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ORIGINAL ARTICLE

Antibacterial and antifungal activity of curcumin and methylene blue associated with laser on bacterial and fungal strains

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ABSTRACT

Objective: To analyze the effect of methylene blue and 10% curcumin in fungi and bacteria through an *in vitro* study using photodynamic therapy (PDT).
Methods: Curcumin and methylene blue were photosensitized by a Photon Lase III laser applied for 90 s in a dark environment within a laminar flow chamber. *Enterococcus faecalis* and *Candida albicans* strains were cultured and standardized. Then, a minimum inhibitory concentration (MIC) assay was conducted for these photosensitizers, with concentration variations and incubation to evaluate their antimicrobial activity.
Results: With PDT, Curcumin had significant antibacterial activity against *E. faecalis* (MIC = 250 µg/mL). In contrast, methylene blue had antibacterial activity against *E. faecalis* (MIC < 12.5 µg/mL with PDT) and antifungal activity against *C. albicans* (MIC <12.5 µg/mL with or without PDT). Both agents showed greater efficacy in the presence of the laser. The results suggest that curcumin and methylene blue associated with laser may effectively treat microbial infections.
Conclusion: Further research is needed to evaluate the efficacy and safety of using these agents in animal and human models and their effectiveness against different bacterial and fungal strains.

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INTRODUCTION

Photodynamic therapy (PDT) is a therapeutic technique that involves the administration of a photosensitizer and its activation by a light source to generate reactive oxygen species (ROS) that cause damage to the target cells. PDT is a therapeutic option in various areas of medicine. Because it is selective and non-invasive, it is a promising approach for treating bacterial, viral, and fungal infections, especially those caused by antibiotic-resistant pathogens. Curcumin, a polyphenolic compound found in the rhizome of *Curcuma longa*, has been studied as a promising photosensitizer for PDT because of its unique photophysical and photochemical properties[1-3].

Several studies suggest that curcumin may be an effective therapeutic agent against various infectious pathogens, including bacteria, viruses, and fungi. PDT using curcumin as a photosensitizer has been investigated in several *in vitro* and *in vivo* studies with encouraging results. Curcumin can be topically or systemically administered and activated by different wavelengths of light, including visible and infrared light[2,4-7].

Because of its wide-ranging properties, methylene blue is a photosensitizing chemical compound that is used in various applications, including photodynamic therapy. It can absorb light at specific wavelengths and transfer this energy to the molecular oxygen present in tissues, which leads to the production of ROS, which are toxic to cells and can cause irreparable damage. It is worth noting that its activation only occurs when exposed to light of a specific wavelength, allowing the treatment to be targeted to specific areas and minimizing damage to surrounding tissues. Currently, methylene blue is used in several areas of medicine, including oncology, dermatology, and ophthalmology, to treat conditions such as cancer, infections, and vascular diseases[8].

However, many issues must be addressed before PDT with curcumin can be widely used in clinical practice. These include the appropriate choice of dosage regimen, determination of the optimal wavelength for curcumin activation, optimization of curcumin concentration, and identification of the molecular mechanisms underlying the antimicrobial effects of PDT with curcumin[4,5]. This study aimed to use PDT to analyze the effect of methylene blue and 10% curcumin on the inhibition of fungal and bacterial cell culture growth through an *in vitro* study.

METHODS

High-performance liquid chromatography of the photosensitizer

Curcumin is a compound naturally found in turmeric root and is known for its antioxidant and anti-inflammatory properties. Chromatography is a common technique for analyzing curcumin and other compounds in complex matrices such as herbs and spices.

The equipment used in chromatography was a Shimadzu Prominence HPLC system (DGU-20A3, LC-20AD; SIL-20AHT, CTO-20A; SPD-20A, CBM-20A HPLC), which is a high-performance and reliable system for the separation of complex compounds. The column used was a Phenomenex Luna 5µm, a reverse-phase column with high separation efficiency.

A 3 µL curcumin sample (at a concentration of 7 µg/mL) was injected. The flow rate of the mobile phase was 0.200 mL/min. Detection was performed at two wavelengths, 290 and 365 nm, which are the standard wavelengths for detecting curcumin. The program used for chromatography involved water as phase A and acetonitrile as phase B, with a gradient concentration of phase B from 2% to 100% in 20 min. The oven temperature was maintained at 40 °C during the analysis (Figure 1).

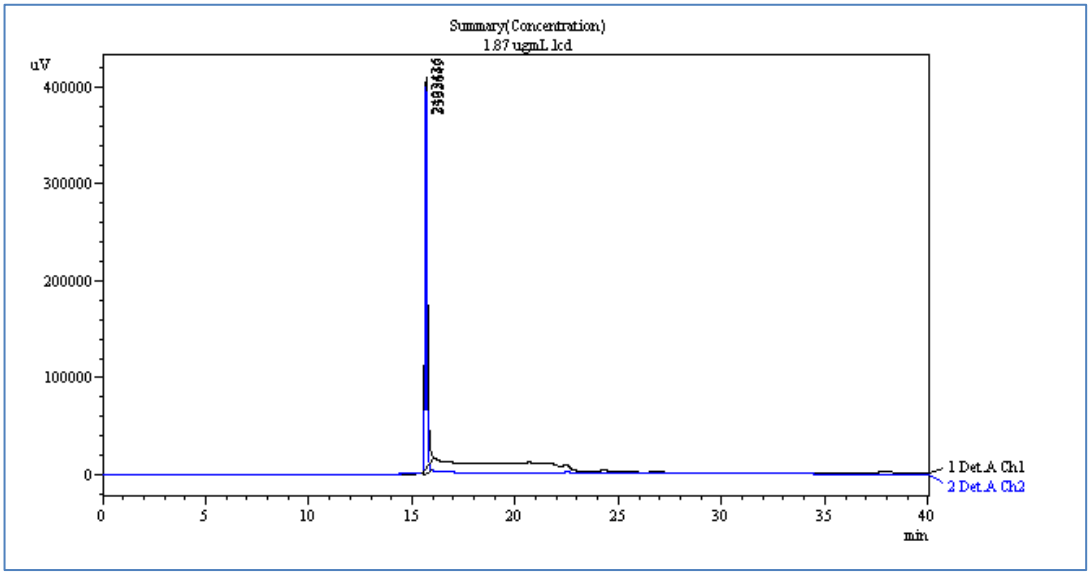

Figure 1 — Chromatogram showing a curcumin retention time of 15.68 min, confirming the sample was purified at > 99%.

Laser

A low-power laser with adjusted wavelength and energy settings was used in photodynamic therapy for effective application. Photon Lase III laser model therapy EC equipment was used to photosensitize curcumin and methylene blue.This equipment has GaAlAs active medium and a wavelength of 660 nm, which corresponds to the high absorption wavelength range of these photosensitizers.

A 100 mW laser power was used and measured before each experimental procedure. The irradiation area on the plate was determined, and the laser was continuously irradiated for 90 s with the laser tip opening above the microwell. Irradiation was performed in the dark in a laminar flow chamber (Figure 2).

Figure 2 — Determination of the irradiation area on the plate.

Microorganism preparation and determination of the minimum inhibitory concentration (MIC)

The bacterial strain *Enterococcus faecalis* ATCC 29212 was previously cultured in a bacteriological incubator at 35 ± 2 °C for 24 h, according to the protocol suggested by the Clinical & Laboratory Standards Institute (CLSI) in 2015[9]. From the grown colonies, standardized suspensions were prepared in sterile saline solution (0.85% w/v NaCl) to obtain an absorbance between 0.08 and 0.13 at a wavelength of 625 nm, corresponding to the McFarland scale (1-2 × 10^8CFU/mL). The microorganism suspension was diluted in Mueller-Hinton broth to prepare the bacterial inoculum for a 5 × 10^5 CFU/mL. The MIC values for the photosensitizer (i.e., Curcumin), the laser (90 s), and the photosensitizer associated with the laser (90 s) were determined.

For this purpose, the inhibitory agents were subjected to serial dilutions of a 2-fold ratio, in which the concentrations of curcumin ranged from 500 µg/mL to 125 µg/mL and those of methylene blue ranged from 50 µg/mL to 12.5 µg/mL. The experiment was performed in triplicate, and the microplates were placed in a bacteriological incubator at 35 ± 2 °C for 24 h. After this period, the results were observed with added resazurin blue (0.1%), followed by incubation at 35 ± 2 °C for 20 min. The color of the dye changes from blue to pink based on the viability of the microorganism (Figure 3).

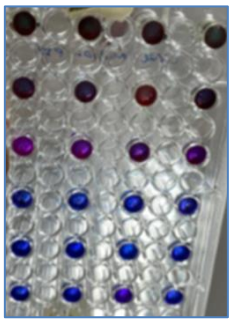

Figure 3 — Observation of results with addition of resazurin blue (0.1%), followed by incubation at 35 ± 2 °C for 20 min. The color of the dye changes from blue to pink based on the viability of the microorganism.

The fungal strain *Candida albicans* ATCC 10231 was tested according to the procedures of document M27-A3 (2008) of the CLSI with adaptations[10]. Microorganisms were exposed to curcumin and methylene blue with and without laser in brain heart infusion (BHI) medium. The yeasts were initially subcultured in tubes containing Sabouraud agar, incubated in an oven at 35 ± 2 °C for 48 h, prepared in sterile saline solution at 0.85%, and standardized in a spectrophotometer with λ = 530 nm until a transmittance value of 90% was obtained. The inocula were diluted in BHI medium to achieve a final concentration of microorganisms between 0.5 x 10^3 and 2.5 x 10^3 CFU/mL.

The MIC values for the photosensitizer (i.e., Curcumin), laser (90 s), and the photosensitizer associated with the laser (90 s) were determined according to CLSI. Initially, the antifungal activity of the photosensitizers without the action of the laser was determined. The yeast was exposed to serial dilutions of curcumin and methylene blue at concentrations ranging from 500 µg/mL to 125 µg/mL and 50 µg/mL to 12.5 µg/mL, respectively. The experiment was performed in triplicate, and the microplates were placed in an incubator at 35 ± 2 °C for 24 h. The results were observed by adding resazurin blue (0.1%), followed by incubation at 35 ± 2 °C for 20 min.

RESULTS

The results showed that curcumin exhibited significant antibacterial activity against *E. faecalis* ATCC 29212, with a MIC of 250 µg/mL in the presence of laser and >500 µg/mL in the absence of laser. Methylene blue also showed significant antibacterial activity against *E. faecalis*, with a MIC of <12.5 µg/mL in the presence of laser and 25 µg/mL in the absence of laser. Regarding *C. albicans* ATCC 10231, methylene blue exhibited significant antifungal activity in the presence or absence of laser, with MIC <12.5 µg/mL (Table 1).

DISCUSSION

This study aimed to evaluate the antibacterial and antifungal activities of curcumin and methylene blue

Table 1 — Minimum inhibitory concentration (MIC) in µg/mL of photosensitizing agents against *Enterococcus faecalis* and *Candida albicans* used after laser application.

		Curcumin	Methylene blue
E. faecalis ATCC 29212	With PDT	250	< 12.5
	Without PDT	> 500	25
C. albicans ATCC 10231	With PDT	> 500	< 12.5
	Without PDT	> 500	< 12.5

associated with laser on bacterial and fungal strains. The chromatography results were consistent with the presence of curcumin in the sample and the effective separation of curcumin from the complex matrix. The reported peak area suggests that the concentration of curcumin in the sample was relatively high.

The MIC determines the effectiveness of an antimicrobial agent on a specific microorganism and is defined as the smallest amount of a compound capable of preventing the growth of 90% of the target microorganism population. In practice, microorganisms are inoculated with different concentrations of the antimicrobial agent. Subsequently, the plates were incubated for a determined period, and at the end of this period, the development of microorganisms is evaluated at each compound concentration. The lowest concentration that can inhibit the growth of 90% of the bacterial population is identified as MIC[11].

In the present study, an important reduction in MIC was observed when photodynamic therapy was used against the bacterium *E. faecalis* ATCC 29212. Furthermore, when methylene blue was associated with PDT, the reduction in MIC was even more significant, indicating that curcumin and methylene blue associated with laser may effectively reduce bacterial and fungal colonies *in vitro*.

Laser may increase the efficacy of antibacterial and antifungal agents, probably because of the increased penetration of photosensitizers into microbial cells and the generation of ROS that cause cell damage. A similar finding was observed in a study that demonstrated the efficacy of PDT associated with curcumin in significant morphological changes of promastigotes forms of *Leishmania major* and *Leishmania braziliensis*. However, more research is required to investigate mitochondrial activity in tests[9-13].

However, it is essential to note that this study was performed *in vitro*, and further research is required to evaluate the efficacy and safety of curcumin and methylene blue associated with laser in animal and human models. In addition, the efficacy of the treatment needs to be evaluated against different types of bacterial and fungal strains, as well as the influence of different laser parameters such as power and exposure time. Despite these limitations, the results of this study provide promising information regarding the potential use of curcumin and methylene blue associated with laser in treating bacterial and fungal infections[4,5,10,11,13-16].

Moreover, the efficacy of curcumin and methylene blue photosensitizers associated with PDT was evaluated and compared with the isolated use of photosensitizers for inhibiting bacterial and fungal growth. The results indicated that PDT effectively reduced the MIC in both tested strains compared with the isolated use of photosensitizers. This suggests that PDT is a promising therapeutic approach for treating microbial infections, especially in cases where conventional antibiotics are limited or ineffective[5,11,14,15,18,19].

Finally, the results of this study suggest that curcumin and methylene blue are potential therapeutic agents for treating bacterial and fungal infections. In addition, the association of photosensitizers with PDT may be an effective therapeutic alternative in cases of infections resistant to conventional antibiotics. However, further studies are required to investigate the safety and clinical efficacy of these therapeutic agents in different animal and human models. Moreover, optimizing the application conditions of photosensitizers with respect to irradiation time and dosage is essential for obtaining more precise and reliable results.

CONCLUSION

This study showed that the photosensitizing agents curcumin and methylene blue, when used in conjunction with PDT, were more effective in inhibiting the growth of the fungal and bacterial organisms C. *albicans* ATCC 10231 and *E. faecalis* ATCC 29212, respectively. The efficacy of this inhibition observed through the decrease in the MIC with both photosensitizers may be due to the more significant penetration of photosensitizers into the cells of microorganisms and the generation of ROS. Therefore, the results suggest that curcumin and methylene blue associated with laser may effectively treat microbial infections.

However, more studies are necessary to adequately prove the effectiveness of curcumin and methylene blue associated with PDT in combating fungal and bacterial infections.

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