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BIOMEDICAL SCIENCES

Promising effect of propolis and a by-product on planktonic cells and biofilm formation by the main agents of human fungal infections

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Abstract: Few antifungals available today are effective in treating biofilms. Thus, it is urgent to discover new compounds, such as natural products, that provide improvements to existing treatments or the development of new antifungal therapies. This study aimed to perform a comparative analysis between the green propolis extract (PE) and its by-product, a waste of propolis extract (WPE) through a screening with *Candida* sp., *Fusarium* sp. and *Trichophyton* sp. The antifungal property of PE and WPE was assessed by the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) determination in planktonic cells. The influence of both extracts on the inhibition of biofilm formation in these fungi was also tested. The WPE MIC and MFC values (68.75 to 275.0 µg/mL) were three to twelve times lower than the values obtained for PE (214.06 to 1712.5 µg/mL). PE was more efficient than WPE in inhibiting the biofilm initial phase, especially in *C. albicans*. Meanwhile, WPE had dose-dependent behavior for the three fungi, being more effective on filamentous ones. Both PE and WPE showed excellent antifungal activity on planktonic cells and demonstrated great efficacy for inhibiting biofilm formation in the three fungi evaluated.

Key words: antifungal activity, new biological product, prevention, propolis reuse, treatment, waste products.

INTRODUCTION

More than 300 million people are infected with fungi in an acute or chronic way, resulting in death, prolonged illnesses, blindness, psychological problems or reduced work capacity (Website Life 2020). These fungal infections are also known as mycoses and can affect the skin and its attachments to invasive, serious and fatal systemic infections. Superficial and skin mycoses are very common, especially in tropical countries. Among others, onychomycosis, one of the most frequent nail disorders, has an estimated prevalence of 5.5% of the general population around the world (Gupta et al. 2017). Onychomycosis' main etiologic agents are dermatophyte fungi, such as the *Trichophyton* species, followed by yeasts of the genus *Candida* and non-dermatophyte molds (NDM) such as *Fusarium* spp. (Gupta et al. 2020). Another common fungal infection is vulvovaginal candidiasis (VVC), caused by yeasts of the genus *Candida*, which affects the reproductive tract of immunocompetent women, with a high global incidence and profound negative impact on quality of life (Willems et al. 2020). On the other hand, representing more serious infections, with a high impact on health, leading to high mortality rates are the systemic nosocomial infections, also caused by yeasts,

mainly *Candida albicans*. Currently, the origin of these infections has been associated with the organization of these fungi as biofilms formed on medical devices. Frequently these biofilms are multispecies or polymicrobial involving different species of fungi and bacteria (Bernard et al. 2020). Likewise, the mechanisms involved in the etiopathogenesis of both onychomycosis and VVC have been associated with biofilms, which also reflects the difficulty of treatment (Gupta et al. 2016, Rodríguez-Cerdeira et al. 2019).

Biofilms are structured microbial communities, surrounded by a self-produced extracellular polymeric matrix (ECM), which are adhered to inert or living surfaces. Fungal biofilms, formed by both yeasts and filamentous fungi, are developed in several stages, representing different and progressive stages of maturation (Uppuluri et al. 2010). The ability to form biofilm is an important virulence factor for pathogenic fungi, as cells organized in this way, unlike their planktonic counterparts, have different phenotypes and greater resistance to antimicrobials, as well as to environmental conditions, which can be associated with the persistence of infections (Costa-Orlandi et al. 2017). It is proven that cells that dissociate from biofilm have a greater association with serious infections and high mortality rates. In addition, more than 65% of human infections involve the formation of biofilms and more than 500,000 deaths per year are related to biofilm (Sardi et al. 2014).

Currently, the therapeutic arsenal available for these fungal infections is limited and presents restrictions such as they are not directed to specific cellular targets, they usually have insufficient tissue penetration, in addition to frequent drug interactions and toxicity. In addition, fungi may have several mechanisms of resistance or tolerance to these agents (Sanguinetti et al. 2015, Martinez-Rossi et al. 2018). Importantly, one of the main features of biofilms is to confer high resistance to microorganisms to antimicrobial agents. This occurs due to the presence of structural factors, such as the ECM, which provides a physical barrier of protection for the biofilm cells, and is responsible for several other mechanisms, such as the alteration or overexpression of target molecules, efflux pumps, *quorum sensing* molecules and presence of persistent cells. The antifungal susceptibility of fungal biofilms can also be influenced by external factors such as temperature, pH, availability of oxygen and other environmental stresses (Costa-Orlandi et al. 2017). Unfortunately, few antifungals available today are effective in treating biofilms (Ramage et al. 2012). For this reason, it is highly relevant and urgent to discover new compounds that aim to fill this gap, providing improvements to existing treatments or the development of new antifungal therapy strategies. In this sense, natural products are promising and deserve to be better evaluated.

Propolis is a well-known natural product that has been widely studied and obtained very promising results in the development of pharmaceutical formulas such as emulgel, polymeric systems and nanoparticles which proved some properties such as anti-inflammatory, antioxidant and antimicrobial activity (Balata et al. 2018, Afra et al. 2020, Oliveira et al. 2020) among others. Propolis extract (PE) has already shown efficiency regarding antifungal activity both in planktonic cells and in the ability to inhibit biofilm formation in some fungi (de Castro et al. 2013, Galletti et al. 2017, Veiga et al. 2018, Gucwa et al. 2018). In addition, it has recently been proposed to use a by-product (WPE) obtained from PE, which apparently maintains the original antifungal properties of propolis (de Francisco et al. 2018). Thus, the present study aims to make, for the

first time, a comparative analysis between extracts of PE and WPE by screening with three of the main genera of fungi that are frequently isolated from human fungal infections.

MATERIALS AND METHODS

Fungal strain and growth conditions

Fungal strains from the American Type Culture Collection of *Candida albicans* (ATCC 90028), *Candida glabrata* (ATCC 2001), *Candida tropicalis* (ATCC 750), *Candida parapsilosis* (ATCC 22019) and the clinical isolates *Fusarium oxysporum* and *Trichophyton rubrum*, deposited in the Microbial Collections of Paraná Network-TAX online, under registration number CMRP2925 and GenBank (MG692504.1) and CMRP2912, respectively, were tested. For confirmation of the strains, the yeast was cultured in CHROMagar™ Candida (DifcoTM, Detroit, United States), to check the culture purity. The filamentous fungi were isolated from patients with onychomycosis and their identification was performed using classic methods, including the examination of colonies and microscopic morphology (De Hoog et al. 2018). These fungi were maintained in culture and a freeze-dried state in the Mycological Collection of the Laboratory of Medical Mycology of the State University of Maringá (UEM), Brazil. Before the experiments, these isolates were subcultured in Sabouraud Dextrose Agar (SDA; DifcoTM, Detroit, United States) overnight at 37 °C or 25 °C. The cellular density was adjusted based on hemocytometer cell counts before each assay.

Propolis and by-product extracts

Brazilian green propolis was obtained from an apiary of *Apis mellifera* L. bees, in the northwest of Parana state (23º24'2" S, 52º1'50" W), Brazil, and located inside a eucalyptus reserve, surrounded by native forest with a predominance of *Baccharis dracunculifolia* (Asteraceae). This research was registered in Brazil with SISGEN N° AC7A2F5. Propolis extract (30%, w/w; PE) was prepared using ethanol 96% (v/v) by turboextraction. After the process of extraction, PE was obtained by filtration (filter paper grade 3) and the remaining product on the filter surface (propolis by-product; WP) was collected (de Toledo et al. 2015, Rosseto et al. 2017, de Francisco et al. 2018). Afterwards, WP was also subjected to turboextraction using the same parameters and the WP:ethanol ratio of 50:50 (w/w). The final dispersion was filtered through the same type of filter paper, resulting in the extract of propolis by-product (WPE) (de Toledo et al. 2015, Francisco et al. 2019).

Both PE and WPE were investigated as their physicochemical characteristics: pH, relative density, dryness residue, ethanol content and total polyphenols content (de Toledo et al. 2015, Rosseto et al. 2017, de Francisco et al. 2018). Moreover, they were also analyzed by High Performance Liquid Chromatography (HPLC) using a methodology previously validated (Rosseto et al. 2017, Corrêa et al. 2020). In brief, PE and WPE were evaluated using an HPLC system consisting of two pumps, with automatic flow controller, detector of diode array, stationary phase oven, and an integrator system (Agilent, Santa Clara, USA). For PE or WPE sample preparation, an aliquot of 1.0 mL of extract was submitted to extraction using 25 mL of ethyl acetate. The acetate fraction was submitted to drying in a water bath (40 °C) and the residue was dissolved in 10.0 mL of methanol. The sample was filtered through modified PTFE membrane filter (pore size of 0.45 µm, Millipore, Bedford, MA, USA) and an aliquot of the filtrate was injected in a fixed loop injector (Rheodyne vs 7125, 50 µL). The prepared samples of PE and WPE were analyzed using a reversed phase Platinum C18 column (150 mm × 4.6 mm i.d., particle size 5 µm,

Hypersil BDS, Alltech, USA), at 20 ± 0.1 °C (Agilent, Santa Clara, USA). The isocratic mobile phase was composed of methanol at 70% and aqueous solution acetic acid (2%, v/v) at 30%. The flow-rate was 1.0 mL/min and the absorbance of the eluate was monitored at wavelength λ = 310 nm. For both extracts, the marker chrysin (97% purity, analytical standard, from Sigma-Aldrich®, St. Louis, USA) was analyzed using the calibration curve previously validated (Rosseto et al. 2017).

Determination of PE and WPE antifungal properties in planktonic cells

The antifungal activity of PE and WPE against all strains was determined by minimum inhibitory concentration (MIC) based on the Clinical & Laboratory Standards Institute protocol M27-A3 (CLSI 2008), with certain modifications for natural products (Dalben-Dota et al. 2010). The serial dilution of both extracts was performed at a ratio of two, with the concentration ranging from 13700.0 to 26.75 µg/mL of total phenol content (TPC) for PE and from 2200.0 to 4.3 µg/mL of TPC for WPE. The test was carried out in Roswell Park Memorial Institute 1640 medium (RPMI Medium 1640; Gibco, Grand Island, NY, United States), with L-glutamine (with sodium bicarbonate) and 0.165 M 3-(N-morpholino) propanesulfonic acid (pH 7.2) as a buffer (Sigma–Aldrich, St. Louis, United States), and 2% glucose, in 96-well flat-bottomed microtitration plates (Orange Scientific, Braine-l'Alleud, Belgium). Resazurin sodium salt ($C_{12}H_6NNaO_4$, R7017-5G, Sigma-Aldrich, Brazil) was diluted according to the manufacturer's instructions adding 0.002 g of resazurin to 10 mL of distilled water and at a final concentration of 2 %, 30 µL of the dye was added to each well after 24 hours of incubation maintaining a further 24 h, with reading in a total final period of 48 h.

The MIC was defined as the concentration of each extract that reduced 100% of the growth compared with the fungal growth in the absence of the drug. The minimum fungicidal concentration (MFC) was determined by seeding aliquots from the suspensions after exposition to both extracts on SDA plates and incubating them at 37 °C (yeast) or 25° C (filamentous fungi) for 24 h. The MFC was defined as the lowest concentration of the extracts in which no recovery of microorganisms was observed.

Determination of PE and WPE antifungal properties in biofilm formation

The strains of *F. oxysporum* and *T. rubrum* were grown on SDA for seven days at 25 °C. The colonies were gently scraped, harvested in sterile phosphate-buffered saline, pH 7, 0.1 M (PBS) and filtered to separate the conidia from the hyphae. *C. albicans* was grown on SDA for 24 h at 37 °C, followed by inoculation in Sabouraud Dextrose Broth (SDB; DifcoTM, Detroit, United States) and then was incubated for 18 h at 37 °C by agitating at 120 rpm. After incubation, the cells were harvested via centrifugation at 3000 × g for 10 min, at 4 °C, and were washed twice with 15 mL of PBS. All inocula were adjusted to a final concentration of 1×10^7 conidia/cell mL^{-1} RPMI 1640 medium, and 200 µL of this suspension was placed into 96-well flat-bottomed microtitration plates. The plates were then incubated at 37 °C in a shaker at 110 rev min^{-1}, for 2 h. Non-adherent cells were removed by washing with sterile PBS, followed by addition of 200 µL of PE and WPE (at MIC, 2x MIC and 4x MIC concentrations in RPMI 1640 medium). The plates were incubated at 37 °C for 24 h in a shaker at 110 rev min^{-1} to allow biofilm formation. Thereafter, 30 µL of resazurin 2 % was added to each well and the plate was re-incubated at 37 °C for 24 h. Negative controls (200 µL of only RPMI 1640 medium) and untreated controls (200 µL of RPMI 1640 medium and preformed biofilm) were also included. For the determination of minimal biofilm inhibitory

concentration (MBIC), the resazurin color change was read. For minimal biofilm eradication concentration (MBEC) and the total number of viable cells, the biofilms were washed twice with sterile PBS, and then vigorously scraped and transferred to a conical tube. This process was carried out five times, totalizing 1000 µL, and after vortexed for 1 min to disaggregate cells from the matrix. Then, subjected to 30% sonication for 50 seconds and 35% sonication for 10 seconds, for *C. albicans* and filamentous fungi, respectively. Serial dilutions were made in PBS, and aliquots of 10 µL were placed onto SDA, and incubated at 37 °C for 24 h for *C. albicans* and 25 °C for 48h for *F. oxysporum* and *T. rubrum*. The number of cultivable cells was expressed as colony-forming units per milliliter (CFU/mL) and the results were presented in terms of log of CFU/mL.

Characterization of biofilm in formation treated with PE and WPE

The biofilms were assembled under the same conditions described in the previous session and treated with the concentrations in which there was the greatest reduction in CFU of each extract. After the recovery of the total biomass, followed by the separation of the extracellular matrix (ECM) from the biofilms by mechanical filtration methods in a 0.22 µm membrane (Kasvi, São José dos Pinhais, PR, Brazil), the ECM analysis was carried out regarding the quantification parameters of extracellular deoxyribonucleic acid (eDNA), extracellular ribonucleic acid (eRNA), total protein and total polysaccharides. This filtrate containing the ECM was quantified in relation to each one of the four components based on the technique described by Veiga et al. (2018), with some modifications. The components were measured using the optical density (OD) through spectrophotometry by Nanodrop 2000™ (Nanodrop 2000 UV-Vis Spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA). Optical densities were read at 260 nm wavelengths for nucleic acids, and the ratio was 260/280 and 260/230 nm to estimate the concentration of total proteins and polysaccharides, respectively. Only for negative control, the diluent, sterile PBS, was used.

Statistical analysis

All tests were performed in triplicate, and on three independent days. Data with a non-normal distribution were expressed as the mean ± standard deviation (SD). Significant differences among means were identified using the ANOVA test followed by Bonferroni multiple-comparison test. The data were analyzed using Prism 5 software (GraphPad, San Diego, CA, USA). Values of $p < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

This is the first in-depth study that compares the action of crude propolis extract (PE) with a by-product (WPE) that would be discarded in a normal production process. The physicochemical characteristics of PE and WPE are displayed in Table I. Moreover, the HPLC analysis of extracts was performed using a valid method which showed a good separation of main substances of PE (fingerprint) (Corrêa et al. 2020). In this work, chrysin was utilized as a representative standard (marker) for both extracts and the analytical curve was $y = 163.2 x - 9.5861$ ($r = 0.9999$) (Rosseto et al. 2017, Corrêa et al. 2020). The chrysin content in PE and WPE extracts (eluted at about 5.0 min) was 0.8319 ± 0.0098% and 0.0972 ± 0.0048% (v/v), respectively. These results corroborate with the literature, showing physicochemical characteristics and indicating PE and WPE can be used in the present study (Rosseto et al. 2017, Corrêa et al. 2020). Therefore,

Table I. Physicochemical characteristics of propolis extract (PE) and propolis by-product (WPE). Values are expressed as means ± standard deviation.

Analyses	PE	WPE
pH	5.35 ± 0.0153	5.15 ± 0.1728
Relative density (g/ml)	0.8585 ± 0.0002	0.8747 ± 0.000033
Dryness residue (%, w/w)	13.33 ± 0.1617	8.60 ± 0.2113
Ethanol content (%, w/w)	67.87 ± 1.5878	44.05 ± 1.8247
Total polyphenols content (%, w/w)	2.74 ± 0.1141	0.44 ± 0.0182

these compounds were used in a screening with three of the main agents of superficial and cutaneous fungal infections. The tests were carried out both on a suspension of fungal cells (planktonics) and evaluating a possible impact of both compounds in the beginning of biofilm formation by the same fungi.

The MIC and MFC values of WPE were three to twelve times lower than the values obtained with PE extract in planktonic cells

Interestingly, as shown in Table II, we obtained equivalent values for MIC and MFC for both extracts in all strains tested. Regarding PE, our results are in line with other studies regarding the determination of MIC, but they are better in relation to MFC. Inhibitory and fungicidal concentrations ranged between 214.06 to 1712.5 µg/mL of TPC. Corrêa et al. (2020) with *C. albicans* isolated from VVC patients and another batch of Brazilian propolis extract and found MIC values ranging from 837 to 1675 µg / mL of TPC, compatible with our findings. However, these authors found much higher values for MFC (3350 to 6700 µg / mL of TPC). In fact, the chemical composition of propolis is variable since it is influenced by geographic region and may have different results even according to the production lot in the same region, as well as the type of bee and the season (Anjum et al. 2019). This variation can significantly interfere in antifungal properties (Negri et al. 2014).

PE exhibited strong fungicidal activity against *Fusarium* spp. agreeing with Galletti et al. (2017) that showed that 1093.75 µg / mL of TPC was able to inhibit 90% of the tested isolates and, likewise, MIC and MFC were coincident for all species. Besides that, Veiga et al. (2018) report low MIC values of PE, which were identical to the MFC against 29 isolates of *T. rubrum*. Thus, our data corroborate that PE exerts an important fungistatic action as well as fungicidal action, strain and dose dependent (Galletti et al. 2017, Veiga et al. 2018, Corrêa et al. 2020).

We also noticed that MIC and MFC values obtained for filamentous fungi were lower than those obtained for yeasts, data that corroborate with studies of Falcão et al. (2014) with Portuguese propolis, where the MIC for *T. rubrum* was lower than for *C. albicans* and *A. fumigatus* and Cuban propolis tested with *T. rubrum* and *C. albicans* (Monzote et al. 2012).

Regarding the WPE, our results ranged between 68.75 to 275.0 µg / mL of TPC. Few are the studies of propolis by-products and a preliminary comparison between PE and WPE, de Francisco et al. (2018) tested reference strains of *C. albicans*, *C. parapsilosis* and *C. tropicalis*, finding good results and also huge similarities between these two extracts. However, there is no previous data to compare the antifungal activity of WPE for the other fungi tested in this screening. Although without data for comparison, our results are very encouraging,

Table II. Determination of the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of the propolis (PE) and by-product (WPE) extracts in planktonic cells.

Strains	PE		WPE	
	µg/mL in TPC			
	MIC	MFC	MIC	MFC
C. albicans	1712.5	1712.5	137.5	137.5
C. glabrata	1712.5	1712.5	275.0	275.0
C. parapsilosis	856.25	856.25	68.75	68.75
C. tropicalis	1712.5	1712.5	275.0	275.0
F. oxysporum	428.13	428.13	68.75	68.75
T. rubrum	214.06	214.06	68.75	68.75

TPC: total phenol content.

as surprisingly, the values we obtained for MIC and MFC from WPE were three to twelve times lower than the values obtained with PE, showing that WPE was more efficient in inhibiting the growth of these fungi in planktonic form. This probably occurred due to the fact that the WPE had a lower dry residue content (Table I), and also resin dissolved, as a result of its extraction process. As the WPE goes through yet another ethanol extraction process, the resin content in the extract decreases while the wax content increases, making it more malleable and non-polar (de Francisco et al. 2018). Thus, we assume that the resin present in the PE can control the polyphenols release, as previously observed (Bruschi et al. 2004, 2007), and makes it difficult for the polyphenols to be available to penetrate inside the fungal cell. This can justify the better results obtained with the WPE, despite the fact that it has a lower total polyphenol content than the PE.

The first study that compared the extracts of PE and WPE suggests that WPE can be used in different areas, including the pharmaceutical and food industries, as it is a rich source of bioactive compounds and for having demonstrated high efficiency in the physicochemical aspects, in antioxidant activity, in the elimination of radicals and antifungal activity, as well as in cell viability. In addition, the high amount of WPE produced by the propolis industry must be taken into account, emphasizing that its reuse is essential from an economic and, mainly, environmental point of view (de Francisco et al. 2018). Our results not only corroborate this study, but also reinforce that this by-product, which would be neglected, has great potential, since it demonstrated excellent antifungal activity against fungi that are of great importance in the context of human diseases and infections.

PE was more efficient in inhibiting the initial phase of biofilm, especially in *C. albicans* while WPE had more effective on filamentous fungi

Considering the particularities of fungi in terms of behavior and response to antifungals when organized in biofilm form (Uppuluri et al. 2018), in this study we evaluated the ability of PE and WPE to prevent the development of biofilms produced by *C. albicans*, *F. oxysporum* and *T. rubrum*. For the determination of the MBIC we used three concentrations of these extracts defined from the MIC determined in planktonic cells: MIC, twice the MIC value (2x MIC) and four

times the MIC value (4x MIC). In all tested fungi the MBEC was equivalent to CFU (Figure 1).

Assessing the impact on the viability of *C. albicans* biofilm exposed to PE, in the MIC concentration, we observed that this extract was able to significantly reduce the number of CFU by 4 logs and completely inhibited the yeasts of the biofilms treated with higher concentrations of PE (4x MIC). WPE was not so efficient, however, it significantly reduced biofilm in all tested concentrations. Regarding *F. oxysporum*, PE significantly reduced 2 logs of CFU in the first treatment concentrations and completely inhibited the growth of the fungus with 4x MIC treatment. WPE also caused a significant reduction in all concentrations tested, but less efficiently than PE. Finally, for *T. rubrum*, we observed that both PE and WPE caused reductions in CFU, which were dose-dependent and, statistically significant, in relation to untreated control, as well as between treatment concentrations, for both extracts.

PE was more efficient than WPE in inhibiting the initial phase of biofilm, especially in *C. albicans*. This result is opposite to the behavior found in relation to planktonic cells and this ability is probably associated with the release profile of the bioactive compounds of the two extracts. Rosseto et al. (2017) showed that the release of polyphenols from WPE was significantly faster than PE. In fact, due to the extraction process, WPE has a lower resin content than PE (de Francisco et al. 2018) and therefore, it is possible to hypothesize that the resin may prolong the release and action of polyphenols on adhered fungal cells that would initiate biofilm, which would explain the better results of PE extract.

When comparing the efficiency of the extracts between the fungi, we noticed that for the three fungi the WPE had a similar behavior, in the sense of dose dependent effectiveness, but

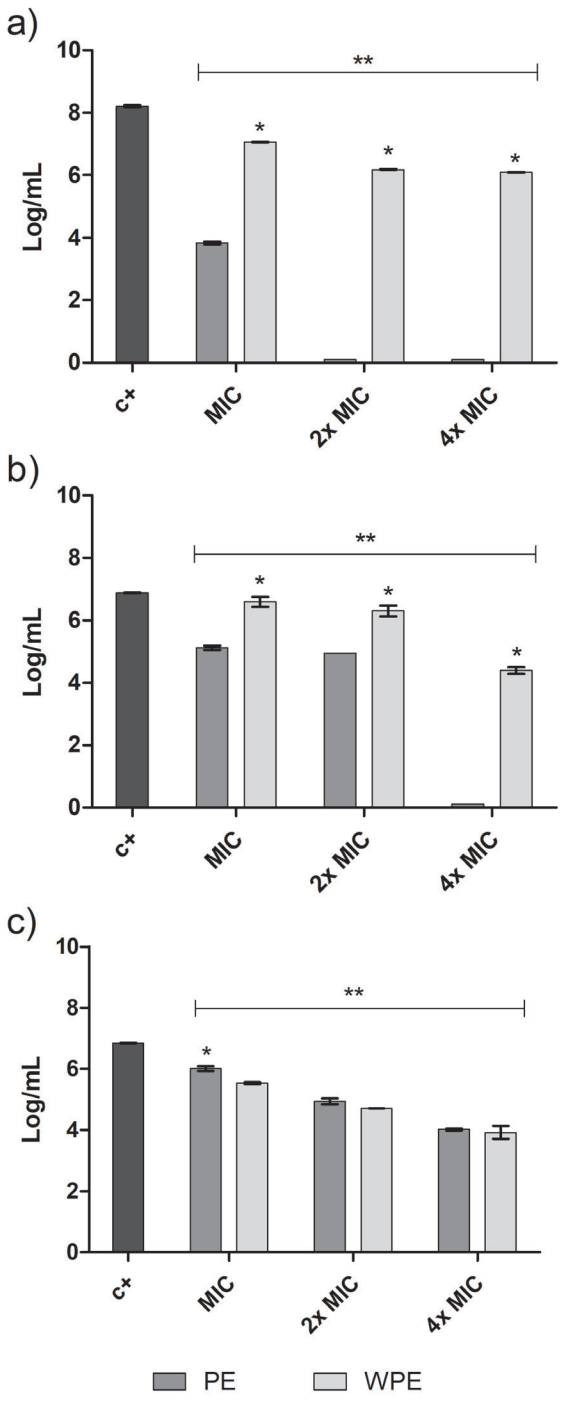

Figure 1. Colony-forming units (log / mL) of biofilms in formation of *C. albicans* (a), *F. oxysporum* (b) and *T. rubrum* (c) treated with propolis extract (PE) and propolis by-product extract (WPE). * Statistical difference between PE and WPE extracts. ** Statistical difference between controls and treaties. MIC - minimum inhibitory concentration.

it was more effective on the filamentous fungi, where 4x MIC was able to inhibit the formation of biofilm in the order 2.5 log compared to the control, without treatment. We infer that the differences found in the CFU values of each extract between the three fungi evaluated are due to the physiological differences between each fungal genus, as well as in response to other drugs (Fernandes et al. 2016).

Our findings are in accordance with Freires et al. (2016) who also evaluated the action of two PE extracts at the beginning of biofilm formation and on mature biofilms from *Candida* spp. and observed that the extracts disturbed the biofilm structures, since amorphous areas with cell damage were observed in the two studied biofilm stages. Tobaldini-Valerio et al. (2016) also showed that PE was able to inhibit biofilm formation and destroy mature biofilms from *Candida* spp. These results suggest that PE should be considered a promising candidate for the treatment of oral candidiasis and VVC, since the action of the extract in inhibiting the biofilms formation caused by VVC isolated yeasts has already been proven (Capoci et al. 2015). In addition, PE could play a role in preventing systemic candidiasis by treating medical devices (Freires et al. 2016).

In the case of *F. oxysporum*, PE not only reduced the number of CFU but, at higher concentrations, was able to completely inhibit biofilm formation. Similar results were found by Galletti et al. (2017), that used PE in the treatment of mature biofilms from clinical isolates of *Fusarium* spp. and they also found a significant decrease in viable cells. These results were confirmed by epifluorescence microscopy, in which the authors noticed disorganization and damage to the structure of biofilms, which suggested that PE can be used as a topical treatment for onychomycosis.

In relation to *T. rubrum*, the main agent of onychomycosis, PE was less efficient than for the other fungi, but even so it had an excellent performance, as it was able to significantly decrease the CFU number of the fungus involved in biofilm formation. Our results corroborate with the study by Veiga et al. (2018), which revealed that PE had a good antifungal performance, both in planktonic cells and in mature biofilms of species of *Trichophyton* spp.

The ECM compounds varied according to the concentration of the extract and the strain tested

Regarding to the ECM of biofilms in formation, this study suggests that the three fungi have very different characteristics, for example, while in *F. oxysporum* biofilms there was a high production of proteins, *T. rubrum* produced a greater amount of polysaccharides (Figure 2). But, fortunately independently, propolis was able to inhibit the formation of ECM in all tested strains. The matrix compounds (proteins, polysaccharides, eDNA and eRNA) varied according to the concentration of the extract and the strain tested.

For the three fungi, there was a significant reduction in total proteins in the two tested concentrations of both extracts when compared to the untreated control. It is also observed that the amount of *F. oxysporum* total proteins corresponds to almost double the concentrations of *C. albicans* and *T. rubrum*. (Figure 2).

There was a significant increase of polysaccharides in biofilms treated with both extracts, in relation to the untreated control of *C. albicans*, and this increase was greater at the higher concentrations of the extracts. On the other hand, for *F. oxysporum* and *T. rubrum* the amount of polysaccharides significant decreased after treatment with the two concentrations of both extracts, although the polysaccharide

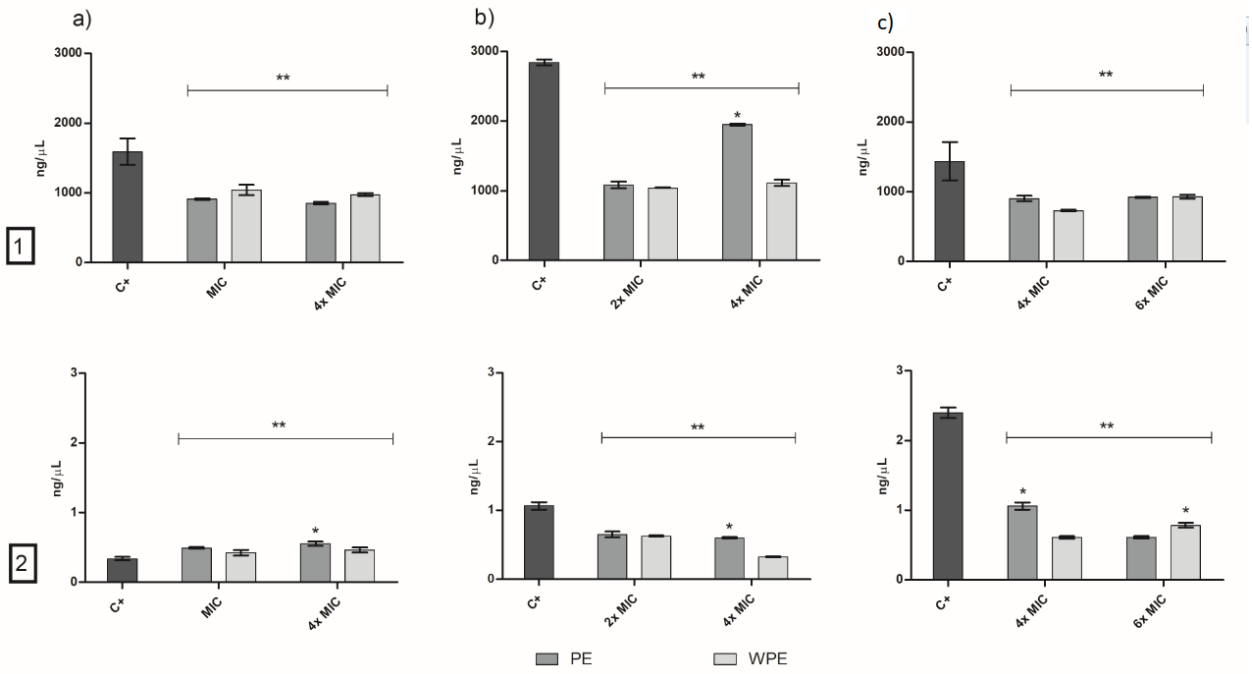

Figure 2. Proteins (1) and polysaccharides (2) quantification for biofilm matrix analysis of *C. albicans* (a), *F. oxysporum* (b) and *T. rubrum* (c) performed by NanoDrop spectrophotometer. * Statistical difference between propolis extract (PE) and propolis by-product extract (WPE). ** Statistical difference between controls and treaties. MIC - minimum inhibitory concentration.

concentrations of the filamentous fungi are higher than those observed for yeast (Figure 2).

As for eDNA and eRNA, both behaved similarly to each other, but with specificity for each of the three fungi tested. There was a significant increase in the production of both nucleic acids in the three fungi, however in response to different concentrations of the extracts (Figure 3). Correlating these findings with those in Figure 1, it is possible to attribute this increase to cell degradation, with possible leakage of cytoplasmic material as suggested by Corrêa et al. (2020). These findings corroborate those of Gucwa et al. (2018) who associated the action of the Polish PE with a depolarization of the cell membrane in *C. albicans*, which would have favored cell leakage. The increase of eRNA may be associated with protein synthesis, probably related to cell death, because according to de Castro et al. (2013) propolis is involved in apoptosis-induced *C. albicans* cell death.

It is proven that the organization of fungi in the form of biofilm gives these microorganisms some advantages, among others, the resistance to antifungals that can reach up to 1000 times greater than their respective planktonic counterparts (Ramage et al. 2012). This scenario is complex and multifactorial and few antifungal agents are effective in this situation. Kuhn et al. (2002) evaluated the antifungal susceptibility of *Candida* biofilms to various conventional drugs and found efficacy only with echinocandins and liposomal amphotericin B, drugs indicated for the treatment of systemic infections. However, these drugs are not used in the prevention or treatment of cutaneous mycoses. *Fusarium* spp. are also highly resistant to azole antifungals, which are generally not active against this fungus, and the response to terbinafine varies by species (Galletti et al. 2015). In addition, other factors are involved, such as less susceptibility to cold, heat, UV light and some fungicides by

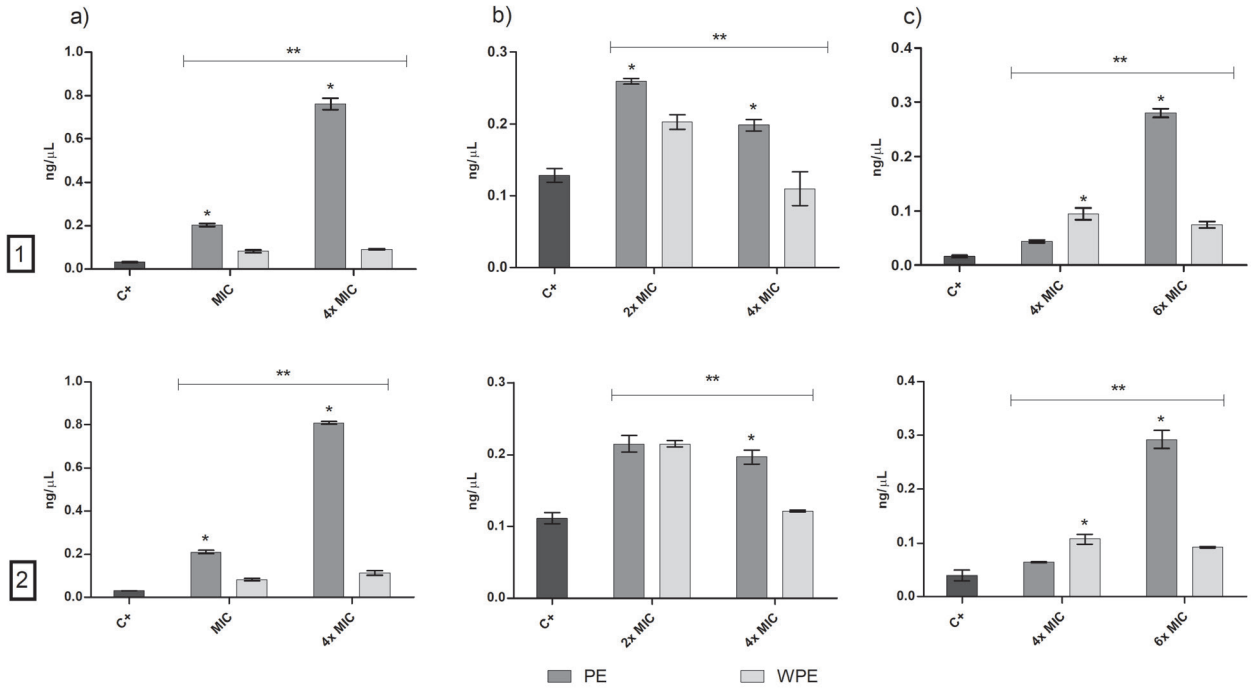

Figure 3. Extracellular DNA (1) and RNA (2) quantification for biofilm matrix analysis of *C. albicans* (a), *F. oxysporum* (b) and *T. rubrum* (c) performed by NanoDrop spectrophotometer. * Statistical difference between propolis extract (PE) and propolis by-product extract (WPE). ** Statistical difference between controls and treaties. MIC - minimum inhibitory concentration.

F. oxysporum biofilms, compared to planktonic cell counterparts (Peiqian et al. 2014).

In the present study, it was possible to show the potential of these compounds, mainly of PE, for the inhibition of biofilm formation. This ability could play a role in preventing candidiasis as suggested by de Castro et al. (2013), as well as in the application in the treatment of medical and dental devices.

Some pathogenic fungi, mainly yeasts, form biofilms in prostheses and catheters, causing widespread fungal infections, with high mortality rates (Costa-Orlandi et al. 2017). Propolis has already been described as a promising anti-cariogenic agent and can be considered a good oral antiseptic for caries prevention (Djais et al. 2020). In addition, it could be used to prevent the formation of biofilms in dental plaques and the development of oral candidiasis, as the treatment options available are also limited (Aslani et al. 2018). Some studies, such as those by Galletti et al. (2017) and Veiga et al. (2018) have already shown that propolis has low or negligible cytotoxicity on human cells. In addition, it is able to permeate the nail without the need for facilitating vehicles, indicating that it is a promising natural product for the treatment of onychomycosis.

CONCLUSIONS

The present study proved that both the propolis extract and the by-product showed excellent antifungal activity against the three fungi evaluated, which are of great importance in the context of human diseases and infections. In addition to the important antifungal action on planktonic cells of each fungus, these compounds demonstrate great potential for inhibiting the formation of biofilms. Thus,

besides being promising as a topical treatment for onychomycosis, it is possible to infer its potential for the prevention of cutaneous candidiasis, as well as its application in the treatment of medical and dental devices.

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