Curcumin/H$_2$O$_2$ photodynamically activated: an antimicrobial time-response assessment against an MDR strain of Candida albicans

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Abstract. - OBJECTIVE: Human candidiasis is typically treated with antifungal drugs, but the rise of drug-resistant strains of Candida spp. poses a serious problem, making treatment difficult. At the same time, photodynamic therapy (PDT) has drawn increasing attention from researchers for its potential to effectively inhibit multidrug-resistant pathogenic fungi and for its low tendency to induce drug resistance. This study’s goal was to examine how a multidrug-resistant oral isolate of Candida albicans responded to a PDT that used a curcumin/H$_2$O$_2$ formulation as a photosensitizer and was exposed to various light sources.

MATERIALS AND METHODS: A commercial product containing curcumin/H$_2$O$_2$ 3% was used as a photosensitizer and evaluated in a PDT treatment that can use two different light sources: traditional irradiation with 7 W light at $\lambda = 460$ nm or a new, never evaluated, polarized light source of 25 W with a wavelength range of $\lambda = 380-3,400$ nm. The antimicrobial activity of these procedures was assessed on a clinical oral isolate of Candida albicans, in terms of agar susceptibility test, growth curve behavior, and biofilm inhibition.

RESULTS: Both light sources were able to activate the photosensitizer formulation, suggesting a fungistatic activity vs. this C. albicans MDR strain. An interesting difference was observed in the cell-generation-time (CGT$_{oo}$) after PDT treatment, where the polarized light was more active compared to the source of 460 nm. In fact, CGT$_{oo}$ was 16 and 8 hours, respectively.

CONCLUSIONS: The PDT evaluated here presented an inhibition window time, a crucial point for clinicians, who could activate an additional prophylactic treatment to resolve the clinical management of Candida infections in the oral cavity.

Key Words: Candida albicans, Oral infections, Photosensitizing agents, Photodynamic therapy, Curcumin, Hydrogen peroxide.

Introduction

Candidiasis is an opportunistic infection due to Candida spp., which can affect the oral cavity, vagina, penis, or other parts of the body. Candida is the only fungal genus that unequivocally contributes to the aetiology of the most common infections of the oral mucosa. Oropharyngeal candidiasis (OPC) can affect the skin and the mucous membranes, with a very different clinical manifestations (pseudomembranous, erythematous, hyperplastic), responsible for local symptoms such as dysgeusia, dysphagia, odynophagia, glossodynia, burning sensation. Pseudomembranous candidiasis is common in chronically ill patients and infants. It is presented as white, soft, slightly elevated plaques, most commonly on the tongue and buccal mucosa. Plaques are composed by desquamated epithelium, debris of necrotic tissue, keratin, leucocyte, fibrin, and bacteria. This plaque can be wiped away and, when it happens, leaves an erythematous area in the tissues affected. Erythematous candidiasis often occurs after the use of antibiotics or corticosteroids for prolonged therapy. It presents as painful erythematous areas (red lesions without plaques) and can involve the tongue with central papillary atrophy, and the palate. Chronic hyperplastic candidiasis, also called candidal leukoplakia, presents with white plaques that are not scrapable, and can involve the lips, tongue, and buccal mucosa. These plaques could be homogenous or nodular, it is a potentially malignant lesion. Candida associated clinical lesions include denture stomatitis (overall on the hard palate), angular cheilitis, and median rhomboid glossitis, linear gingival erythema.

Species of oral Candida include C. albicans, the most frequent, in 30-50% of healthy patient’s mouths and 60% of patient’s mouths over the age
of 60 years, then C. glabrata, C. guillermondii, C. krusei, C. parapsilosis, C. pseudotropicalis, C. stellatoidea, and C. tropicalis.1,3

Although Candida albicans is the most common cause of candidiasis, non-albicans species have significantly increased over the past ten years. Understanding the type of yeast that is causing the infection is crucial since it affects the course of treatment; for instance, some non-albicans isolates may be inherently resistant to azoles, particularly fluconazole.6

In immunosuppressed patients (for example, people with diabetes or other autoimmune diseases, oncologic patients), if not treated effectively, candidiasis can spread throughout the body, causing systemic infections with a high rate of morbidity and mortality.7-10

Candida infections are treated with antifungal medications such as nystatin, clotrimazole, amphotericin B, miconazole.11 In recent decades, the increase in antimicrobial resistance to conventional antimycotics has become a major health problem that requires the development of new drugs.12-15 This is hugely affecting clinical and hospital environments around the world.12,16 The main factors associated with this resistance are global demographic changes, the ageing population, the increase in invasive anticaner therapies, as well as long-term antifungal treatments.17 Therefore, the lack of effective antifungal agents, the high toxicity of the drugs on the market, especially in liver and kidney patients, and the emergence of resistance have prompted researchers to experiment with alternative therapies, one of which is represented by photodynamic therapy (PDT).18 Photodynamic therapy requires three components: molecular oxygen, the photosensitizer (PT) and light at the wavelength corresponding to the absorption peak of the PT.19,20 In fact, the light-activated PT initiates a cascade of processes that lead to the formation of reactive oxygen species (ROS) responsible for the destruction of pathogenic microorganisms.19 PDT’s undeniable advantages include its lack of side effects, lower drug resistance, ease of use, and patient safety (PDT acts with a high therapeutic index between infected and uninfected cells).21,22

Among the various photosensitizers, perhaps the most studied against Candida species reported different photosensitizer such as toluidine blue, methylene blue, malachite green, indocyanine green, photodytazyme, chlorella, chlorophyllin, phycocyanin, 5-aminolevulinic acid and riboflavin.23

In spite of numerous articles on PDT that use a wide range of source lights and further PTs, some questions and doubts remain open: 1) the presence of PT resistant strains, 2) whether PDT acts as a fungicide or fungistatic, and 3) if there is a time window in which the majority of cells are in metabolic shock, a phenomenon caused by PT-induced radicals under PFT. This period of no growth may provide an opportunity for oral clinicians to implement, at the same time, a new antimicrobial strategy.24

PDT is widely used in oral medicine and dentistry, however the search for new PTs effective against the most common oral pathologies is still remarkable. Curcumin (CUR), extracted from the rhizomes of the Curcuma longa L. plant, is gaining attention in the scientific literature for its anti-inflammatory, antibacterial, antiviral, and anticancer properties.25,26 Furthermore, numerous studies in the literature have highlighted the immunomodulating properties of CUR in patients with HIV, Alzheimer’s disease and multiple sclerosis.27,28

A key property for its use as a photosensitizer is that the CUR shows the light absorption in the visible spectra peak, around 400-500 nm. Curcumin’s antimicrobial property as a PT in PDT against Candida albicans has been demonstrated in numerous in vivo studies, but no comparative in vitro studies with different wavelength lights using different PTs have been reported in the literature yet. The aim of our in vitro study is to evaluate the effectiveness of a commercial chemical formulation (curcumin + 3% hydrogen peroxide) with two light sources: (i) A clinical isolate of a multidrug-resistant strain (MDR) of Candida albicans was exposed to 460 nm standard light or 380–3400 nm high irradiation polarized light. This approach could be interesting and useful for oral clinicians to predict, by laboratory evaluation, the times and ways to treat infections due to C. albicans MDR strains.

Materials and Methods

A clinical Candida albicans multidrug-resistant isolate, CA97, was used to perform the antimicrobial test. This strain was previously characterized for its response to different antifungal drugs. We have characterized it in our previous studies both by cultural and molecular procedures.24 In fact, it was found to be resistant to three different azoles (Fluconazole, Voriconazole, and Ketoconazole), especially due to a mutation in the ERG11 gene.25,26
Photodynamic Devices Used in This Work

Two different commercial devices were used in these experiments:

(i) Bioptron AG (Wollerau, Switzerland)
    Polarized light, $\lambda = 380-3400$ nm, power = 25 W;

(ii) FlashMax® P7, CMS Dental, Elmevej 8, Glyngore, 7870 Roslev, Danmark, $\lambda = 460$ nm, power = 7 W.

These apparatuses are usually described for photodynamic therapy for a variety of oral illness or for oral composites polymerization\(^\text{35,36}\), moreover the emitted $\lambda 460$ nm light is normally suggested in the clinical protocols to stimulate curcumin as Photosensitizers\(^\text{37}\).

In all experiments, the photosensitizers were light stimulated by a 1-minute as FlashMax® P7 manufactured instructions.

Photosensitizer

We used Curcumin plus hydrogen peroxide 3 % v/v (FlashMax® P7 QroxB2®). All compounds were used following the manufacturer’s instructions (light irradiation time, light wavelength, Photosensitizer concentration). Curcumin, bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, is a natural yellow-orange dye derived from the rhizome of Curcuma longa L.

Curcumin Base Formulation Absorption Spectrum

The spectral properties of the Curcumin/H\textsubscript{2}O\textsubscript{2} base product were studied by using a UV-visible spectrophotometer JASCO V600 – Bio, (JASCO Europe, Cremella, Italy) in the range of 200-700 nm, by using an optical path $L = 10 \times 10$ and mm glass cuvette (Hellman Analytics, Munich, Germany), following the manufacturer’s instructions. We have investigated the absorption spectra of commercial formulation as such, and concentrations diluted in water 1/10-fold. Calculating the UV-vis spectra was required to assess the perfect assonance between light emission profiles and photosensitizer excitation motifs for use optimally in photodynamic therapy.

In Vitro Antimicrobial Protocol

A set of different laboratory procedures were used to evaluate the antifungal activity by using modified standard protocols adapted for photodynamic treatment. The base procedures were carried out using the disk diffusion method, which was determined in accordance with the European Committee for Antimicrobial Susceptibility Testing (EUCAST). At the same time, the behavior of the C. albicans growth curve in the absence or presence of photodynamic treatment was evaluated. In addition, the biofilm inhibition activity was evaluated by the protocol described by Montana University’s Center for Biofilm Engineering\(^\text{38,39}\).

The Disk Diffusion Method (Kirby-Bauer)

An initial evaluation was performed by using a modified Kirby-Bauer procedure following the operative scheme described in Figure 1. 15 mL of agarized medium (Sabouraud agar Microbiol, Uta, Cagliari, Italy) at $55^\circ C$ was put into a $\varnothing 90$ mm Petri dish and, prior to agar solidification, five sterile iron rivets, $\varnothing 10$ mm in diameter and 2 mm thick (Firm, Milan, Italy), were put into the agar hot solution and then removed from the medium when it solidified (about $30^\circ C$). A C. albicans suspension in Sabouraud Broth in the lag phase, about 15 hours after growth, was used as an inoculum with a microbial concentration of $5 \times 10^6$ (CFU/mL). The yeast was inoculated onto the plate surface using a sterile swab using the already mentioned standardized inoculum. 0.05 mL of photosensitizer solution was put in each well positioned inside the agar thickness. At this point, the light was irradiated on the well surface by using the strict conditions suggested by the manufacturing instructions. Petri dishes were incubated in air at $37^\circ C$ for 48 h. After incubation, the diameter of the possible inhibition alone was measured. The final value was represented as the geometrical mean of three different experimental repetitions.

Susceptibility Tests in Liquid Media

C. Albicans growth curves

This procedure was useful for monitoring the growth and proliferation of Candida in real time, and any antimicrobial activity could be explained with high sensitivity by a growth curve motif. In this context, different growth curves, considering the strain under or without photodynamic treatment, were performed by using a classical microplate technique\(^\text{40}\). The positive control was performed by using an aliquot of 5 $\mu l$ of $10^7$ C. albicans mL\(^{-1}\) cell suspended in Sabouraud broth (Microbiol Cagliari, Italy). Each well was then overlayed with 245 ul of liquid media for a total volume of 250 $\mu l$. This corresponded to a
final concentration of 2*10^5 Candida cells mL^-1 per well. On the test wells, the photodynamic procedure was applied after 5 ml inoculum deposition on the well bottom, by using the times and conditions previously described. After treatment, each well was overlayed with 245 ul of Sabouraud broth media. Different tests were performed: (a) complete photodynamic treatment, (b) only light, and (c) only H2O2, all determinations were performed in triplicate. The negative control was performed by using 250 ul of the growth medium, without any fungal inoculum. The microplates were incubated at 37°C for 72 hours and monitored for absorbance at 660 nm using a BMG LABTECH microplate-reader (Allmendgrun 877799 Ortenberg, Germany). Data was recorded for 72 hours every 10 hours after 1 minute of shaking at 355 rpm.

Growth Curves Analysis
The data mean obtained from three different experiments was used to calculate the standard deviation, and each experimental point was used to predict the Candida OD generation time (CGT_{OD}) considering that an OD of 0.125 corresponded to approximately 1.5 *10^7 cells/ml. Obviously, the positive effect of a photodynamic treatment can determine an increase in CGT_{OD}. In this work, periodic sampling was done to calculate the generation time using the equation:

\[
\text{CGT}_{OD} = \frac{t}{3,3 \log \left( \frac{b}{B} \right)}
\]

were:
CGT_{OD} is generation time of C. albicans cells evaluated as optical density;
t is the time interval between measurements b and B (in the exponential (log) growth curve phase);
B is the OD measured at the start of log growth curve phase;
b is the OD at the end of log growth curve phase.
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**Biofilm Assessment**

The ability of PDT to inhibit biofilm formation was evaluated by using a set of microplates prepared with the same culture conditions and photodynamic treatments already described in the previous steps. For the biofilm evaluation, we used the protocol described by Montana University’s Centre for Biofilm Engineering. In brief, the microplates were incubated at 37°C for 96 hours in air, to permit the biofilm formation. The plate samples were subsequently washed three times with Phosphate-buffered saline GIBCO®PBS (Thermo Fisher Scientific, Waltham, MA, USA) to eliminate planktonic cells; the biofilm was then stained with 100 μl of 0.1% w/v of crystal violet solution (Microbial, Uta, Italy) for 10 min at 25°C. After three washes with PBS solution, 200 μl of 30% v/v acetic acid was added in every well to solubilize the dye from the bacterial biomass. The biofilm amount was measured with a plate reader spectrophotometer BMG LABTECH (Allmendgrün 877799 Ortenberg Germany). The percentage of the biofilm inhibition, in comparison with the *C. albicans* positive control, was evaluated by using the following formula:

\[
\text{Bin}\% = \frac{\text{OD}_{\text{PDT}} - \text{OD}_{\text{cont}}}{\text{OD}_{\text{PDT}} - \text{OD}_{\text{cont}}} \times 100
\]

Bin\%: percentage of biofilm inhibition;

\(\text{OD}_{\text{PDT}}\): 600 nm optical density of dye solution after photodynamic treatment;

\(\text{OD}_{\text{cont}}\): 600 nm optical density of dye solution.

**Statistical Analysis**

All values with a standard deviation (SD) less than 10% of the mean value for the same experimental condition were deemed significant. The differences between the tested formulations were compared using the *Fisher* test by using an online web calculator, with *p* set at 0.05.

**Results**

**Curcumin Base Formulation Absorption Spectrum and Light Devices Suitability**

As described in previous works, the photosensitizer has to show suitable optical properties, i.e., it must be able to absorb light efficiently at the wavelength used for excitation. In other words, it should possess a high extinction coefficient (ε). The used formulation represents a mixture of curcumin and other compounds, such as peroxides. Thus, ε is a function of the formulation composition more than the single curcumin, and
Figure 3. The inhibition halos were obtained under different treatment conditions. Irradiated curcumin base formulation shows the highest inhibition results to demonstrate the synergic activity of Curcumin plus hydrogen peroxide and light.

for this reason, we have analyzed the absorption spectrum of the entire commercial product. The pure curcumin in water, following previous studies, Zsila et al.45, showed an absorption peak in the visible area of about 350 nm to 450 nm45, while our compound reflects a continuous absorption zone from 350 to 700 nm. This increase in the absorption wavelength area allows the use of both light generators used in this work (Figures 1, 2).

**Disk Diffusion Method (Kirby-Bauer)**

By using the agar diffusion method, the anti-fungal activity of PDT was assessed against the aforementioned *C. albicans* MDR strain. The results showed that two PDT treatments were very effective against this pathogen; for example, both treatments displayed a good level of inhibition (Ø = 47 ± 1 mm), whereas curcumin without PDT or only hydrogen peroxide 3% were represented with the lowest levels of inhibition halos, Ø = 37 and 22 mm, respectively. This early finding revealed the synergy of the PDT components, as shown in Figure 3. Despite this, an evaluable number of yeast colonies were observed after being observed inside the inhibition halo area after 72 hours incubation time.

**C. Albicans Growth Curve Behavior Under PDT Treatment**

The decreased rate of cell growth over time, with or without PDT therapy, can be represented by the growth curve. This strategy might be a sensitive and trustworthy way to assess step-by-step this antifungal method’s effectiveness46.

First, as seen in Figure 4, in the light of fungistatic assessment, there has been a significant difference between the performed experimental settings and the antimicrobial activity. Further evidence for yeast growth inhibition for this PDT treatment, suggests that the biofilm formation rate after 96 hours of incubation is the same in all formulations tested; thus, the system was unable to inhibit the *C. albicans* biofilm for an extended period of time (Table I). Furthermore, as previously assessed through the Kirby Bauer procedure, the PDT treatment has suggested a temporal inhibition of *C. albicans* growth in that after 72 hours, some yeast colonies have been observed inside the inhibition halo area. In this context, the growth curve analysis demonstrated this behaviour again on the yeast cell growth under PDT. For both treatments, 460 nm and polarised light, Figure 4 epitomises an inhibition window of about 36 hours. Other vari-
Figure 4. The behaviour of the *C. albicans* MDR strain’s growth curves with PDT. The formulation of curcumin-H$_2$O$_2$ was tested under two different light sources. Regular light was at 460 nm (A), while polarised light was at 380-3,400 nm (B).
Table I. *C. albicans* growth curve parameters, evaluated under various photodynamic treatment condition.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>log phase start time (hours)</th>
<th>Inhibition windows time (hours)</th>
<th>CGTOD* (hours)</th>
<th>Log phase slope (Φ) OD/hours Y = Φ*X</th>
<th>Bin% ***</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin/H2O2 + λ 460 nm</td>
<td>60</td>
<td>36</td>
<td>8.90</td>
<td>0.0264</td>
<td>0</td>
</tr>
<tr>
<td>Curcumin/H2O2 + λ 380-3,400 nm</td>
<td>60</td>
<td>36</td>
<td>16.36</td>
<td>0.0164</td>
<td>0</td>
</tr>
<tr>
<td>Curcumin/H2O2</td>
<td>48</td>
<td>24</td>
<td>8.4**</td>
<td>0.0256**</td>
<td>0</td>
</tr>
<tr>
<td>Positive control</td>
<td>24</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>λ 460 nm</td>
<td>24</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>λ 380-3,400 nm</td>
<td>48</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Expressed during log phase, considering that at = 0.125 OD, about \( \sim 1 \times 10^7 \) *C. albicans* cells/ml. **Mean of two experiments performed in triplicate, SD max = 0.5%. ***Evaluated at 96 hours.

Discussion

First for all, this study highlights the good results of antifungal activity of polarized light in PDT. In fact, it was most active in that GTGOD and the curve growth slope Φ is about half in the comparison with 460 nm treatment (Table I). This could be due to various conditions, e.g., the major power of polarized light being 25 W vs 7 W of 460 nm. Another advantageous point could be the polarized light apparatus’ major irradiation wavelength range, which is capable of stimulating different molecular status in the photosensitizer formulation, such as the peak around 380 nm (Figure 2), but also the major ability of polarized light to enter the yeast cell wall[47]. Thus, by surprise, an apparatus normally used for oral composite polymerization proved the most performant. This study, although we consider it preliminary, suggests other ways to investigate the basilar and molecular mechanisms of PDT in the treatment of oral candidiasis. First of all, it could be interesting to evaluate if various PDT treatments described in the literature are indeed fungicides and not fungistatic. In fact, following the official antimicrobial protocols published by EUCAST protocols[48], the normal observation time ranges from 24 to 48 hours, while we have observed a candida regrowth time of 60 hours. It should, therefore, be necessary to extend the time of *in vitro* analysis to assess the effective fungicide activity of a PDT treatment. We consider these points extremely significant for the transfer of PDT *in vitro* results to *in vivo* patient management[48,49]. For example, the inhibition window is the time range between PDT treatment and microbial regrowth during which the immunity response and adjunctive clinical treatments are still effective. The inhibition window in oral candidiasis could be crucial in-patient management, e.g., antymycotics are most effective when the cells are under stress due to oxidative injury due to PDT. But this advantage could be frequently overridden before patients can reasonably get the drug. However, the study must be considered again preliminary for the following reasons: (I) it could be interesting to evaluate laboratory formulated with different curcumin concentrations. This could highlight a possible minimum inhibition concentration, thus differences inside clinical isolates of *C. albicans*. In this context, it could be necessary to evaluate a large group of strains and correlate this with the host clinical status. In the second place, curcumin is also associated with cancer treatment and could be investigated for its activity in *C. albicans* infection in cancer patients by evaluating some biomolecular markers[49,50].

Conclusions

In this work, we have observed, with a different perspective, an already noted clinical proce-
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dure for antifungal clinical treatment in oral tissues, in infections due to C. albicans. By using a commercial formulation of curcumin/H$_2$O$_2$ as photosensitizer and two different light sources, the work has shown a crucial strongly time-dependent of PDT evidencing this as an effective fungistatic agent. This study, even if preliminary, indicates a fungistatic activity of curcumin/PDT where the oral clinician can operate with adjunctive prophylactic tools during the yeast inhibition time, for the purpose of better candidiasis treatment.

**Conflict of Interest**
The authors declared no conflict of interest

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**Ethics Approval and Consent to Participate**
Not applicable.

**Availability of Data and Materials**
The data that support the findings of this study are available on request from the corresponding author.

**Authors’ Contribution**

**References**


