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In vitro antifungal activity of curcumin mediated by photodynamic therapy on *Sporothrix brasiliensis*

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ABSTRACT

Background: Sporothrix brasiliensis is a pathogenic dimorphic fungus that affects humans and animals causing sporotrichosis. The treatment of this disease with conventional antifungals commonly results in therapeutic failures and resistance. Therefore, this study aimed to evaluate the *in vitro* effect of curcumin (CUR) mediated by photodynamic therapy (PDT) in its pure state and incorporated into pharmaceutical formulation in gel form, on the filamentous and yeast forms of *S. brasiliensis*. Methods: Cells from both forms of the fungus were treated with pure curcumin (PDT-CUR). For this, CUR concentrations ranging from 0.09 to 50 μM were incubated for 15 min and then irradiated with blue LED at 15 J/cm^2. Similarly, it was performed with PDT-CUR-gel, at lower concentration with fungistatic action. After, a qualitative and quantitative (colony forming units (CFU)) analysis of the results was performed. Additionally, reactive oxygen species (ROS) were detected by flow cytometry. Results PDT with 0.78 μM of CUR caused a significant reduction ($p < 0.05$) in cells of the filamentous and yeast form, 1.38 $\log_{10}$ and 1.18 $\log_{10}$, respectively, in comparison with the control. From the concentration of 1.56 μM of CUR, there was a total reduction in the number of CFU ($\geq$ 3 $\log_{10}$). The PDT-CUR-gel, in relation to its base without CUR, presented a significant reduction ($p < 0.05$) of 0.83 $\log_{10}$ for the filamentous form and for the yeast form, 0.72 $\log_{10}$. ROS release was detected after the PDT-CUR assay, showing that this may be an important pathway of death caused by photoinactivation. Conclusion PDT-CUR has an important *in vitro* antifungal action against *S. brasiliensis* strains in both morphologies.

1. Introduction

Sporothrix brasiliensis is a dimorphic fungus, it is pathogenic and responsible for a subcutaneous mycosis, sporotrichosis. This fungus can be inoculated in humans and animals through trauma to the skin and subcutaneous tissues [1]. Sporotrichosis caused by *S. brasiliensis* is a zoonosis transmitted to humans through bites and scratches from infected cats. This species is the main etiologic agent of mycosis outbreaks involving these hosts [2].

Human sporotrichosis can present different clinical manifestations, including cutaneous, lymphocutaneous, disseminated and systemic forms [3]. This spectrum justifies the search for adequate strategies to treat the localized forms installed and prevent the spread and evolution to more severe cases of the disease. Itraconazole, potassium iodide,

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terbinafine and amphotericin B are the main drugs currently recommended for the treatment of sporotrichosis [4]. However, these drugs are variable in terms of therapeutic efficacy, in addition, their prolonged use leads to significant side effects. Another important issue is that resistance to these antifungals has already been reported [5]. In this sense, there is a need for less toxic alternatives that result in rapid clinical cure and are low cost [6].

Photodynamic therapy (PDT) is a non-invasive method that has been used for the treatment of several infectious diseases, with good results [7–9]. PDT is based on three main components: a photosensitizing agent (PS), a light source, with a specific wavelength and complementary to the PS, in addition to oxygen. These three components, in combination, are capable of causing target cell death from the release of reactive oxygen species (ROS) and other related pathways [10]. These molecules, once generated, cause damage to DNA and membranes, among other cellular structures of microorganisms [11].

Curcumin (CUR) is a natural compound obtained from the *Curcuma longa*, a plant that has a wide range of pharmacological properties, such as antioxidant, anti-inflammatory and antimicrobial action [12]. This compound has a bright yellow color and can be used as a PS in PDT, since it is activated by a blue wavelength, in the range of 440 to 485 nm [13]. Despite some reports of the antifungal action of CUR, this compound has been poorly evaluated in PDT against human pathogenic fungi [14]. Thus, the aim of the present study was to evaluate the *in vitro* antifungal activity of PDT-mediated by CUR (PDT-CUR) using pure CUR and also incorporated into a pharmaceutical formulation in gel form (PDT-CUR-gel), over the forms filamentous and yeast-like strains of *S. brasiliensis*.

2. Materials and methods

2.1. Microorganism and growth conditions

Strain 8902/1 of *Sporothrix brasiliensis* was used, it had isolated from a clinical case of sporotrichosis. The fungus was evaluated in the filamentous form and after thermal conversion to the yeast phase. The isolate was kept in tubes containing Sabouraud dextrose agar (SDA - KASVI®), incubated at 25ºC for seven days. The thermoconversion from filamentous to yeast form was obtained in Brain and Heart Infusion agar (BHI - KASVI®) incubated at 37 °C for five days. Conidia from cultures of both forms were suspended in saline (0.85%) and the inoculum, for all assays, was adjusted in a Neubauer chamber to a final concentration of 1×10^6 conidia/mL.

2.2. Photosensitizer

The photosensitizer (CUR) was prepared by the Nucleus of Research in Photodynamic Systems (NUPESF) of the State University of Maringá. Powdered CUR (Inlab® Confiança; purity: 90–110%) was diluted in DMSO (Sigma®; purity > 99.5%) for subsequent analyses. The initial concentration was adjusted to 5000 μM, then dilutions were made (50–0.09 μM) with sterile distilled water and all were stored at 25 °C in the dark.

The pharmaceutical gel formulation with incorporated CUR (CUR-gel) was prepared by the Research and Development Laboratory of Drug Release Systems (LABSLiF) at the State University of Maringá. Polycarbophil polymer (PC) was used at a concentration of 1.0% (m/m). The polymer dispersion was carried out in purified water, under mechanical agitation at 200 rpm and at room temperature. After complete dispersion, CUR (0.1%, w/w) was added to the systems and then the pH was neutralized with triethanolamine.

2.3. Light source

The light source (Fig. S1) was composed of an array of 20 LEDs available for different wavelengths (from 410 to 660 nm). The LED systems were optical and mechanical designed (internal mirror walls) to obtain a homogeneous irradiation (>90%) area for the irradiation of cellular cultures using multi-well plates. The spectral irradiance of the LED chamber was obtained using a radiometer (Gooch & Housego, OL 756) coupled to an optical fiber (Gooch & Housego, OL 730 7q-1.0) and a 50.8 mm diameter integrating sphere (Gooch & Housego, IS- 270) to provide a power density as illustrated in Fig. S2.

The *in vitro* Blue LED system used in the experiments has the spectral Irradiance described in Fig. 3. The emission spectrum of the LED has a peak at 450 nm and a bandwidth of 20 nm. The total Irradiance is given by the area of the curve (integration of the spectral irradiance). To control Irradiance (mW/cm^2) an automated controller Fig. S4, which allowed the adjustment of the fluence (J/cm^2), through the current control (PWM regulator) in the LED system and the time of illumination. Thus, the time was adjusted to obtain fluences of 10, 15 and 20 J/cm^2.

2.4. Preparation of filamentous inoculum

The *S. brasiliensis* strain was cultivated in SDA and incubated at 25ºC for seven days. Then, the culture was collected in saline solution (0.85%) with an inoculation loop. The hyphae were separated by filtration through a syringe with a layer of compressed cotton, and the inoculum adjusted exclusively with conidia to 1×10^6 conidia/mL.

2.5. Yeast inoculum preparation

A suspension was prepared with a small portion of the culture transformed into yeast, removed with a microbiological loop in sterilized saline (0.85%) and then the inoculum was adjusted to 1×10^6 conidia/mL.

2.6. In vitro photodynamic inactivation with cur on yeast and filamentous S. brasiliensis

Aliquots of 100 μL of suspensions of 1×10^6 conidia/mL of filamentous or yeast were transferred to 96-well microplates (KASVI®) and incubated with different concentrations of CUR (0.09; 0.19; 0.39; 0.78; 1.56; 3.125; 6.25; 12.5; 25; 50 μM) for 15 min, in the dark, in a humid chamber, at 25 °C. Then, the microplates were irradiated with blue LED at different energy doses: 10, 15 and 20 J/cm^2. Two controls were included: conidia with CUR (all concentrations), without irradiation (dark control) and conidia irradiated with 10, 15 and 20 J/cm^2, without CUR (light control). Aliquots of all situations were transferred to Petri dishes containing SDA for the filamentous form and BHI for the yeast form, aiming at a qualitative and quantitative analysis. These plates were incubated at 25ºC and 37ºC, respectively, for five days to count the number of colony forming units (CFU), which was expressed in $\log_{10}$.

The fungistatic activity of pure curcumin (PDT-CUR) was defined as the lowest concentration of CUR capable of inhibiting the visible growth of the fungus, that is, CUR provided a reduction in the number of CFUs up to 99.9%. The fungicidal action was considered when the reduction was ≥ 3 $\log_{10}$ CFU/mL, that is, reduction greater than 99.9% [15].

2.7. In vitro photodynamic inactivation with CUR-gel on filamentous and yeast forms of S. brasiliensis

CUR at a concentration of 0.78 μM (fungistatic activity of pure CUR), incorporated into three grams of gel, was transferred to a 6-well microplate (KASVI®), with the addition of 1 mL of dimethylsulfoxide (DMSO) and added to 1 mL of sterile distilled water. Then, 1 mL of inoculum was added, the set was incubated for 15 min and then irradiated with blue LED at 15 J/cm^2. The interpretation of the results was carried out according to the criteria adopted in the experiments PDT-CUR. A gel base not supplemented with CUR was also tested under the same conditions.

2.8. Analysis of generation of reactive oxygen species (ROS) by flow cytometry

The quantification of ROS generated by PDT-CUR was performed using the total ROS detection probe, 2′,7′-dichloro-dihydrofluorescin diacetate (DCFH-DA) as a probe for total ROS detection. DCFH-DA permeates through yeast and filamentous fungal cells and into the cell membrane, is deacetylated to a non-fluorescent dihydrofluorescein. This binds to ROS eventually present, emitting fluorescence, which can be detected. The conditions tested were: positive control with H_2O_2 (10 μM), suspension of fungi exposed to 1.56 and 0.78 μM of CUR and negative control (fungus not treated with H_2O_2 and not subjected to PDT-CUR). After PDT, the contents of the wells of each tested condition were collected and centrifuged (5 min/4000 rpm). The pellet of each sample was washed 1x with phosphate buffered saline, 0.1 M, pH 7.4 (PBS) and resuspended with 600 μL of PBS. Next, DCFH-DA (3 μL; 10 μM) was added to the pellet and kept for 1 hour in the dark at 35ºC. The material was analyzed in a FACSCalibur flow cytometer, using the FL1-H filter (wavelength of 488 nm). The results of this experiment were expressed in histograms that indicate the proportional displacement of the cells that are producing ROS.

2.9. Statistical analysis

The analysis of the PDT-CUR results was performed using the one-way ANOVA test followed by the Tukey test for comparison between groups. Statistical analysis of the PDT-CUR-gel was performed using the unpaired T Test. All analyzes were performed using Graphpad Prism 5 software, and p-value < 0.05 was considered statistically significant.

3. Results

Thermoconversion of *S. brasiliensis* 8902/1 from filamentous to yeast was successfully achieved (Fig. 1). The filamentous colonies are white with a membranous appearance, while the yeast colonies are yellowish-beige and have a shiny creamy appearance. Microscopically, the mycelia show thin, branched hyaline hyphae with clusters of conidia, which are small and ovoid, arranged at the ends of a simple conidiophore. Yeasts are unicellular and may have one or more buds. Its shape can vary from oval to globose, or resembling a "cigar" shape.

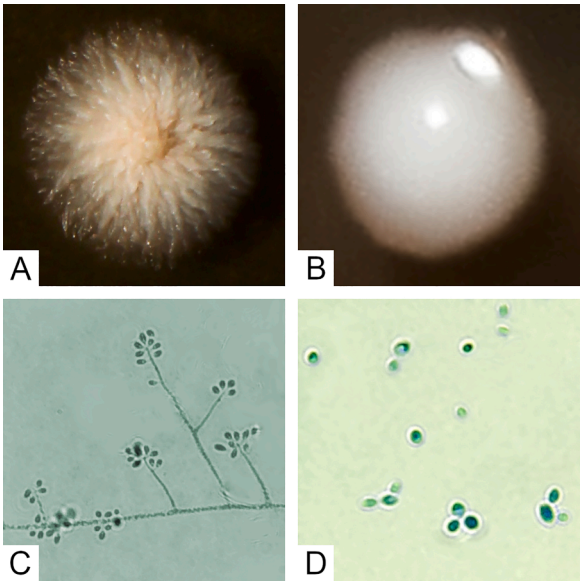

Fig. 1. Macro and micromorphological characteristics of *Sporothrix brasiliensis*. 1A) Aspect of the filamentous colony in SDA at 25 °C. 1B) Aspect of the yeast colony in BHI at 37 ºC. 1C) Micromorphology of the filamentous form at 25 ºC, showing typical aspects with septate, hyaline hyphae and oval conidia in the shape of a "daisy". 1D) Micromorphology of the yeast form at 37 ºC, with the presence of simple rounded cells with budding.

Aiming to define the experimental conditions for PDT, four blue LED fluences (0, 10, 15 and 20 J/cm^2) and five concentrations of pure CUR (0.19, 0.78, 3.125, 12.5, 50 μM) were demonstrated. The lowest values for blue LED fluence and pure CUR concentration, required to cause significant reduction in both fungal morphotypes, were 15 J/cm^2 and 0.78 μM, respectively, as shown in Fig. 2.

To determine the lowest concentration of pure CUR necessary for the inhibition of the fungal cells, under fluence of 15 J/cm^2, ten concentrations of pure CUR (ranging from 0.09 to 50 μM) were evaluated. Thus, from a qualitative point of view, 0.78 μM of CUR was sufficient for pure PDT-CUR to cause inhibition of visible growth of *S. brasiliensis* both in filamentous and yeast forms (Fig. 3A and B), demonstrating a fungistatic profile for both fungal forms. This result was confirmed by the quantitative analysis (Fig. 3C and D), in which the concentration of 0.78 μM of pure CUR was sufficient to significantly reduce ($p < 0.05$) by 1.38 $\log_{10}$ (of 4. 44 $\log_{10}$ to 3.06 $\log_{10}$ CFU/mL) in the filamentous form and 1.18 $\log_{10}$ (from 4.64 $\log_{10}$ to 3.46 $\log_{10}$ CFU/mL) with the fungus in the yeast form. From the concentration of 1.56 μM of pure CUR, the reduction was $> 3\log_{10}$ CFU/mL, (99.9%) of the fungus in both morphotypes.

Fig. 4 shows the results obtained with PDT-CUR-gel, there was a significant reduction ($p < 0.05$) of 0.83 $\log_{10}$ (5.25 $\log_{10}$ to 4.42 $\log_{10}$ CFU/mL) for the filamentous form and of 0.72 $\log_{10}$ (4.31 $\log_{10}$ to 3.59 $\log_{10}$ CFU/mL) for the yeast form. In this same experiment, the action of PDT-CUR, tested for evaluation parallel to the gel, the reduction was in the same range shown in Fig. 3. Thus, PDT-CUR-gel showed fungistatic activity against both forms of the fungus.

The antifungal action of PDT-CUR was confirmed by PDT-induced ROS production (Fig. 5). ROS release was significantly detected when fungal cells were exposed to 0.78 and 1.56 μM pure CUR compared to control (whitout curcumin). It is interesting to note that the ROS release rate was similar between the tested concentrations, when comparing intra morphological forms.

4. Discussion

In the present study, the *in vitro* action of PDT-CUR on a strain of *S. brasiliensis* tested in the filamentous and yeast forms was evaluated. Interestingly, 0.78 μM of pure CUR was sufficient to inhibit the visible growth of both fungal morphotypes. At this concentration, PDT-CUR causes an important fungistatic action against *S. brasiliensis*, similar to the action obtained by conventional treatments, such as that performed with itraconazole, which also have a fungistatic action [16]. From 1.56 μM, PDT-CUR showed fungicidal action against the fungus, proven by the reduction of $\geq 3\log_{10}$ in the number of CFUs, an effect similar to that caused by amphotericin B, the most potent antifungal drug available on the market [17]. This indicates that CUR is a promising PS for PDT on *S. brasiliensis* and the fact that these concentrations are considered low encourages future studies with this PS.

This is the first time that the action of PDT-CUR has been investigated in fungi of the genus Sporothrix. The action of PDT on *Sporothrix* spp. has already been tested previously, but using another PS, such as methylene blue (MB) [18] and the precursor of protoporphyrin IX, 5-aminolevulinic acid [19] and in both situations, this technique has shown promise as a potential alternative for the treatment of sporotrichosis [14]. However, comparing those results with thee found in the present study, the pure CUR seems to be even more promising, since the determined fungicide concentration was approximately 2-fold lower than the MB concentration defined by Nunes Mário et al. 2020 [18] and 10^6-fold lower than the 5-aminolevulinic acid concentration used by Chen *et al.* 2020 [19].

Indeed, the antifungal action of non-irradiated CUR has been previously demonstrated. Huang *et al.* 2016 [20] reported that pure,

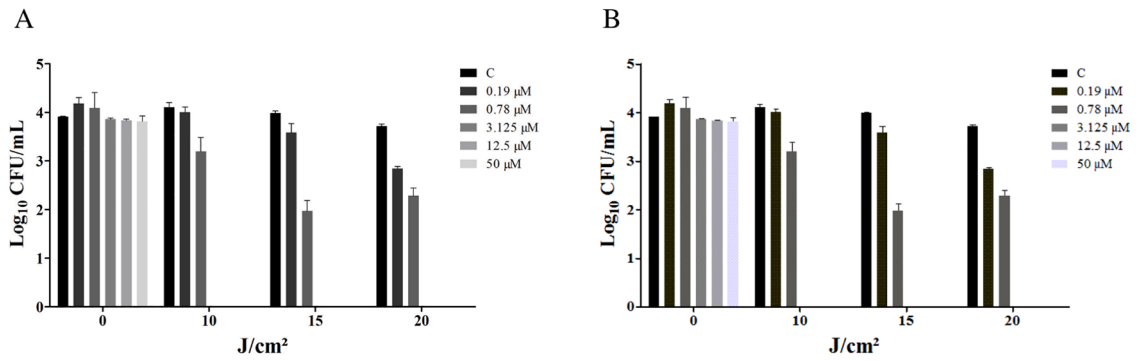

Fig. 2. Reduction in the number of CFU/mL of *S. brasiliensis* after exposure to different CUR concentrations and light doses. The results for the filamentous (A) and yeast (B) form of the fungus are presented. The fungi were treated with different concentrations of CUR for 15 min, then exposed to blue LED irradiation for 15 min at doses of 0, 10, 15 and 20 J/cm^2. Data are presented as mean ± standard deviation of the multiple tests performed.

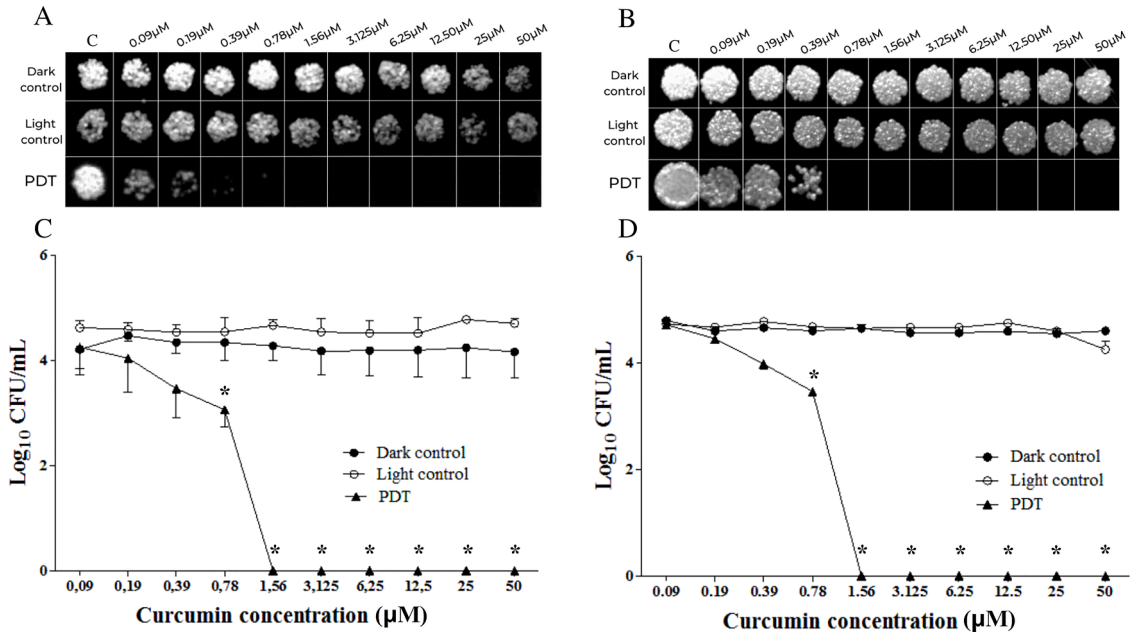

Fig. 3. Determination of the antifungal activity of *in vitro* pure PDT-CUR from *S. brasiliensis* in filamentous and yeast form. Various concentrations of CUR were tested and irradiated with LED (450 nm and irradiance of 17.0 mW/cm^2) using a fluence of 15 J/cm^2. A- Qualitative analysis for the filamentous form of the fungus. B- qualitative analysis for yeast form. C- Count of CFUs for filamentous form. D- Count of CFUs for the yeast form.

non-irradiated CUR also exerted an important fungicidal action, according to the authors, apparently due to the accumulation of chitin in the cell wall of *S. schenckii*. This fact also was associated with decreased virulence of the fungus in mice infected. Our results revealed an interesting finding that the antifungal properties of CUR (0 J/cm^2) were not effective in killing the fungus. However, when CUR was used in combination with irradiation (PDT-CUR), it was able to reduce the fungal viability, as shown in Fig. 2. A probable explanation may be correlated with the concentration of CUR in the study. The studies that use CUR alone work with much higher concentrations than when CUR is combined with phototherapy. An example of this can be observed in the investigation carried out by Huang et al. (2016) [20], in which they found that the minimum inhibitory concentration (MIC) required for complete inhibition of *S. schenckii* growth by pure CUR is 64 µg/mL. Notably, this concentration is almost one hundred times greater than the concentration defined in the present study in which there was the action of the CUR together with the irradiation of the LED.

According to the literature, CUR has some limitations, such as low permeability and low solubility in water [21], so the CUR powder was solubilized with DMSO [22]. However, aiming to simulate a possible pharmaceutical formulation for topical use, we tested the action of CUR incorporated into a gel. Fig. 4 shows that there was a significant reduction in colony counts with CUR incorporated in the gel, for both forms of S. brasiliensis, however, the action of the gel was less efficient than the pure CUR. This issue can be improved in further studies, conducted specifically aiming at the development of pharmaceutical forms addressed to the treatment of infected humans and animals. This proposal deserves attention, as *in vivo* studies involving CUR-gel for the treatment of vulvovaginal candidiasis in mice showed promising results on fungi of the genus *Candida* [23]. The present study is only a preliminary investigation of a gel, it was carried out superficially in order to simply complete a panel of *in vitro* tests, it is likely that the PDT-CUR-gel also exhibits fungicidal action, although the fungistatic action demonstrated in Fig. 4 is enough to prove the antifungal potential of this technique also with pharmaceutical forms.

It is known that PDT has multiple mechanisms of action, including the release of ROS. In our study, we were able to demonstrate the participation of reactive oxygen species as one of the possible death mechanisms of PDT-CUR on *S. brasiliensis* (Fig. 5). This reaction occurs from the absorption of light by the PS, which passes from its ground state

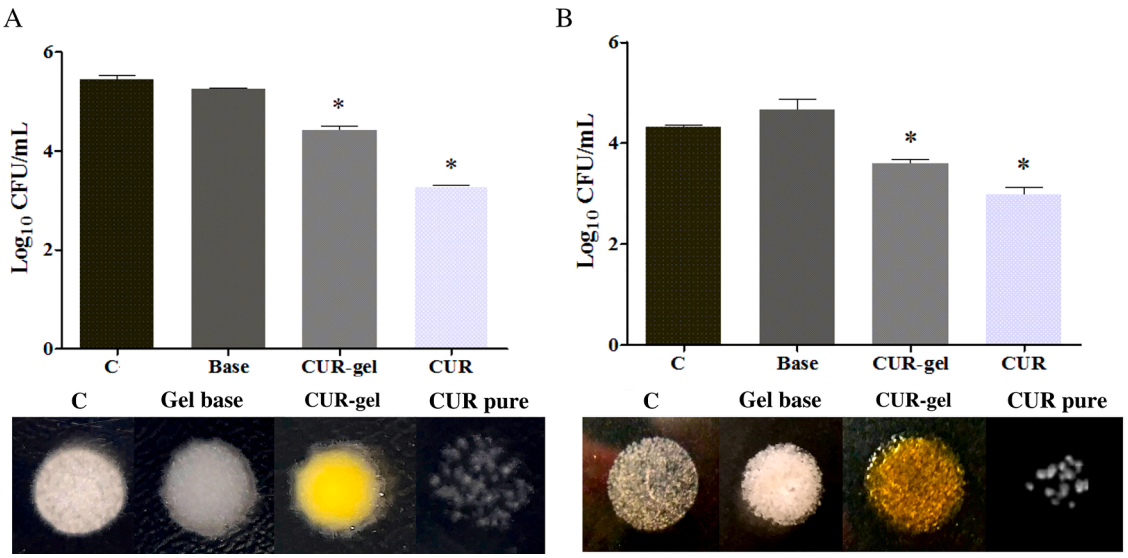

Fig. 4. *In vitro* PDT using CUR-gel compared to pure CUR, both at a concentration of 0.78 μM. Followed by incubation for 15 min and blue LED irradiation at 15 J/cm^2. In A, the result is presented for the filamentous form of the fungus and in B for the yeast form.

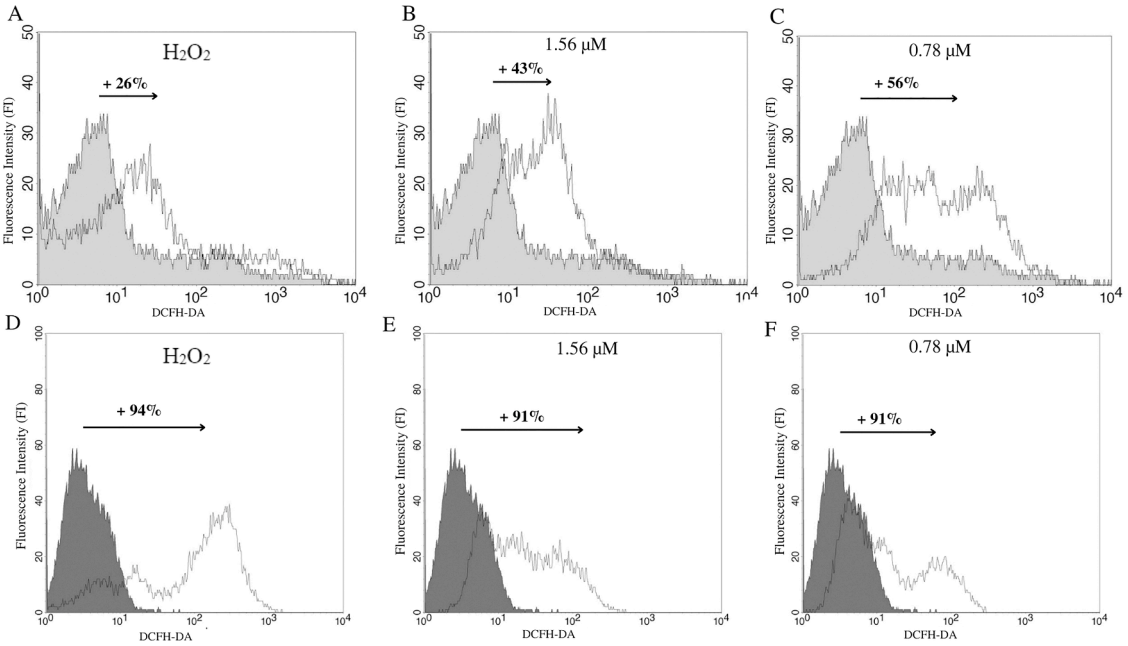

Fig. 5. Detection of ROS with DCFH-DA generated after PDT-CUR on the filamentous and yeast form of *S. brasiliensis*. Three groups are shown: negative control, positive control with H_2O_2, fungal cells exposed to 1.56 and 0.78 μM of CUR. All groups were compared with the negative control (fungal cells not exposed to PDT-CUR, represented in graphs in gray). Histograms A, B and C show the fluorescence intensity of DCFH-DA and the percentage of displacement of groups exposed to PDT-CUR in relation to negative controls for the filamentous form and D, E and F for the yeast form.

(singlet state) to an electronically excited state (triplet state). So, the PS promotes the direct transfer of energy to the oxygen present in the substrate, forming ROS [24]. Indeed, the photoinactivation of *S. brasiliensis* cells had the active participation of ROS, but probably not exclusive since the rates of ROS detected were similar between the concentrations tested. Thus, this study opens up a range of possibilities for testing the mechanism of action that proves the complete pathway for the cause of death of *S. brasiliensis* by the action of PDT-CUR.

The present study showed for the first time that PDT-CUR exerts an important *in vitro* fungicidal action against *S. brasiliensis*, and may be a promising candidate for the topical treatment of this fungal infection, especially in sensitive patients or in cases resistant to conventional antifungal drugs.

Declaration of Competing Interest

None.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.pdpdt.2023.103659.

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