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Abstract: This study assessed the cytotoxicity of Antimicrobial Photodynamic Inactivation (aPDI), mediated by curcumin, using human keratinocytes co-cultured with *Candida albicans*. Cells and microorganisms grew separately for 24 hours and kept in contact for more 24 hours. After this period, aPDI was applied. The conditions tested were: P+L+ (experimental group aPDI); P-L+ (light emitting diode [LED] group); P+L- (curcumin group); and P-L- (cells in co-culture without curcumin nor LED). In addition, keratinocytes and *C. albicans* were grown separately, were not placed in the co-culture and did not receive aPDI (control group). Cell proliferation was assessed using Alamar Blue, MTT, XTT and CFU tests. Qualitative and quantitative analyses were performed. Analysis of variance (ANOVA) was applied to the survival percentages of cells compared to the control group (considered as 100% viability), complemented by multiple comparisons using the Tukey test. A 5% significance level was adopted. The results of this study showed no interference in the metabolism of the cells in co-culture, since no differences were observed between the control group (cultured cells by themselves) and the P-L- group (co-culture cells without aPDI). The aPDI group reached the highest reduction, which was equivalent to 1.7 $\log_{10}$ when compared to the control group. The P+L-, P-L+, P-L- and control groups were not statistically different ($\rho>0.05$). aPDI inhibited the growth of keratinocytes and *C. albicans* in all tests, so the therapy was considered slightly (inhibition between 25 and 50% compared to control group) to moderately (inhibition between 50 and 75% compared to control group) cytotoxic.

Keywords: Antimicrobial Photodynamic Inactivation, Curcumin, Co-culture, *Candida*

1. **Introduction**

Colonization by microorganisms and subsequent biofilm formation on surfaces are relevant factors in the fungal development and pathogenesis of candidal infection, which may be considered a public health problem. Despite the multifactorial etiology, contamination by fungal species and their adherence to the surface of the prosthesis seems to be the primary factor for the onset and spread of denture stomatitis.[1] The treatment of fungal infections can be topical or systemic. Topical agents such as nystatin and miconazole are effective in relieving the clinical signs and symptoms of stomatitis associated with *Candida* species.[2] Nevertheless, the diluent effect of saliva and the cleansing action of the oral musculature tend to decrease the concentration of these drugs.[1] Thus, the re-colonization of the oral mucosa by the microorganism is common after treatment, which leads to a recurrent infection. Systemic antifungal agents such as amphotericin B and fluconazole are effective, but these substances do not remove the microorganisms from the denture surface.[3]

It has been reported that the use of topical and systemic antifungals cannot promote the desired effects in the treatment of denture stomatitis, as the etiological agent may develop resistance to these drugs. *Candida* species have the capacity to develop drug resistance mechanisms through genetic mutations.[4,5] For that reason, the correct choice of antifungal medication after the proper diagnosis is crucial for successful treatment.

Taking into consideration the aspects mentioned above, Antimicrobial Photodynamic Inactivation (aPDI) seems to be a good alternative for the treatment of fungal infections. Briefly, aPDI uses light energy to activate a photosensitizer (PS) in the presence of oxygen.[6] The interaction between PS and light results in the production of reactive oxygen species (ROS) that cause oxidative damage to the cells.[7] One of the advantages of aPDI is the reduced collateral effects because it can be applied specifically to the target cells.[8,9]

Various types of PSs have been studied in aPDI.[8,10-13] Curcumin is a naturally occurring, intensely yellow pigment that is isolated from rhizomes of the *Curcuma longa* L. plant.[14] An increasing number of investigations have suggested that the effects of curcumin may possibly be enhanced by combining it with light.[13,15,16] A previous in vitro study showed that aPDI mediated by curcumin was effective in reducing the

viability of planktonic cells and biofilms of *C. albicans*.[15] At the same time, this is a limitation of aPDI, as the host cells are also susceptible to the action of ROS. Ribeiro et al.[17] showed the efficiency of curcumin combined with blue light emitting diode (LED) light in causing the photoinactivation of bacteria. However, this therapy also caused a significant reduction of L929 cell metabolism.

In this scenario, aPDI seems to be a promising method for the treatment of superficial infections. This therapy could overcome the difficulties found with conventional treatments, such as antifungal resistance. However, this treatment must be evaluated regarding to biocompatibility, mainly in co-culture, as the aPDI has been applied in cancer treatment, having a high level of power to eradicate the irradiated cells. aPDI produces reactive oxygen species, which may interact with various cell structures, such as proteins, nucleic acids and membranes, causing irreversible damage to microorganisms.[18] Only after the analysis of the biocompatibility may aPDI be considered a therapeutic modality for the treatment of denture stomatitis and other fungal infections associated with opportunistic fungi. For that reason, this study aimed to evaluate the cytotoxicity of aPDI, mediated by curcumin and LED light, using human keratinocytes co-cultured with *C. albicans*.

2. Material and Methods

2.1. PS and light source used for aPDI

Natural curcumin (Fluka Co.) was obtained from Sigma-Aldrich (St. Louis, MO, USA). A solution of curcumin (200 µM) was prepared in dimethyl sulfoxide (DMSO, keeping its final concentration at 10%) and then diluted in saline solution to obtain the concentration to be tested (40 µM of curcumin). The 10% concentration of DMSO was selected since it had no effects on the viability of *Candida*[15,16]. The pre-irradiation time was 20 minutes. A LED-based device that provided a uniform emission from 440 nm to 460 nm, with a maximum emission at 455 nm, was used. The light dose of 5.28 J/cm^2 was used, corresponding to 4 min of irradiation.[15]

2.2. *C. albicans* biofilm

The microorganism used in this study was an American Type Culture Collection (ATCC; Rockville, MD, USA) strain of *C. albicans* (ATCC 90028). The cells were

maintained in a freezer at 70 ºC until the assays. For the tests, 25 µL of *C. albicans* were seeded in Petri dishes containing the culture medium Sabouraud Dextrose Agar (SDA) with chloramphenicol (0.1 g/L), and the plates were incubated at 37 °C for 48 hours. The yeast was inoculated in 5 mL of Yeast Nitrogen Base (YNB) and grown overnight aerobically at 37 °C for 16 hours. To obtain the standardized suspensions of *Candida*, each culture was harvested after centrifugation at 4000 rpm for 5 minutes, washed with sterile distilled water and resuspended in 2 ml of sterile saline to 10^7 CFU/mL by adjusting the optical density of the suspension to 0.38 at 520 nm in a spectrophotometer (Biospectro, Equipar Ltda, Curitiba, Paraná, Brazil). After adjustment of the concentrations, aliquots of 1000 µL of the suspension obtained for *C. albicans* were placed on the transwell membranes of a 12-well plate, and 1000 µL of Roswell Park Memorial Institute (RPMI) medium were added in each well. The plates were incubated for 90 minutes at 37 °C under agitation at 75 rpm (adhesion layer). After this period, the biofilm formed on the membrane was washed twice with 1000 µL of sterile PBS to remove non-adherent cells, the medium buffer and metabolites. Subsequently, 1000 µL of sterile RPMI were added onto the biofilm. Plates were incubated again at 37 °C in an orbital shaking incubator (75 rpm) for 24 hours.

2.3. Cellular culture

Keratinocytes (HaCaT 0341) [Banco de Células do Rio de Janeiro, RJ, Brazil] were propagated in the culture medium (Dulbecco's modified Eagles medium [DMEM]). The culture was maintained at 37 ºC in an atmosphere of 5% CO_2 and 95% air. For the tests, the cells (1 x 10^4 cell/mL) were seeded into 12 well culture plates and incubated for 24 hours at 37ºC in an atmosphere containing 5% CO_2.

2.4. Co-culture

After 24 hours, the interaction between *C. albicans* and keratinocytes was performed, where the cells were grown in co-culture for more 24 hours in order to form a biofilm for 48 hours (Scheme 1).

2.5. Experimental groups

To evaluate the cytotoxic effect of aPDI the following experimental conditions were tested:

P+L+ (aPDI group): cells in co-culture exposed to curcumin irradiated for four minutes, corresponding to 5.28 J/cm^2;

P+L- (curcumin group): cells in co-culture exposed only to curcumin and not to LED light;

P-L+ (LED light group): cells in co-culture exposed only to LED light and not to curcumin;

P-L- (not treated group): cells in co-culture without curcumin nor LED light;

Control group: keratinocytes and *C. albicans* were grown separately, were not placed in co-culture and did not receive aPDI.

2.6. Tests performed for analysis of cell viability

MTT assay

For the analysis of cellular metabolism, the mitochondrial activity of the keratinocytes was measured using the MTT assay. For each group, the transwell membrane was removed from each compartment, thus removing the *Candida* biofilms. Thereafter, keratinocytes were washed with 1000 µL of PBS, and 800 µL of the MTT solution (5.0 mg/mL) were added in each well. The plates were incubated at 37 ºC with 5% CO_2 and 95% air for four hours in the dark. After this period, formazan crystals were solubilized in 800 µL of acidified 2-propanol with HCl 0,04N. After the solution was stirred and its homogeneity checked, aliquots of 150 µL of each compartment, in quadruplicate, were transferred to a plate of 96 wells to perform the reading. The absorbance was measured with a microplate reader at a test wavelength of 570 nm.

XTT assay

A XTT assay was used to determine the metabolic activity of the *Candida* biofilm. For each group, the transwell membranes were removed from each compartment and were put in another plate. At the first moment, non-adherent cells were removed through washing with sterile 1000 µL of PBS. Then, the XTT solution was prepared with 790 mL of sterile PBS; 200 mL of XTT (Sigma Aldrich) dissolved in PBS to a final concentration of 1 mg/mL and filter-sterilized with a 0.2-mm pore size filter; and 10 mL Menadione (Sigma Aldrich) prepared in acetone to a 0.44 mM concentration. For the assay, 800 µL of the XTT solution was added onto the biofilm formed on the transwell membranes. After incubation with the XTT solution for four hours in the dark, and after the solution was stirred and the homogeneity checked,

aliquots of 150 µL of each compartment, in quadruplicate, were transferred to a plate of 96 wells to perform the reading. The absorbance at 492 nm was determined using a microplate reader.

Alamar Blue® assay

The cell proliferation of keratinocytes and *C. albicans* was also assessed using the Alamar Blue® assay. The assay presents an indicator of fluorimetric growth based on metabolic activity detection. To perform the assay, the transwell membranes containing the biofilm were transferred to a new sterile 12-well plate so that the test could be conducted separately for the cells that had adhered to the plate bottom, and the *C. albicans* biofilm on the membrane. The medium existent in both cells was removed, and the cells were washed once with 1000 µL of PBS. Finally, 100 µL of the Alamar Blue solution diluted in 900 µL RPMI medium with 10% fetal bovine serum were added, totaling 1000 µL of solution in each well. The plates were incubated at 37 ^{o}C with 5% CO_2 and 95% air for four hours in the dark. After incubation with the Alamar Blue solution, and after the solution was stirred and the homogeneity checked, aliquots of 150 µL of each compartment, in quadruplicate, were transferred to a plate of 96 wells to perform the reading. The reading for the fluorescence of the samples was measured using the Fluoroskan Ascent FL fluorometer (Labsystems, Helsinki, Finland) with a filter combination of 544 nm/590 nm.

Survival fractions of C. albicans

For the quantification of fungal viability, after treatment, the membranes containing the biofilm were separated from co-culture. The membrane was removed from the holder with the help of a scalpel handle. Each membrane was placed, separately, in Falcon tubes containing sterile PBS. The tubes were sonicated in an ultrasonic device for five minutes to complete the detachment of the fungal cells. Then, the serial dilution process was performed in sterile PBS by transferring 100 µL of stock solution into tubes containing 900 µL of the same solution. Dilutions from 10^{-1} to 10^{-4} were obtained, and one aliquot of 25 µL of each dilution was plated, in duplicate, in one of the quadrants of a Petri plate containing SDA with chloramphenicol. Next, the plates were incubated at 37 °C for 48 hours. After incubation, yeast colony counts of each plate were quantified using a digital colony counter (CP 600 Plus, Phoenix Ind Com

Equipamentos Cientıficos Ltda, Araraquara, SP, Brazil). The colony-forming unit per milliliter (CFU/mL) was determined.

2.7. Analysis of results

Qualitative and quantitative analyses were performed. In the quantitative analysis, analysis of variance (ANOVA) was applied to the percentages of living cells compared to the control group (considered as 100% viability). This analysis was complemented by multiple comparisons using the Tukey test. Normality and homogeneity of the variances were checked using the Shapiro-Wilk and Levene tests. A 5% significance level was adopted for all statistical tests performed. In addition, in the qualitative analysis, the results of each experimental group were compared with those of the control group (considered as 100% viability), and the proposed treatments were classified according to the cytotoxic effect as: not cytotoxic (inhibition below 25%); slightly cytotoxic (inhibition between 25 and 50%); moderately cytotoxic (inhibition between 50 and 75%); and intensely cytotoxic (inhibition higher than 75%).[19]

3. Results

3.1. Quantitative analysis

The results are summarized in Table 1. For the Alamar Blue assay, ANOVA of two factors was applied: treatment (different groups) and cell type (keratinocytes and the *C. albicans*). In this case, there was a significant interaction effect. Multiple comparisons using Tukey's test determined which groups were statistically different from the others (Table 1). Figure 1 (A, B, C) show the results of the fluorescence and absorbance values from the cellular metabolism assays for the Alamar Blue (keratinocytes and *C. albicans*), MTT (queratinócitos) and XTT (*C. albicans*) tests.

For the Alamar Blue assay, the mean values of cell viability for *C. albicans* was not different from that found for the keratinocytes (Table 1). Furthermore, with regard to the keratinocytes, even for the same test, it was observed that the P+L+ (aPDI experimental group), P+L- (curcumin group) and P-L+ (LED light group) groups showed differences that were statistically significant compared to the P-L- group (group not treated). Concerning *C. albicans*, in the Alamar Blue assay, cell viability was lower in the P+L+ group compared to the other groups. The results of the P+L- and P-L+

groups were similar to each other, and all groups showed a statistically significant difference from the P-L- group.

For the MTT assay, which evaluated the cell viability of the keratinocytes, it was observed that the P+L+ group was statistically different from the other groups. The P+L- and P-L+ groups showed no difference between them and were similar to the P-L- group. In the analysis of cell viability of *C. albicans* (XTT assay), no significant difference was observed between the P+L+ and P-L+ groups. However, the P-L+ group showed no significant difference to the P+L- group, all of which were different P-L-. The results of this study showed no interference in the metabolism of the cells in co-culture, as no differences were observed between the control group (cultured cells by themselves) and the P-L- group (co-culture cells without aPDI).

The data of CFU/mL and survival fraction (%) are shown numerically in Table 2. For this analysis, the number of *C. albicans* colonies was transformed into base 10 logarithm ($\log_{10}$). The analysis of variance applied to the transformed data showed a significant treatment effect ($\rho<0.001$). To identify the difference among the treatments, the Tukey test was applied. The results are shown in Table 2. The aPDI group showed the highest reduction in CFU/mL, which was equivalent to 1.7 $\log_{10}$ when compared to the control group. The P+L-, P-L+, P-L- and control groups were not statistically different.

3.2. Qualitative analysis

The results were also evaluated in accordance with ISO-standard 10993-5[19], which states that inhibition of <25% counts as non-cytotoxic, 25–50% as slight, 50–75% as moderate and >75% as highly cytotoxic. For the Alamar Blue and MTT assays, the cell viability of P+L+ was between 50 and 75% compared to the control group (aPDI was classified as slightly cytotoxic). For the XTT, the cell viability of *C. albicans* was between 25 and 50%, and aPDI was classified as moderately cytotoxic compared to the control group. The results of this study also showed that for all tests, the P+L- and P-L+ groups decreased the number of viable cells compared to the control group, although they had been classified as non-cytotoxic for the Alamar Blue test. In addition, for the MTT assay, all groups except for the P-L- were classified as slightly cytotoxic,

with the cell viability of the keratinocytes being between 50 and 75%. For the XTT assay used for *Candida albicans*, only the P-L- group was considered non-cytotoxic, whereas the P+L- and P-L+ groups showed intermediate inhibition between 25 and 75% of the viable cells being considered slightly cytotoxic. In addition, for the XTT assay, the P+L+ was the most toxic group, scored as moderately cytotoxic.

4. Discussion

Cell culture toxicity assays are the international standard for the initial screening of biomaterials. In this study, the co-culture method was used because monolayer cell cultures are distant from the structure and function of the natural mucosa having multilayered cellular architecture. The interaction between human cells and fungal cells can occur, inhibiting or potentiating the effect of certain substances. Keratinocytes, for example, can act as immunocytes[20,21] and can secrete a variety of pro-inflammatory cytokines.[21,22] These cytokines play a critical role in the development of protective immunity against intracellular pathogens.[21,23] In addition, their contact with microorganisms also leads epithelial cells to produce a variety of antimicrobial proteins, including defensins.[21,24] Thus, the system of co-culture between pathogens and host cells allows for the study of relations between the two organisms, and under controlled conditions, this can lead to the discovery of interaction mechanisms, pathogenicity and resistance at the cellular level.[25] Studying interactions in co-culture has some advantages, such as the ability to manipulate cells in a defined environment and to retrieve and study specific cell types.[26] In addition, it is better to reproduce cell behavior in situ and to provide a physiological basis for future studies of the molecular and genetic mechanisms of disease. Other advantages of the co-culture method are less expensive; relatively less ethical constraints; increased comparability and reproducibility and higher control over the experimental conditions. The transwell membranes were used because epithelial cells in direct contact with fungal cells in vitro has caused extensive oral epithelial cell death by apoptosis.[27] Co-cultivation cell culture systems in a transwell two chamber system allows the separated culture of one cell type on top of a porous membrane and the other cell type at the bottom.[28] Moreover, these systems enable an investigation of immune responses stimulated by the pathogens themselves or their secreted virulence factors including determination of cytokine

release and monitoring of enhanced neutrophil transmigration.[29] For this reason, this study assessed the cytotoxicity of aPDI using human keratinocytes co-cultured with *C. albicans*. The results of this study showed no interference in the metabolism of the cells in co-culture, as no differences were observed between the control group (cultured cells by themselves) and the P-L- group (co-culture cells without any treatment).

Studies have been conducted in order to verify the effectiveness of aPDI in the elimination or reduction of *Candida albicans*[9-15]. Some parameters are important when aPDI is performed, such as concentration of PS, light fluence and pre-irradiation time. The parameters used in this study were previously established based on data obtained by Dovigo et al.[15], in which the highest percentages of reduction of *Candida albicans* biofilms were achieved. Therefore, aPDI was investigated using 40 µM of curcumin, 20 minutes of pre-irradiation and LED illumination at a fluence of 5.28 J/cm^2. Based on the analysis of the results of the current study, both keratinocytes cells and *C. albicans*, the P+L+ group (experimental group aPDI) showed a statistically significant difference when compared to the P-L-group (group not treated). For the Alamar Blue (keratinocytes cells and *C. albicans*) and MTT assays (keratinocytes cells), cell viability was between 50 and 75% compared to the control group (aPDI was classified as slightly cytotoxic). For the XTT, the cell viability of *C. albicans* was between 25 and 50%, and aPDI was classified as moderately cytotoxic compared to the control group. Toxicity of aPDI is due to the production of reactive oxygen species that cause oxidative damage to the target cells, including microbial cells and human cells.[7] The results of this study are in agreement with other studies.[16,30] Andrade et al.[30] evaluated the effects of aPDI mediated by curcumin against the planktonic and biofilm cultures of reference strains of *C. albicans, Candida glabrata* and *Candida dubliniensis*. The authors observed a complete inactivation of fungal cells in the suspension and an inhibitory effect on the viability of the biofilm cells. Similarly, Dovigo et al.[16] evaluated aPDI mediated by curcumin against clinical isolates of *C. albicans, Candida tropicalis* and *C. glabrata,* in both planktonic and biofilm forms. The authors demonstrated that the association between curcumin at the concentration of 20 µM and light dose of 5.28 J/cm^2 was effective for the inactivation of a planktonic solution of *C. albicans*. However, for reducing the cell metabolism of the fungal biofilm, it was necessary to have a higher concentration of curcumin (40 µM) with the same light dose.

In this study, aPDI was toxic to keratinocytes, agreeing to Ribeiro et al.[31] that evaluated the cytotoxicity of aPDI with Photogem associated to blue LED on L929 and MDPC-23 cell cultures. The authors observed that aPDI caused severe toxic effects in normal cell culture, characterized by the reduction of the mitochondrial activity, morphological alterations and induction of necrotic cell death. On the other hand, according to Zeina et al.,[32] the aPDI could be used as a safe alternative to conventional antimicrobial treatments, as this method produced little cytotoxic effect on keratinocytes. Dovigo et al.[13] concluded that curcumin-mediated aPDI proved to be effective for the in vivo inactivation of *C. albicans* without harming the host tissue of mice. In the same way, Carmello et al.[8] described the photoinactivation of *C. albicans* in a murine model of oral candidosis, mediated by Photodithazine®. The authors observed that aPDI was effective for the inactivation of *C. albicans* without causing any harmful effects on host tissues. Importantly, although we used the model of co-culture, this study was conducted in vitro, where the conditions found clinically were not simulated. Thus, although the results have shown that aPDI may represent a promising alternative for the treatment of candidiasis, data interpretation should be made with caution.

The results of this study also showed that for all tests, the P+L- and P-L+ groups decreased the number of viable cells compared to the control group, although they had been classified as non-cytotoxic for the Alamar Blue test. The light may be absorbed by the respiratory chain components, and the primary effects occur in the mitochondria.[33] Damage to mitochondria in particular can lead to apoptosis even at relatively low light doses.[34] In addition, study[35] have reported that the wavelength used in the present study causes oxidative stress that may be associated with the photoexcitation of endogenous intracellular porphyrins. The absorption of these photons would promote energy transfer to oxygen, culminating in the production of ROS.[35] These factors could explain the reduction of the proliferation of keratinocytes and *C. albicans* cells observed in this study in the P-L+ group. In order to minimize the damage to the adjacent healthy tissues, the light source should be carefully applied, and the PS, in turn, should be applied topically only on the infected areas. The results of this study are consistent with other results.[35,36] In a recent study, only light caused significantly longer comet tails, an indication of DNA damage of *C. albicans* when compared to the negative control.[35] Likewise, de Souza et al.[36] found a significant reduction in the number of CFU/ml of *C. tropicalis* when only light was applied, suggesting a possible susceptibility of this

species to laser light. On the other hand, some authors found that the use of light alone did not affect the viability of microbial cells.[8,13,37] These differences may be related to the light source and dose used in different studies.

It was also observed that the curcumin, in the absence of light (P+L-), inhibited cell growth of both cells when co-cultured. Curcumin is a natural component of low molecular weight, which has antioxidant and anti-inflammatory effects. It has been reported that this compound has a cytotoxic effect on cancer cells.[38,39] These effects on inflammatory and cancer cells could explain the cytotoxic effect of curcumin found in this study. Moreover, antifungal effect of curcumin alone has been reported[40], with a minimum inhibitory concentration (MIC value) of 64 mg/L against *C. albicans*. On the other hand, in previous studies, it was shown that the use of curcumin only did not cause any damage to the yeast cells.[8,35] The differences among the results could be explained by the method used in different studies (*in vitro* and co-culture). In addition, different results may be related to different concentrations of curcumin.

Despite the toxic effect of curcumin (P+L-) and light (P-L+) alone, the highest reduction in CFU/mL was found for the P+L+ group. This fact can be explained by the interaction between light and the photosensitizer. The activation of the photosensitizing by light can cause photochemical reactions and induce cellular damages. aPDI exhibits its effects as result of generation of toxic reactive oxygen species by the activation of PS by light at a certain energy level.[41] The reduction in CFU/mL for the P+L+ group was equivalent to 1.7 $\log_{10}$ when compared to the control group. Similar results were found by Mima et al.[37], who conducted an in vivo oral candidiasis study in immunossuppressed mice to evaluate the efficacy of aPDI using Photogem at 400, 500 and 1000 mg/L for 30 minutes followed by illumination with LED light (305 J/cm^2) at 455 or 630 nm. For each Photogem concentration, aPDI resulted in 1.05, 1.59, and 1.40 $\log_{10}$ reductions, respectively, of *C. albicans* colony counts. On the other hand, only the use of 80 μM of curcumin associated with 37.5 J/cm^2 of LED light was able to promote a drop of 4.65 log 10 in *C. albicans* viability in a murine model of oral candidosis[13].

In this study, the MTT (which measured the viability of keratinocytes) and XTT (which measured the cell viability of fungal cells) tests showed the most significant results of cell death in comparison with the Alamar Blue assay (performed for both types of cells). For the MTT assay, all groups except for the P-L- group were classified as slightly cytotoxic, with the cell viability of keratinocytes being between 50 and 75%. For the same assay, the P+L+ group (experimental group aPDI) showed a statistically

significant difference when compared to other groups. This group was classified as slightly cytotoxic but showed only 51.7% of viable cells (borderline value). Therefore, it can be suggested that the aPDI has changed cellular metabolism of keratinocytes. On the other hand, histological analysis revealed that aPDI had no adverse effects on the adjacent tissue in a murine model of oral candidiasis using the same parameters used in this study[13]. The mechanisms of action of aPDI have been previously explained in this study. For the XTT assay used for *C. albicans*, only the P-L- group was considered non-cytotoxic, whereas the P+L- and P-L+ groups showed intermediate inhibition between 25 and 75% of the viable cells being considered slightly cytotoxic. In addition, the P+ L+ group was the most toxic group, scored as moderately cytotoxic. In the Alamar Blue test, considering each group separately, aPDI had a greater cytotoxic effect on cell viability. These results are in agreement with the data reported Andrade et al.[30], who demonstrated that, when organized in biofilm cultures, aPDI, with the same parameters (40 μM of curcumin, 20 minutes of pre-irradiation and LED illumination at a fluence of 5.28 J/cm^2), promoted significant reduction in cell metabolism.

Thus, the P+L+ group for both types of cells (keratinocytes and *Candida*) was classified as slightly cytotoxic because the cell viability was between 50 and 75% compared to the control group. The other groups had less than 25% of cell inhibition, and the groups were classified as non-cytotoxic. The assays measure mitochondrial and metabolic activity. The difference is that the Alamar Blue is a fluorimetric test, whereas MTT and XTT are colorimetric tests. According to Nociari et al.,[42] the Alamar Blue assay is a one-step extremely simple, reproducible, economical and non-toxic procedure used to evaluate cell-mediated cytotoxicity. The single-step procedure permits the measurement of a large number of samples and contributes to the high accuracy of the technique. According to the above-mentioned aspects, the highest value of cytotoxicity found for the MTT and XTT tests could be explained according to the sensitivity and high variability of these tests.

Even though the results show cytotoxic effect of aPDI on epithelial cells, they are considered very important due to the difference between cells. Keratinocytes were cultured in monolayers, while fungal cells were grown as biofilms. *Candida* spp. multiplies as a community of adherent cells covered in a self-produced extracellular matrix. These biofilms show innate resistance to several drug classes and are capable of tolerating elevated concentrations of antifungal agents[43,44]. In addition, previous studies reported that microorganisms organized in biofilms are less susceptible to aPDI

compared with planktonic cells[15,45]. According to Pereira et al.,[45] the effects of aPDI occurred predominantly in the outermost layers of the biofilms, suggesting a difficulty of PS to penetrate into the microbial biofilms. This question may explain why aPDI was not able to eliminate all the *Candida* biofilm cells in the present study.

Concerning to measure of CFUs, similar results with the Alamar Blue test were observed. On the other hand, the data were different from XTT test. The XTT assay is a colorimetric method of quantifying fungal growth by measuring the cell metabolism. The CFU count method, considered the gold standard of microbiological assays, evaluates the ability of cell proliferation, thus even cell submitted to sublethal doses of an antifungal treatment are able to recover and grow during the incubation period (48 hours). It may justify the results of the present investigation, since candidal biofims submitted to curcumin and light alone have shown reduced metabolic activity (XTT assay) but not reduced colonies growth (CFU/mL). Therefore, it is important to complement the tests used to evaluate cell viability instead of relying on the results of a single assay. It is also important to note that different parameters for the cytotoxicity analysis should be used because the cytotoxic substances can act in different ways on cellular metabolism. In addition, it should be emphasized that the results obtained from preliminary cytotoxicity tests have limitations with respect to their direct correlation with clinical situations. Therefore, the clinical implications of the results from this study should be investigated to explore the potential application of this protocol for candidiasis treatment, mainly in immunocompromised patients.

5. Conclusion

According to the findings, and within the limitations of this study, it can be concluded that:

1. aPDI inhibited the growth of keratinocytes and *C. albicans* in all tests;
2. Light (P-L+ group) and curcumin (P+L- group) alone reduced the metabolism of cells compared to the control group, but not the ability of *C. albicans* to growth;
3. The cell interaction in co-culture did not influence the metabolism of keratinocytes and *C. albicans*.

Conflict of interest statement

None declared

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Table 1 – Percentages and standard deviation obtained by different tests.

Treatment	Alamar Blue				MTT		XTT	
	keratinocytes %		*Candida* %†		%		%	
P+L+	72.2 (1.4)	a	66.1 (3.6)	a	51.7 (3.9)	a	39.9 (7.2)	a
P+L-	76.0 (4.4)	a	81.1 (4.2)	b	64.3 (4.6)	b	61.3 (8.5)	b
P-L+	76.9 (2.3)	a	83.9 (3.7)	b	62.3 (3.0)	b	52.2 (2.4)	ab
P-L-	94.0 (0.8)	b	94.0 (1.4)	c	76.3 (4.9)	b	90.3 (5.0)	c
Control	100.0	b	100.0	c	100.0	c	100.0	c

Means followed by the same letter in the column are not significantly different (Tukey's test: p>0,05)

† *Candida* mean always equal to the mean keratinocytes to the same treatment

Table 2: CFU/mL (Log_{10}) e standard deviation of the values obtained for each group.

Treatment	CFU Log_{10}(CFU/mL)		%
P+L+	5.39 (0.15)	a	76,6 (6,1)
P+L-	6.51 (0.25)	b	92,6 (8,2)
P-L+	6.49 (0.18)	b	92,3 (7,8)
P-L-	6.83 (0.33)	b	96,9 (6,8)
Control	7.07 (0.48)	b	100

Means followed by the same letter in the column are not significantly different (Tukey test: $\rho>0.05$).

Scheme 1: *Candida albicans* and keratinocytes in co-culture.

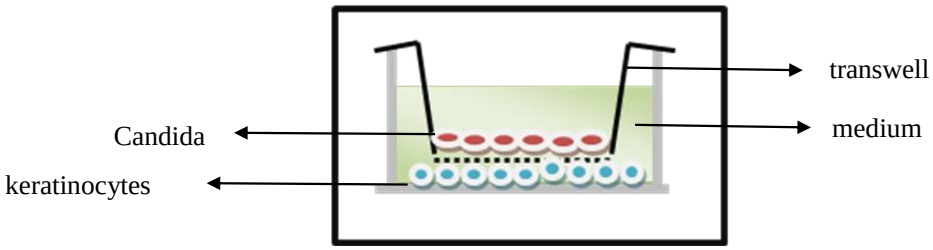
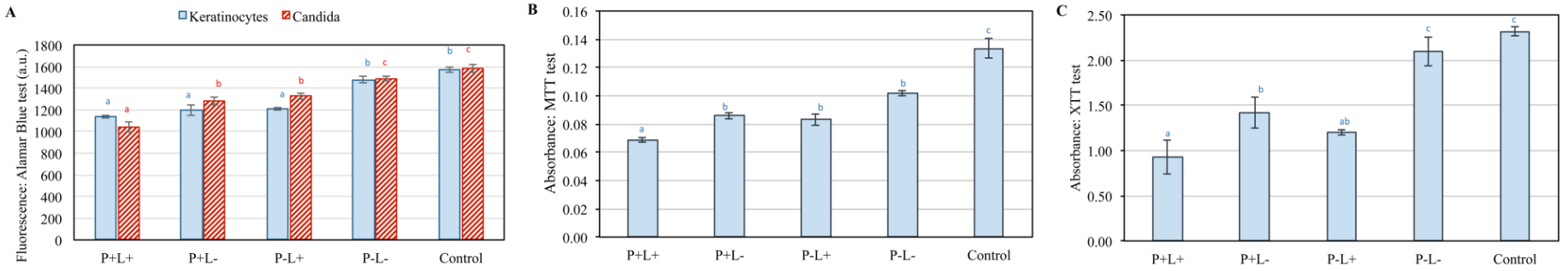


Figure 1: Mean values of metabolic activity of keratinocytes and *Candida albicans* in different tests.



A: Alamar Blue (keratinocytes and *Candida*), B: MTT (keratinocytes) and C: XTT (*Candida*). The vertical bars indicate standard deviations. The same lower case letter denotes non-significant differences among groups ($p > 0.05$).