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Photodynamic Therapy in *Candida* spp. and Epithelial Cell Cultures: An *In vitro* Study

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Authors' contributions

This work was carried out in collaboration among all authors. Author SAM did design the study, managed the literature searches, performed the statistical analysis, wrote the protocol, wrote the first draft of the manuscript and collected the data of the manuscript. Authors ABT and RFC did assist the literature searches and data collection. Authors SDDO and DCM did assist the data collection. Author KC did design the study, wrote the protocol, performed the statistical analysis and corrected the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The objective of this study was to determinate the photodynamic effect using methylene blue and diode laser on *Candida* spp. and epithelial cells *in vitro.*
Study Design: An *in vitro* study was carried on using cultures of *Candida* spp. and HEp-2 cells.
Place and Duration of Study: This study was developed at the School of Biosciences of the Department of Microbiologic Sciences and at the Laboratory of Pneumology of the Biomedical Research Institute at São Lucas Hospital, both at the Pontifical Catholic University of Rio Grande do Sul, located in Porto Alegre, Brazil, between January and December.
Methodology: Cultures of *Candida* spp. and HEp-2 cells were submitted to aPDT with methylene blue (100 µg/mL) and indium-gallium-aluminum-phosphide (InGaAlP) diode laser at 100 J/cm^2, 270 J/cm^2 and 450 J/cm^2.
Results: All of these three doses caused significant inactivation of *Candida* spp. ($P<0.05$). At 450 J/cm^2, the viability of *Candida* spp., based on colony forming units (CFUs), was reduced by 72.42%, followed by lesser effects with 270 J/cm^2 and 100 J/cm^2, 45.87% and 22.83%, respectively. PDT decreased CFUs by 50.44% in *C. albicans*, while other *Candida* species showed a 41.18% decline in CFUs ($P<0.05$). With regard to the average effect of the three doses tested in PDT group, HEp-2 cell viability, based on trypan blue exclusion, declined to 70.81%, which was significantly lower than that observed in the control group (86.21%).
Conclusion: Methylene blue plus laser exposure (100 J/cm^2, 270 J/cm^2 and 450 J/cm^2) caused significant inactivation of *Candida* spp. Photodynamic inactivation of the epithelial cells based on cell viability was 2.24-fold lower than the inactivation of *Candida* spp., which suggests a safety margin for *in vivo* application.

Keywords: Candida; Photochemoterapy; Lasers; Methylene Blue.

1. INTRODUCTION

The fungus *Candida* sp., mainly the species *C. albicans* is frequently found as a commensal in digestive and vaginal tracts of the human host. *C. albicans* is part of the normal microbiota of individuals in more than 60% of the population, who do not show any clinical manifestation of disease, thereby considered as carriers of the fungus [1,2]. Oral candidosis is considered the "sick patient's illness"[3] because it needs one or more predisposing factors for its establishment [4]. Effective treatment of candidosis requires correction of the predisposing factor, since the absence of this factor makes it difficult for the fungal disease to occur [5]. Species of *Candida* have become very prevalent in systemic infections. In addition, resistance of *Candida* spp. to traditional antifungals (as polyenes, azoles, and echinocandins) has increased because of excessive and uncontrolled use of these drugs. The number of pathogenic fungi has been increasing due to the high frequency of hospital infections in immunocompromised patients and there are currently few safe antifungals, most of which are fungistatic agents. Furthermore, intensive use of antibiotic therapy progressively leads to the emergence of resistant strains [6-9].

Antimicrobial photodynamic therapy (aPDT) consists of the combination of a generally exogenous photosensitizing agent administered topically or systemically and a visible light source with the objective of causing microbial inactivation [10,11]. This technique should not be considered as a replacement for antibiotics, but as a new treatment modality for localized infections and as an effective complementary treatment for oral infections [12,13] especially in cases of resistant microorganisms. The sensitivity of microorganisms to aPDT indicates the applicability of the technique *in vivo*, for localized and superficial infections of known microbiota [14].

Isolated application of laser (light amplification by stimulated emission of radiation) did not affect fungal cells viability [15-17], where the application of 21 J of energy to *Candida* spp. yeasts and hyphae did not change their viability [16]. However isolated application of the laser caused a reduction in the CFUs of *C. tropicalis*, which suggested the susceptibility of this species to laser light [18,19] and also a susceptibility of *C. krusei* [19].

The aim of the present study was to determine the antifungal efficacy *in vitro* of aPDT with methylene blue and an indium gallium aluminum phosphide (InGaAlP) diode laser in cultures of *Candida* spp., and its effect on cultures of epithelial cells as well.

2. MATERIALS AND METHODS

2.1 Sample

The sample comprised cultures of *Candida* spp. and cultures of epithelial cells. *Candida* cultures were obtained from 38 adult patients, of both genders with diagnosis of oral candidosis [20]. The specimens were collected with a dry, sterile swab, the material was cultivated in Sabouraud-4% dextrose agar (Merck, Darmstadt, Germany) with chloramphenicol (16 mg/mL, Neo Química, Anapolis, Brazil), and incubated at 30ºC for 48 h. The species were identified by phenotypic tests (germ tube formation, microculture in rice agar, CHROMAgar™ *Candida* and assimilation of carbohydrates). In the identification tests, *C. albicans* ATCC (American Type Culture Collection) 28367, *C. dubliniensis* CBS (Centraalbureau voor Schimmel cultures) 7987 and *C. krusei* ATCC 6250 served as standards.

The identification of C. albicans was confirmed by PCR (Polymerase Chain Reaction), using the oligonucleotides CAL5 (TGTTGCTCTCTCGG GGGCGGCCG) and NL4CAL (AGATCATTATG CCAACATCCTAGGTTAAA), in the 5' to 3' sequence, which produced an amplification product of 175 bp. [21,22].

HEp-2 ATCC CCL-23® cell line was grown in DMEM medium (Dulbecco's Modified Eagle Medium, Sigma, St Louis, USA), supplemented with penicillin (100 UI/mL, Sigma), streptomycin (100 μg/mL, Sigma), gentamycin (50 μg/mL, Sigma) and 10% fetal bovine serum (Gibco, Grand Island, USA). The cultures were incubated and maintained in a humidified incubator at 37°C, 5% CO_2 atmosphere. After 7 days, the cells were washed with PBS and trypsinized with trypsin/EDTA solution (0.25% trypsin/0.02% EDTA) for 10 min. at 37°C and afterward centrifuged (8500 x g for 3 min.) to obtain a cell suspension [23].

2.2 Laser and Photosensitizer

The diode laser (InGaAlP) used was Thera Lase (DMC Equipment, São Carlos, Brazil): 685 nm, continuous emission, 35 mW. The doses were 100 J/cm^2 (2.8 J, 1 min 21 s), 270 J/cm^2 (7.5 J, 3 min 37 s) and 450 J/cm^2 (12.6 J, 6 min) considering an area of 0.028 cm^2. The photosensitizer used was methylene blue (Sigma) at a concentration of 100 μg/mL [24] with an incubation time of 5 minutes. These three doses could provide a viable treatment time *in vivo*.

2.3 aPDT in *Candida* spp.

After growth in YPD medium (yeast peptone dextrose, Sigma) at 30°C for 48 h, serial dilutions were made with *Candida* spp. cultures using sterile saline. Aliquots of 100 μL of the 1:1000 dilution were used for the application of the treatments, carried out in duplicate in a 96-well culture plates, with single application. The doses were: 100 J/cm^2, 270 J/cm^2 and 450 J/cm^2.

2.3.1 aPDT test groups

100 μL of 1:1000 dilution of *Candida* sp. were used for the treatments (in the 3 doses) plus 90 μL of PBS and 10 μL of methylene blue (100 μg/mL, 5 min).

2.3.2 Control groups

100 μL of 1:1000 dilution of *Candida* sp. and 100 μL of PBS, where no treatment was applied. Each laser dose had its corresponding control group, where they were handled and evaluated simultaneously.

Just after the treatments, the cell suspensions were centrifuged (3 min, 8500 x g) and washed 3 times to remove residual methylene blue. After centrifugation, aliquots of 100 μL of each microtube were seeded on Sabouraud dextrose agar and incubated at 30ºC for 48 h, and CFUs were counted. Viability (percentage survival) was determined considering the percentage of CFUs of the test group in relation to that of the corresponding control group.

During the treatments, the cultures were protected from light using an opaque plastic box.

2.4 PDT in Epithelial Cells

After cultivation, HEp-2 cells were resuspended in PBS and the suspension was transferred to 96-well culture plates, in aliquots of 100 μL at a density of 2 x 10^5 cells/mL.

The samples were distributed into groups, and the treatments performed in triplicate:

PDT group (application of laser and methylene blue) and *Control group – methylene blue* (application of methylene blue without laser): 100 μL of the epithelial cell suspension, 90 μL of PBS and 10 μL of methylene blue (100 μg/mL, 5 min).

Control group - laser (application of laser without methylene blue) and *Control group -untreated*:

100 µL of the epithelial cell suspension, 100 µL of PBS.

The laser doses employed were 100 J/cm^2, 270 J/cm^2 and 450 J/cm^2. During the treatments, the cultures were protected from light using an opaque plastic box. After the treatments, the samples were centrifuged (3 min, 8500 x *g*) and washed 3 times to remove residual methylene blue. Afterward 100 µL of trypan blue (0.4%, w/v) were added to the cell suspension and the cells counted in a Neubauer chamber (Optik Labor, Darmstadt, Germany) using a light microscope (MC80DX, Zeiss, Germany) with a 40 x objective. Viability of the epithelial cells was determined by trypan blue exclusion, counting stained and unstained cells in 16 fields.

2.5 Statistical Analysis

The results obtained were analyzed by descriptive statistics, Student's *t*-test for paired samples and analysis of variance (ANOVA), complemented by Tukey's test for multiple comparisons, at the 5% level of significance.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Viability of *Candida* spp.

From 38 samples of *Candida* spp. collected from patients, 24 were identified as *C. albicans* and 14 as *C*. non-*albicans*. Comparing the number of CFUs between the test and control groups, significant difference (*P*<0.05) was found for the three doses employed (Table 1, Fig. 1).

By calculating the mean percentage of viable *Candida sp*. CFUs after PDT at different doses, the following results were obtained: 77.17% (±18.32) of viable CFUs for 100 J/cm^2; 54.16% (±19.73) of viable CFUs for 270 J/cm^2 and 27.58% (±12.26) of viable CFUs for 450 J/cm^2.

When considering the different *Candida* species, the mean viability of *C. albicans* was 49.56% (±26.12), while for *C.* non-*albicans*, this value was 58.82% (±26.35). These values showed statistically significant differences, that is regardless of dose, *C. albicans* presented a significantly lower mean percentage of CFUs than *C.* non-*albicans* species (Fig. 2).

C. albicans was more sensitive to PDT compared to *C.* non-*albicans* species. No significant difference (*P*>0.05) in viability was observed between the two groups when compared at different doses (Table 2).

3.1.2 Viability of epithelial cells

At 100 J/cm^2 the mean viability of epithelial cells in PDT group was 77.23% (±3.31). The minimum and maximum values of cell viability were respectively 75.07% and 81.04%. In control group 1 (laser) the minimum and maximum viability values were respectively 77.1% and 96.19%, with mean 86.53% (±9.55). Control group 2 (methylene blue) presented a mean viability of cells of 78.83% (±6.30), with minimum value of 73.66%, and a maximum of 85.84% of cell viability. In control group 3 (without laser and without methylene blue) the mean viable cells were 90.23% (±0.70) and the minimum and maximum values were respectively of 89.59% and 90.97% of viable epithelial cells.

At 270 J/cm^2 the mean viability in PDT group was 69.82% (±6.08), and the minimum and maximum values of cell viability were 64.09% and 76.19%. In control group 1 (laser) the minimum and maximum viability values were respectively 68.32% and 93.51%, with a mean of 79.29% (±12,91) of viable cells. Control group 2 (methylene blue) presented a mean of viable cells of 80.27% (±4.08) with a minimum value of 76.59% and a maximum of 84.66% of cell viability. In control group 3 (without laser and without methylene blue) the mean of viable cells was 80.56% (±14,58) with minimum and maximum values being respectively 64.67% and 93.33% of viable epithelial cells.

Table 1. Colony-forming units (CFUs) of *Candida* sp. after PDT application at doses of 100 J/cm^2, 270 J/cm^2 and 450 J/cm^2.

Dose	***Candida* spp. (n=38)**				***P****
	Test group		**Control group**		
J/cm^2	**Mean**	**Standard deviation**	**Mean**	**Standard deviation**	
100	78.28	27.79	99.78	23.52	**<0.05**
270	52.93	19.54	100.74	29.71	**<0.05**
450	28.87	15.71	103.54	26.30	**<0.05**

**t-test for paired samples*

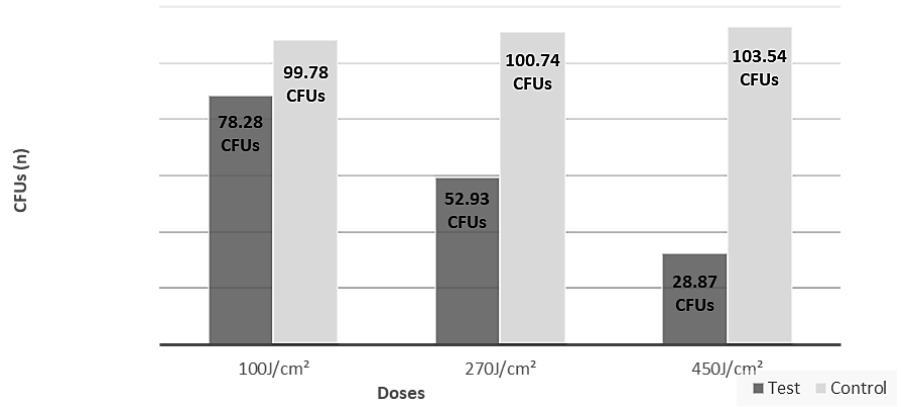

Fig. 1. Mean of *Candida* spp. colony-forming units (CFUs) after PDT application at doses of 100 J/cm², 270 J/cm² and 450 J/cm²

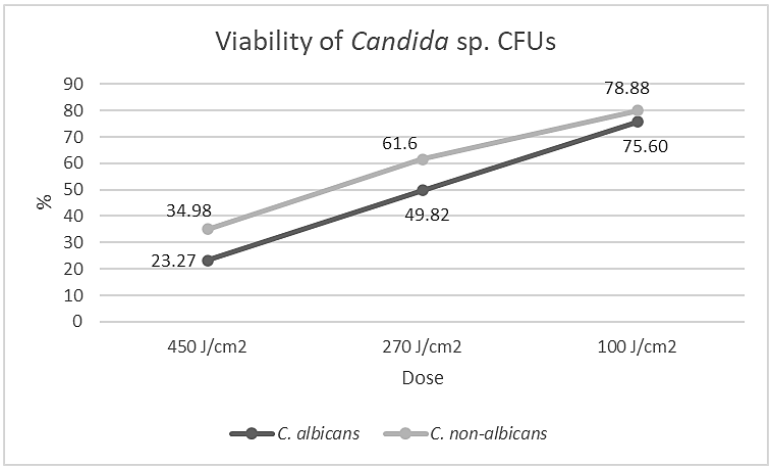

Fig. 2. Viability of *Candida* spp. after aPDT at doses of 100 J/cm², 270 J/cm² and 450 J/cm²

Table 2. Viability of *Candida* spp. after aPDT application at doses of 100 J/cm², 270 J/cm² and 450 J/cm²

PDT Dose	Viability				Total *Candida* sp.	
	C. albicans (n=24)		*C.* non-*albicans* (n=14)			
	Mean (%)	Standard deviation	Mean (%)	Standard deviation	Mean (%)	Standard deviation
100 J/cm²	75.60	17.68	79.88	19.74	77.17 [A]	18.32
270 J/cm²	49.82	18.03	61.60	20.95	54.16 [B]	19.73
450 J/cm²	23.27	6.34	34.98	16.22	27.58 [C]	12.26
Mean	49.56 [b]	26.12	58.82 [a]	26.35	52.97	26.47

Means followed by different uppercase letters and means followed by different lowercase letters differ significantly. ANOVA in randomized blocks, Tukey's test at the 5% level of significance

At 450 J/cm² the epithelial cells presented a mean viability of 65.39% (±1.77) in PDT group. The minimum and maximum values of cell viability were 63.58% and 67.12% in this group. In control group 1 (laser) the minimum and maximum viability values were respectively 74.18% and 93.09%, with a mean of 85.65% (±10.08) of viable cells. Control group 2 (methylene blue) presented a minimum value of 53.66% and a maximum of 84.64% of cell viability, with a mean of cell viability of 66.47% (±16.17). In control group 3 (without laser and without methylene blue) minimum and maximum values were respectively of 79.67% and 96.11%

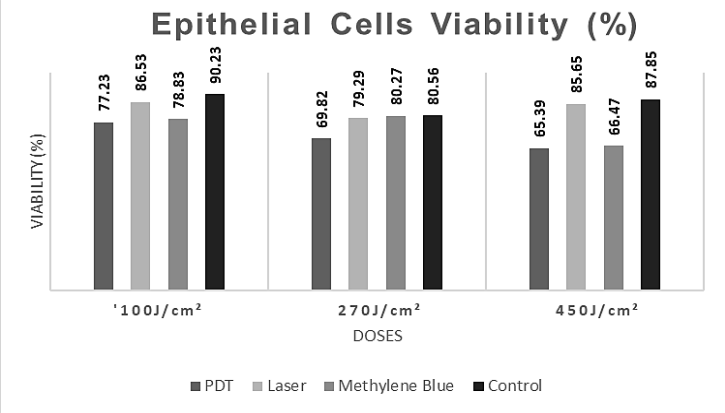

Fig. 3. Viability of epithelial cells in different treatments at 100 J/cm², 270 J/cm² and 450 J/cm².

Table 3. Viability of epithelial cells at laser doses of 100 J/cm², 270 J/cm² and 450 J/cm².

Treatment	Dose						Total	
	100 J/cm²		270 J/cm²		450 J/cm²			
	Mean (%)	Standard deviation	Mean (%)	Standard deviation	Mean (%)	Standard deviation	Mean (%)	Standard deviation
PDT	77.23	3.31	69.82	6.08	65.39	1.77	70.81 ^{B}	6.29
Laser	86.53	9.55	79.29	12.91	85.65	10.08	83.82 ^{A}	10.08
Methylene blue	78.83	6.30	80.27	4.08	66.47	16.17	75.19 ^{AB}	11.07
Control	90.23	0.70	80.56	14.58	87.85	8.22	86.21 ^{A}	9.44
Total	83.20	7.57	77.49	10.02	76.34	14.07	79.01	11.00

PDT: methylene blue and laser; Laser: application of laser without methylene blue; Methylene blue: application of methylene blue without laser; Control: without methylene blue or laser. Means followed by different uppercase letters differ significantly. ANOVA, Tukey's test at the 5% level of significance

of viable epithelial cells, with a mean of 87.85% (±8.22) of viable cells.

Fig. 3 shows the viability of epithelial cells in different treatments at 100 J/cm², 270 J/cm² and 450 J/cm².

In comparing the different treatments, independent of dose, a significant difference in viability (P<0.05) was found for the epithelial cells, but there was not significant difference (P>0.05) among the doses used (Table 3)

3.2 Discussion

aPDT acts to inactivate *Candida* spp. causing irreversible damage and cell death. Laser and photosensitizer association generates reactive oxygen species (ROS) with two types of photochemical reactions: type I, which occurs after photosensitizer degradation by direct action of light and generation of free radicals or superoxide ions that interact with biomolecules. Type II reaction occurs when the photosensitizer reacts with molecular oxygen and forms singlet oxygen, highly reactive and the main cytotoxic agent of antifungal aPDT. ROS can affect many types of organic molecules (nucleic acids, lipids and amino acids) and they are responsible for irreversible damage to cell membranes (cytoplasmic, mitochondrial, lysosomal and nuclear). Many of the cellular responses are centered on mitochondria inducing cellular apoptosis through the release of cytochrome c and activation of caspases. ROS disrupt *Candida* spp. cytoplasmatic membrane and cause an increase in cellular permeability and subsequent damage to intracellular targets [24-29].

The effect of aPDT on cultures of *Candida* spp. was determined and cytotoxicity was found to be directly proportional to the laser dose applied. aPDT at a single dose of 450 J/cm² was considered effective in the inactivation of *Candida* spp. Kömerik et al. [30] showed that PDT at 340 J/cm² did not cause clinical or histological changes in the oral mucosa of rats. In humans laser doses of 250 to 300 J/cm² have been applied to the skin in anticancer PDT [31] with recovery of the epithelium after a few weeks.

However in the oral cavity these authors did not apply more than 200 J/cm^2. aPDT at 100 J/cm^2 was found to be less than effective causing a 22.83% decrease in CFUs of *Candida* spp. in the present study. Perhaps successive application of 100 J/cm^2 would lead to a greater degree of inactivation of *Candida* spp. Guidolin et al. [32] in their systematic review on PDT (mainly using Photofrin™ as a photosensitizer) and colorectal cancer in human case series (n=137) observed that doses ranged from 32 J/ cm^2 to 500 J/cm^2 with the most commonly used light dose being around 200 J/cm^2. Complications observed were a skin photosensitivity reaction. Cure/complete ablation of tumor was reported in 40% and partial response was reported in 43.2% of cases.

Energy provided by the highest dose in the present study was 12.6 J. Wilson et al. [33] used 12.2 J on bacterial biofilms *in vitro* and demonstrated effective inactivation of *Streptococcus sanguinis*. Langmack et al. [34] used 108 J in humans by applying 12.6 J/cm^2 to treat basocellular carcinoma. Observed side effects were moderate erythema and slight peeling. Although the dose used was low (12.6 J/cm^2), the energy applied was high (108 J) due the long period of application (30 min). As another treatment option in cases of denture stomatitis (a common oral fungal infection caused by upper oral dentures) aPDT using Light-emitting diode - LED (the most popular light source) on acrylic dentures could prevent or treat this fungal infection [35] instead of applying aPDT to the oral mucosa.

In the present study, aPDT was applied to yeasts of *Candida* spp. (cultures in YPD medium). According to Jackson et al. [16] yeasts as well as hyphae of *Candida* spp. are sensitive to this treatment, although hyphae are more sensitive. The authors employed an energy of 21 J, that is 1.66 times greater than the 450 J/cm^2 of the present study, and they determined that the sensitivity of hyphae was 175 times greater than that of yeast. The greater susceptibility may be associated with differences between hyphae and yeast in the expression of target sites for cytotoxic substances and protective antioxidants [25]. As 72.42% of yeasts in the present study were sensitive to aPDT at 450 J/cm^2, it is possible that hyphae which are the pathogenic form of candidosis are even more sensitive. Such supposition should be investigated by *in vitro* studies with the induction of hyphae. Also, according Costa et al. [36], *Candida* spp. in biofilms have been shown to be less susceptible to aPDT than fungi in the planktonic phase, which may be due to biofilm heterogeneity, protection of yeast by extracellular matrix material, and reduced penetration of the photosensitizer into a biofilm. A systematic review by Wiench et al. [37] showed that of the studies carried out with planktonic cells, only one showed eradication of *C. albicans* and the others showed only partial elimination. In yeast biofilms, there are partial and a statistically significant cell growth inhibition. aPDT *in vivo* with toluene blue exhibited some effects against *Candida* spp. but its clinical use for oral infections still requires further investigations. Zhang et al. [38] observed rapid inactivation of mature mixed biofilm (*C. albicans* and *C. tropicalis*) *in vitro* using toluene blue (1 mg/mL) and LED (500 or 750mW for 1 or 2 minutes), inactivating more than 90% and 99% of fungi respectively.

In this present study regardless of the dose used, *C. albicans* was significantly more sensitive to aPDT compared to non-*albicans* species, with inactivation levels of 50.44% and 41.18% respectively. This is a favorable result since *C. albicans* is the predominant fungal species in the oral cavity [1,39]. Strakhovskaya et al. [40] found that *C. guilliermondii* was 1.6 to 1.7 times more sensitive than *C. albicans*. Dovigo et al. [41] found that aPDT had a fungicidal effect on fungal suspensions, being effective in inactivating *C. albicans, C. dubliniensis and C. tropicalis* and promoting a significant reduction in the viability of *C. krusei.* Souza et al. [18] observed that aPDT (10.5 J, 28 J/cm^2) decreased the number of CFUs by 91.6% for *C. krusei*, 88.6% for *C. albicans*, 84.8% for *C. dubliniensis* and 82.3% for *C. tropicalis*. This result differed from the findings of the present study, in which a greater amount of energy was applied - 12.6 J provided by a dose of 450 J/cm^2 eliminating 76.73% of *C. albicans* and 65.02% of *C.* non-*albicans*. Lavaee et al. [42] showed that aPDT using methylene blue as photosensitizer (1.92 J/cm^2 for 60 seconds) showed an antifungal effect only on *C. albicans*. Combination of methylene blue and silver nanoparticles did not have any effect on *C. albicans*, but did on *C. glabrata and C. parapsilosis* concluding the effect of aPDT was strain and photosensitizer dependent.

For the three different doses used, the mean viability of epithelial cells was 70.81%, that is an inactivation of 29.19% of cells by PDT. PDT of epithelial cells at 450 J/cm^2 resulted in mean viability of 65.39%. In other words, the most effective dose in inactivating *Candida* spp. in the

present study is also seems to allow a safety margin for its application *in vivo*.

In the present study, despite having a similar effect to that of methylene blue alone, PDT caused more damage to epithelial cells than without treatment. Zeina et al. [23] found that application of visible light alone with an energy of 452 J did not affect the keratinocyte proliferation [24]. Viability of epithelial cells in the present study did not show a significant difference between the untreated group (86.21%) and laser group (83.82%), indicating that the isolated application of laser did not affect cell viability even at 450 J/cm^2, while PDT did.

As the yeast *Candida* sp. is a eukaryotic microorganism like epithelial cells and both have a greater number of target organelles that bind to the photosensitizer, compared to bacteria, this could explain the greater sensitivity of bacterial cells compared to yeast and host cells. Cell volume should also be considered, as *Candida* spp. and keratinocytes are 25 to 50 times larger than bacteria such as *Staphylococcus* spp. and *Streptococcus* spp. The mortality rate of keratinocytes was on average 18 times lower than that of *Candida* spp. and 200 times smaller than that of bacteria [17]. Such finding is in agreement with the results of the present study, since HEp-2 epithelial cells were more resistant than *Candida* spp. for PDT. Furthermore, more studies are needed to prove that PDT is valuable for use in daily clinical practice, since aPDT against *Candida* spp. has superior performance compared to conventional antifungal therapies [25].

4. CONCLUSION

aPDT with methylene blue and InGaAlP laser at 100 J/cm^2, 270 J/cm^2 and 450 J/cm^2 caused substantial inactivation of *Candida* spp., where *C. albicans* was more sensitive compared to other species. HEp-2 epithelial cells were less sensitive to PDT than *Candida* spp., showing photodynamic inactivation 2.24 times lower than that of fungal cells, which suggests the existence of a safety margin for *in vivo* application. More *in vitro* and animal model studies are needed to better understand the results obtained.

CONSENT AND ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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