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ABSTRACT

In this study, *Candida albicans* in its planktonic form, was used as target microorganism. This species frequently appears in superficial and invasive infections because of its pathogenic potential, and it is becoming highly resistant. Bacteriochlorin was used as a photosensitizer, presenting absorption around 780 nm and low toxicity in the absence of light. The objective was to evaluate its photodynamic inactivation potential in *Candida albicans*. The photosensitizer was synthesized from the extraction of bacteriochlorophylls derived from non-sulfurous purple bacteria and the converted to bacteriochlorin. The inoculum of *Candida albicans* was grown for 24 hours and adjusted to the concentration of 10^6 CFU/mL. The device used for the emission of light was a homemade device with LEDs of 780 nm wavelength. The quantitative evaluation of viable cells was performed by spread plate in Sabouraud Dextrose Agar. The results showed that this new bacteriochlorin is not much stable in its absorption peak, being necessary a better chemical characterization to verify its antimicrobial potential.

Keywords: Photodynamic inactivation, bacteriochlorin, *Candida albicans*.

1. INTRODUCTION

In recent decades there has been significant progress in the treatment of diseases caused by bacteria, fungi, viruses, and protozoa. Although there are several methods of microbial control, microorganisms have acquired resistance to these methods.[1] Fungi, which were regarded as harmless until a few years ago, today are aggressive and spread in silence. Fungal pathogens cause severe chronic conditions (such as allergic bronchopulmonary aspergillosis), life-threatening invasive diseases (such as pneumonia), and complex chronic respiratory conditions (such as asthma). Such pathogens can also cause recurrent infections such as oral and vaginal candidiasis. Most fungal infections are related to health conditions resulting from immunosuppression, with high mortality rates.[2,3]

The microorganisms have the characteristic to adapt themselves to environment changes, regulating its gene expression and the cellular activities, reversibly. Also, they can generate genetically modified variants resistant to adverse conditions. Under selective pressure, such as antimicrobial therapy, these variants may arise and diffuse in a relatively short space of time.[4]

The yeast *Candida albicans* commonly appears in the digestive tract of most healthy people; however, it can also trigger symptomatic infections, especially in immunocompromised hosts. Its pathogenic potential is mainly due to its ability to adhere to mucous membranes and epithelia, the production of filamentous structures and thermo-tolerance. The species is usually susceptible to all antifungal drugs, but prolonged use of these may result in resistance.[5,6,7]

Photodynamic inactivation (PDI) is a type of photochemotherapy based on three main factors: photosensitizer (PS), light source, and molecular oxygen. One of the essential points for photodynamic action is that the PS absorbs energy at the appropriate wavelength for a change in energy level, inducing local toxicity that can cause oxidative cellular damage.

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Cell death can be induced through two different reaction types. In the reaction type I, the components of the microenvironment and the PS, in the activated state, carry out an electron or hydrogen transfers, resulting in radical ions that react with the oxygen in the ground state, generating oxidized products. In the reaction type II, the FS, in the triplet state, transfers energy to the molecular oxygen present in the environment, resulting in singlet oxygen.[8,9]

The bacteriochlorins, which are derived from bacteriochlorophylls, present as primary candidates for PS. They belong to a class of sensitizers which has low toxicity in the absence of light as these compounds exhibit relatively intense absorptions in the region of 750-800 nm.[10,11,12,13]

Bacteriochlorophylls can be found naturally in some microorganisms, including purple bacteria. These microorganisms use Mg^{2+} complexes present in the bacteriochlorophyll-A, as light absorptive chromophores, necessary for photosynthesis under anaerobic conditions.[14,15]

2. MATERIAL AND METHODS

2.1 Photosensitizer

The PS used in this study was bacteriochlorin obtained from non-sulphur purple bacteria, this bacteriochlorin was modified synthetically, where Trisma was added, obtaining the photosensitizer used in this study. First, 0.5 mg of bacteriochlorin (PS) was diluted in 500 µL of dimethyl sulfoxide (DMSO) until its complete solubilization, to a concentration of 1402 µM. From the stock solution, the PS was then adjusted to the desired concentrations being diluted in phosphate buffered saline (PBS). The absorbance of bacteriochlorin trisma in different concentrations and the comparison between the absorbance of the PS and the LED 780 nm can be seen in Figure 1.

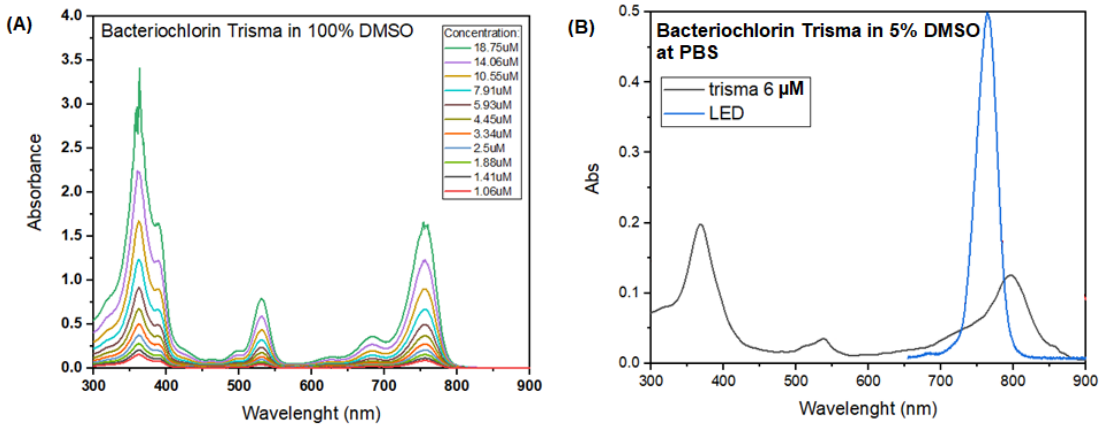

Figure 1: Absorbance of bacteriochlorin trisma in 100% DMSO in different concentrations (A), and the comparison between the absorbance of 6 µM of bacteriochlorin and the LED 780 nm (B).

2.2 Culture standardization

C. albicans (ATCC 90028) was grown for 24 hours in Tryptic Soy Broth (TSB). After growth, the suspension was centrifuged at 3500 rpm for 15 minutes. The supernatant was removed, and the pellet was resuspended in 2 mL of PBS. The suspension was adjusted using a spectrophotometer to reach a concentration of 10^6 CFU/mL (absorbance value of 0.06 at 600 nm).

2.3 Photodynamic inactivation

To analyze the photodynamic action of bacteriochlorin in planktonic yeast, experimental groups were studied using different light doses and PS concentrations. These experimental groups were incubated for 30 and 60 minutes with the PS so that it could penetrate through the cell walls. The penetration of bacteriochlorin trisma in *C. albicans* can be seen in

Figure 2. The photodynamic inactivation (PDI) experiments were conducted by the illumination of the microplate wells containing *C. albicans* and PS. The illumination was done by a homogeneous system of LEDs at 780 nm with an intensity of 30 mW/cm^2.[16]

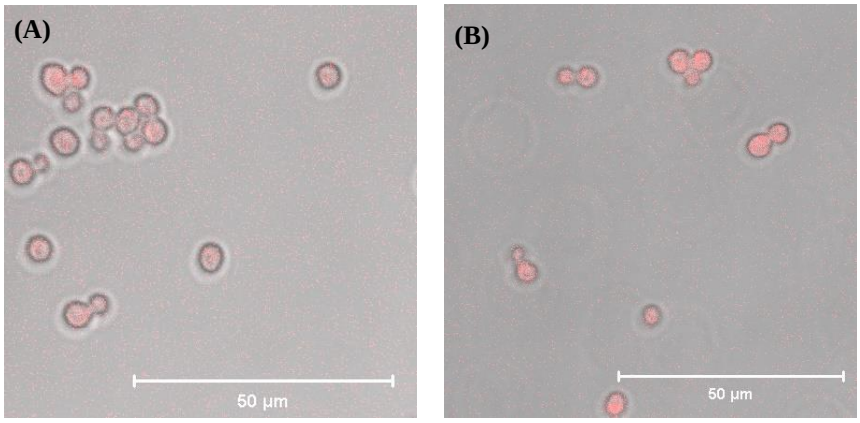

Figure 2: Penetration of bacteriochlorin trisma in *Candida albicans* in 30 minutes (A) and 60 minutes (B). Red signal comes from bacteriochlorin trisma fluorescence with excitation at 532 nm.

2.4 Quantitative evaluation of viable bacterial cells of biofilms

In order to evaluate the viable yeast cells, the spread plate method was used. Serial dilutions up to 10^{-5} were performed in PBS from the suspension after all treatments of the experimental groups. Subsequently, 25 µL of the dilutions and the original suspension were plated in Petri dishes with Sabouraud dextrose agar using sterile Drigalski handles. Afterward, the plates were incubated at 36 ± 1 °C for 24 hours for the growth of *C. albicans* cells that were alive and then counting colony forming units (CFU) in each experimental condition.

3. RESULTS AND DISCUSSION

3.1 Spectrophotometry of bacteriochlorin trisma

Bacteriochlorin trisma in both 100% PBS and 5% DMSO shows absorption peak near 800 nm. This similarity is due to the amount of PBS present in solution, since the sample with 5% DMSO has 95% PBS. DMSO is a substance toxic to cells, if used in high concentrations, and can cause unwanted damage to healthy cells. The tests were carried out with 5% DMSO, since RANDHAWA (2008) describes the values above that as highly toxic to the cells of *C. albicans*, being able to provoke cell death even before the PDI.[17] The results of the spectrophotometry are shown in Figure 3.

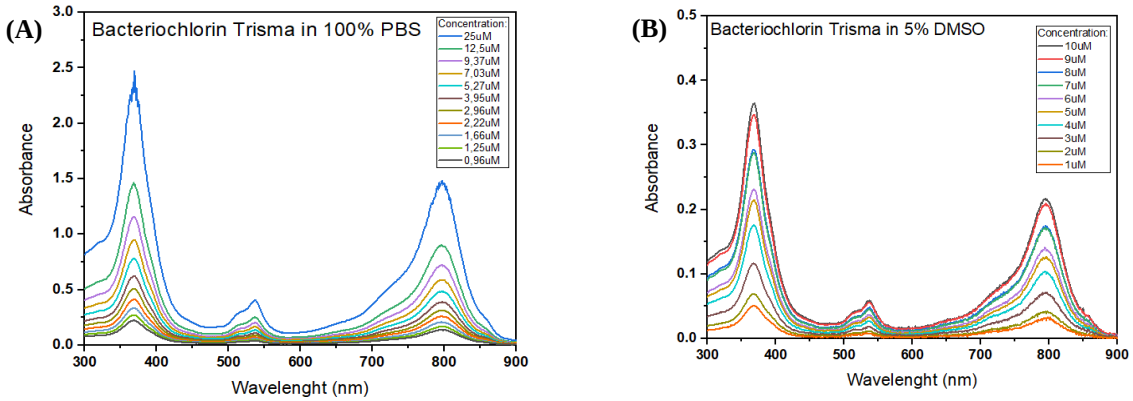

Figure 3: Spectrophotometry of bacteriochlorin trisma in 100% PBS (A) and in 5% DMSO at PBS (B).

The comparison between the absorbance of bacteriochlorin trisma in 5% DMSO and the LED 780 nm is showed in Figure 4.

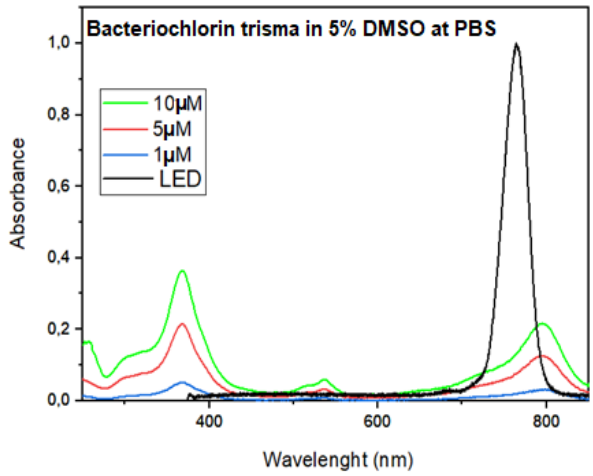

Figure 4: Absorbance of different concentrations of bacteriochlorin trisma in 5% DMSO at PBS and the LED 780 nm.

Therefore, when using homemade device, bacteriochlorin trisma will not receive the full dose of light applied. The device emits light at a wavelength of 780 nm, while the absorption peak of the PS under these conditions is 800 nm, which may reduce the potential of PDI due to the differences between the absorption and the emission spectrum.

3.2 Photodegradation of bacteriochlorin trisma

Photodegradation of bacteriochlorin trisma in 4 hours of illumination can be seen in Figure 5. The photodegradation of PS in 100% DMSO, although starting at a higher absorbance, is 2 times faster than its photodegradation in 5% DMSO. The tests were carried out with bacteriochlorin trisma at a concentration of 25µM and maintained the previously observed pattern, with the lowest concentration of DMSO in 800 nm and the highest concentration in 770 nm.

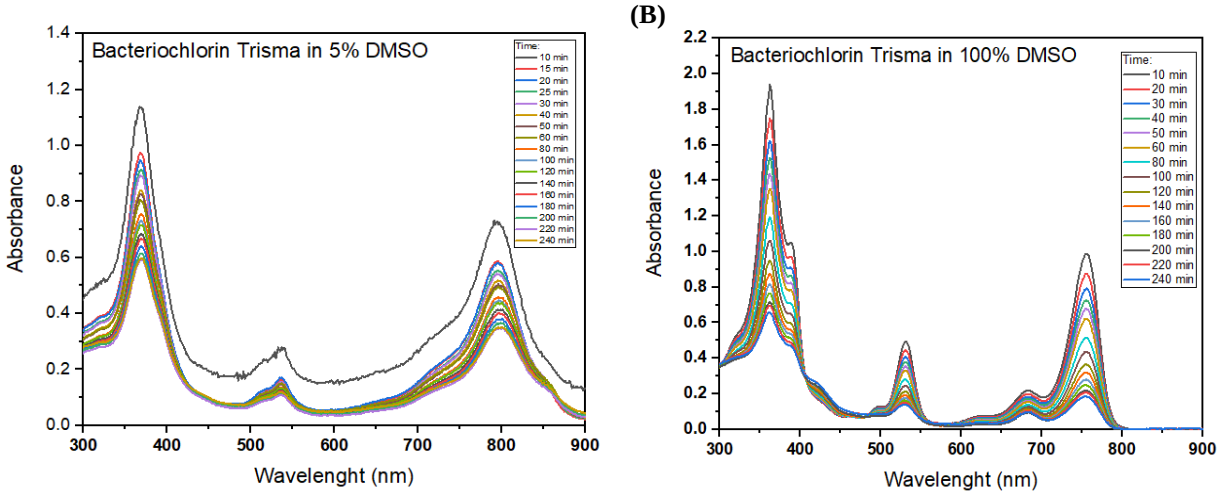

Figure 5: Photodegradation of bacteriochlorin trisma at 25µM concentration in 5% DMSO at PBS (A) and in 100% DMSO (B).

SILVA et al. (2016) tested the photodegradation of Benzo[*cd*]pyren-5-one, a new photosensitizer, in two solvents, benzene and DMSO. On this occasion, it was also possible to observe that the photodegradation in DMSO occurred 2.5 times faster than in the other solvent. This change may be related to the use of polar aprotic solvent, DMSO, which tends to selectively increase the rate of an oxidative reaction involving polar intermediates.[18]

3.3 Photodynamic inactivation of *Candida albicans* in planktonic form

The bacteriochlorin trisma was tested using PDI against *C. albicans* in different concentrations and different doses of light, according to the results in figures 6, 7 and 8.

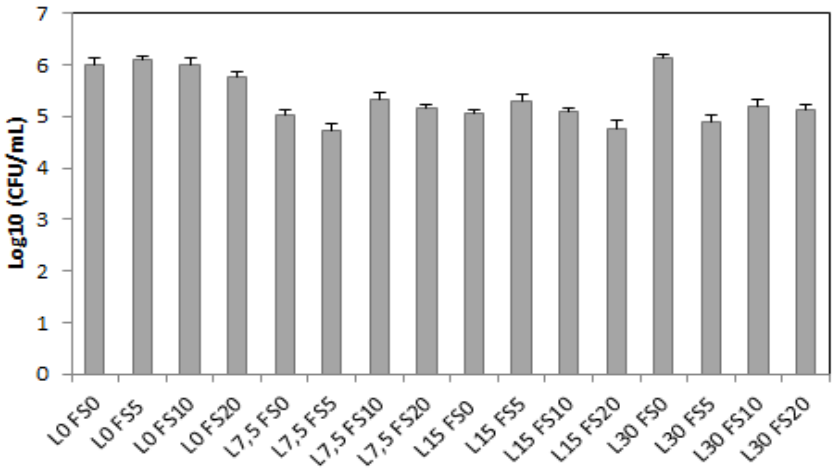

Figure 6: *C. albicans* exposed to 0, 7,5, 15 and 30 J/cm^2 of light (L), and 0, 5, 10 and 20 µM of photosensitizer (PS).

The different doses of light show similar results. During the execution of the experiment, the formation of precipitate in the PS solution at PBS was observed, indicating solubility problems. It is possible that the different concentrations of bacteriochlorin trisma were not effectively applied to *C. albicans* cells, justifying the similarity between the results. The highest concentration in which the formation of agglomerates was non-existent or undetectable was 10 µM. In addition, in the PS solution at PBS, a concentration of 5% DMSO was established. The following experimental group was based on these results.

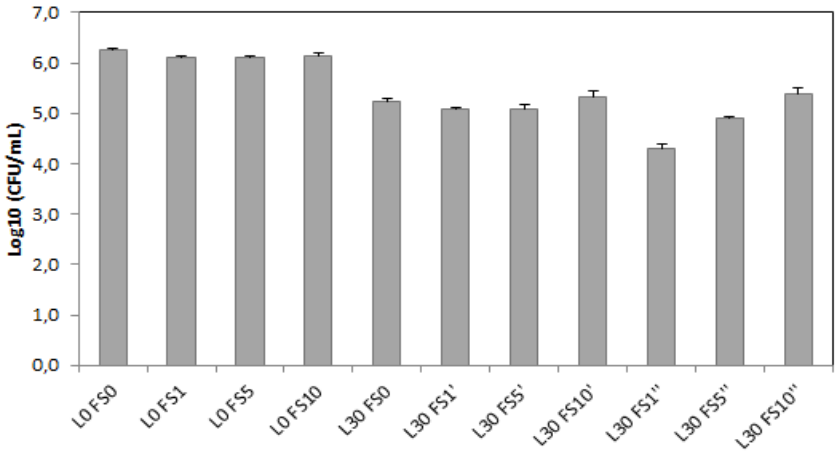

Figure 7: *C. albicans* exposed to 0 and 30 J/cm^2 of light (L), and 0, 1, 5 and 10 µM of photosensitizer (PS).

In this experiment, two incubation periods, 30 (') and 60 (") minutes, were tested in an attempt to solve the solubility problem. A particular difference is observed between the two incubation periods, especially in the lower PS concentrations. Probably the lowest concentrations of bacteriochlorin trisma solubilize more quickly in the medium and may have greater penetration power in the cells of the microorganism.

The third experimental group was developed to apply the three conclusions obtained previously: (a) higher solubility at lower concentrations of PS in 5% DMSO; (b) incubation period of 60 minutes; (c) higher light dosage in order to compensate the difference between the emission and the absorption spectrum.

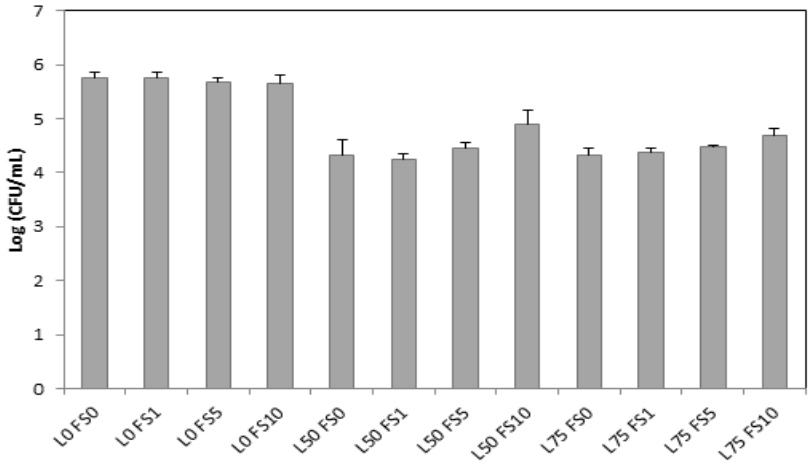

Figure 8: *C. albicans* exposed to 0, 50 and 75 J/cm^2 of light (L), and 0, 1, 5 and 10 μM of photosensitizer (PS).

The results obtained with PS in 1 μM concentration are more efficient when compared to the other concentrations. This is due to the lower precipitation rate of bacteriochlorin trisma when used in smaller volumes. It is also noted that the increase in light doses did not imply a higher rate of microbial control. As the LED emission bands and the bacteriochlorin trisma absorption bands are not aligned, it is difficult to infer the amount of light that actually excites the PS inside the *C. albicans* cells. The excitation may be being very low, inefficient.

4. CONCLUSION

The bacteriochlorin trisma molecule was unstable about the light absorption peak, estimated at a wavelength of 780 nm, but measured in bands above 800 nm. The solvent used was decisive for this displacement. This characteristic impaired the excitation of the PS compromising the effectiveness of PDI, which, in its theoretical basis, reinforces the need for alignment between the emission spectrum of the light source and the absorption spectrum of the PS. The solubility problems of the bacteriochlorin trisma molecule make it difficult to correctly infer the concentration of photosensitizer that is applied to the cell. This aspect also affects the results of PDI, since the technique is based on the appropriate combination of the dose of light and the concentration of the PS.

ACKNOWLEDGEMENTS

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