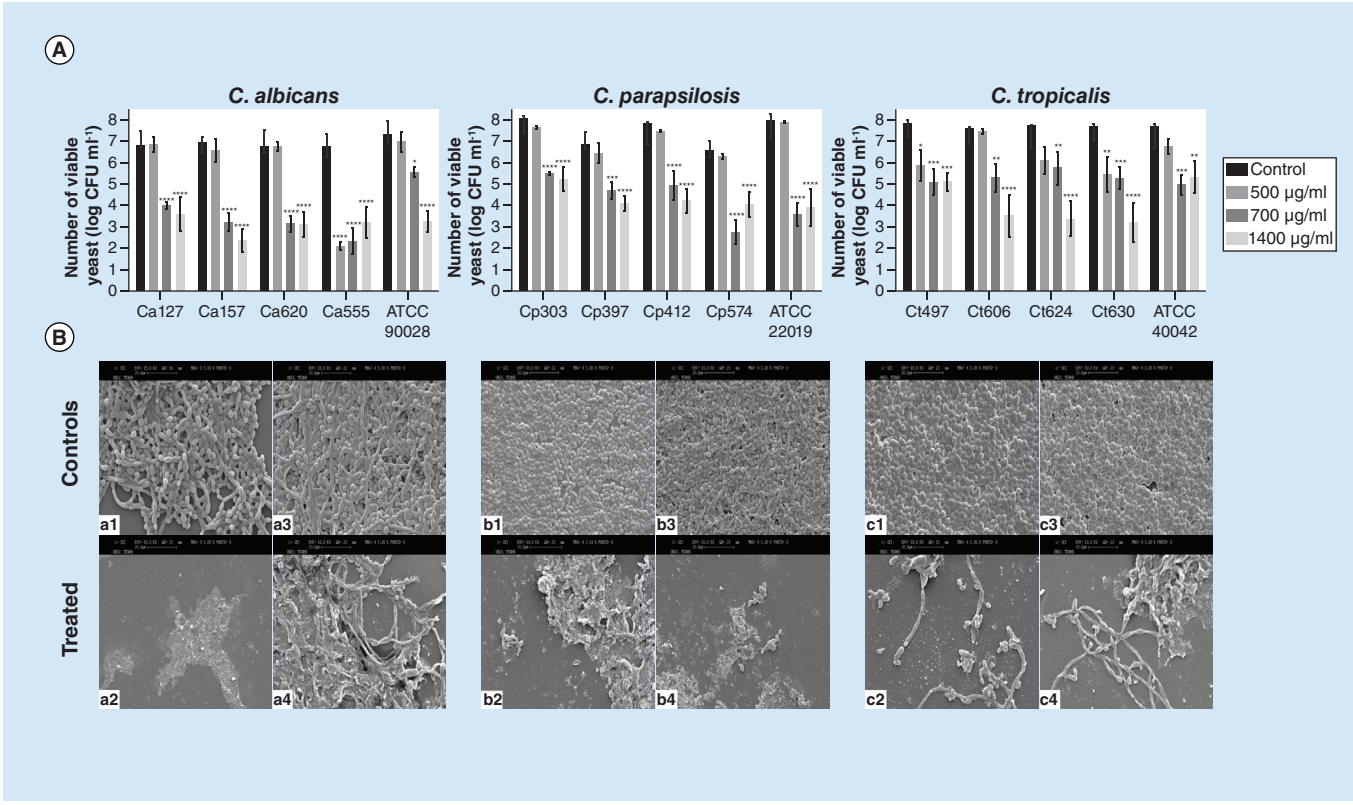

Figure 4. Logarithm of number of *Candida* cells biofilms (A) and scanning electron microscopy images (B) of propolis extract effect on preformed *Candida* species biofilms. *Candida* cells, were allowed to form biofilm for 24 h, then propolis was added and incubated further for 24 h at 37°C. Error bars represented in graphics indicate the standard deviation. In the images **(a1 & a2)** represent isolate clinical *C. albicans* **(a3 & a4)** ATCC *C. albicans* 90028 **(b1 & b2)** isolate clinical *C. parapsilosis* **(b3 & b4)** ATCC *C. parapsilosis* 22019 **(c1 & c2)** isolate clinical *C. tropicalis* **(c3 & c4)** ATCC *C. tropicalis* 40042.

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ATCC: American Type Culture Collection.

this extract. A time-kill assay was performed to determine the kinetic effect of PE on *C. albicans*, *C. parapsilosis* and *C. tropicalis* growth **(Figure 1)**. Results revealed approximately 90% of reduction on its growth for all species, with an effective reduction on *Candida* cells cultivability. In fact PE was able to reduce around three logs (99.9%) of *C. albicans* and *C. parapsilosis* and an approximately 1.5 log of *C. tropicalis*. These results are in accordance with the susceptibility results where, *C. tropicalis* was the species with the highest MIC value.

As previous works only showed an effective activity of PE against planktonic *Candida* species cells [36,52] this work intends to extend this knowledge, by evaluating the PE's effect on *Candida* species virulence traits, such as yeast-filamentous transition and biofilm formation ability. It is known that the formation of hyphae helps *C. albicans* to penetrate the host tissues with subsequent invasiveness that leads to the establishment of infection [53]. Thus, the ability of PE to inhibit the formation of filamentous forms was evaluated and the results revealed that PE was able to block 90% of the yeast-filamentous forms in *C. albicans* and *C. tropicalis* **(Figure 2)**. This inhibition of yeast-filamentous' forms transition by PE, presents a very attractive option to control *Candida* infections. It was previously reported that the morphological switch from yeast to hyphae cells is important in many processes, such as biofilm formation [54]. Thus, the high capacity of PE to efficiently inhibit yeast-hyphae transition may be associated

with its ability to prevent biofilm formation. Furthermore, this product has received the attention of clinicians and researchers due to its diverse pharmacological activities and low toxicity [20,55].

Biofilm formation by microorganisms is a mechanism that allows them to become persistent colonizers, to resist clearance by the host immune system and antibiotic's effect [56]. Interestingly, it was observed that PE was able to inhibit biofilm formation **(Figure 3)** and to destroy mature biofilms **(Figure 4)** of *C. albicans*, *C. parapsilosis* and *C. tropicalis* strains. It is important to highlight that PE at 450 and 700 µg/ml of TPC was able to inhibit approximately 90% of biofilm formation **(Figure 3A)** and 1400 µg/ml of TPC was able to reduce preformed biofilms in 99.9% **(Figure 4A)**. Previous studies have shown that different concentrations of propolis (0.25–1.25%) were able to reduce 40–45% of the *in vitro C. albicans* biofilm formation [57]. Moreover, Capoci *et al.* (2014) also revealed a small reduction on *C. albicans* biofilm formation (<0.5 log) at concentration of PE lower than MIC [24]. However, the promising results obtained for *C. tropicalis* and *C. parapsilosis* were never stated before. The SEM images corroborate the biofilm disruption **(Figures 3B & Figure 4B)**, also demonstrated by cultivable cells determination **(Figures 3A & Figure 4A)**, reinforcing the PE's capability to inhibit filamentation **(Figure 2)**.

Conclusion & future perspective

This study showed that PE is a potent antifungal agent with effect on *Candida* planktonic cells and biofilms. It is important to highlight, that these effects were not only observed against *C. albicans* but on other NCAC species, namely *C. tropicalis* and *C. parapsilosis*. This is a very promising data, considering that NCAC species has shown to be highly resistant to the conventional antifungal agents.

Such properties of PE as inhibitor of *Candida* virulence represent an alternative and innovative pathways of chemotherapy for pathogens that are resistant to classical antimicrobial agents.

Financial & competing interests disclosure

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EXECUTIVE SUMMARY

- Propolis presents potential antifungal activity.
- Propolis is a stronger inhibitor of filamentous forms formation.
- Propolis is able to reduce and destroy *Candida* species biofilms.
- Propolis is a promising alternative to antifungal traditional therapy.

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