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ORIGINAL PAPERS

Investigation on the in vitro anti-Trichophyton activity of photosensitizers

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Abstract

Onychomycosis is the most common disease caused by fungal nail infections, and often caused by dermatophytes. This infection is very resistant to antifungal treatments, and promising Photodynamic Therapy (PDT) mediated treatments has been presented as a multitarget tracking. Optimization of PDT guide for uptake time, concentration of photosensitizers (PS) and the light dose to inactivate *Trichophyton mentagrophytes*. Curcumin derivatives, porphyrin Chlorin e6 (CHL-E6) and Chlorin-P6-6-*N*-butylamide-7-methyl-ester (CHL-butyl) were evaluated. PS photobleaching was observed on the hyphae photosensitized over the time, correlating the PS concentration and light dose of antifungal PDT. Porphyrin, Curcumin, Chl-e6 and Chl-butyl concentrations of 2.5 µg/mL, 0.025 µg/mL, 10 µg/mL and 5 µg/mL respectively, under illumination of 10.5 J/cm^2 were the best antifungal conditions found in the study. Curcumin, in low concentrations, and chlorin were the PSs with higher activity anti-*T. mentagrophytes*.

Keywords Photodynamic therapy · Curcumin · Porphyrin · Chlorin · Onychomycosis

1 Introduction

Onychomycosis is a fungal infection caused by *Trichophyton rubrum* and *T. mentagrophytes* in keratinized tissues. It is caused by opportunistic pathogens due to immunological conditions or foot care1 [1, 2] A study on the emergence of terbinafine resistant *T. mentagrophytes* required 28 strains from a total of 45 clinical isolates demonstrated resistance to antifungal drugs. This infection occurs in more than 10% of the population, and it is more common in older adults; with a prevalence of 20% and 60% of persons age 60 and 70, respectively [3]. Conventional treatment includes antifungals in oral formulations, in addition systemic formulations (itraconazole and terbinafine) and surgery is also used.

The physico-chemical properties of the drug are decisive in its quality and may result in interactions, adverse effects and therapeutic efficacy [3, 4] However, resistant strains to antifungals are a concern for the failure of these fungicide treatments. Due to these limitations, researches involving new compounds and technologies are necessary to treat onychomycosis caused by dermatophyte fungi, and for that, PDT has been be considered a potential technique to mitigate this disease [5].

In PDT, a PS accumulation at the target site of a microorganism is activated by visible light from the selected region and forms reactive oxygen species (ROS) with a cytotoxic effect [6, 7]. PDT applied for the treatment of onychomycosis can be considered as a very selective and multitarget treatment, since the PS reaches its target cell and the lightcan be directed to the site of the fungal lesion. PDT has several advantages over systematic treatments due to the selectivity of microbial cells, in addition to the fact that the treatment is local and non-invasive, thus reducing the chances of generating antimicrobial resistance from microorganisms [8–14]. PDT of onychomycosis is considered a local treatment. The PS and light can be directed to the region of the lesion where the fungus is found. The toxic selectivity of cells in PDT has

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advantages over systematic treatments due to the generation of antimicrobial resistance.

Different PSs were tested for presenting photodynamic action dependent on photophysical properties. Each FS absorbs light at different wavelengths and produces reactive oxygen species depending on the factors involved as well as the microorganism to be targeted.

Successful studies covering in vitro fungi inactivation using PSs such hypericin, aminolevulinic acid (protoporphyrin IX precursor), rose bengal, methylene blue, porphyrins and phthalocyanines have been published [15, 16]. The PSs accumulate inside hyphae after incubation and are activated by a light source at a specific absorption wavelength of each PS, and just as part of the energy dissipated is fluorescence, which is observed to monitor the location of the PS or even photobleaching during the PDT treatment.

In this context, the purpose of this study is to present results that support the development of an efficient protocol for the inactivation of *T. mentagrophytes (*in vitro) comparing four different classes of PSs: porphyrin, curcumin natural mixture (curcuminoids) and semi-synthetic chlorophyll derivatives (chl-e6 and chl-butyl). Four different photosensitizers were chosen to evaluate optimal conditions of use of each in PDT and which would be best for anti-Trichophiton applications.

2 Material and methods

2.1 Microrganism

The *T. mentagrophytes* ATCC 11,481 from a dermatophytosis of humans in England by INCQS (National Institute for Quality Control in Health)—401 811 was donated by FIOCRUZ (Fundação Oswaldo Cruz, Rio de Janeiro, Brazil) was used in this study.

The *T. mentagrophytes* ATCC 11,481 from a human dermatophytosis isolated in England by the INCQS (National Institute for Quality Control in Health)—401 811 was donated by FIOCRUZ (Fundação Oswaldo Cruz, Rio de Janeiro, Brazil) was used to carry out this study. Fungal growth was carried out in Petri dishes containing Sabouraud Dextrose Agar (Difco—BD & Co, USA) at 37° C for 7 days. The optical density of fungal cells 0.16 at 530 nm represented 1×10^9 CFU/mL.

2.2 Photosensitizers

In this study, four PSs were evaluated porphyrin, curcumin mixture (isolated from *Curcuma Longa*), chl-Butyl and chl-e6 (Fig. 1). Photogem® (Limited Liability Company, Moscow, Russia) was used as a porphyrin derivative (main absorption bands at 369 nm and 500- 635 nm. Curcumin mix (PDT Pharma Ltda, Brazil) was obtained from *Curcuma Longa* as a mixture of three curcuminoids presented a absorbance peak at 420 nm [11, 17]. Natural curcumin produced by PDT Pharma is made up of 57.64% curcumin and 16.5% demethoxycurcumin and 25.99% bis-demethoxycurcumin. The chlorin derivatives chl-e6 and chl-butyl were semi-synthetic and characterized at the Department of Chemistry of the Federal University of São Carlos, according to the literature [18] and presents red-shifted absorption bands at 660 nm (both).

2.3 Light sources

LED devices with 24 diodes were developed by the Laboratory of Technological Support—LAT / USP (São Carlos Institute of Physic, IFSC / USP) titled *Biotable* for irradiation. Two reds light source ($\lambda_{em} = 660$ nm) and ($\lambda_{em} = 630$ nm) both with output intensity at 30 mW/cm^2 and a blue light source ($\lambda_{em} = 450$ nm) with intensity at 35 mW/cm^2, respectively.

The light source used for the illumination of the samples was dependent on the absorption spectrum of the PS. The blue light source (λ em = 450 nm) was used on curcuminoids, and porphyrin and chlorins were irradiated with a red light source ($\lambda = 630$ and 660 nm, respectively).

2.4 Fluorescence confocal microscopy

Fluorescence images microscopy were performed on an inverted fluorescence confocal microscope (Zeiss—LSM780, Zeiss, Jena, Germany) with an excitation laser at 405 nm to verify the PS uptake versus the incubation time. GaAsP made the acquisition performed by two high sensitivity photomultipliers with acquisition bandwidth ranging from 490 to 600 nm (Channel 1—autofluorescence) and 600–700 nm (Channel 2—PS fluorescence). The images were collected using suitable optical filters to exclude the laser light. All images were obtained using Zen 2010 software (Zeiss, Jena, Germany). Cell suspensions was washed with sterile distilled water for five times with vortex to eliminate Sabouraud Dextrose Broth, and the PS (150 µg /mL) was added to the cell solution.

The incubation time was analyzed in different periods, depending on the compound. The fluorescence images were acquired after 5, 10, 15 and 20 min of incubation of porphyrin and curcumin in the hyphae, while the chlorophyll derivatives (chlorin e6 and chlorin chl-e6-butyl) were 30, 60 and 90 min. After incubation time the fragments were washed again, arranged on slides and covered by coverslips for analysis. The hyphae were irradiated and post-PDT fluorescence images of the fragments were obtained to assess the photobleaching of the PSs.

2.5 Photodynamic therapy studies

The colony-forming units (CFU) was used as indicator of PDT efficacy in the inactivation of the *T. mentagrophytes*.

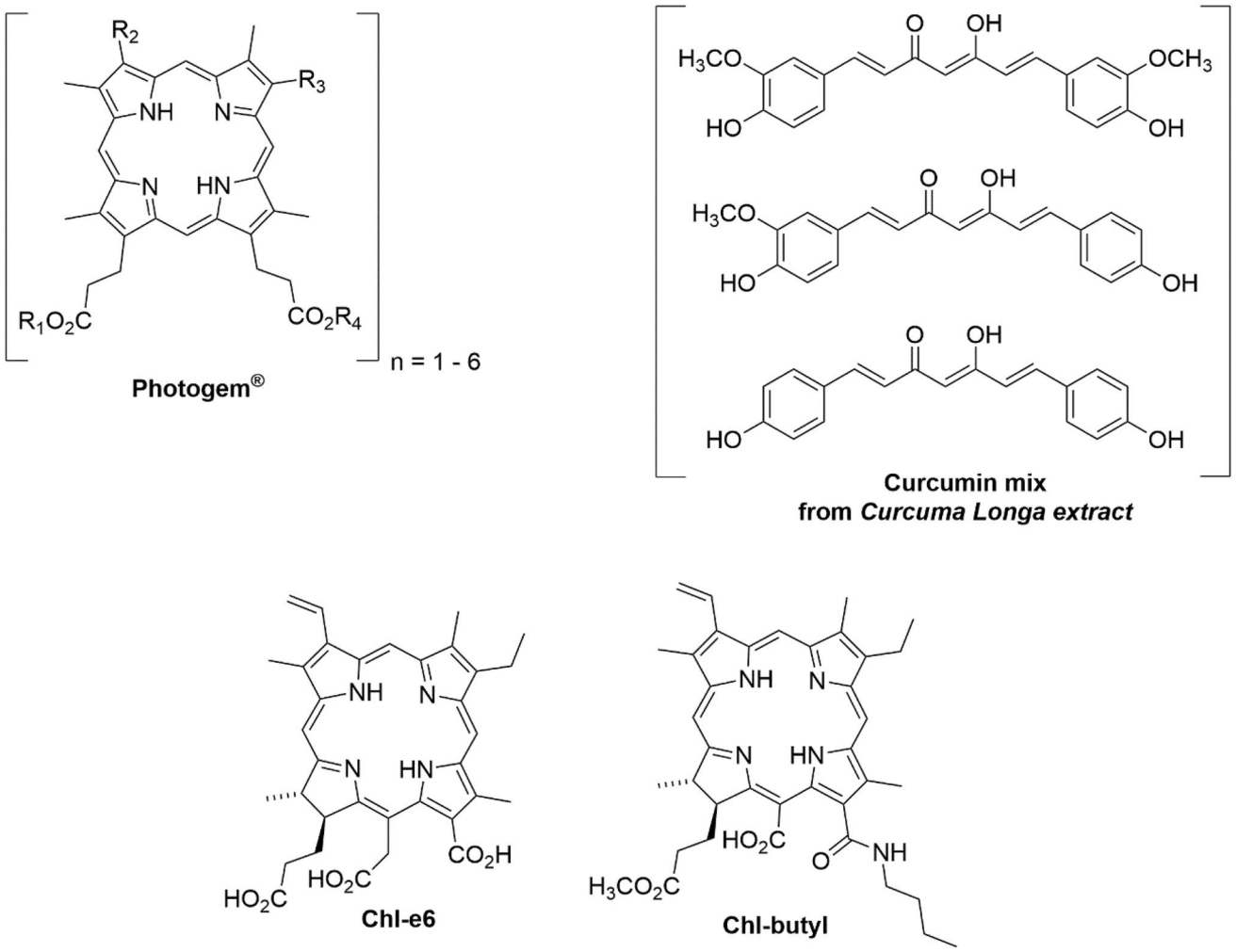

Fig. 1 Chemical structures of the photosensitizers used in this study: Photogem®**,** Curcumin mix, Chl-e6 and Chl-butyl

2.6 Treatment efficacy

The hyphae fragments were transferred to a 10 mL tube with sterile distilled water and homogenized by vortex for 30 s. This procedure was performed three times, and the suspension was adjusted to 10^9 cells/mL. The absorbance of the supernatant was determined using a spectrophotometer (Cary UV–Vis 50, Varian). Subsequently, aliquots of 150 µL cell suspensions (*T. mentagrophytes*) were added into 24-well culture plates and incubated with 150 µL PS. The experimental evaluation will describe and represente P + photosensitizer, P − no photosensitizer, L + presence light, L − no light. The incubation time at 20 min using porphyrin or curcumin, whereas for chlorins the incubation time was 60 min. All the tests were performed in triplicate.

We evaluated the *T. mentagrophytes* growth without light and photosensitizer after 5, 10, 15, and 20 min in the control group (P-L-). The PS cytotoxicity in dark (P + L-) was evaluated. PSs were added in the cell suspension in separated experiments. The highest concentrations of the evaluated PS, were 10 µg/mL for Photogem®, 0.10 µg/mL for curcumin mix and 10 µg/mL for semi-synthetic chlorins (Chl-e6 and Chl-butyl). The effect of light (P-L +) in the absence of PS on fungal toxicity was evaluated. The cell suspension in distilled water was illuminated with a light source with a maximum fluence of 42 J/cm^2. The intensity of 30 mW/cm2 and 35 mW/cm^2 at 630 nm, 660 nm and 450 nm were performed. The photodynamic effect (P + L +) was evaluated at different light doses and PS concentrations: Photogem® (1.25, 2.5, 5 and 10 µg/mL), curcumin (0.0125; 0.025; 0.05 and 0.10 µg/mL), Chl-e6 (0.6, 1.25, 2.5, 5 and 10 µg/mL) and Chl-butyl 0.6, 1.25, 2.5, 5 and 10 µg/mL. The energy doses 10.5; 21; 31.5 and 42 J/cm^2 were evaluated for all PS. The counting of colonies was performed after a week of incubation at 37 °C.

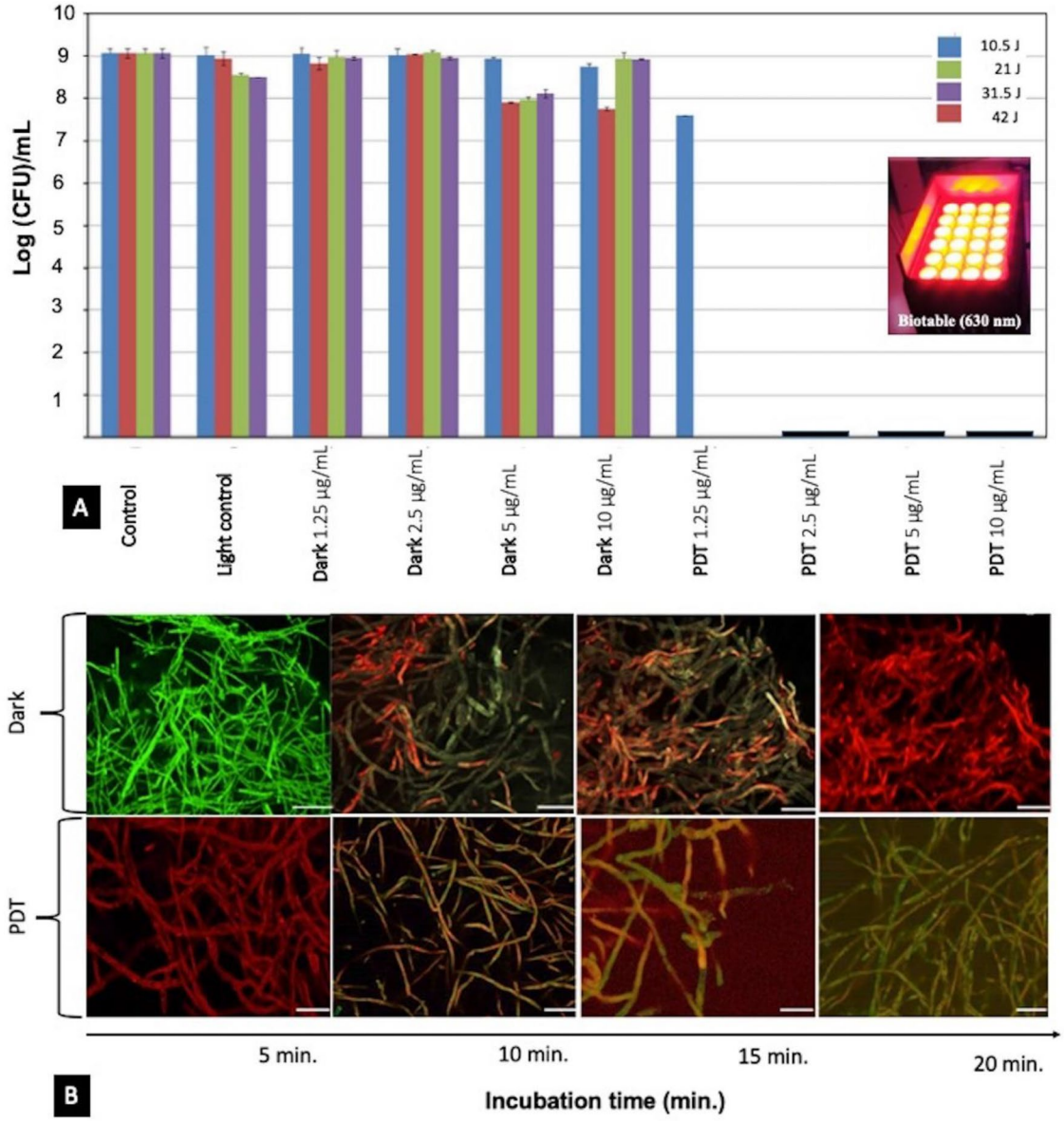

Fig. 2 Fungal death rates obtained for the fragments *T.mentagrophytes* undergoing PDT using Photogem® as a photosensitizing agent. **A** Confocal microscopy image at 150 µg/mL for 5, 10, 15 and 20 min **B**. The scale bar refers to 200 µm

3 Results and discussion

First, we studied the incubation time, PS concentration and light dose of four different PSs (Photogem®, Curcumin, Chl-e6 and Cl-butyl) using fluorescence guide and microbial counts. We observed that even at low concentrations of each PS (from 0.025 to 1.25 mg/mL), short incubation time (from 10 to 20 min.) and low doses of light (from 10.5 to 21 J) there is antifungal activity. due to PS uptake and photobleaching, as seen in fluorescence images. The potential of PDT using Photogem®, Curcumin and Chl-e6/Cl-Butyl was dose-dependent on irradiation. All control groups (light or PS) did not show significant fungal inactivation, which helps to prove the photodynamic efficiency of this treatment protocol.

3.1 Antimicrobial photodynamic inactivation

The fungal death rates of *T. mentagrophytes* submitted to PDT with Photogem® are shown in Fig. 2. The four concentrations of Photogem® (1.25, 2.5, 5 and 10 µg / mL) were evaluated using four doses of light (10.5, 21, 31 and

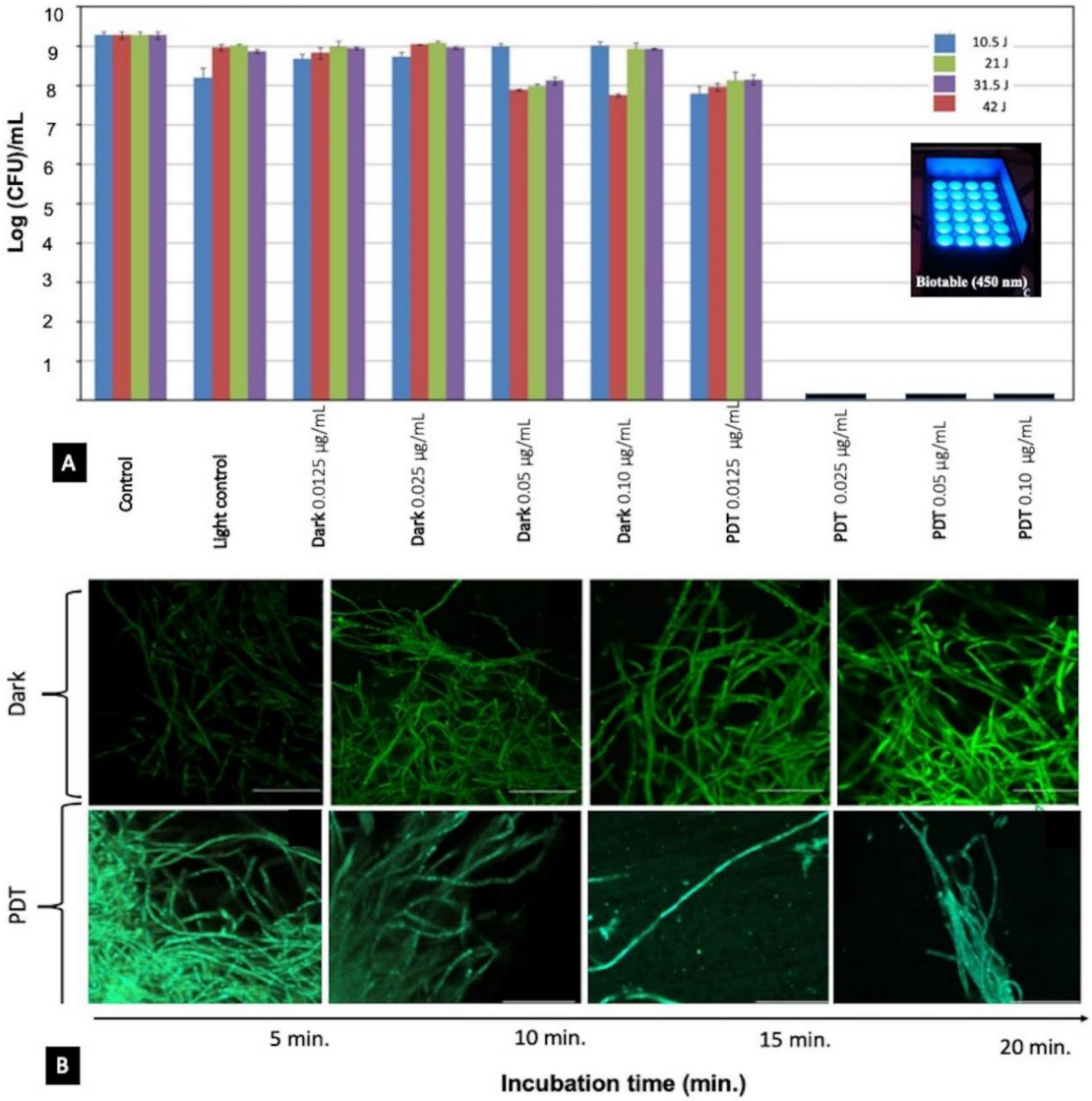

Fig. 3 Fungal death rates obtained for the fragments *T.mentagrophytes* undergoing PDT using curcumin as a photosensitizing agent. **A** Confocal microscopy image at 150 µg/mL for 5, 10, 15 and 20 min **B**. The scale bar refers to 200 µm

42 J). We observed that only the lowest concentration of Photogem® (1.25 µg / mL) illuminated with the lowest light dose of 10.5 J) showed a low microbial reduction of only 1.5 CFU / mL. Concentrations between 2.5 and 10 µg / mL of Photogem® required total antifungal inactivation. To our delight, the concentration study required that 2.5 µg / mL Photogem® and 10.5 J is the most suitable condition for its use in PDT. Smijs [19] demonstrated that a morphological damage was found in hyphae when treated by Photogem® whithout light. However, only PDT (PS + light) leads to a significant fungal damage that leads to death.

The effects of PDT on *T. mentagrophytes* fragments using curcumin (natural mixture of curcuminoids) as photosensitizer is shown in Fig. 3A. Different curcumin concentrations (0.0125, 0.0250, 0.050 and 0.10 µg / mL) and light doses (10.5, 21, 31 and 42 J) were evaluated. A 100% reduction in the target microorganism was obtained with 0.025 mg /mL curcumin and 10 J light dose, concentrations which are 100 times lower than Photogem® (Fig. 3). The singlet oxygen quantum yield formation of curcumin is dependent on the medium where it is found, however, studies have shown that at very low concentrations it presents a high stability in the presence of light, which can represent low photodegradation, thus obtaining good results of photodynamic inactivation [20].

Subsequently, two semi-synthetic chlorin derivatives were selected for the study because they are derived from chlorophyll *a* (Chl a), and are widely used in PDT against

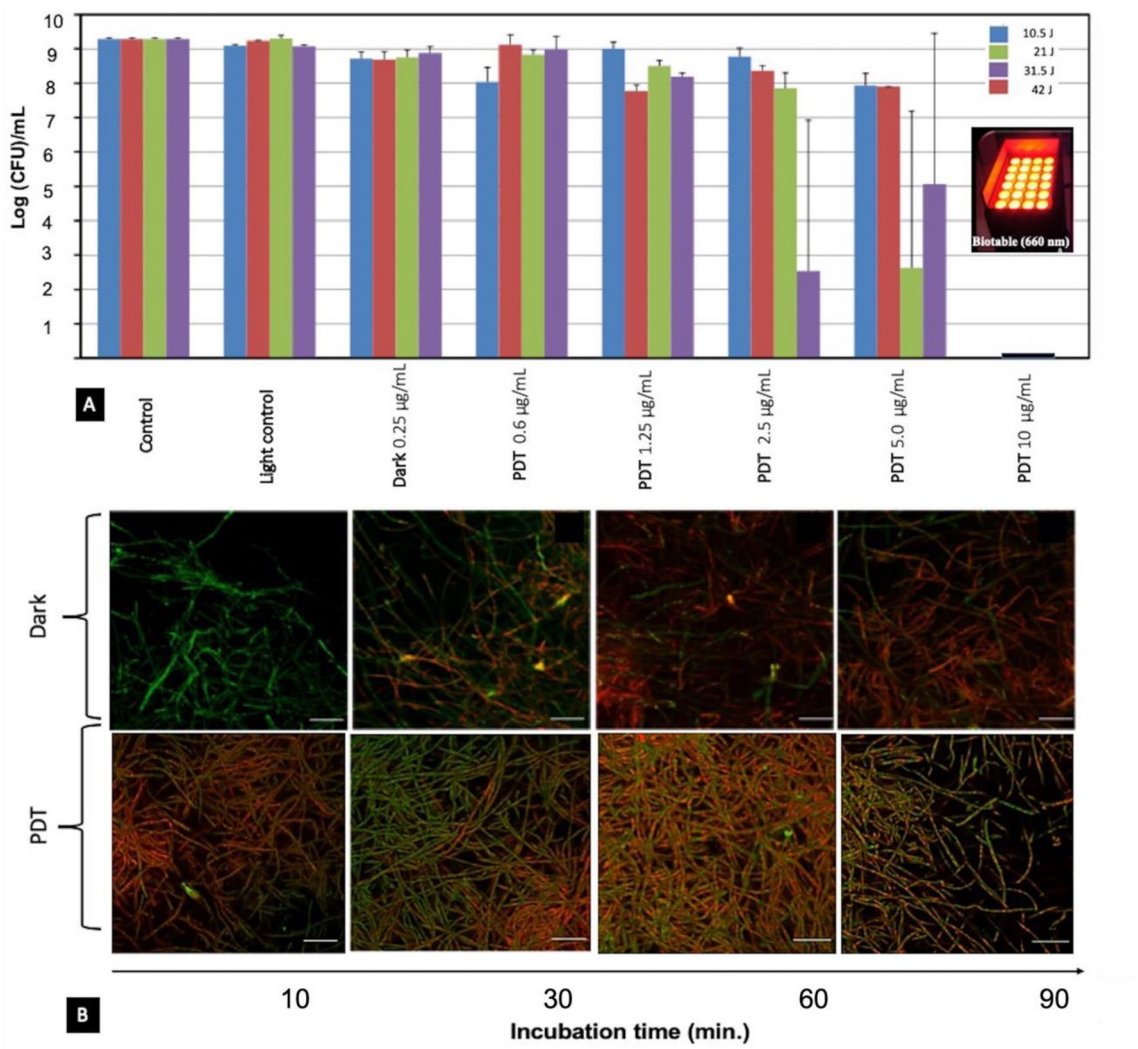

Fig. 4 Fungal death rates obtained for the fragments *T.mentagrophytes* undergoing PDT using chl-Butyl as a photosensitizing agent. **A** Confocal microscopy image at 150 µg/mL for 10, 30, 60 and 90 min **B**. The scale bar refers to 200 µm

microorganisms. Results of antifungal PDT using Chl-e6 and Chl-butyl in four concentrations (0.6, 1.25, 2.5 5 and 10 µg/mL) and fluence at 660 nm (10, 21, 31 and 42 J) are shown in Figs. 4 and 5. A complete photoinactivation was observed while Chl-butyl and Chl-e6 was used at 10 mg/mL and 10.5 J, and 1.25 mg/mL and 21 J, respectively.

3.2 Microorganism uptake and photobleaching

In Fig. 2B it is observed by fluorescence an accumulation of PS with better incorporation and distribution with the increase in the time of immersion of hyphae in Photogem®, which occurs in 20 min. When the PS is irradiated in the hyphae, after the absorption of light, the accumulated PS emit fluorescence which decreases with its irradiation. The PS's fluorescence was monitored here to notice the photodynamic effect by the photobleaching of the PS. The distribution of Photogem® in the hyphae was observed in just 5 min of incubation (2B), and the optimized distribution and homogeneity with the increase in the incubation time (20 min). With the photobleaching of the PS there was a reduction in its concentration and the monitoring of the effectiveness of the PDT. The second line of Fig. 2B refers to the fragment of the irradiated microorganisms after incubation at different incubation times. Changes in the morphology of the hyphae were observed and, although there is no rupture of the cell wall, the number of the pathogen decreases after treatment [19].

The curcumin mixture was detected in the fungi at all incubation times, being more difficult to observe than the hyphae incubated in the Photogem® solution (Fig. 3B). There is a subtle contrast between the fungus and curcumin's fluorescence, which show greenish-blue and green fluorescence, respectively. Curcumin is a lipophilic molecule that

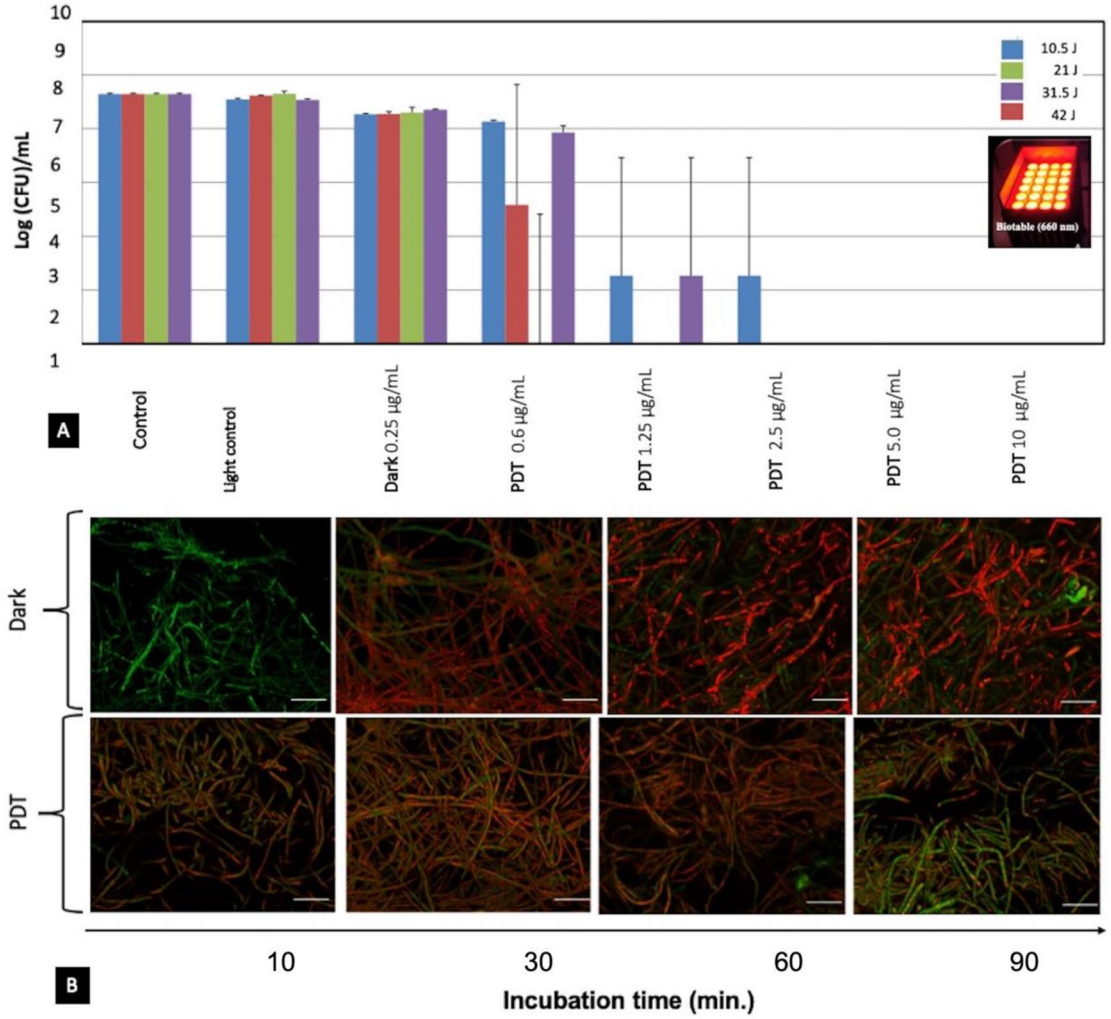

Fig. 5 Fungal death rates obtained for the fragments *T.mentagrophytes* undergoing PDT using chl-e6 as a photosensitizing agent. **A** Confocal microscopy image at 150 µg/mL for 10, 30, 60 and 90 min **B**. The scale bar refers to 200 µm

can interact with the hyphae's walls and later be transported inside the hyphae [22]. The absorption of curcumin by fungi can also be related to the fixation of substances for their nutrition.

Fragments of microorganisms immersed in the 150 µg / mL chl-butyl solution were observed for 10 to 90 min (Fig. 4B and 5B). The pathogen emitted green autofluorescence, and red fluorescence was emitted by chl-butyl chlorin (630–700 nm). The penetration of PS into the microorganism in the first five minutes of PDT is probably due to a change in the fungus' cell wall that favors absorption. It can be confirmed by an increase in fluorescence in the red region. The intracellular targeting of PS in microorganisms is still a challenge, considering its difficulty on penetrating the target cell. Chlorin derivatives required a low affinity for the fungus compared to curcumin and Photogem® for the same incubation time.

Filamentous fungi affect the horny layer of nails in onychomycosis and are not good for topical treatments due to the low permeability of the nail plate, however in this study, we have not shown that PSs reach filamentous fungi by observing the fluorescence and photodegradation of these photosensitive captions under confocal microscopy, in addition to the reduction in the number of pathogens. The results of this study can serve as a guide for future research in clinical trials, which indicate the use of Photogem®, Curcumin, Chl-e6 and Chl-butyl under optimized conditions of 2.5 µg/mL, 0.025 µg/mL, 10 µg/mL, and 5 µg/mL, respectively in all conditions 10.5 J/cm^2. The most effective incubation time was 20 min for porphyrin and curcumin, while for chlorins and their derivatives the time was 60 min. Although these results are promising for the treatment of onychomycosis, the diversity of fungi that cause the disease must be

considered. However, the major limitation for treatment of onychomycosis is that most PS do not penetrate into the keratin nail matrix.

4 Conclusion

We concluded that curcumin was the most effective PS against the fungus *T. mentagrophytes*, followed by Photogem® and chlorin derivatives. The inactivation of the fungus with curcumin required a concentration 100 times lower than that of Photogem®, Chl-e6 and Chl-butyl proven by an uptake and photobleaching of the PS. This study indicated the possibility of using curcumin in low concentrations for a quick and effective treating of onychomycosis.

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