Evaluation of dual application of Antifungal Photodynamic Therapy - afPDT in *Candida albicans* Fluconazole resistant using 1,9-Dimethyl-Methylene Blue zinc chloride double salt (DMMB) and red LED (Λ630 ηm ± 1 ηm): *in vitro* study.

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Abstract

This study aimed to evaluate, in vitro, the efficacy of antifungal photodynamic therapy - afPDT using DMMB associated with the red LED (41.2mW, λ630ηm ± 1 μm) in planktonic cultures of *Candida albicans*. The tests were performed using the ATCC 90228 strain grown at 37°C for 24-h, according to a growth curve of *C. albicans*. The colonies were resuspended in sterile saline adjusted to a concentration of 2 x 10^8 cells / mL, with three experimental protocols being tested (Protocol 1, 2 and 3) with a fixed concentration of 750 ηg/mL obtained through the IC_{50}, and energy density 20 J/cm^2. Protocol 1 was carried out using conventional afPDT, Protocol 2 was applied double afPDT in a single session, and Protocol 3 was applied double afPDT in two sessions with a 24-h interval. The results showed logarithmic reductions of 3 and 4 logs of total fungal load in protocols 3 and 2 respectively, with Protocol 2 the best result recorded with a reduction of 99.991% ($p < 0.0001$). Our results indicated that double application of afPDT was the most effective in inhibit the proliferation of *Candida albicans* (99.991% inhibition).

Keywords: *C. albicans*; Taylor Blue; PDT; Double application; Photoinactivation.

1. Introduction

Fungi are disease-causing agents in humans being the Candida genus the most studied yeast species. This fungi can cause a many diseases such as infections in superficial mucous membranes in which their form of yeast changes to filamentous one (vulvovaginal and oropharyngeal candidiasis) that can become highly invasive threatening human lives [1]. In addition, *C. albicans* resistance rates for some antifungals used in its control are high: amphotericin B (2.9%), fluconazole (5.9%), itraconazole (4.2%) and voriconazole (2.5 %) [2,3].

Infections caused by fungi represent a fundamental challenge for public health, with great socioeconomic importance, mainly due to resistant species of traditional antifungals, requiring the development of scientific research that seeks new therapeutic modalities including PDT to combat these microorganisms more efficiently [4].

Increased resistance to *candida ssp*. to microbiocidal antifungals in medical practice and their side effects, arouse interest in new therapies such as afPDT (Antifungal Photodynamic Therapy), which does not generate resistance from the microorganism and are effective in controlling the pathology [5,6]. amPDT has been advocated as a therapeutic alternative to antimicrobial agents, including fungi, in order to suppress more superficial species, since excessive use of chemotherapeutic drugs increases the resistance of microorganisms [7].

amPDT follows variable standards that define the nature of its protocol models, whose basic parameters such as: pre-irradiation time (clinically acceptable), concentration of the photosensitizer, energy density and the number of applications of the technique, must be according to the patient's needs and the type of target cell [8]. The low cost, simplicity of the technique, the inability to generate microbial resistance and the possibility of multiple and repeated applications arouse the interest of the technique and its clinical application [9]. However, the photodynamic effect depends on certain factors: the concentration of the photosensitizer used, pre-irradiation time (PIT), combined with density-based light irradiation parameters that will activate the photosensitizer to produce EROS reactive oxygen species [10-13].

Photosensitizers derived from phenothiazines have been widely used in research involving PDT (Photodynamic Therapy). Phenothiazines are tricyclic heteroaromatic compounds, blue dyes, such as the toluidine blue dye, methylene blue and Taylor blue, which have demonstrated efficiency in amPDT [8]. Low concentrations of the photosensitizer do not produce cytotoxic action on healthy cells, being sufficient to cause damage to microbial cells [14]. The choice of DMMB as a photosensitizer for protocol use in amPDT has shown high efficacy even when used in nano-concentrations, regardless of the type of microorganism [8]. Upward modulation of energy density can modulate the photodynamic effect of the therapy, amplifying or reducing its effect [15].

The current literature presents several afPDT protocols for the treatment of *Candida ssp*. with little clinical viability including long pre-irradiation time and high energy densities which makes them difficult to use it in clinical reality [6, 16]. It was hypothezised that the use of afPDT could be effective in killing *C. albicans*. The present study aimed to evaluate, in vitro, the effectiveness of Antifungal Photodynamic Therapy using the DMMB associated with the Red LED (λ630ηm ± 1 ηm) on flucanozole resistant *Candida albicans*.

2. Materials and Methods

2.1. Preparation of the initial inoculum

The tests were performed using the strain of *Candida albicans* ATCC 90028 (Labchecap Laboratório de Análises Clínicas e Imagem, Salvador, BA, Brazil). The culture was kept in an ultra-freezer (ThermoFisher, STP, AS) until the beginning of the experiment, being incubated in a bacteriological oven (UltraSafe HF 212, PR, Brazil) at 37◦C for 24-h according to the growth curve of *C. albicans* [17]. The colonies were resuspended in sterile 0.85% NaCl saline, at a concentration of 2 x 10^8 cells/mL, quantified by counting in the Neubauer chamber, using serial dilutions from 10^{-1} to 10^{-3} [18]. Concentrates containing isolated C. albicans were cultivated in Sabouraud broth medium (TM Media, Rajasthan - India) for growth and Agar Sabouraud medium (Himedia Laboratories, Nashik – India) for colony visualization and counting.

2.2.Photosensitizer

The phenothiazine dye used was the DMMB (1,9-Dimethyl-Methylene Blue zinc chloride double salt or DMMB, molecular weight 416.05, content 80% Sigma-Aldrich, St. Louis, MO, USA). The photosensitizer was diluted in a laminar flow chamber (Pachane, SP, Brazil) using sterile distilled water and the compound was diluted to a concentration of 100 µg/mL, followed by 0.22 µm membrane filtration (KASVI, PR, Brazil) to disinfect it. The concentrations used were in accordance with the calculated IC_{50} [19]. In all the following protocols, the five-minute pre-irradiation time (PIT) was adopted, which is necessary for the cell to absorb the compound.

2.3. Determination of the IC_{50} of the DMMB

Groups were carried out with concentrations of DMMB in tubes, except for the control group that used phosphate-saline (PBS, Gbico Life Technologies, Brazil), the others, contained, respectively, 250, 500,1000 and 5000 ηg/mL in a stock dilution. The concentrations of DMMB were established through stock concentration and added to the test tubes, the solutions were inoculated with 100 µL of culture medium Agar Sabouraud, where 100 µL of each dilution of the *C. albicans* + DMMB set was pipetted in triplicate. In addition, 100 µL of the inoculum were removed from the control well and transferred directly to corresponding Petri dishes.

2.4. Antifungal Photodynamic Therapy

2.4.1. Preparation of the suspension of C. albicans

The strain was activated in a bacteriological oven (UltraSafe HF 212, PR, Brazil) during 24-h at 37°C and adjusted to 2 x 10^8. Being prepared according to the initial inoculum (**Item 2.1**) performed for the DMMB.

2.5. Light source

The prototype of the Red LED calibrated and certified by the Department of Physics of the Federal University of Bahia was used. The energy density of 20 J/cm^2 was used in the irradiated groups. The parameters can be seen below (***Tab.1***), together with their operation (***Fig. 1***).

Table 1
Parameters of the LED prototype light source.

Parameters	LED
Wavelength	ʎ630 ηm ± 1
Mode	CW
Well area (cm²)	1.5 cm^2
Irradiation time	14.5 min
Power (mW)	41.2 mW/cm^2
Dose (J/cm^2)	20 J/cm^2

For the determination of the energy density to be used, the light absorption of the Sabouraud and PBS culture media was verified using a Power meter device (Thorlabs PM30, Newton, NJ, USA). Initially, it was verified if there would be loss of energy caused by the Sabouraud broth culture medium when irradiated by the LED, and then in PBS, where this variable was not detected, not affecting the delivery of energy density.

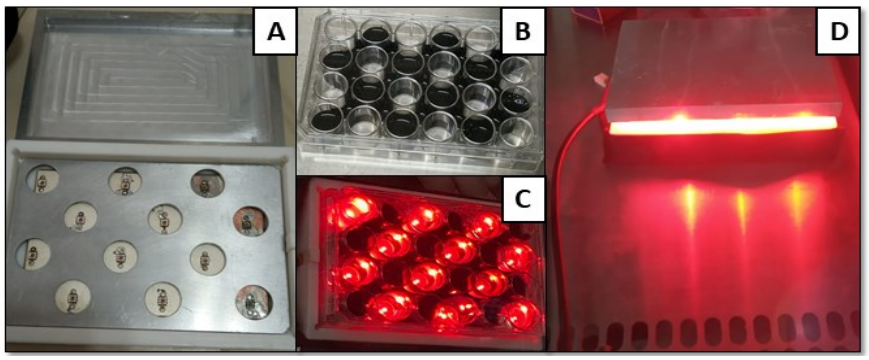

Fig. 1. Images of the operation of the LED prototype. (**A**) Prototype with the lid open, showing its irradiation LEDs. (**B**) 24-well plate with alternating wells used in the prototype. (**C and D**) Irradiation of the wells in the LED prototype.

2.6. afPDT procedures

2.6.1. Protocol 1: single application in single session

For the experiment, 24 well plates (CELLSTAR® Cell Culture Microplates, Salzburg, Austria) were used for each analysis group (**Tab. 2**). 250 µL of fungal inoculum containing 2.25 mL of Sabouraud broth medium (2.5 mL total) was pipetted, this being the 10^{-1} dilution, and then serial dilutions up to 10^{-8} were performed in the following wells. The inoculations for growth were carried out in Petri dishes containing Sabouraud agar medium and incubated in a bacteriological oven for 24-h at 37°C.

Table 2
Experimental groups for Protocol 1.

Experimental groups	Concentration (ŋg/mL)	Energy Density (J/cm^2)
Control	X	X
DMMB	250	X
DMMB	500	X
DMMB	750	X
DMMB	1000	X
LED light	X	20
afPDT	DMMB (250)	20
afPDT	DMMB (500)	20
afPDT	DMMB (750)	20
afPDT	DMMB (1000)	20

Group 1: Control - 33.3 uL of the inoculum were transferred from each well/dilution of its corresponding 24-well plate to each space between the divisions of the Petri dish, spread with the aid of the Drigalsky loop on Sabouraud agar medium and taken to the bacteriological oven at 37ºC for 24-h for later CFU counting [20].

Group 2: Photosensitizer - After serial dilutions (10^{-1} to 10^{-8}), one 24-well plate was used for each one of the chosen concentrations of the photosensitizer (250, 500, 750 and 1000 ŋg/mL). The Pre-Irradiation Time (PIT), which corresponds to the period required for the cell to absorb the photosensitizer, was five minutes [21]. After this period, similar procedure carried out in the control group was followed [20].

Group 3: LED light - Each dilution well was irradiated simultaneously by the red LED prototype (**item 5.5.2**) with a density of 20 J/cm^2, then similar procedure carried out in the control group was followed [20].

Group 4: afPDT - The process of adding the DMMB to the photosensitizer group was repeated in each dilution well. Before plating, each well containing DMMB + PIT (5 min) was irradiated with the red LED prototype (20J/cm^2). Following irradiation similar procedure carried out in the control group was followed [20].

2.7. Definitions of Protocols 2 and 3

Alternative Protocols 2 and 3 were performed after Protocol 1, where the non-inoculated material was used for the subsequent experiment, so different controls were used. The procedures were performed with three groups (Control, DMMB and afPDT) and it was not necessary to analyze the isolated light (**Tab. 3**). All the procedures of the two alternative protocols had their initial part identical to that of Protocol 1 (**Item 2.6.1**), with variations in the procedures described below.

The samples were washed three times with PBS buffer before protocols 2 and 3, to avoid the accumulation of photosensitizer after the second addition of the dye, using a centrifuge (Eppendorf AG, 5804R, Brazil) at 5.000 RPM for 10 min with resuspension in PBS, all resuspension procedure was carried out in a laminar flow chamber, with its fungal material at the end of the third wash, resuspended in Sabouraud broth.

The photosensitizer group and afPDT had a single concentration of 750 ηg/mL. Before all plating, all material from each 24-well plate was transferred to a 50 mL tube, where it was homogenized and divided into two identical 25 mL volumes, one of the tubes followed the experiment in Protocol 2, and the other was stored in a bacteriological oven at 37°C for 24-h to follow Protocol 3.

2.6.2. Protocol 2: double application in single session

This protocol used 750 ηg/mL of DMMB on Photosensitizer and afPDT groups. This concentration was chosen as it was the best concentration/response based on the reduction of the total fungal load of Protocol 1 (**Item 2.6.1**), with the lowest cytotoxicity ratio of DMMB isolated in the IC_{50}, with the response after application of afPDT. The organization of the groups can be seen below (**Tab. 3**).

Table 3
Experimental groups in Protocols 2 and 3.

Experimental groups	Photosensitizer	Light source/Energy Density (J/cm^2)
Control	X	X
DMMB	DMMB (750 ηg/mL)	X
afPDT	DMMB (750 ηg/mL)	LED 20 J/cm^2

Group 1: Control - The fungal samples were washed and resuspended in 1.25mL of Sabouraud broth, this being the 10^{-1} dilution of the control in the initial well of the 24-well plate, repeating the serial dilution process up to 10^{-8}. Finally, the plating was identical to that performed in Protocol 1, as well as it is storage and counting of CFU/mL (**Item 2.6.1**).

Group 2: Photosensitizer - The samples were washed and resuspended, this being the 10^{-1} dilution. After these processes, the volume corresponding to 750 ηg/mL was added and the five-minute PIT was awaited. Finally, plating and storage was carried out as described previously (**Item 2.6.1**).

Group 3: afPDT - The fungal cultures corresponding to the initial afPDT followed the same processes as the previous groups until the serial dilution (**Item 2.6.1**), where a volume corresponding to 750 ηg/mL of DMMB was added in each well/dilution and awaited the 5 min PIT. After the period, the second application of the LED light was carried out doing the afPDT (Protocol 2). Finally, plating and storage was carried out as previously described (**Item 2.6.1**).

2.6.3. Protocol 3: single application in two sessions (24-h)

The Protocol 3 groups were a reflection of Protocol 2, differentiating the 24-h interval from repetition of the afPDT. The organization of the groups can be seen previously (**Tab. 3**).

Group 1: Control - The tube containing the Protocol 1 samples stored in a bacteriological oven for 24-h was used repeating the entire process (**Item 2.6.1**), however, before the washing process, a control plating was performed for obtaining results of the total fungal load after 24-h of incubation, this plating followed the same pattern as previously performed (**Item 2.6.1**), as well as all the following serial dilution and plating procedures.

Group 2: afPDT - Following the same process as the control group (**Item 2.6.1**), where the plating was also performed before the washes, the entire washing process, serial dilution, and formation of the afPDT was repeated, thus completing the second application (Protocol 3). Finally, the samples were again plated and stored in a bacteriological oven for later CFU/mL counting after 24-h (**Item 2.8**).

2.7. CFUs count

After 24-h of incubation at 37ºC, the Petri dishes were subjected to the colony counting method. By this procedure, the number of CFU was calculated. For the counting of CFUs, Petri dishes containing colonies within the range and repeatability precision of 25 to 250 colonies were selected. For each dilution (10^{-1} to 10^{-8}), the number of colonies was counted and tabulated for further statistical analysis.

3. Results

The CFU / mL count was performed, and all values were tabulated on the Graphic Pad Prisma 8.1 (GraphPad Software, San Diego, CA), and the One-way ANOVA tests were performed together with the Tukey test and non-linear regression with multivariate analysis, obtaining significance ($p < 0.0001$).

3.1. IC_{50} of the DMMB in *C. albicans*

CFUs were quantified and statistical analyzes were performed to obtain the IC_{50}. The values obtained from the fungal growth were tabulated and normalized for the application of the non-linear regression test ($p < 0.0002$) (**Fig. 2**).

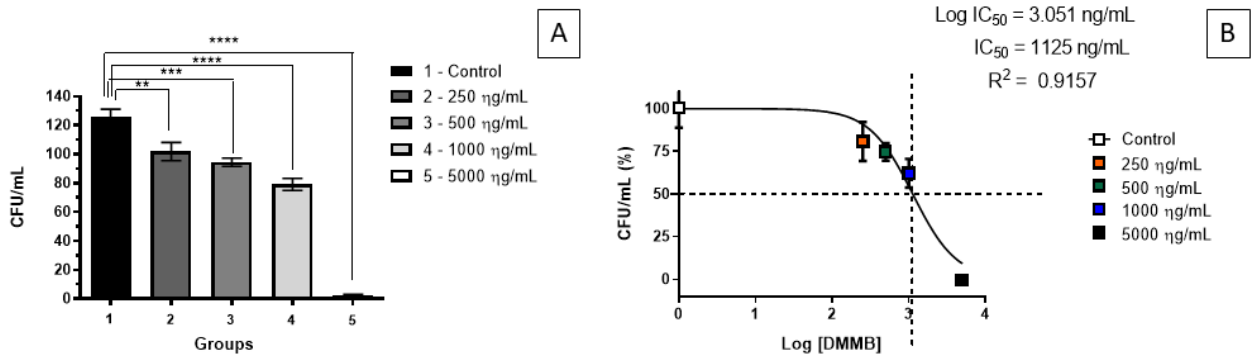

Fig. 2. Graphic representation of DMMB IC_{50} cytotoxicity. (**A**) Reduction of CFU / mL of *C. albicans* with increased concentration of DMMB from the dose of 250 µg / mL ($p < 0.01$), also

showing a significant reduction ($p < 0.0001$) in CFU/mL, especially at doses greater than 500 µg/mL. (**B**) Non-linear regression with increasing DMMB concentration in *C. albicans* cultures. The IC_{50} was obtained at a concentration of 1125 µg/mL and R^2 was 0.9157.

3.2. Protocol 1: initial afPDT

The results obtained after the CFU/mL counts demonstrated a 59 % reduction in the fungal load at the concentration of 500 ηg mL applied in afPDT ($p < 0.0001$). Using 750 ηg/mL and 1000 ηg/mL of the photosensitizer, afPDT achieved respective logarithmic reductions of 99.7 % and 99.9 % ($p < 0.0001$), respectively. The number of fungal cells quantified in the isolated LED groups, did not show statistical difference ($p > 0.05$), while the DMMB groups, only showed significance from 750 ηg/mL ($p < 0.05$). The results can be seen in **Fig. 3**.

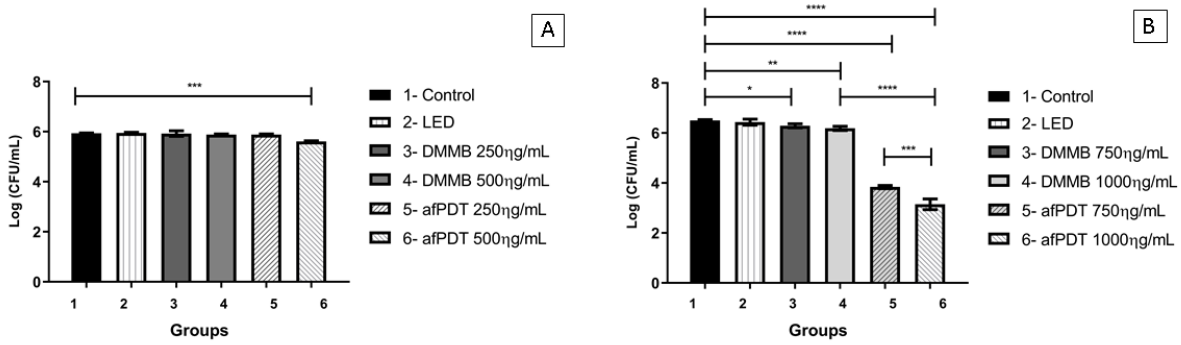

Fig. 3. Results of the CFU count of afPDT using concentrations below the IC_{50}**.** (**A**) Log CFU/mL count using the concentrations of 250 and 500 ηg/mL. (**B**) Log CFU/mL count using the concentrations of 750 and 1000 ηg/ml. Can possible observe the intergroup statistical differences * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

3.3.Protocols 2 and 3

The results obtained after the CFU/mL count demonstrated reductions in both protocols carried out. The total fungal load after the first afPDT (Protocol 1) using 750 ηg/mL caused a significant reduction ($p < 0.0001$), equivalent to 99.9% of the total load. After the application of Protocol 2, the reduction increassed reaching 99.991% reduction in the total load with high statistical significance ($p < 0.0001$). Protocol 3 showed a reduction of 99.95 % in relation to the control, however, it did not show statistical significance ($p > 0.05$) in relation to Protocol 1, the graphical representation of these results can be seen below (**Fig. 4**).

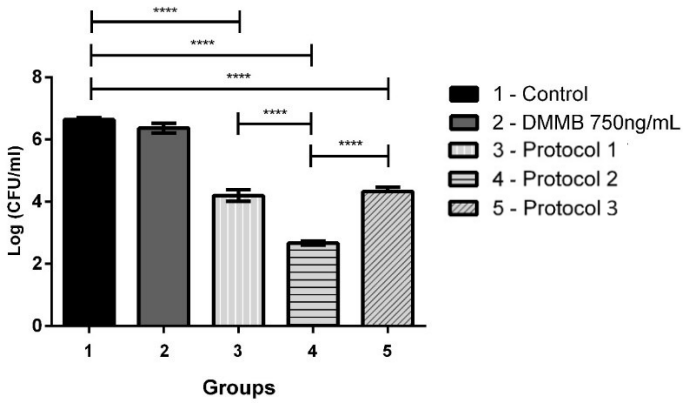

Fig. 4. Graphical representation of all afPDT. Reductions in Log (CFU/mL of *C. albicans* with the use of protocols 1, 2 and 3 at the concentration of 750 ηg/mL of DMMB, also showing a significant reduction ($p < 0.0001$) in the log (CFU/mL) in all afPDT groups.

The results obtained with the DMMB groups, demonstrated that there were no significant reductions ($p > 0.05$) of fungal cells when using the concentration of 750 ηg/mL below the IC_{50} (1125 ηg/mL), both in the initial afPDT (Protocol 1) and in protocols 2 and 3, (**Fig. 5**), demonstrating that there was no accumulation of photosensitizers capable of inhibiting fungal growth in isolation, which can be observed more clearly below.

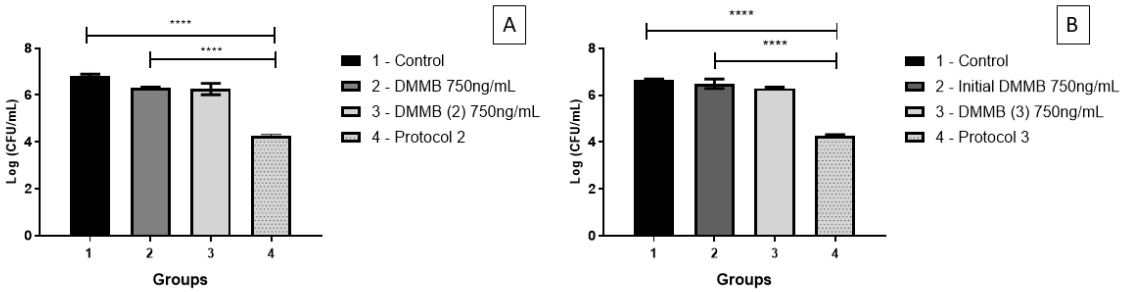

Fig. 5. Log count (CFU/mL) demonstrating the non-accumulation of DMMB in protocols 1 and 2**.** (**A**) Log CFU count/mL using the concentration of 750 ηg/mL isolated and protocol 2. (**B**) Log CFU count/mL using the concentration of 750 ηg/mL isolated and protocol 3.

4. Discussion

The current literature shows variable levels of effectiveness of using antifungal compounds against *Candida albicans.* It is also important to consider that as most of them causes considerable side effects in patients and have fewer available targets for drug action and some drugs may cause resistance, being this indicative of the need for other therapeutic approaches [22]. Therefore, pursuing alternative therapies that are effective against the microorganism and do not generate new resistance is relevant [23, 24]. Thus, PDT has shown itself a therapeutic alternative capable of reducing the microbiological load of different types of microorganisms [6, 8, 17, 20].

The innovative aspect of the present study is the use of a double exposure to afPDT of a resistant pathogen (C. *albicans*). Our results showed that the double application (Protocols 2 and 3) was more effective when compared to the conventional protocol of single application (Protocol 1) showing greater inhibition of the culture of *C. albicans* compared to other studies using phenothiazinic photosensitizers of the same family of the DMMB [25 – 27].

The photodynamic effect observed on this study may be explained as part of proteomic studies in *C. albicans,* where an irradiated phenothiazinic agent with higher energy doses was used a 3 J/cm^2, these modifications consist of the appearance of new more acidic points when compared to the control not exposed to light, when the energy was increased to 10 J/cm^2, these proteome modifications were intensified, resulting in the appearance of even more acidic spots, these appearances indicate a dependent energy dose on the photodynamic effect of the photosensitizer. To reinforce this theory, protein stains were found to remain after low fluency treatment, undergoing modification when treatment intensity was increased [6]. Analyzing the amino acids affected by afPDT, it has been shown that His, Trp, Tyr, Met e Cys, are the most affected by Singlet Oxygen ($_1O^2$), histamine being the one that has undergone the greatest degradation [28], as the formation of these compounds involve the elimination of amino and guanidine groups, oxidized proteins become more acidic, unbalancing the medium and its functions [29, 30].

The use of different protocols in the present study aimed to evaluate the best way that afPDT could act on the microorganism. As is universally known, PDT can reduce loads instantly after its application, but its capability is not demonstrated over time due to its immediate effect. This is because the technique is used as a control/help to the human body to control infections [31].

The DMMB is capable of damaging membrane structures of mitochondria, lysosomes and supporting proteins such as teichoic and lipotheichoic acids [32]. Our results showed 99.991 % inhibition in planktonic culture of this fungus, using the photosensitizer in nano

concentration, corroborating results previously reported in the literature on the efficacy of the therapy [16]. In addition, the presence of the DMMB photosensitizer available in the cellular environment, adds photodynamic effects harmful to the structures of the microorganism, regardless of its subcellular location and at very low concentrations [14, 33].

The results achieved by this study and its innovative character with sequenced double exposure aim to enhance the effectiveness of antifungal photodynamic therapy. In addition, it provides an effective clinical protocol model, as it defines and uses photosensitizer concentrations lower than the IC_{50} (item 2.3) obtained from the product, below the maximum cytotoxicity index of the DMMB in relation to the planktonic culture of *Candida albicans*; which makes it a primordial, safe, selective, and viable choice for future use in clinical practice [34].

It is important to notice that the application of the afPDT in a single session (Protocol 1) showed satisfactory results (99.7 % reduction) in the fungal load of *C. albicans* in comparison with those already found in the literature. It is possible to find some results in the literature with similar methodologies, but considered clinically unfeasible, reductions of 77 % [28], 88.6 %, 90 % [26], 99.9 % [6], and 99.999 % [16]. The satisfactory results are due to the use of DMBB in nano concentrations below the IC_{50} and pre-irradiation time of 5 min, making the protocols viable with reality *in vivo*, obtaining 99.9% reduction of fungal load in its best group of irradiations in single session (Protocol 1).

Comparing the protocols performed, it is possible to observe an increase in efficiency in fungal reduction of 90.2 % of protocol 2 (double application) compared to protocol 1 (single application), demonstrating a clear increase in potential with significance ($p<0.001$). At the same time, comparing protocol 2 with protocol 3 (double application in 24-h double session), although there is no statistical significance ($p>0.05$), it is necessary to consider that 24-h under adequate growth conditions (**item 2.4.1**) C. albicans maintained a "controlled" growth, which can be considered an option to maintain stable infection levels [35].

Previous studies [6, 16] presented a percentage reduction in the fungi *culture of Candida albicans* 99.9 % and 99.999 % respectively, however, the protocols used had high concentrations of the photosensitizer in micrograms, energy density 37 J/cm^2 and 33-minute PIT, which would not make them suitable for clinical use. Thus, our protocol with the photosensitizer in ng/mL, energy density of 20 J/cm^2 and PIT of 5 min, presented efficacy of 99.991% on the culture of *C. albicans,* like those described above and with more effective protocol parameters, lower risk of toxicity and possible of being clinically tested *in* vivo.

It is observed in the literature, a growing number of PDT publications that address protocol models of similar single irradiations, but little or nothing refers to double exposures of afPDT. Its purpose is to fight highly resistant microorganisms, be they bacteria, fungi, viruses, or parasites. These are microorganisms with high resistance patterns due to their natural morphological physical barriers and that can be acquired [36]. In addition to structures present, such as eflux pumps and peptide glycan layers, cell wall, and cellular organization with nuclear wrap, which greatly hinder the treatment of patients, especially those who require the use of chemotherapy drugs, in addition to their adverse effects [37, 39].

5. Conclusion

Our results indicated that double application of afPDT was the most effective in inhibit the proliferation of Candida albicans (99.991% inhibition).

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