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Evaluation of the Effects of Photodynamic Therapy With Methylene Blue on Different *Candida* Species *In Vitro*

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Abstract

Introduction: Oral candidiasis is the most prevalent opportunistic infection of the oral cavity. The most common cause of this infection is *Candida albicans*. Considering the side effects of conventional antifungal therapies, this study aimed to evaluate the efficacy of photodynamic therapy with new methylene blue (a photosensitizer) in inhibiting the growth of *C. albicans*, *C. tropicalis*, *C. glabrata*, and *C. krusei in vitro*.

Methods: In this experimental study, 200 samples of standard suspension (0.5 McFarland) were prepared from *C. albicans*, *C. tropicalis*, *C. glabrata*, and *C. krusei* (50 samples from each species). The samples of each species were divided into five groups (n = 10), including photodynamic therapy with a photosensitizer, with or without laser irradiation, nystatin treatment, laser therapy, and control. Next, cultivation of samples was performed on Sabouraud dextrose agar, and the colony-forming units were determined after 24 hours of incubation at 37 °C. Data were analyzed in SPSS version 22 by means of the Kruskal-Wallis test ($P < 0.05$).

Results: The most sensitive and resistant species to nystatin therapy were *C. glabrata* and *C. krusei*, respectively. On the other hand, *C. krusei* was the most sensitive species to photodynamic therapy, and *C. glabrata* was the most resistant type to this treatment. The highest therapeutic effectiveness was attributed to nystatin therapy, although photodynamic therapy was also effective. Laser therapy was recognized as the least effective method.

Conclusion: Photodynamic therapy with new methylene blue, as a suitable adjunct therapy, can be effective in the management of candidiasis. It may also be a potential novel treatment for immunocompromised patients with oral candidiasis.

Keywords: Photodynamic therapy; New methylene blue; *Candida*; Laser.

Introduction

Oral candidiasis is the most prevalent opportunistic infection of the oral mucosa. The most common cause of this infection is *Candida albicans*, accounting for 60%-70% of all cases.[1] Nystatin and amphotericin B are routinely administered for the treatment of *C. albicans* infections. However, not only do these medicines have a bitter taste, but they also lead to nausea and intolerance in patients.[2] Moreover, there has been a growth in the resistance of *Candida* species to various antifungal treatments, including azoles, particularly in immunocompromised patients (e.g., AIDS patients and patients undergoing chemotherapy).[3] *Candida albicans*, *C. krusei*, *C. stellatoidea*, *C. kefir*, and *C. glabrata* are among resistant *Candida* species.[4] Therefore, there is a need for the development of new therapeutic methods for these fungal infections.[5-7]

Photodynamic therapy is one of the new therapeutic techniques which use a photosensitizing substance, activated by exposure to light (at a wavelength that is absorbed by it). Activation of the photosensitizer leads to multiple chemical reactions, such as the generation of reactive oxygen species (free radicals) and other reactive molecules, culminating in oxidative stress, followed by tissue and cellular damage at the target sites.[8] Generally, photodynamic therapy is a two-stage process. In the first stage, the photosensitizer is exposed to the target organ or microorganism for a short period to bind to target

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cells. In the second step, light (at a wavelength that the sensitizer can absorb) is emitted to the target tissue. Emission at a specific wavelength can be accomplished by laser or non-aligned sources, such as light-emitting diodes (LEDs). One advantage of laser therapy is the lack of light scattering, as it focuses beams of radiation on the target tissue.

Most previous studies have evaluated the effects of photodynamic therapy on *C. albicans* in animal models or *in vitro*.[9-15] Since there is no study on the effectiveness of photodynamic therapy and laser therapy in eradicating resistant *Candida* species, including *C. glabrata*, *C. tropicalis*, and *C. krusei*, this study aimed to investigate the effects of photodynamic therapy on the growth of these resistant *Candida* species *in vitro*.

Materials and Methods

Study Design

This double-blind experimental study was conducted in the mycology laboratory of Isfahan University of Medical Sciences, Isfahan, Iran, during 2018-2019. The study population consisted of 200 samples of four *Candida* species (50 samples per species), including *C. albicans* (ATCC 10231), *C. glabrata* (ATCC 90030), *C. tropicalis* (PFCC 89-1456), and *C. krusei* (DSM 70079), prepared by Pasteur Institute of Iran (Tehran, Iran). The exclusion criteria were non-standard fungus-containing vials, non-compliance with the storage conditions, and lack of a microbiologist's approval of the sample.

Microorganisms and Culture Conditions

Freeze-dried *Candida* species were subjected to the process of reviving. To prepare a 0.5 McFarland standard, the isolates were passaged twice on Sabouraud dextrose agar (SDA) plates 24 hours before the preparation of *Candida* suspensions. To prepare SDA plates, 65 g of agar was dissolved in 1 mL of distilled water. Suspensions containing *Candida* strains (1×10^6 cells/mL) were standardized by a spectrophotometer at 530 nm (Biochrom WPA Lightwave II UV, Cambridge, UK). To ensure the sterility and darkness of the environment, all the steps were performed under a laminar flow cabinet at 28 °C.

Preparation of the Photosensitizer

To prepare the new methylene blue photosensitizer, new methylene blue (4 mg) was mixed in 4 mL of distilled water to obtain a solution with a concentration of 1 mg/mL, according to the manufacturer's instructions.

Intervention

The samples of each fungal species (n = 50) were divided into five groups (n = 10 per group):

- Group 1 (control group): In this group, 0.1 mL of *Candida* suspension was mixed in 0.1 mL of sterile normal saline in a microplate, and no intervention was performed.
- Group 2 (nystatin therapy): In this group, 0.1 mL of 100 000 U nystatin (Jaber Ebne Hayyan Pharmaceutical Company, Tehran, Iran) and 0.1 mL of *Candida* suspension were mixed in a microplate.
- Group 3 (photosensitizer): In this group, 0.1 mL of new methylene blue was added to 0.1 mL of *Candida* suspension in a microplate.
- Group 4 (laser therapy): In this group, 0.1 mL of sterile normal saline was added to 0.1 mL of *Candida* suspension in a microplate. The samples were exposed to continuous laser emissions at 660 nm.
- Group 5 (photodynamic therapy): In this group, 0.1 mL of new methylene blue was added to 0.1 mL of *Candida* suspension in a microplate. Next, the samples were exposed to continuous laser emissions at 660 nm.

In this study, a diode device (Polaris 2/SW PM2-25/M1/AP; version 4/0; Star Company, Bielsko-Biala, Poland) was used for laser emissions. This task was continuously performed under aseptic conditions for 100 seconds under a laminar flow cabinet in the dark, without contact with the sample (cross-sectional area, 1 cm^2). The power of the laser device was 100 mW, with an irradiation dose of 10 J/cm^2 and a wavelength of 660 nm.

Colony Count

Viable cells were counted per milliliter by a person, who was blinded to the study design, using the pure plate method. In this method, 0.1 mL of the diluted suspension was poured into a sterile plate by a sampler. Next, 25 mL of cooled sterile SDA medium was added to the suspension and thoroughly mixed (Behdad Co., Tehran, Iran) (Figure 1). The plate was then incubated for 24 hours at 37 °C. Forty-eight hours after incubation, *Candida* colonies were counted by a colony counting device (HYC-560 Digital Colony Counter, Hanyang Science Lab Co., Ltd., HYSC, Korea) (Figure 2). The formula used for calculating the number of colonies per milliliter was as follows:

$$Colony\ \ forming\ \ units = Dilution\ \ factor \times Volume$$

Figure 1. Culture Media Prepared by the Pure Plate Method

factor × Number of colonies counted

A lower count of living cells indicated the greater effectiveness of the treatment method in destroying *Candida* species.

Data Analysis

Descriptive statistics, the Kruskal-Wallis test, and the Mann-Whitney post-hoc test were used to analyze the data at a significance level of $P < 0.05$ in SPSS version 22.

Results

According to Figure 3, which presents the average colony-forming units of fungal species in separate treatment methods, nystatin therapy was found to be the most effective method in reducing the number of colonies. Paired comparisons between the treatment methods for each *Candida* species are presented in Table 1.

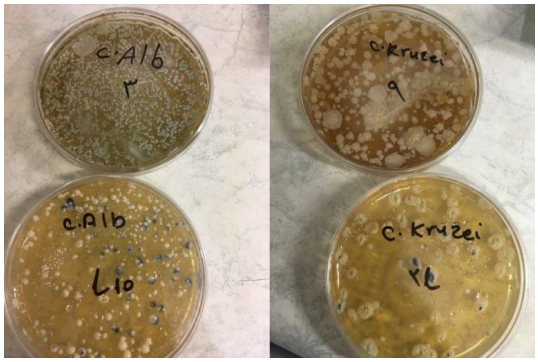

Figure 2. Culture Media Indicating the Growth of Different *Candida* Species

Candida albicans

Significant differences were observed between the control group and the nystatin and photodynamic therapy groups ($P < 0.001$), indicating the greater growth-inhibitory effects of these methods against *C. albicans* compared to other methods; nystatin therapy was even more effective than photodynamic therapy (Figure 1). However, the photosensitizer therapy and laser therapy groups were not significantly different from the control group, indicating the inefficacy of these methods in inhibiting the growth of this species.

Candida krusei

Nystatin therapy, photosensitizer therapy, and photodynamic therapy could effectively inhibit the growth of *C. krusei*. Although photodynamic therapy showed a stronger anti-candidiasis effect, there was no significant difference between these groups ($P > 0.05$) (Figure 3). Also, laser therapy did not show adequate potency to inhibit the growth of this species ($P > 0.05$). This finding suggests that new methyl blue could clearly inhibit the growth of *C. krusei*; the addition of laser radiation to this modality could enhance its effects. Nevertheless, laser therapy alone did not show adequate therapeutic efficacy against this *Candida* species.

Candida glabrata

Compared to the control group, none of the treatment methods could exert significant growth inhibitory effects against *C. glabrata*, except nystatin therapy ($P < 0.001$).

Candida tropicalis

The nystatin therapy and photodynamic therapy

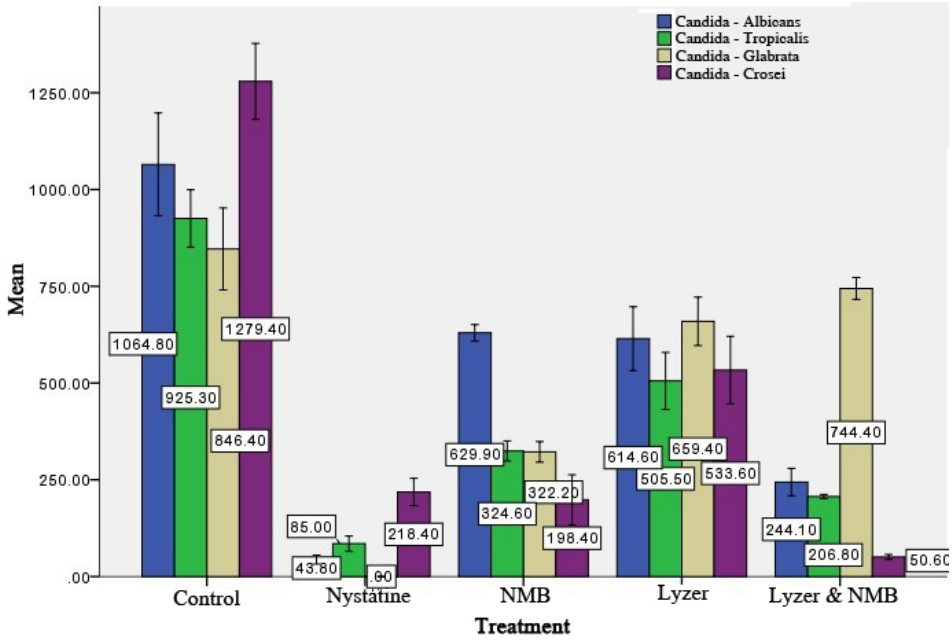

Figure 3. The mean number of colony-forming units stratified by different fungal species subjected to different treatment methods

groups were significantly different from the control group regarding the suppressed growth of *C. tropicalis* ($P<0.001$). However, there was no significant difference between the two former therapeutic methods, which could effectively inhibit the growth of *C. tropicalis* ($P>0.05$). As shown in Table 1, photodynamic therapy was not significantly different from photosensitizer therapy or laser therapy in terms of inhibitory effects against the *Candida* species assessed ($P>0.05$).

According to Figure 4, it can be concluded that nystatin therapy could significantly inhibit the growth of all four studied *Candida* species compared to the control group, suggesting its considerable growth inhibitory effects (Table 1 and Figure 4). The most sensitive and the most resistant species against nystatin therapy were *C. glabrata* and *C. krusei*, respectively. On the other hand, *C. krusei* was the most sensitive species to photodynamic therapy, while *C. glabrata* showed the highest resistance to this therapy.

Based on the results, there was no significant difference between nystatin therapy and photodynamic therapy groups regarding the growth inhibitory effects against the studied *Candida* species, except *C. glabrata* (Table 1), indicating the comparable effectiveness of these two therapeutic methods. Nevertheless, *C. glabrata* was resistant to photodynamic therapy.

Discussion

Candidiasis is one of the most common infections of the oral cavity. However, under certain circumstances, it may become pathogenic, causing oral candidiasis.[16,17] Conventional treatments for oral candidiasis lesions

Table 1. Comparison of the Effects of Various Therapeutic Methods on *Candida* Species

	Candida albicans **(*P* Value)**	***Candida tropicalis*** **(*P* Value)**	***Candida glabrata*** **(*P* Value)**	***Candida krusei*** **(*P* Value)**
Control-nystatin therapy	<0.001	0.024	<0.001	<0.001
Control-photosensitizer therapy	1.0	<0.001	0.101	0.408
Control-laser therapy	1.0	1.0	1.0	1.0
Control-photodynamic therapy	<0.001	<0.001	1.0	<0.001
Nystatin therapy-photosensitizer therapy	<0.001	1.0	1.0	<0.001
Nystatin therapy-laser therapy	0.044	0.045	0.201	0.001
Nystatin therapy-photodynamic therapy	1.0	1.0	<0.001	1.0
Photosensitizer therapy-laser therapy	1.0	1.0	1.0	1.0
Photosensitizer therapy-photodynamic therapy	1.0	1.0	0.196	1.0
Laser therapy-photodynamic therapy	1.0	0.002	1.0	0.408

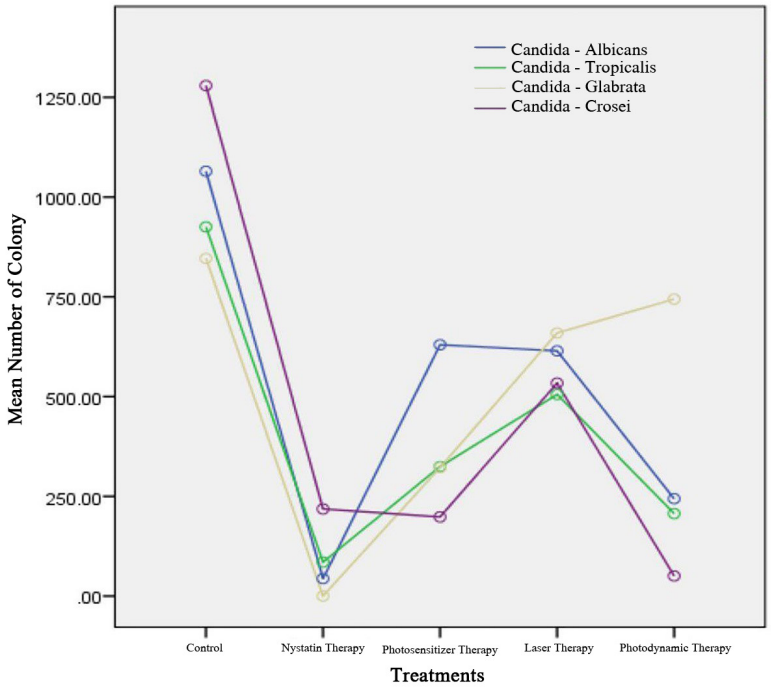

Figure 4. Summary of the Study Findings

include topical medications, such as nystatin and amphotericin B. However, these medicines are usually ineffective for immunocompromised patients, necessitating the prescription of ketoconazole, fluconazole, or itraconazole.[18]

The use of topical and systemic antifungals, especially their long-term use, definitely increases the rate of drug resistance in patients. Approximately 80% of AIDS patients are resistant to conventional antifungal drugs,[19] encouraging scientists to constantly seek novel alternative treatments, including photodynamic therapy. Photodynamic therapy is a technique involving laser radiation in the presence of a dye, which increases oxidation in target cells and kills *Candida* species by destroying the cell wall and inactivating cellular proteins.[20] On the other hand, outpatient treatment requires mouth washing with nystatin five times a day for at least one week, which requires a high level of patient compliance. Given the bitter taste of this medicine, the relatively high rate of non-compliance is usually considered the main reason for the inefficacy of nystatin. However, photodynamic therapy can be performed in private practice and can even replace nystatin therapy for uncooperative patients.

This *in vitro* study aimed to evaluate the inhibitory effects of photodynamic therapy by means of new methylene blue as a photosensitizer against a number of *Candida* species. A total of 200 standard cell suspensions of *C. albicans*, *C. tropicalis*, *C. glabrata*, and *C. krusei* were exposed to either nystatin therapy, photosensitizer therapy, laser therapy, or photodynamic therapy (using new methylene blue as a radiation-absorbent substance). Our literature review revealed that most previous studies have considered the effects of photodynamic therapy in combination with indocyanine green only against *C. albicans* species. However, there is no report on the effects of photodynamic therapy in association with new methylene blue against *C. krusei*, *C. glabrata*, and *C. tropicalis*; therefore, comparison of our findings with those of other studies is not possible.

A number of important factors can influence the outcomes of laser therapy and photodynamic therapy, including the type and concentration of dye, physiological status of target microorganisms, duration of radiation, and output energy of the laser device.[21] There is no specific or approved method for determining laser-related parameters. In the present study, a device power of 100 mW, radiation time of 100 seconds, and energy density of 10 J/cm^2 were considered, according to the findings of a study by Azizi et al.[9] Nonetheless, Azizi et al employed both pulse-based and continuous methods and only evaluated *C. Albicans* species. Comparatively, in the present study, the continuous radiation method was employed to evaluate four *Candida* species (*C. albicans*, *C. krusei*, *C. glabrata*, and *C. tropicalis*).

Laser dosimetry is another factor affecting the outcomes of photodynamic therapy. Several studies have investigated the effects of different laser densities and types of radiation (i.e., pulsed and continuous) on photodynamic therapy.[4] Further, the maximum absorption of the photosensitizer must agree with the wavelength of emitted light so that the highest rate of free radical production is warranted for eradicating microorganisms. For example, the maximum absorption of indocyanine green occurs at 810 nm.[19,22]

According to the results of the present study, the antifungal effects of photodynamic therapy, combined with the new methylene blue dye, significantly reduced the number of *Candida* colonies. Previous studies reported lower reductions in *Candida* colony counts compared to the present study,[11] which might be justified by differences in laser irradiation parameters. In the current study, photodynamic therapy was performed with new methylene blue as a photosensitizer, which exhibited the greatest growth inhibitory effects against *C. krusei*, followed by *C. albicans*. In another study, Wilson et al. reported that helium-neon irradiation (635 nm) in conjunction with toluidine blue reduced the growth of *C. albicans* by 77%, *C. tropicalis* by 65%, *C. stellatoidea* by 63%, and *C. kefir* by 40%.[11] Moreover, Bliss et al used Photoferin dye and observed a similar reduction in the growth of *C. albicans* and *C. krusei* to our study. Also, *C. glabrata* showed resistance to this dye, which is consistent with our findings.[23]

In a study by Fekrazad et al, photodynamic therapy by the application of indocyanine green and new methylene blue significantly reduced the growth of *C. albicans* compared to the control group *in vitro*, which is in line with the results of the present study.[10] In another study by Azizi et al, the effects of indocyanine green and new methylene blue with different laser dosimetry settings (pulsed or continuous) were investigated for only *C. albicans in vitro*. Photodynamic therapy using both dyes induced significant antifungal effects compared to the control group, which is consistent with the results of the present study.[9]

In another study, Fani and Araghizadeh[24] examined the antifungal effects of low-power laser in combination with toluidine blue and methylene blue against four *Candida* species. They found that the samples irradiated in the presence of a dye had the lowest colony growth rate. Toluidine blue and methylene blue effectively inhibited the growth of *C. albicans* colonies by 88.6% and 83.2%, respectively; conversely, laser irradiation had relatively lower antifungal properties in the absence of a dye.

Therefore, the application of a new product of photosensitizers for more successful treatment of fungal infection is an important aspect of PDT. According to the studies by Fekrazad et al,[10] Azizi et al,[9] and Daliri et al,[14] photodynamic therapy with methylene blue effectively reduces the number of candida species. Furthermore, the

fungicidal activity of photodynamic therapy depends on photosensitizer type and appropriate light wavelength.

In PDT, the activation of a photosensitizer can be achieved by exposure to low-power visible light with a specific wavelength. Red light between 630 and 700nm used to activate most photosensitizers and methylene blue has maximum activation and light absorption in a 660 nm wavelength.

Based on our findings and the results of previous research, it can be concluded that *Candida* species respond variably to photodynamic therapy; therefore, further comprehensive studies are needed on these species to determine various factors affecting their response rates. Additionally, in the present study, it was found that photodynamic therapy with new methylene blue had significantly higher efficacy in suppressing the growth of *Candida* species compared to the control group. This method showed lower efficacy than nystatin therapy, although the difference was insignificant (except for *C. glabrata*). Nevertheless, this method can be an effective complementary treatment used alongside conventional nystatin therapy. It may even be a potential alternative therapy, although further studies and modifications are required.

It should be noted that in the present study, only one session of either nystatin therapy or photodynamic therapy (a single session of irradiation) was used, both yielding promising results. However, in conventional treatments, nystatin mouth washing or sucking had no therapeutic effects. Therefore, it is recommended to conduct further *in vitro* studies and apply the therapeutic methods of this study in several sessions to confirm their effectiveness against *Candida* species; this may help improve the efficacy of photodynamic therapy or even replace nystatin therapy.

Due to limitations in the implementation of this study in the clinical setting, future studies are suggested to assess the effects of multiple sessions of nystatin therapy and multi-stage photodynamic therapy on *Candida* species isolated from patients with candidiasis and to extract *Candida* species from the patients' mouths for obtaining more clinically accurate results. Also, it is recommended to conduct similar studies using different laser dosimetry settings, as well as other types of photosensitizers *in vivo*.

Conclusion

The results of the present study showed that photodynamic therapy in combination with new methylene blue could be a suitable complementary therapy for the effective management of oral candidiasis.

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Authors' Contribution

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Validation: Faezeh Khozeimeh, Mehdi Razzaghi-Abyaneh, Marzieh Ghayour.
Visualization: Faezeh Khozeimeh, Atefeh Tavangar, Mehdi Razzaghi-Abyaneh.
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Writing–review & editing: Faezeh Khozeimeh, Marzieh Ghayour.

Competing Interests

The authors declare that they have no conflict of interest.

Ethical Approval

This study was registered and approved by the ethical committee of Isfahan University of Medical Sciences, number (IR.MUI.REC.1397.3.056).

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