Acta Scientiarum

http://www.periodicos.uem.br/ojs/
ISSN on-line: 1807-863X
Doi: 10.4025/actascibiolsci.v43i1.55700

Standardization of resazurin use in susceptibility testing of natural products against yeasts in planktonic cells and in biofilms formation

Isabella Letícia Esteves Barros[1], Flávia Franco Veiga[1], Jakeline Luiz Corrêa[1], Isabele Carrilho Jarros[1], Melyssa Negri[1,2] and Terezinha Inez Estivalet Svidzinski[1,2*]

[1]Programa de Pós-Graduação em Ciências da Saúde, Universidade Estadual de Maringá, Avenida Colombo, 5790, 87020-900, Maringá, Paraná, Brazil. [2]Departamento de Análises Clínicas e Biomedicina, Universidade Estadual de Maringá, Maringá, Paraná, Brazil. *Author for correspondence. E-mail: terezinha.svidzinski@gmail

ABSTRACT. Natural products, such as the ethanolic propolis extract (PE), have been shown to be a safe and effective therapeutic alternative for the treatment of fungal skin and nail diseases. However, the presence of the resin and the physicochemical characteristics of the extract sometimes difficult the reading and determination of breakpoints of the *in vitro* tests, evidencing the need for alternatives that facilitate the reading. The present study aimed to standardize the use of resazurin in tests of susceptibility of PE with planktonic yeast cells and biofilm forms. The antifungal activity of PE was determined by minimum inhibitory concentration (MIC) and we observed that, for all *Candida* spp. tested, the most reproducible MIC results were obtained when resazurin was placed after 24 hours of incubation and remained more 24 hours with yeasts plus PE. For encapsulated yeasts, there was no dye reduction and color transition. Resazurin was also used for the evaluation of minimal biofilm inhibitory concentration and minimal biofilm eradication concentration and it was metabolized and reproduced the action of PE on *Candida* biofilms. In addition, microdilution checkerboard plates were made with the dye, which assisted reading the result of the interaction between PE and nystatin. We observed that the resin, the color and the turbidity of the PE slightly changed the color of the resazurin in high concentrations of the extract and did not impair the reading. Therefore, the resazurin standardization tests were proven to be efficient and grounds that it should be used as an auxiliary methodology for reading and interpretation of the susceptibility tests for non-encapsulated yeasts with natural products, which form turbidity or precipitation, such as propolis.

Keywords: resazurin; microbial sensitivity tests; propolis; biofilms; planktonic cells; standardization.

Received on September 8, 2020.
Accepted on November 3, 2021.

Introduction

Nowadays, skin diseases of various etiologies are the most common and prevalent disorders in the world. For many years, these diseases were neglected and have recently been considered as being of relevance. These problems are more common in tropical countries, where infections such as impetigo, abscess and others are frequent (Hay, 2020). Among them, those of fungal origin, including dermatophytosis as well as *Candida* and *Malassezia* infections are highly prevalent (Hay, 2020), including the onychomycosis (Gupta et al., 2020). The treatment of superficial fungal diseases is challenging for both patients and professionals, due to the difficulty in achieving a cure level and also because of the high rates of recurrence. Another problem is the antifungal arsenal to be restricted to limited options and reports of clinical resistance to these drugs have already been related (Sanguinetti, Posteraro, & Lass-Flörl, 2015). The few treatment options indicate the necessity for safe and effective therapeutic alternatives. Propolis is an interesting natural product and its ethanolic extract (PE) is promising, since it is a source of several active substances that have cumulatively diverse therapeutic properties, such as antimicrobial, anti-inflammatory, and immunomodulatory (Oryan, Alemzadeh, & Moshiri, 2018). Besides being economical, easily accessible, have low cytotoxicity and broad spectrum of action. Recently, our group showed that PE is capable of breaking down the integrity of the cell membrane as well as the cell wall from the fungi, without causing damage to human cells (Corrêa et al., 2020).

Propolis (bee glue) is a complex mixture of substances collected by honey bees, composed of plant exudates (resin), beeswax, and other substances such as pollen and sugars. The manipulation and interpretation of the *in vitro* tests with PE is of fundamental importance in proving the effectiveness of the extract, before its application to patients. Nevertheless, the reading and determination of the breakpoints, as regarding to visual as by spectrophotometer, are infeasible due to the presence of the resin and its physicochemical characteristics, which is the main source of total polyphenols, an antimicrobial component (Bruschi, Cardoso, Lucchesi, & Gremião, 2003; Bruschi et al., 2006; Teixeira, Message, Negri, Salatino, & Stringheta, 2010). Therefore, there is a need to find alternatives that facilitate the reliable reading of these tests; among others, the use of dyes such as resazurin is an option for optimizing the interpretation parameters. However, it depends on a standardization.

Resazurin sodium salt is a non-toxic, oxidation-reduction indicator that is intracellularly reduced to a fluorescent compound by enzymes in the electron transport system. It is already used to colorimetrically evaluate the viability of microorganisms for different fungi (as in planktonic cells or in biofilm form), with various conventional drugs and for natural extracts (Jahn, Stüben, & Bhakdi, 1996; Fai & Grant, 2009; Monteiro et al., 2012; Punithavathy, Nalina, & Menon, 2012; Van den Driessche, Rigole, Brackman, & Coenye, 2014; Dantas Silva et al., 2017). However, there are no standardized protocols and, besides other parameters, it is important to adjust usage and assess whether this dye interferes with the reading and interpretation of susceptibility results, especially when it comes to natural extracts, due to the physicochemical characteristics of these products. Thus, the present study aims to standardize the use of resazurin in tests of susceptibility of natural products, using yeasts as an experimental model under planktonic and in biofilm forms.

Material and methods

Strains and growth conditions

Candida albicans ATCC 90028, *Candida glabrata* ATCC 2001, *Candida tropicalis* ATCC 750, *Candida parapsilosis* ATCC 22019, *Rhodotorula mucilaginosa* ATCC 64684, *Cryptococcus neoformans* ATCC 90113 and *Cryptococcus gattii* ATCC 32269 were tested. Before the experiments, these isolates were subcultured in Sabouraud Dextrose Agar (SDA; Difco™, Detroit, United States) and CHROMagar™ *Candida* (Difco™, Detroit, United States), to check the culture purity. The isolates were subcultured on SDA overnight at 37°C or 25°C. For experimentation, the cellular density was adjusted based on hemocytometer cell counts before each assay. All tests were performed in triplicate, and on three independent days.

Natural product: propolis ethanolic extract (PE)

The green propolis used in this study was collected from hives located in northern Paraná state, Brazil. For assays, the PE was prepared according to the protocol by Veiga et al. (2018). The physicochemical evaluation of the PE revealed the following characteristics: Alcohol content: 65.73% (V/V), pH = 5.35 ± 0.02 and relative density = 0.8585 ± 0.0002 g mL^{-1}. The dryness residue value was 13.33% ± 0.34 (w w^{-1}) and the total polyphenol content (TPC) value was 1.31% ±0.01 (w w^{-1}).

Determination of antifungal properties of PE using resazurin in planktonic cells

The antifungal activity of PE against all *Candida* spp. strains was determined by MIC based on the Clinical & Laboratory Standards Institute protocol M27-A3 [CLSI] (2008), with certain modifications for natural products (Dalben-Dota et al., 2010). For this test, the serial dilution of PE was performed at a ratio of two, with the concentration of total polyphenols ranging from 0.001 to 0.655 µg mL^{-1}. About 100 µL of each yeast suspension adjusted to 1×10^3 yeasts mL^{-1} was inoculated in all the wells. The test was carried out using the 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 30 µL of the dye was added to each well at three different periods: 0h of incubation maintaining 48 hours with the preparation (A) and after 24 hours of incubation maintaining a further 24 hours (B), with reading in a total final period of 48

hours. We also added the dye after 48 hours of incubation maintaining another 24 hours for resazurin (C), and the reading was observed after a total period of 72 hours of incubation. Minimum inhibitory concentrations (MICs) were determined by direct observation of growth and change of the dye color from blue to pink to purple.

The results of the MIC were defined as the concentration of PE that reduced 100% of the growth compared with the yeast growth in the absence of the drug. The minimum fungicidal concentration (MFC) was determined by seeding on SDA plates, aliquots from the suspensions after exposition to different concentrations of PE. Plates were then incubated at 37°C for 24 hours. The MFC was defined as the lowest concentration of the PE in which no recovery of microorganisms was observed.

The MIC values were considered in accordance when their reading was equal to the visual growth (G). The interpretive breakpoints of resazurin (R) and MFC obtained were used to determine the categorical agreement compared to the results of the reference visual reading (G) method for the three different aforementioned time periods.

Evaluation of resazurin in planktonic cells of encapsulated yeasts

The antifungal susceptibility of *R. mucilaginosa*, *C. neoformans* and *C. gattii* was determined as described in the section above. The serial dilution of the extract was performed at a ratio of two, 100 µL of each yeast suspension adjusted to 1×10^3 yeasts mL^{-1} was inoculated in all the wells and the plates were incubated at 25°C for 24 hours. Thereafter, 30 µL of fresh resazurin solution, was added to each plate well and re-incubated for another 24 hours.

Application of resazurin in microdilution checkerboard assays (synergism)

The microdilution checkerboard plates were prepared in duplicate, as previously described (Cuenca-Estrella, 2004; Garcia, 2010) with certain changes to all *Candida* spp. strains and by using the interaction between the PE and nystatin (Sigma-Aldrich, St. Louis, MO, USA) as a model.

Briefly, the plates were prepared by serially diluting nystatin (0.125–64 µg mL^{-1}) in the x-axis and PE (0.001–0.655 µg mL^{-1}) in the y-axis in a 96-well microtiter plate. About 100 µL of each yeast suspension adjusted to 1×10^3 yeasts mL^{-1} was inoculated in all the wells. The plates were covered with their lids and incubated at 37°C for 24 hours. Thereafter, 30 µL of fresh resazurin solution, was added to each plate well and incubated for another 24 hours at 37°C. A blue to pink color change indicated a reduction of resazurin, and thus, yeast growth. The classical checkerboard reading was performed by a visual reading of yeast growth, without resazurin.

Application of resazurin in the evaluation of minimal biofilm inhibitory concentration (MBIC) and minimal biofilm eradication concentration (MBEC)

All *Candida* spp. strains were grown on SDA for 24 hours at 37°C, followed by inoculation in Sabouraud Dextrose Broth (SDB; DifcoTM, Detroit, United States) and were then incubated for 18 hours 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 phosphate-buffered saline, pH 7, 0.1 M (PBS). The cell suspensions comprising 1×10^7 yeasts mL^{-1} were prepared in RPMI 1640 medium; 200 µL of these suspensions were inoculated into a 96-well polystyrene plate, and incubated at 37°C on a shaker at 120 rpm $min.^{-1}$ for 2 hours. Non-adherent cells were removed by washing with sterile PBS, followed by addition of 200 µL PE (at MIC, 2x MIC and 4x MIC concentrations in RPMI 1640 medium). The plate was incubated at 37°C for 24 hours to allow biofilm formation. Thereafter, 30 µL of resazurin was added to each well and the plate was re-incubated at 37°C for 24 hours. Negative controls (200 µL of only RPMI 1640 medium) and untreated controls (200 µL of RPMI 1640 medium and preformed biofilm) were also included (Capoci et al., 2015; Veiga et al., 2018).

For the determination of MBIC, the resazurin color change was read, whereas, for MBEC, the biofilms were scraped from the respective wells and the suspensions were vigorously vortexed for approximately 2 min. to disaggregate cells from the matrix. Serial dilutions were made in PBS, plated onto SDA, and incubated for 24 hours at 37°C for evaluating the colony-forming units (CFU).

Results and discussion

Determination of MIC in planktonic cells using resazurin at different times

The antifungal activity of propolis has been demonstrated in several studies carried out at different times and with different extracts and compositions (Negri et al., 2014). Researches have shown the antifungal activity of propolis against different species and isolates of Candida, which corroborate our findings (Dalben-Dota et al., 2010; Falcão et al., 2013; Tobaldini-Valerio et al., 2016; Corrêa et al., 2020). However, the visual reading is difficult due

the resinous material, requiring complementary tests such as the MFC determination. Resazurin has been employed aiming to facilitate the reading of susceptibility tests for classical antifungal drugs (Monteiro et al., 2012; Van den Driessche et al., 2014) as well as for other certains microorganisms (Palomino et al., 2002). But still is few utilized in tests with natural compounds addressed to fungi of human interest.

Here, aiming standardize the MIC determination of the PE against pathogenic yeasts, the resazurin was added in the microplates comprising yeast suspension with PE, in the three aforementioned conditions (A, B, and C) in order to verify if the time of contact with the dye interfered in its metabolism and consequently in the reading of the tests and in the results obtained. The readings were performed by visual observation of yeast growth on microplates, observation of dye color change, and MFC.

In this trial, we observed that, for all *Candida* species tested, the most reproducible results of the readings were obtained in condition B, where resazurin was placed after 24 hours incubation and remained for another 24 hours in the medium (Table 1). In addition, we realized a resin precipitate formed at the bottom of the well when PE is in high concentrations (columns 1 of Figure 1 and in line A of Suppl. Figure 1). This precipitate induced a slight change in the color of the resazurin (Figure 1). In order to clarify this problem, we compared with the reading of another natural extract without the precipitate (column 1 of Figure 2). It is important to highlight that it did not prevent a safe visual reading.

Table 1. Determination of the minimum inhibitory concentration of the ethanolic propolis extract (PE) based on the yeast growth reading without resazurin (G), yeast growth reading with color change by resazurin (R), and minimum fungicidal concentration by cell viability (MFC).

Yeast	Time of incubation before resazurin addition								
	A (0h)			B (24h)			C (48h)		
	Type of reading to determine MIC (ug mL^{-1} in TPP)								
	G	R	MFC	G	R	MFC	G	R	MFC
C. albicans	0.010	0.010	0.041	0.041	0.041	0.041	0.041	0.041	0.164
C. glabrata	0.010	0.010	0.164	0.020	0.020	0.082	0.041	0.082	0.328
C. parapsilosis	0.041	0.041	0.164	0.082	0.082	0.164	0.082	0.164	0.328
C. tropicalis	0.020	0.020	0.082	0.041	0.041	0.082	0.041	0.082	0.164
% Categorical agreement		100	0		100	25		25	0

G - growth (visual); R - resazurin (color); MFC - minimum fungicidal concentration (growth inhibition); A - 0h of incubation maintaining 48 hours with the preparation; B - resazurin added after 24 hours of incubation maintaining a further 24 hours with reading after a total period of 48 hours; C - resazurin added after 48 hours of incubation maintaining a further 24 hours with reading after a total period of 72 hours of incubation.

We observed that the MFC values, in most cases, do not agree with the MIC values, indeed Tobaldini-Valerio et al. (2016) reported that in the first 12 hours, PE just inhibits the fungal growth, reflecting in MIC results, furthermore PE would decrease the CFU number and this data is related to MFC. The addition of resazurin after 48 hours of incubation and its reading at 72 hours (C) allowed us to visualize this PE behavior. Therefore, the dye in Condition B presented higher percentages of agreement with the reference reading (visual growth) thus, revealing less discrepancies in the reading parameters and the presented differences were within the acceptable limits for the MIC reading.

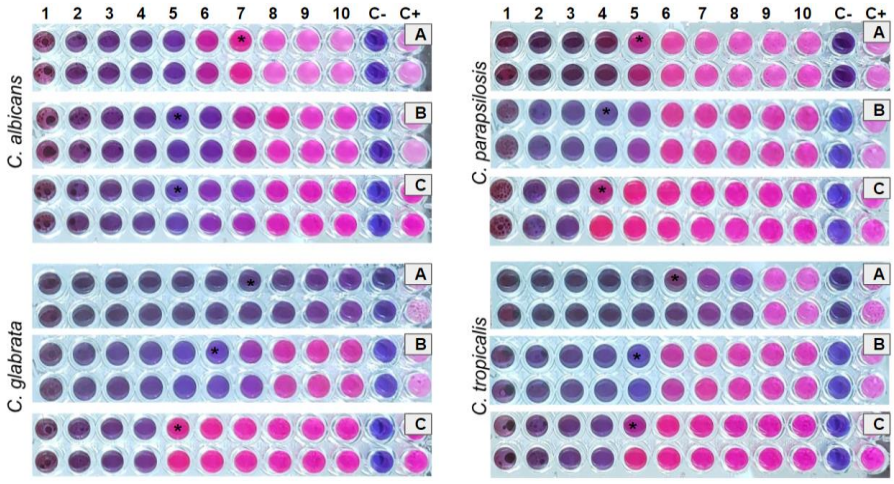
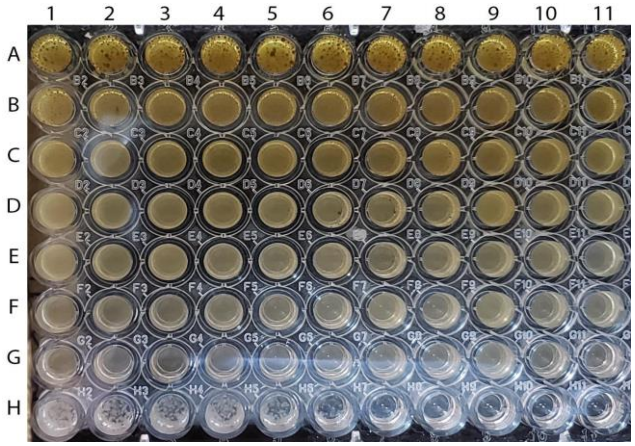


Figure 1. C+ color of yeast suspension with resazurin, without PE; C- color of resazurin without yeast and PE; 1-10 (ten different concentrations of PE ranging from 0.001 to 0.655 µg mL^{-1} in TPP). MIC determination of the PE with resazurin added in the plates of all yeasts at 0h of incubation, totaling 48h with resazurin (A), after 24 hours of incubation, totaling a further 24 hours with resazurin (B), and after 48 hours of incubation plus another 24 hours with resazurin (C). * indicates the MIC of each case, in a visual reading evidencing the divergences and showing that in B condition there was major agreement between the two readings.



Supplementary Figure 1. Microdilution checkerboard plate of *Candida* sp. with interaction between propolis extract and nystatin without the use of resazurin.

Determination of MIC in planktonic cells using resazurin at encapsulated strains

It is already known that resazurin is metabolized and reduced intracellularly to a fluorescent compound by enzymes in the electron transport system (Rampersad, 2012). However, when we evaluated *R. mucilaginosa*, *C. neoformans*, *C. gattii* strains, we observed that it was not possible to obtain the resazurin reading, since there was no color transition of the dye nor in the positive controls, without antifungal drug. So, we carried out a test using an aleatory natural product extract, which does not form a precipitate, and we were able to confirm that encapsulated yeasts did not absorbed resazurin, differently of the *C. albicans* (Figure 2). Despite this, we obtained normal growth and MFC readings. All of them are encapsulated yeasts (Miceli, Díaz, & Lee, 2011), and this may have prevented the entry of the dye and its subsequent metabolization inside the cells. In addition, we noticed that the color of resazurin was neither altered by the color nor by the turbidity of the natural product extract tested.

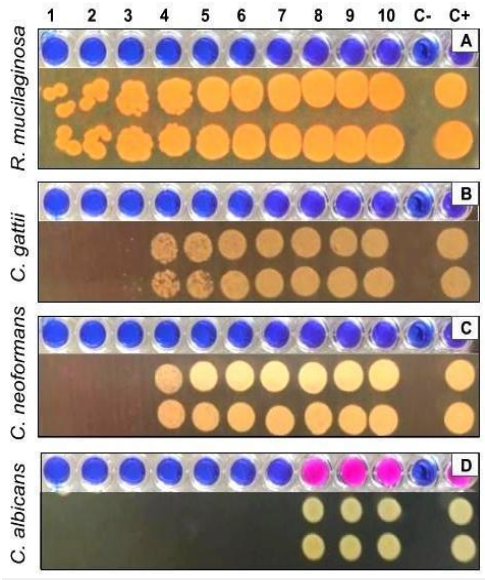


Figure 2. MIC and MFC determinations of an aleatory natural product extract against three encapsulated yeast comparing to *C. albicans*, with resazurin added after 24 hours of incubation totaling a further 24 hours with resazurin. C+ color of yeast suspension with resazurin, without the extract; C- color of resazurin without yeast and extract; 1-10 (ten different concentrations of natural product extract). It was not possible to obtain the resazurin reading for MIC, since there was no color transition of the dye nor in the positive controls, except for *C. albicans*. Nevertheless, it was detected a normal growth and MFC readings.

Use of resazurin in the microdilution checkerboard assays

During the microdilution checkerboard tests, resazurin was added to all yeast plates after 24 hours of incubation and the reading was performed after 48 hours by observing the growth and color change of the dye. This time of contact of the dye was selected considering the best results obtained in the MIC trials.

In all microdilution checkerboard plates (Figure 3), it was possible to determine the MIC of nystatin in line H and PE in column 1 as well as the interaction between the two substances at the center of the plate. In this case, resazurin facilitated the reading and interpretation of the results obtained since, without this dye, the interaction reading between nystatin and PE would be impaired by the color and turbidity of the natural extract (Suppl. Figure 1).

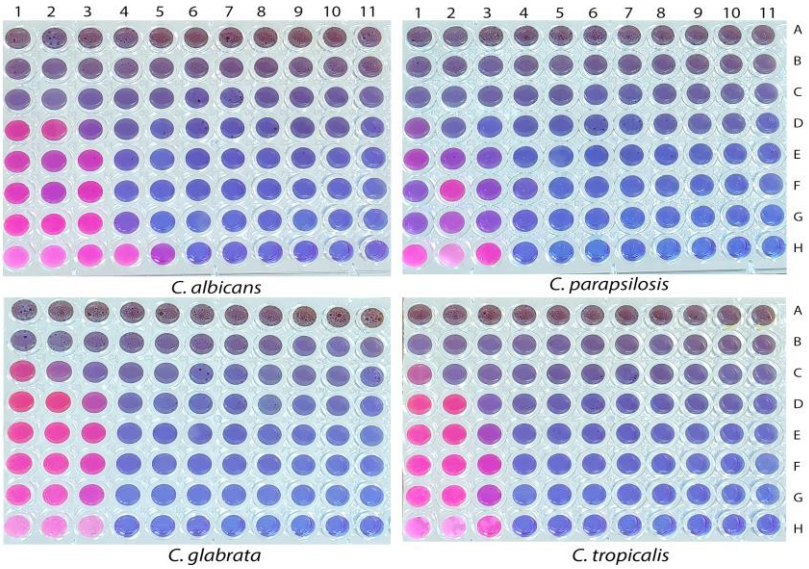


Figure 3. Microdilution checkerboard plates of *Candida* spp. with interaction between the serially diluting of nystatin (0.125-64 µg mL^{-1}) in the x-axis and PE (0.001-0.655 µg mL^{-1}) in the y-axis. A blue to pink color change indicated a reduction of resazurin, and thus, yeast growth. The MIC of nystatin is in line H and PE in column 1, as well as the interaction between the two substances are observed at the center of the plate.

Evaluation of MBIC and MBEC using resazurin

The ability of PE to act and inactivate biofilms formed by different fungi has already been proven by previous studies (Capoci et al., 2015; Tobaldini-Valerio et al., 2016; Veiga et al., 2018). While evaluating the MBIC of *Candida* sp. biofilms, we noticed that resazurin was also metabolized and that it evidenced the action of PE on biofilms formation (Figure 4). Despite this, it is clear a certain difficulty in reading the color transition of the dye at this stage, which can be explained by the biofilm architecture itself. Biofilms are formed by several cell layers, including basal layers of cells with decreased metabolic activity (persistent cells), which can result in difficulty in absorbing and metabolizing resazurin. In addition, the presence of the extracellular matrix that surrounds the biofilms can limit the access of the dye to the cells (Blankenship & Mitchell, 2006; Donné & Dewilde, 2015). Still, the dye allows for visual reading.

In this case, the use of this dye helped us to evaluate the dilution count that would be necessary for each concentration of the extract for accomplishing CFU. Therefore, our results from resazurin readings corroborate with the results of the MBEC of all species, as can be observed in Figure 5, in which the MIC of PE was able to inhibit in two or three logs the biofilm formation of the four *Candida* species, whereas, except for *C. glabrata*, all were eradicated at 2x MIC concentration.

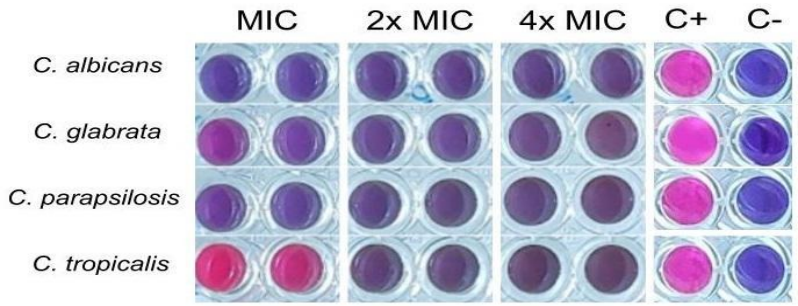


Figure 4. Minimal biofilm inhibitory concentration of PE at MIC, 2x MIC and 4x MIC concentrations on biofilms formed by four *Candida* species. C+ color of yeast suspension with resazurin, without the extract; C- color of resazurin without yeast and extract. A blue to pink color change indicated a reduction of resazurin, and thus, yeast growth.

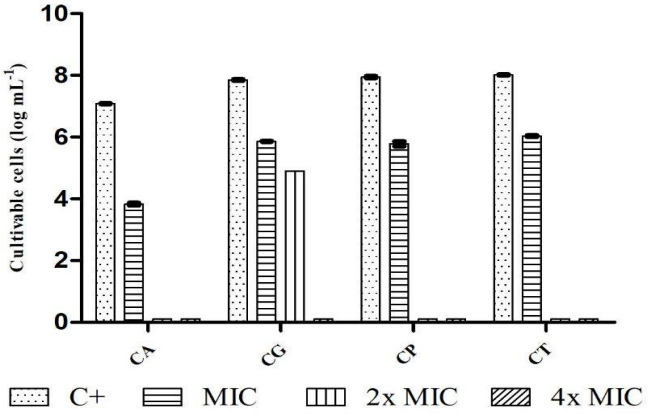


Figure 5. Minimal biofilm eradication concentration of PE on biofilms formed by *Candida* spp. CA - *Candida albicans*; CG - *Candida glabrata*; CP - *Candida parapsilosis*; CT - *Candida tropicalis*; C+: growth of yeast suspension without PE.

Conclusion

The addition of resazurin facilitated the reading and interpretation of the antifungal susceptibility tests. The resin, the color and the turbidity of the PE slightly changed the color of the resazurin in high concentrations of the extract and did not impair the reading. Thus, the resazurin standardization tests were proven to be efficient in the tests performed and grounds that it should be used as an auxiliary methodology for reading and interpretation of the susceptibility tests with natural products, which form turbidity or precipitation, such as propolis, against the principal microorganisms responsible by skin and nail diseases.

Acknowledgement

We thank the *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES) as well as *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq) and *Fundação Araucária* for supporting this study. We thank Angélica Sayuri Mizutani for reviewing the English of this manuscript. We have no conflict of interest to declare. This study was financed in part by the *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* - Brasil (CAPES) - Finance Code 001.

References

Blankenship, J. R., & Mitchell, A. P. (2006). How to build a biofilm: a fungal perspective. *Current Opinion in Microbiology, 9*(6), 588-594. DOI: http://dx.doi.org/10.1016/j.mib.2006.10.003

Bruschi, M. L., Cardoso, M. L. C., Lucchesi, M. B., & Gremião, M. P. D. (2003). Gelatin microparticles containing propolis obtained by spray-drying technique: preparation and characterization. *International journal of pharmaceutics*, *264*(1-2), 45-55. DOI: http://dx.doi.org/10.1016/s0378-5173(03)00386-7

Bruschi, M. L., Lara, E. H. G., Martins, C. H. G., Vinholis, A. H. C., Casemiro, L. A., Panzeri, H., & Gremião, M. P. D. (2006). Preparation and antimicrobial activity of gelatin microparticles containing propolis against oral pathogens. *Drug Development and Industrial Pharmacy, 32*(2), 229-238. DOI: http://dx.doi.org/10.1080/03639040500466312

Capoci, I. R. G., Bonfim-Mendonça, P. S., Arita, G. S., Pereira, R. R. A., Consolaro, M. E. L., Bruschi, M. L., ... Svidzinski, T. I. E. (2015). Propolis is an efficient fungicide and inhibitor of biofilm production by vaginal *Candida albicans*. *Evidence-Based Complementary and Alternative Medicine, 2015*(1), 1-9. DOI: http://dx.doi.org/10.1155/2015/287693

Clinical and Laboratory Standards Institute [CLSI]. (2008). *Reference method for broth dilution antifungal susceptibility testing of yeasts: approved standard - Third Edition* (CLSI document M27-A3). Wayne, PA: CLSI

Corrêa, J. L., Veiga, F. F., Jarros, I. C., Costa, M. I., Castilho, P. F., Oliveira, K. M. P., ... Negri, M. (2020). Propolis extract has bioactivity on the wall and cell membrane of *Candida albicans*. *Journal of Ethnopharmacology, 1*(256), 1-11. DOI: http://dx.doi.org/10.1016/j.jep.2020.112791

Cuenca-Estrella, M. (2004). Combinations of antifungal agents in therapy–what value are they? *Journal of Antimicrobial Chemotherapy, 54*(5), 854-869. DOI: http://dx.doi.org/10.1093/jac/dkh434

Dalben-Dota, K. F., Faria, M. G. I., Bruschi, M. L., Pelloso, S. M., Lopes-Consolaro, M. E., & Svidzinski, T. I. E. (2010). Antifungal activity of propolis extract against yeasts isolated from vaginal exudates. *Journal Of Alternative And Complementary Medicine* , *16*(3), 285-290. DOI: http://dx.doi.org/10.1089/acm.2009.0281

Dantas Silva, R. P., Machado, B. A. S., Barreto, G. A., Costa, S. S., Andrade, L. N., Amaral, R. G., ... Umsza-Guez, M. A. (2017). Antioxidant, antimicrobial, antiparasitic, and cytotoxic properties of various Brazilian propolis extracts. *PLoS ONE*, *12*(3), e0172585. DOI: https://dx.doi.org/10.1371/journal.pone.0172585

Donné, J., & Dewilde, S. (2015). The challenging world of biofilm physiology. *Advances In Microbial Physiology*, *67*(1), 235-292. DOI: http://dx.doi.org/10.1016/bs.ampbs.2015.09.003

Fai, P. B., & Grant, A. (2009). A rapid resazurin bioassay for assessing the toxicity of fungicides. *Chemosphere*, *74*(9), 1165-1170. DOI: http://dx.doi.org/10.1016/j.chemosphere.2008.11.078

Falcão, S. I., Vale, N., Cos, P., Gomes, P., Freire, C., Maes, L., & Vilas-Boas, M. (2014). In vitro evaluation of Portuguese propolis and floral sources for antiprotozoal, antibacterial and antifungal activity. *Phytotherapy Research*, *28*(3), 437-443. DOI: http://dx.doi.org/10.1002/ptr.5013

Garcia, L. S. (2010). Synergism Testing: broth microdilution checkerboard and broth macrodilution methods (p. 140-162). In L. S. Garcia (Ed.), *Clinical Microbiology Procedures Handbook, 3rd Edition* (p. 140-162). Washington, DC: ASM Press.

Gupta, A. K., Stec, N., Summerbell, R. C., Shear, N. H., Piguet, V., Tosti, A., & Maria Piraccini, B. (2020). Onychomycosis: a review. *Journal of the European Academy of Dermatology and Venereology, 34*(9), 1972-1990. DOI: https://dx.doi.org/10.1111/jdv.16394

Hay, R. J. (2020). Skin disease in the tropics and the lessons that can be learned from leprosy and other neglected diseases. *Acta dermato-venereologica, 100*(1), 235-241. DOI: http://dx.doi.org/10.2340/00015555-3469

Jahn, B., Stüben, A., & Bhakdi, S. (1996). Colorimetric susceptibility testing for *Aspergillus fumigatus*: comparison of menadione-augmented 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide and Alamar blue tests. *Journal of Clinical Microbiology*, *34*(8), 2039-2041. DOI: https://dx.doi.org/10.1128/jcm.34.8.2039-2041.1996

Miceli, M. H., Díaz, J. A., & Lee, S. A. (2011). Emerging opportunistic yeast infections. *The Lancet Infectious Diseases, 11*(2), 142-151. DOI: http://dx.doi.org/10.1016/s1473-3099(10)70218-8

Monteiro, M. C., Cruz, M. de la, Cantizani, J., Moreno, C., Tormo, J. R., Mellado, E., ... Vicente, F. (2012). A new approach to drug discovery: high-throughput screening of microbial natural extracts against *Aspergillus fumigatus* using resazurin. *Journal of Biomolecular Screening*, *17*(4), 542-549. DOI: http://dx.doi.org/10.1177/1087057111433459

Negri, M., Salci, T. P., Shinobu-Mesquita, C. S., Capoci, I. R. G., Svidzinski, T. I. E., & Kioshima, E. S. (2014). Early state research on antifungal natural products. *Molecules*, *19*(3), 2925-2956.

Oryan, A., Alemzadeh, E., & Moshiri, A. (2018). Potential role of propolis in wound healing: Biological properties and therapeutic activities. *Biomedecine & Pharmacotherapie, 1*(98), 469-483. DOI: https://dx.doi.org/10.1016/j.biopha.2017.12.069

Palomino, J.-C., Martin, A., Camacho, M., Guerra, H., Swings, J., & Portaels, F. (2002). Resazurin microtiter assay plate: simple and inexpensive method for detection of drug resistance in *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy*, *46*(8), 2720-2722. DOI: https://dx.doi.org/10.1128/AAC.46.8.2720-2722.2002

Punithavathy, P. M., Nalina, K., & Menon, T. (2012). Antifungal susceptibility testing of *Candida tropicalis* biofilms against fluconazole using calorimetric indicator resazurin. *Indian Journal Of Pathology & Microbiology*, *55*(1), 72-74. DOI: http://dx.doi.org/10.4103/0377-4929.94861

Rampersad, S. N. (2012). Multiple applications of Alamar Blue as an indicator of metabolic function and cellular health in cell viability bioassays. *Sensors* , *12*(9), 12347-12360. DOI: http://dx.doi.org/10.3390/s120912347

Sanguinetti, M., Posteraro, B., & Lass-Flörl, C. (2015). Antifungal drug resistance among *Candida* species: mechanisms and clinical impact. *Mycoses*, *58*(Suppl 2), 2-13. DOI: http://dx.doi.org/10.1111/myc.12330

Teixeira, E. W., Message, D., Negri, G., Salatino, A., & Stringheta, P. C. (2010). Seasonal variation, chemical composition and antioxidant activity of Brazilian propolis samples. *Evidence-Based Complementary and Alternative Medicine*, *7*(3), 307-315. DOI: http://dx.doi.org/10.1093/ecam/nem177

Tobaldini-Valerio, F. K., Bonfim-Mendonça, P. S., Rosseto, H. C., Bruschi, M. L., Henriques, M., Negri, M., ... Svidzinski, T. I. E. (2016). Propolis: a potential natural product to fight *Candida* species infections. *Future Microbiology*, *11*(8), 1035-1046. DOI: http://dx.doi.org/10.2217/fmb-2015-0016

Van den Driessche, F., Rigole, P., Brackman, G., & Coenye, T. (2014). Optimization of resazurin-based viability staining for quantification of microbial biofilms. *Journal of Microbiological Methods*, *98*(1), 31-34. DOI: http://dx.doi.org/10.1016/j.mimet.2013.12.011

Veiga, F. F., Gadelha, M. C., Silva, M. R. T., Costa, M. I., Kischkel, B., Castro-Hoshino, L. V., ... Svidzinski, T. I. E. (2018). propolis extract for onychomycosis topical treatment: from bench to clinic. *Frontiers in Microbiology*, *9*(1), 1-13. DOI: http://dx.doi.org/10.3389/fmicb.2018.00779