Effectiveness in total reduction of *Candida albicans* promoted by PDT with hypocrellin B:Lanthanum

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ABSTRACT

In this work we described the potentiality of the Hypocrellin B (HB) modified with the presence of lanthanum (La^{3+}) ions, in eliminate *Candida albicans* in suspension. The results showed that the presence of lanthanum ions promotes a red shift of the HB absorption band and an enhancement in singlet oxygen quantum yield in 32%. Also in this work we obtained that the best molar ration between HB and La concentrations was 1:2. No photobleaching was observed in our experimental conditions. Antimicrobial activity was studied exciting *C. albicans* suspension with a 460 nm LED and a 660 nm laser both with 330 mW/cm^2 irradiance. Best irradiation time, PS concentration and ROS production profile were determined showing that using 460 nm LED with 10 μM of PS, only 30 s of irradiation time was sufficient to reduce 100 % *C. albicans* colonies.

Keywords: PDT, Hypocrellin, *Candida albicans*

1. INTRODUCTION

Candida albicans is an opportunistic human fungal pathogen and it is responsible for candidiasis [1]. Due to the increased number of immunocompromised patients, the infections associated to the pathogenic microbes, among which *C. albicans* have increased in the recent years[2]. For this reason, antifungal resistance is considered a challenge of the millennium. Recently, photodynamic therapy (PDT) has been presented[3] as a new antimicrobial treatment modality. PDT starts when a dye absorbs light at its resonant wavelength. Simultaneously, this dye generates ROS (reactive oxygen species) upon photochemical reaction with non-absorbing molecules present in the environment. These ROS are responsible for microbial apoptosis [4] [5]. The photochemical reactions occur under electron transfer (type I), generating radical products, and/or energy transfer (type II), generating singlet oxygen [6]. PDT also depends on factors such as the light exposure interval, the light exposure regime, the concentration of photosensitizer, environmental conditions. [7].

The photodynamic effects of different photosensitizing drugs [8] [9] and the fungicide effect of methylene blue (MB), as a photosensitizing drug on *C. albicans* were demonstrated [10] [3].

The purpose of this preliminary study was to investigate the effect of the PDT, using Hypocrellin B (HB) complexed with Lanthanum ions (La^{3+}), a new antimicrobial PDT photosensitizer. HB, Figure 1, is a naturally-occurring perylenequinonoid dye extracted from the fungus *Hypocrella bambusae*, is one among several dyes currently used for PDT [5] [11] [12] [13]. Hypocrellins B are hydrophobic, and exhibit weak absorption in the phototherapeutic window (600–900 nm). These factors limit the clinical application of hypocrellins. Hence there is wide interest to improve red absorption[14] and amphiphilicity of hypocrellins. Studies in the literature have shown that the PDT performance of HB can be improved modifying its structure for example complexing it with lanthanum ions [15, 16]. To evaluate HB:La^{3+} complex activities against in vitro suspension of *C. albicans* we compare its performance with one of MB.

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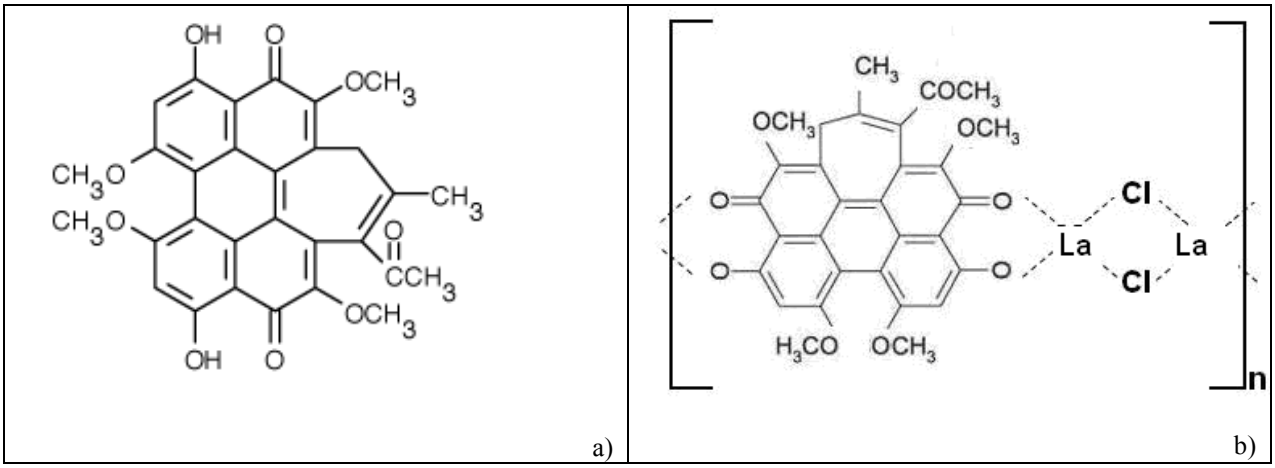

Figure 1. Molecular structure of a) hypocrellin B ($C_{30}H_{24}O_9$) and b) Hypocrellin B:Lanthanum.

2. MATERIALS AND METHODS

2.1. Hb:La^{3+} Complexes and Best Molar Ratios

Hypocrellin B (HB) was purchased from Shaanxi Tianze Bio-Technology CO., LTD. Lanthanum chloride ($LaCl_3.7H_20$) and ethanol (CH_3CH_2OH), used as a solvent, were purchased from Sigma-Aldrich Corporation Ltd. and were of analytical grade. HB and lanthanum chloride (La^{3+}) were separately diluted in ethanol, forming two solutions of equal molarity. The HB and La^{3+} solutions were then mixed in molar ratios of 1:0.5, 1:1, 1:2 and 1:3, with the goal of determining the number of moles of La^{3+} that HB can chelate. All solutions were prepared with the same final concentration, 70μM. This allowed us to quantitatively compare the optical properties of the complexes.

Due to the poor solubility of Hypocrellin B in water, highly concentrated (1mM) Hypocrellin B (HB) or Hypocrellin B:Lanthanum (HB:La^{3+}) solutions were prepared in ethanol and then diluted in PBS for applications.

The solution of MB was prepared by the dissolution of the powder (Sigma Ltd, Poole, UK) in distillated water in a concentration of 10mM, which was filtered through a sterile filter membrane (0.22μm, Millipore, São Paulo, Brazil). The photosensitizer was added to the yeast suspension in proportion of 1/100 that resulted in a final concentration of 100μM

2.2. Optical Characterization of the Complexes - Absorption and Emission Spectra

The absorption spectra of all samples were measured in the range of 300-700nm at room temperature using a Varian Cary 17D Spectrometer. They were plotted in terms of molar absorptivity (ε):

$$\varepsilon = \frac{OD}{b.c} \qquad (1)$$

where OD is the optical density, b is the optical path length (0.1 cm) and c is the concentration of samples (70.10^{-6} M). ε is given in units of $M^{-1}.cm^{-1}$.

Emission spectra were obtained by exciting the samples with a 150 W Xenon lamp in a 1-mm optical path length cuvette. Emissions from the samples were analyzed with a 0.5m Spex monochromator and a PMT detector. The signal was amplified with an EG&G 7220 lock-in amplifier and processed by computer.

Infrared emission around 1270nm was measured with an emission system by Edinburg Instruments. Such system consists of a Q-switched Nd:YAG laser (Continuum Surelite III) emitting 5ns pulses (FWHM) at the second harmonic with a 10-Hz repetition rate, a Hamamatsu R5509 photomultiplier tube, and a 1-cm optical path length cuvette.

2.3. *Candida albicans photoinactivation*

Candida albicans ATCC 90028 was sub-cultured aerobically onto Sabouraud dextrose agar for 48h at 37°C. Yeasts were harvested from the agar and then suspended in phosphatase buffer saline (PBS) at a concentration of approximately 10^6 colony forming units (CFU)/mL. Transmittance of the cuvette fluid was adjusted to achieve a concentration of 72 ± 5% in an optical spectrophotometer at 530nm[17].

The irradiation sources used in this study were a 100mW diode laser (Photon Laser III, DMC, São Carlos, Brazil) and a 460mW LED (Quantum, Ecco Fibras, Campinas, Brazil). Irradiation parameters and details are described in table 1.

Table 1 – Light parameters used in this study

	Laser	LED
Wavelengths (nm)	660±5	460±30
Spot size (cm^2)	0,3	1,29
Output power (mW)	100	430
Irradiance (mW/cm^2)	333	
Irradiant exposure (s)	0 - 360	
Dose (J/cm^2)	0 - 120	

The suspension of each strain was divided into different groups (table 2). The control group (L-PS-) was untreated with either light or photosensitizer (PS). In the PS group (L-PS+), yeast suspensions were stained with either HB or HB:La in a final concentration of 10μM and MB with concentration of 100μM. The solutions were kept in contact with the cells for 5 min in order to evaluate its toxicity in dark conditions. In the PDT group (L+PS+), aliquots of yeast suspension with PS were put in a 96-well flat-bottom microtiter plate and then irradiated with light, in fluence rates of 333 mW/cm² (table 1). Samples were also subdivided in groups to compare different periods of illumination, from 0 to 360 seconds; thereafter, all samples were serially diluted in PBS to generate dilutions of 10^{-1} to 10^{-4} times the original concentration[18].

Ten-μL aliquots of each dilution were streaked onto a Sabouraud agar plate in triplicate and incubated to allow colony formation[19].

Table 2 – Work groups.

Groups	Characteristics
L-PS-	Control Group , untreated with either light or photosensitizer
L-PS+ L-HB+ L-HB:La+ L-MB+	HB, HB:La (HB:La^{3+}) or MB treated
L+PS+ LB+HB+ LB+HB:La+ LR+HB+ LR+HB:La+ LR+MB+	HB, HB:La (HB:La^{3+}) or MB and light treated LB - Blue LED LR - Red laser

2.4. Statistics

Yeast colonies were counted and converted into CFU for analysis. All samples were subjected to this process and statistical analysis of the experimental data was performed using one-way analysis of variance (ANOVA). Mean comparisons were carried out using Tukey's test, which keeps the overall significance level at 5% ($p<0.05$)[20].

3. RESULTS

3.1. Optical properties of Hb:La^{3+} Complexes

Hypocrellin B in ethanol has a very large absorption band that stretches from 400 nm to 600 nm, characterized by three absorption peaks centered on 461, 548 and 589 nm. This observation is consistent with the fact that the absorption spectra of perylenequinonoid pigments at visible wavelengths comprise three bands: one with lower wavelength and higher intensity due to a $\pi \rightarrow \pi^*$ transition, and two others resulting from intramolecular proton transfer.

After the addition of lanthanum ions, changes in HB absorption spectrum were observed, indicating successful complexation (Figure 2a). The absorption peaks showed a red shift to 471, 552 and 603 nm.

The absorption bands of the HB:La^{+3} complex are similar to those of HB, covering all visible wavelengths. At wavelengths longer than 600 nm, however, absorptivity increases remarkably. This is good for photodynamic therapy (PDT), since the higher the incident wavelength, the higher its penetration in tissues. The best molar ratio among all the analyzed complexes was 1 mol of HB for 2 moles of lanthanum ion (1HB:2La^{+3}), which means that there are two binding points on the HB molecule. The number of bonds between the ions and the molecule depends not only on the nature of the molecule, but also on the nature of the metal ions being used. 1HB:2La^{+3} structure have the two lanthanum ions bind with the phenolic hydroxyl oxygen and the carbonyl oxygen of HB, and the bridge between these ions is done by Cl^- ions[16].

Figure 2b shows the HB:La^{+3} complex emission spectra under excitation at 460 nm. It can be seen that all solutions show a large emission band ranging from about 550 to 750 nm. The shortest wavelength emission band of HB, around 617 nm, is due to a $\pi \rightarrow \pi^*$ transition and the emission band around 660 nm is due to intramolecular proton transfer[14]. We observe that HB or HB:La^{+3} solutions display nearly mirror symmetry relationship to the visible range of their absorption. Generally, the mirror symmetry relationship could be expected to exist in one species. It can be observed also that fluorescence maximum occurs at 617 nm for HB and at 627 nm for 1HB:2La^{+3}.

Figure 3 shows absorption of 1HB:2La^{+3} PBS solution containing *C. albicans*. We observed that occurs some changes in absorption spectra in the presence of PBS *C. albicans* suspension. We observe that the $\pi \rightarrow \pi^*$ transition at 471 nm doesn't suffer influence of the new environment. However the absorption spectrum for 1Hb:2La^{+3} with PBS *C. albicans* suspension shows an enlargement of absorption band around 580 nm. In the emission spectrum of 1HB:2La^{+3} PBS solution containing *C. albicans* we observe a blue shift comparing to 1HB:2La^{+3} ethanol solution from 627 to 620 nm. We suggest that Lanthanum ions can be caged by ions present in the PBS solution and free Hypocrellin B molecules appears in solution which causes changes both in the absorption spectrum and the emission spectrum.

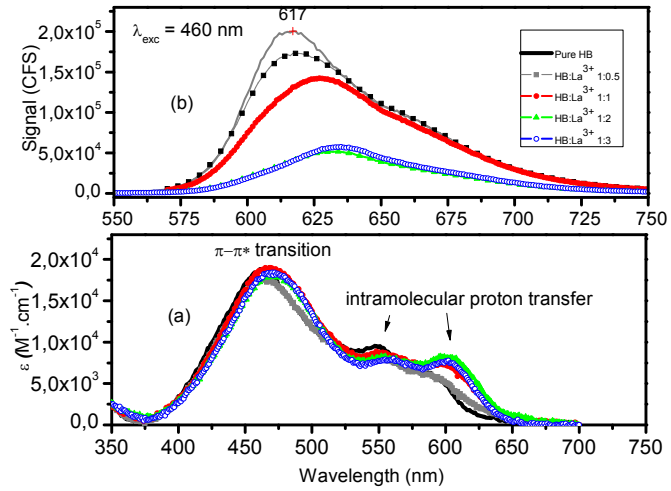

Figure 2. Absorption (a) and emission (b) spectra of HB:La^{+3} complex in ethanol at different molar ratio.

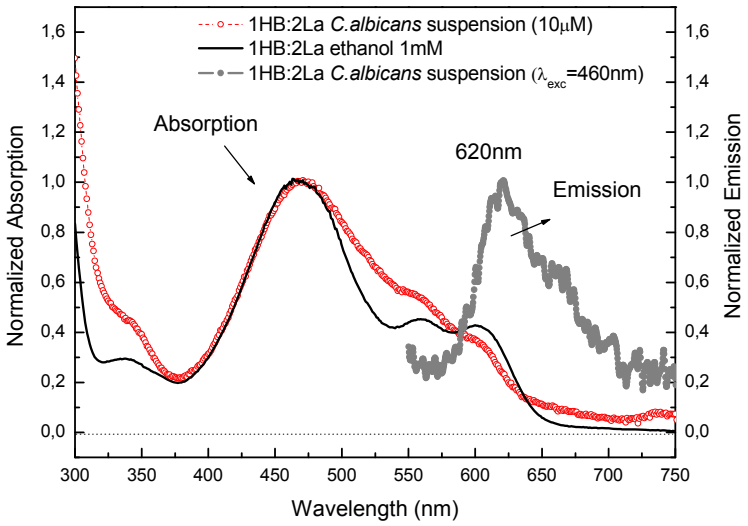

Figure 3**.** Absorption and emission normalized spectra obtained for 1HB:2La^{3+}complex with PBS *C. albicans* suspension.

3.2. Singlet Oxygen Generation Quantum Yields

Figure 4 presents the ethanol solutions emission spectra at the infrared region of the electromagnetic spectrum, under excitation at 532 nm. Methylene Blue (MB) was employed as a reference (Φ_Δ^{MB} = 0.52) for determining the singlet oxygen quantum yields of the ethanol solutions. Indeed, emission at this wavelength is due to the transition from singlet oxygen to ground state oxygen:

$$^1\Delta_g \rightarrow {}^3\Sigma_g^- + h\nu\ (1270\ nm). \qquad (1)$$

It is very important to emphasize that the MB solution was also prepared in ethanol, as had been done with the samples. Finally, singlet oxygen luminescence efficiency, η_L, may vary with the environment. Therefore, the following equation was used to calculate the values of Φ_Δ:

$$\Phi_\Delta^S = \frac{I_S}{I_R}.\frac{A_R}{A_S}.\Phi_\Delta^R \qquad (2)$$

where I is the signal intensity at 1270 nm, A is the absorbance at 532 nm (the excitation source) and the indexes S and R mean sample and reference, respectively.

As shown in Figure 4, the addition of lanthanum ion to HB in ethanol can improve the Φ_Δ value of HB (from 0.47 to 0.62).

Singlet oxygen was also detected chemically by monitoring the bleaching (decrease in A at 440 nm) as can be seen in figure 5 of p-nitrosodimethylaniline (RNO) [21]. Since singlet oxygen ($^1O_2$) does not react chemically with RNO, this bleaching is a consequence of $^1O_2$ capture by the imidazole ring which results in the formation of a trans-annular peroxide intermediate [$^1O_2$] capable of inducing the bleaching of RNO (-RNO). In the absence of RNO, [$^1O_2$] decomposes or rearranges into the final oxygenation product $^1O_2$: $^1\Delta_g$. For this measurement it was used 10μM 1HB:2La^{+3} diluted in water solution.

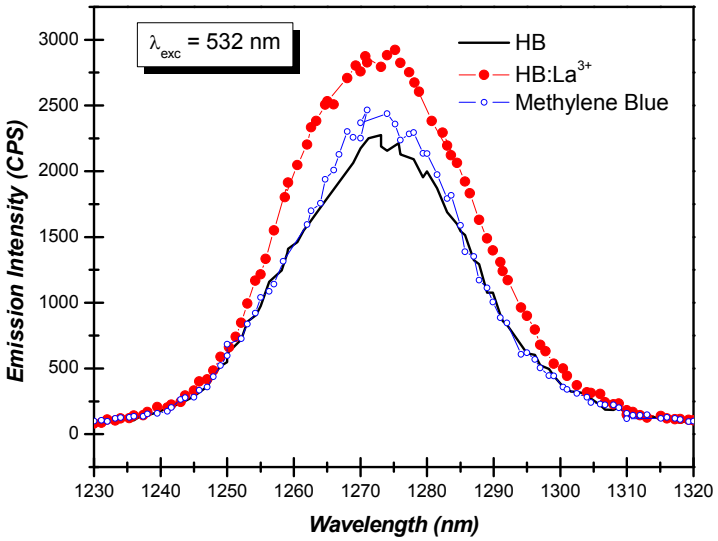

Figure 4. Luminescence spectra at infrared of samples in ethanol under excitation at 532 nm.

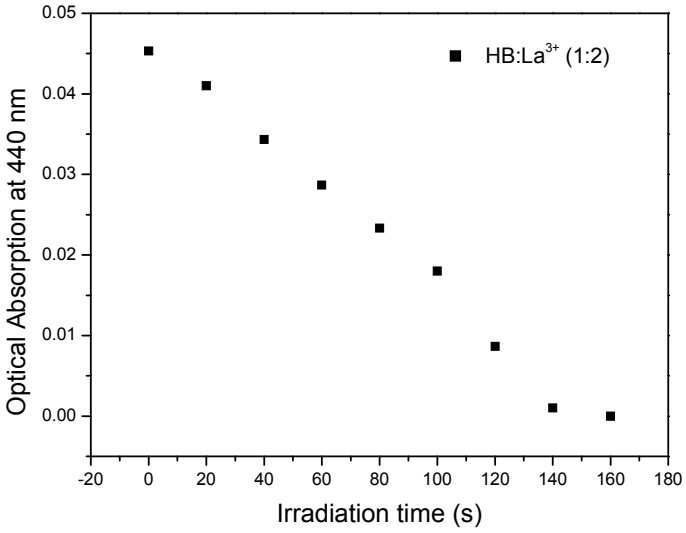

Figure 5. Changes in the concentration of RNO on irradiation time of aqueous solutions containing HB:La^{+3}.

3.3. *Candida albicans photoinactivation*

For *Candida albicans* in vitro tests, HB and 1HB:2La^{3+} solutions were diluted with PBS to obtain 10μM solution. This concentration was chosen irradiating solutions of 0,5; 1; 5 e 10 μM HB:La^{3+} with a LED at 460 nm during 10 s with intensity of 333 mW/cm^2. It was observed from the figure 6 that the photodynamic effect occurs for concentration of 10 μM and lower concentrations do not show significant difference following irradiation compared to their control groups without irradiation.

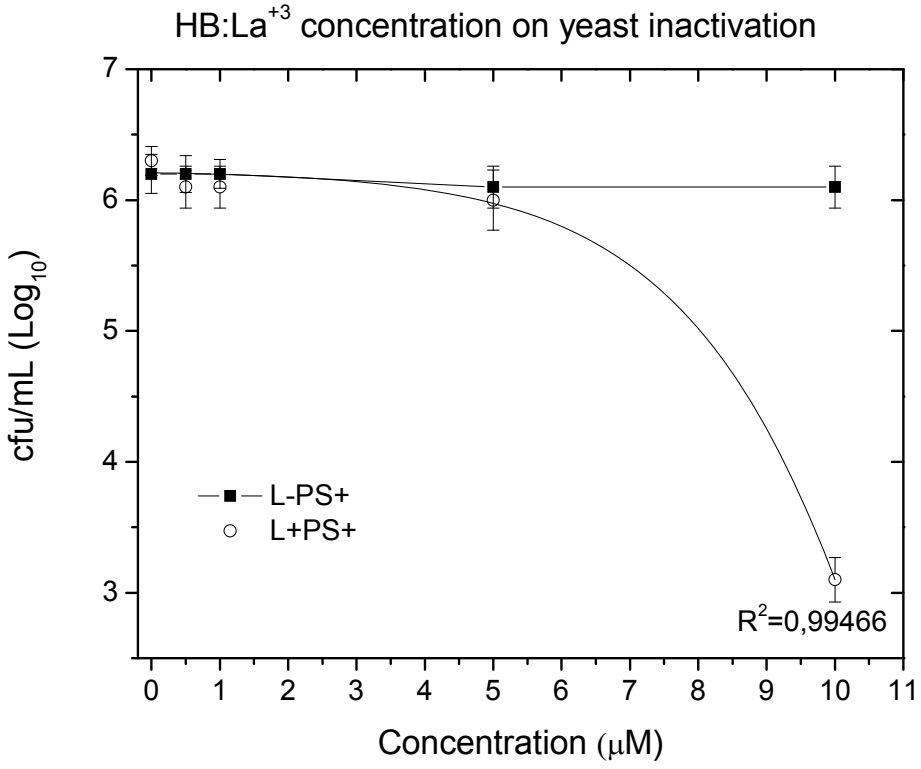

Figure 6. Effect of 1HB:2La^{3+} concentration on photoynamic activity against *C. albicans*. Points in the graphic are means and standard deviation of ufc/mL

Ten-μM 1HB:2La^{3+} staining *C. albicans* suspension in PBS was pumped with a 440mW power, 460nm LED and a 100mW power 660nm laser from 0 to 360s. The results are shown in figure 7.

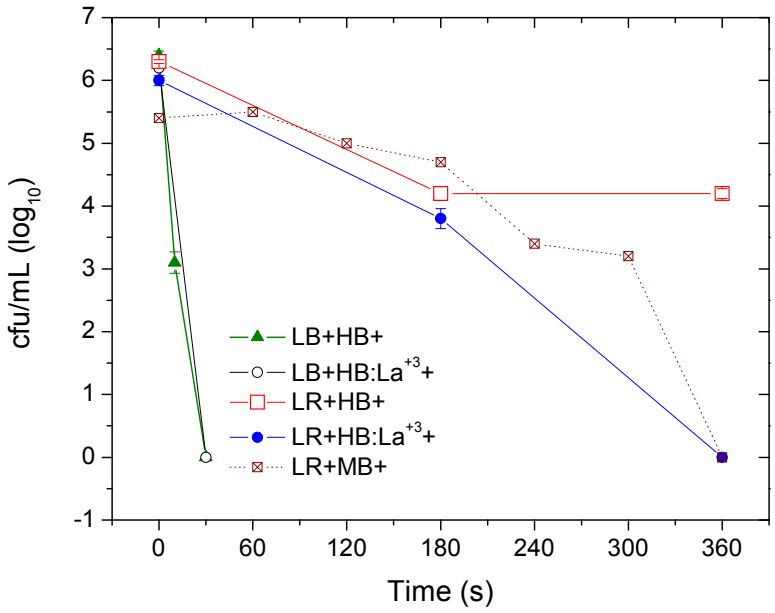

Figure 8. *C. albicans* PDT action stained by 10μM (1HB:2La^{3+} and HB) associated to irradiation at 660nm and 460nm. Error bars represent standard deviations.

The results show that the presence of 10μM 1HB:2La^{3+} solution in *C. albicans* suspension for 5min, did not present dark citotoxicity, since control group did not show significant differences between number of viable cells compared to L-PS+ groups. Using the 460nm LED with 10μM of photosensitizer solution (HB and HB:La^{3+}), only 30s of irradiation were sufficient to eradicate the microorganisms. On the other hand, 660nm laser irradiation presented a pronounced photodynamic effect using 1HB:2La^{3+}. After 180s of irradiation, the microbial reduction was 3 logs for both photosensitizers., at 360s of irradiation, the group stained by HB did not presented any increase on microbial reduction. However, the 1HB:2La^{+3} group presented a total elimination of the target cells. The photodynamic action of 1HB:2La^{3+} was similar to MB under excitation at 660 nm. Furthermore, photodynamic action was observed with both HB and 1HB:2La^{3+}. Nevertheless, using the 660nm laser, even the very small absorption coefficient at this wavelength, HB:La^{3+} resulted in stronger PDT action when compared to its precursor. This effect results from the increased absorption coefficient in the red region when lanthanum ions are incorporated in the HB solution.

4. CONCLUSIONS

Optical spectroscopy was employed in this work to determine the properties of the photosensitizers Hypocrellin B (HB) and the Hypocrellin:Lanthanum (HB:La^{+3}) complex. HB:La^{+3} exhibits a red-shifted absorption spectrum, with increased absorbance above 600 nm. The singlet oxygen emission spectra for HB and HB:La^{+3} were measured, and HB:La^{+3} showed an enhancement of 32%.

The main results of this work were to improve the photodynamic properties of Hypocrellin B and to present the potential of the 1HB:2La^{3+} complex for PDT. Successful inactivation of *Candida albicans* following continuous light irradiation at 460nm and 660nm was demonstrated for 1HB:2La^{3+}. Only 10 s at 460 nm LED was sufficient to promote a reduction of 3 logs, showing high potentiality of the 1HB:2La^{+3} as photosensitizer. No dark toxicity was observed on the samples stained by 1HB:2La^{+3} until 10min of contact. Nevertheless, using 660nm laser, 1HB:2La^{3+} resulted in an increase in the PDT action compared to its precursor. This effect results from the increase in the absorption coefficient in the red region when lanthanum ions are incorporated in HB solution. Action of 1HB:2La^{+3} was similar to MB with excitation at 660 nm.

The results reported herein thus indicate promising antifungal actions of hypocrellin B:Lanthanum. Further evaluation of in vivo antifungal activities in an animal model is needed and change in the light excitation to ~600 nm is indicated.

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