

# *In Vitro* Effects of Photodynamic Therapy with Three Photosensitizers on *Candida* spp from Complicated Vulvovaginitis and Asymptomatic Women

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## Abstract

**Aim:** To evaluate photodynamic therapy (PDT) *in vitro* to reduce the growth of *Candida* spp, and its synergy with the antifungals fluconazole and ketoconazole for inhibition of resistant, DDS and susceptible isolates from asymptomatic carriers and with complicated vulvovaginitis. **Methods:** Between 2017 and 2020, we evaluated 230 women with vulvovaginal candidiasis and 400 asymptomatic. We obtained 130 positive cultures for *Candida* spp from vulvovaginitis and 94 asymptomatic. Yeasts were characterized by classical and molecular tests. Sensitivity to fluconazole and ketoconazole was evaluated by E-test. We used photodynamic light through blue LED, wavelengths between 450 to 470 nm, power of 260 mW, energy fluence of 270 J/cm<sup>2</sup>, for 15 minutes over all colonies of *Candida* spp. Methylene blue (MB) at 450 mg/mL, 2% gentian violet (VG) and 50 µM curcumin (CR) were used in association or not with LED irradiation. Suspensions of *Candida* spp of 10<sup>6</sup> CFU/mL, subjected to the different assays, were introduced in 96-well microplates, incubated for 48 hours at 35°C and the readings at 530 nm. The samples were finally cultivated in Petri plates containing Sabouraud dextrose agar to assess the growth inhibition. All procedures were in triplicate. **Results:** *C. albicans* was prevalent in vulvovaginal candidiasis, however, we also isolated non-*albicans* species such as *C. glabrata*, *C. tropicalis* and *C. parapsilosis*.

There was a substantial reduction (66.6% to 83.8%) of the CFU/mL of the isolates treated with FDT. Gentian violet at 2% alone reduced the growth of CFU/ml of *Candida* spp from 69% to 75%. Among isolates of vaginitis and asymptomatic carriers, after using FDT, we found a reduction in resistant phenotypes and DDS for fluconazole in percentages from 20% to 100% for *C. albicans*, from 50% to 100% *C. glabrata*, 33.3% to 100% *C. parapsilosis* and 100% *C. tropicalis*. For ketoconazole in the same isolates, there was a reduction in phenotypes with MIC > 16 µg/mL of up to 50% in *C. albicans*, 50% to 100% *C. glabrata*, 50% to 100% *C. tropicalis*. **Conclusions:** PDT with MB, GV and CR revealed efficacy *in vitro* in reducing the growth of *C. albicans* and non-*albicans*, especially due to chronic recurrent vulvovaginitis.

### Keywords

Photodynamic Therapy, Photosensitizers, *Candida albicans*, Species Non-*Albicans*, Complicated Fungal Vulvovaginitis

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## 1. Introduction

Three out of four women have at least one episode of vaginal candidiasis during their reproductive years, 40% of whom will experience a second episode and 5% of them will have recurrent vaginitis that is characterized by four or more episodes of vaginitis during one year, with clinical diagnosis and confirmation by culture [1] [2] [3]. Vulvovaginitis caused by *Candida* spp can be classified as uncomplicated (CVV), when they are manifested by sporadic or infrequent episodes, with mild or moderate symptoms, and complicated (CVVC), such as recurrent vulvovaginitis (CVVR) [4] [5] [6].

Vulvovaginal infection by *Candida* spp is diagnosed by clinical and epidemiological conditions, without which colonization cannot be distinguished from infection. About 20% to 30% of women have vaginal colonization by *Candida* spp and are asymptomatic [7]. It is accepted that *C. albicans* is responsible for 80 to 92% of cases of candidiasis vulvovaginal [8]. The clinical syndrome caused by non-*albicans* species can be indistinguishable from that caused by *C. albicans* [9]. Increased frequency of other species such as *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. guilliermondii* and *C. krusei*, among others, has occurred [10] [11]. Recurrences have been attributed to factors such as sexual transmission, hygiene habits, intestinal colonization, allergic and idiopathic causes. However, the epidemiological profile of women with recurrent idiopathic vulvovaginitis has not yet been established [12] [13].

Conventional treatments for fungal vulvovaginitis include antifungals such as ketoconazole, fluconazole and other azoles, but therapeutic failures are common, particularly in patients with *C. glabrata* vaginitis [10]. Fluconazole is a broad spectrum antifungal, with excellent therapeutic results for several *Candida* species, except for *C. glabrata* and *C. krusei*, as they have intrinsic resistance to this medication. *C. glabrata* vaginitis is considered the greatest challenge to the anti-

fungals used [6] [14]. The indiscriminate use of broad spectrum antifungal agents has contributed to the increase in recurrent vaginitis, due to the increasing proportion of events caused by non-*albicans* species and often resistant to azole derivatives [15]. The continued and progressive intolerance to antifungals is a problem of extreme gravity and concern that encourages the design of new antifungal drugs and new therapeutic techniques.

Photodynamic therapy (PDT) consists of the use of a photo-sensitive component and an appropriate light source, representing an effective, promising and safe alternative in the treatment of fungal diseases, among others [16] [17] [18] [19] [20]. Studies have shown the susceptibility of *Candida* spp to PDT, however, studies on the susceptibility of strains of *Candida* resistant to fluconazole are restricted [19] [20] [21] [22] [23]. Some aspects related to the results of PDT on *Candida* spp *in vitro* still need to be better elucidated [19]-[24]. The efficacy of PDT associated with sensitizers such as methylene blue and toluidine blue is reported in the treatment of patients with candidiasis caused by fluconazole-resistant *C. glabrata* and *C. albicans* [17] [21] [22] [25].

Photodynamic therapy using methylene blue at a concentration of 250 to 400 mg/ml promotes a reduction in the growth of *C. albicans*, occurring its complete eradication, in the oral cavity, when concentrations of 450 to 500 mg/ml of methylene blue are used [22]. A study evaluated *in vitro*, the action of 1% gentian violet dye associated with laser and observed an important effect of photodynamic therapy against *Candida albicans* [26]. The gentian violet solution has a potent antifungal activity against several species of *Candida*, such as *C. albicans*, *C. glabrata* and *C. parapsilosis* [27]. Martins *et al.* (2009), evaluated the antifungal activity of Curcumin in 23 strains of fungi *in vitro* and an inhibitory effect on several species of *Candida* spp, much more efficient than fluconazole [27]. Results from another study showed that blue LED associated with Curcumin proved to be effective in the photodynamic inactivation of *C. albicans in vitro* [28].

The present study aims to verify *in vitro* the synergistic effect of the combination of photodynamic light associated with sensitizers such as methylene blue, gentian violet or curcumin to inhibit the growth of *Candida* spp from complicated VVC and asymptomatic women.

## 2. Materials and Methods

### 2.1. Study Population

Microbial isolates were from of women with clinical suspicion of vulvovaginitis, coming from Basic Public Health Units, Private Offices and other Gynecology Services, and the study in question was developed in four years (2017-2020), together with the Laboratory of Mycology and the Department of Gynecology of Paulista School of Medicine/Federal University of São Paulo under the scientific opinion of the Research Ethics Committee N° 5505. All participants signed an informed consent form (FICF). Financial resources and responsibility for re-

search costs were borne by the researcher.

Were included in the study adult participants aged 18 to 65 years with chronic and/or recurrent vaginitis and asymptomatic women who attend for the collection of gynecological routine and oncotic colposcopy examination. Were excluded from the study pregnant women with diabetes mellitus, on corticotherapy, antibiotic therapy or hormone therapy, with immunosuppression, carriers of the human immunodeficiency virus (HIV), using an intrauterine device (IUD), or who used vaginal douches or spermicides.

## 2.2. Collection, Isolation and Identification of Yeasts

Vaginal secretion was collected with the help of a non-lubricated speculum from the ectocervix regions and from the bottom of the vagina using a swab moistened in a sterile saline solution. A total of 630 participants were evaluated, 230 clinically compatible with vulvovaginal candidiasis and 400 asymptomatic. The samples were seeded on Sabouraud dextrose agar media supplemented with chloramphenicol (100 mg/mL) and Chromagar *Candida*, obtaining 140 positive cultures from symptomatic women and 94 from asymptomatic women. From pure cultures, yeasts were identified using non-specific primers from the ITS1 and ITS4 regions, CALB1 and CALB2 for *C. albicans*, CGLAB1 and CGLAB2 for *C. glabrata*, CTR1 and CTR2 for *C. tropicalis*, and CPAR3 and CPAR2 for *C. parapsilosis*.

## 2.3. Antifungigram

For antifungal susceptibility tests, fluconazole and ketoconazole from the standard E-Test Kit (AB Biodisk, Solna, Sweden) were used. The isolates were cultivated on Sabouraud-dextrose agar (DIFCO), at a temperature of 37°C, for a period of 48 hours, suspended in sterile saline solution according to a 0.5 McFarland scale and cultured in Petri dishes containing the solidified medium. The E-Test antimycotic strips, after a period of 15 minutes at room temperature, were added in contact with the seeding in agar. The reading of the MICs was made after 24 and 48 hours of incubation at 35°C following the edge of the ellipse until the lowest concentration on the E-Test strip. Inconsistent and contradictory readings or high minimum inhibitory concentration (MIC) values of the antifungal fluconazole were repeated, as instructed by AB Biodisk (1993). In addition to the isolated fungi, *C. parapsilosis* ATCC 22019 was included in each experiment for quality control in the verification of drug activity, since fluconazole MICs for this microorganism are previously known, according to Rex *et al.*, 1996 [29] and NCCLS, CLSI [30] [31] [32] [33]. For ketoconazole there are no established breakpoints. Susceptibility criteria to fluconazole adopted: ≤8 µg/ml (Sensitive, S), 16 and 32 µg/ml (susceptibility-dependent-dose, SDD), ≥64 µg/ml (Resistant, R) [33].

## 2.4. Use of Photodynamic Light (PL) and Photosensitizing Dyes

Irradiation source consisting of LED devices (Light Emitting Diode) type

adapted photopolymerizers, with blue light emitting diodes, wavelengths between 450 and 470 nm, at the power of 260 mW, dosimetry 270 J/cm<sup>2</sup>, during periods of time of 15 minutes was used in the research. The incident light was oriented perpendicularly at a distance of 15 cm and directed from top to bottom, over all *Candida* spp colonies, before and after antifungigram, seeking to evaluate *in vitro* the effectiveness of (PL) in the viability of *Candida* spp inactivation. Photosensitizers such as methylene blue, gentian violet and curcumin, associated or not with LED light irradiation, were used at concentrations of 450 mg/ml (MB), 2% (GV) and 50 µM (CR), respectively. Thus, the different groups were classified: single treatment: LED, GV, MB, CR; treatment in combination: LED + GV, LED + MB, LED + CR; and control (K) without treatment.

*Candida* spp cultures, after tests with LED and photosensitizing dyes, were inoculated in 5 mL of sterilized peptone saline (SPS), being homogenized in a vortex, to obtain concentrations of 10<sup>6</sup> CFU/mL, measured in a spectrophotometer, with 76% transmittance at 530 nm. Suspensions from different groups were introduced separately into 96-well microplates and then subjected to different treatments. Such *Candida* spp growths, at concentrations of 10<sup>-1</sup> and 10<sup>-2</sup> mL, were seeded by the spread-plate method in Petri dishes containing Sabouraud-dextrose agar and incubated under aerobic conditions for 48 hours at a temperature of 35°C. Subsequently, the counts of the respective *Candida* spp colonies were performed.

Means resulting from the measurements of the procedures performed with all isolates and treatments were then calculated. The interpretation of the results was based on the MIC values recommended by CLSI M27 A2, CLSI M27 S3, CLSI M27 S4 and NCCLS [30]-[36] Documents. All tests were developed in triplicate. Fluconazole susceptibility criteria: ≤8 µg/ml (Susceptible, S), 16 and 32 µg/ml (Susceptible-dose-dependent, DDS), ≥64 µg/ml (Resistant, R) [33].

### 3. Results

From the material collected from patients with complicated vaginitis, 120 cultures were obtained (111 chronic and 9 recurrent), 10 from patients with sporadic vaginitis and 94 from asymptomatic women, making a total of 224 positive cultures for *Candida* spp (Table 1). Of these isolates, 181 (80.8%) were identified as *C. albicans*, 14 (6.25%) *C. glabrata*, 13 (5.8%) *C. tropicalis* and 16 (7.15%) *C. parapsilosis*. Among the 9 patients with recurrent vaginitis, the prevalence of non-*albicans* species was observed, being 5 *C. glabrata* (55.5%), 2 *C. albicans* (22.25%) and 2 *C. tropicalis* (22.25%).

Referring to the microbial growth, in Table 2 we observe a marked reduction in the number of colonies of all *Candida* species after treatment with LED + GV, LED + MB, LED + CR and little inhibition with LED, MB, CR used alone. Gentian violet at a concentration of 2% substantially inhibited the growth of *Candida* spp.

After analyzing the susceptibility profile of *Candida* species to fluconazole

**Table 1.** Number of *Candida* species identified from chronic and recurrent vaginitis, asymptomatic and sporadic participants.

Species	Chronic and recurrent	Asymptomatic	Sporadic	Total
	N	n	n	N
<i>C. albicans</i>	102	72	7	181
<i>C. tropicalis</i>	3	10	0	13
<i>C. parapsilosis</i>	7	8	1	16
<i>C. glabrata</i>	8	4	2	14
Total	120	94	10	224

**Table 2.** Average of CFU/ml *Candida* spp counts 48 h after using photodynamic light with GV, MB, CR, or LED, GV, MB and CR photosensitizers alone. Percentages of colony reduction compared to the control group (K) are superscripted in parentheses.

Species	K	LED	LED + GV	LED + MB	LED + CR	GV	MB	CR
Ca (181)	6.12	6.07 <sup>(0.8)</sup>	1.36 <sup>(77.7)</sup>	2.12 <sup>(66.6)</sup>	1.12 <sup>(81.7)</sup>	1.84 <sup>(70)</sup>	6.02 <sup>(1.7)</sup>	5.58 <sup>(8.9)</sup>
Ct (13)	5.92	5.80 <sup>(2.0)</sup>	1.04 <sup>(82.5)</sup>	1.96 <sup>(66.9)</sup>	1.02 <sup>(83.8)</sup>	1.48 <sup>(75)</sup>	5.58 <sup>(5.8)</sup>	5.02 <sup>(5.3)</sup>
Cp (16)	4.98	4.86 <sup>(2.4)</sup>	1.10 <sup>(78)</sup>	1.65 <sup>(66.9)</sup>	1.24 <sup>(75.2)</sup>	1.39 <sup>(72)</sup>	4.50 <sup>(9.7)</sup>	4.26 <sup>(15.5)</sup>
Cg (14)	5.87	5.72 <sup>(2.5)</sup>	1.31 <sup>(77.7)</sup>	1.47 <sup>(75)</sup>	1.25 <sup>(78.7)</sup>	1.82 <sup>(69)</sup>	5.57 <sup>(5.1)</sup>	4.23 <sup>(27.9)</sup>

Mean values of CFU/ml *Candida* spp counts (n = 224) expressed in (log10). Groups: without treatment administration (control group, K) and all groups, after using photodynamic light plus photosensitizers and isolated agents (LED, GV, MB and CR). Ca = *C. albicans*, Ct = *C. tropicalis*, Cp = *C. parapsilosis*, Cg = *C. glabrata*.

*in vitro* (Table 3) compared to the use of photodynamic light and MB, GV and CR sensitizers (Tables 4-6), a reduction in the number of resistant isolates was observed to the antifungal, mainly in resistant species and susceptible-dose-dependent (SDD) from women with chronic, recurrent and sporadic vulvovaginitis.

After treatment with photodynamic light and GV photosensitizing, there was a reduction in the percentage of resistant isolates from women with vulvovaginitis (*C. albicans*, 42.8%; *C. tropicalis*, 100%; *C. glabrata*, 75%) and asymptomatic (*C. glabrata*, 50%), as shown in Table 4. There was also a reduction in the percentage of SDD isolates, originating from vulvovaginitis (*C. parapsilosis*, 33.3%; *C. glabrata*, 100%) and asymptomatic (*C. albicans*, 40%; *C. tropicalis*, 100%).

Treatment with photodynamic light and methylene blue intensified the susceptibility to fluconazole, decreasing the percentage of resistant isolates from women with vulvovaginitis (*C. albicans*, 28%; *C. glabrata*, 75%) and asymptomatic (*C. glabrata*, 50%), according to Table 5. The DDS isolates also had a reduction in isolates from women with vulvovaginitis (*C. glabrata*, 100%) and asymptomatic (*C. albicans*, 20%; *C. tropicalis*, 100%; *C. parapsilosis*, 100%).

The use of curcumin and photodynamic light caused a decrease in the resistant profiles among isolates of vulvovaginitis (*C. albicans*, 57.7%; *C. tropicalis*, 100%, *C. glabrata*, 100%) and asymptomatic (*C. glabrata*, 100%), Table 6.

**Table 3.** *In vitro* fluconazole susceptibility profile of *Candida* spp isolated from asymptomatic women (a) and vulvovaginitis with chronic, recurrent and sporadic (v).

Species	Resistant		DDS		Sensitive	
	n	%	n	%	n	%
<i>C. albicans</i> <sub>a</sub> (n = 72)	0	0	5	6.9	67	92.9
<i>C. albicans</i> <sub>v</sub> (n = 109)	7	6.4	6	5.6	96	88
<i>C. tropicalis</i> <sub>a</sub> (n = 10)	0	0	1	10	9	90
<i>C. tropicalis</i> <sub>v</sub> (n = 03)	1	33.3	0	0	2	66.7
<i>C. parapsilosis</i> <sub>a</sub> (n = 8)	0	0	1	12.5	7	87.5
<i>C. parapsilosis</i> <sub>v</sub> (n = 08)	0	0	3	37.5	5	62.5
<i>C. glabrata</i> <sub>a</sub> (n = 4)	2	50	1	25	1	25
<i>C. glabrata</i> <sub>v</sub> (n = 10)	4	40	1	10	5	50
Total (224)	14	6.25	18	8	192	85.75

**Table 4.** Susceptibility to fluconazole *in vitro* of *Candida* spp isolated from asymptomatic (a) and with vulvovaginitis (v) after photodynamic light with VG. Percentages of reduction in resistant isolates are expressed in *italics*, compared to the values expressed in **Table 3**.

Species	Resistant		SDD		Sensitive	
	n	%	n	%	n	%
<i>C. albicans</i> <sub>a</sub> (n = 72)	0	0	3 <sub>(40%)</sub>	4.3	69	95.7
<i>C. albicans</i> <sub>v</sub> (n = 109)	4 <sub>(42.8%)</sub>	3.7	6	5.6	99	90.7
<i>C. tropicalis</i> <sub>a</sub> (n = 10)	0	0	0 <sub>(100%)</sub>	0	10	100
<i>C. tropicalis</i> <sub>v</sub> (n = 3)	0 <sub>(100%)</sub>	0	0	0	3	100
<i>C. parapsilosis</i> <sub>a</sub> (n = 8)	0	0	1	12.5	7	87.5
<i>C. parapsilosis</i> <sub>v</sub> (n = 8)	0	0	2 <sub>(33.3)</sub>	25	6	75
<i>C. glabrata</i> <sub>a</sub> (n = 4)	1 <sub>(50%)</sub>	25	1	25	2	50
<i>C. glabrata</i> <sub>v</sub> (n = 10)	1 <sub>(75%)</sub>	10	0 <sub>(100%)</sub>	0	9	90
Total (224)	6	2.7	13	5.8	205	91.5

Reduction in the percentage of DDS isolates from vulvovaginitis (*C. parapsilosis*, 66.7%; *C. glabrata*, 100%) and asymptomatic (*C. albicans*, 60%; *C. tropicalis*, 100%; *C. glabrata*, 100%) was also observed.

Regarding ketoconazole, six isolates, two *C. albicans*, two *C. tropicalis* and two *C. glabrata*, four from patients with chronic or recurrent vaginitis and two from asymptomatic women, showed high MICs > 16 µg/mL before submitted to the LED associated with photosensitizers. We did not obtain MIC > 16 µg/mL in *C. parapsilosis*. After undergoing LED + MB, we observed that there was a 50% reduction in MICs > 16 µg/mL in *C. tropicalis*, *C. glabrata*.

Treated under the action of LED + GV, we found that there was a 50% reduction in MICs > 16 µg/mL in *C. albicans*, *C. tropicalis*, *C. glabrata*. After LED +



**Table 5.** Susceptibility to fluconazole *in vitro* from *Candida* spp isolated from asymptomatic (a) and with vulvovaginitis (v) after PL with MB. Percentages of reduction in resistant and SDD isolates compared to the values expressed in **Table 3**.

Species	Resistant		DDS		Sensitive	
	n	%	n	%	n	%
<i>C. albicans</i> <sub>v</sub> (n = 109)	5 <sub>(28%)</sub>	4.5	6	5.6	98	89.9
<i>C. tropicalis</i> <sub>v</sub> (n = 03)	1	33.3	0	0	2	66.7
<i>C. parasilosis</i> <sub>v</sub> (n = 08)	0	0	3	37.5	5	62.5
<i>C. glabrata</i> <sub>v</sub> (n = 10)	1 <sub>(75%)</sub>	10	0 <sub>(100%)</sub>	0	9	90
<i>C. albicans</i> <sub>a</sub> (n = 72)	0	0	4 <sub>(20%)</sub>	5.5	68	94.5
<i>C. tropicalis</i> <sub>a</sub> (n = 10)	0	0	0 <sub>(100%)</sub>	0	10	100
<i>C. parasilosis</i> <sub>a</sub> (n = 8)	0	0	0 <sub>(100%)</sub>	0	8	100
<i>C. glabrata</i> <sub>a</sub> (n = 4)	1 <sub>(50%)</sub>	25	1	25	2	50
Total (224)	8	3.5	14	6.25	202	90.25

**Table 6.** Susceptibility to fluconazole *in vitro* of *Candida* spp isolated from asymptomatic (a) and with vulvovaginitis (v) after PL with CR. Percentages of reduction in resistant and SDD isolates compared to the values expressed in **Table 3**.

Species	Resistant		SDD		Sensitive	
	n	%	n	%	n	%
<i>C. albicans</i> <sub>a</sub> (n = 72)	0	0	4 <sub>(20%)</sub>	5.5	68	94.5
<i>C. albicans</i> <sub>v</sub> (n = 109)	5 <sub>(28%)</sub>	4.5	6	5.6	98	89.9
<i>C. tropicalis</i> <sub>a</sub> (n = 10)	0	0	0 <sub>(100%)</sub>	0	10	100
<i>C. tropicalis</i> <sub>v</sub> (n = 3)	1	33.3	0	0	2	66.7
<i>C. parapsilosis</i> <sub>a</sub> (n = 8)	0	0	0 <sub>(100%)</sub>	0	8	100
<i>C. parapsilosis</i> <sub>v</sub> (n = 8)	0	0	3	37.5	5	62.5
<i>C. glabrata</i> <sub>a</sub> (n = 4)	1 <sub>(50%)</sub>	25	1	25	2	50
<i>C. glabrata</i> <sub>v</sub> (n = 10)	1 <sub>(75%)</sub>	10	0 <sub>(100%)</sub>	0	9	90
Total (224)	8	3.5	14	6.25	202	90.25

CR, here was a 100% reduction in MICs > 16 µg/mL in *C. tropicalis*, *C. glabrata*.

Two *C. albicans* isolates were resistant to fluconazole after the three photodynamic procedures administered (LED + MB, LED + GV and LED + CR) and with high MICs > 16 µg/mL for ketoconazole. Both of these isolates originated from secretions collected from patients with recurrent vaginitis. The trailing phenomenon was also registered in these two *C. albicans* isolates and in two *C. tropicalis*.

#### 4. Discussion

Among women participating in this study, asymptomatic and affected by chronic or recurrent vaginitis, we found high percentages of non-*albicans* species, in



accordance with what was also observed in other studies [4] [11]. We identified isolates with a resistant and dose-dependent profile to fluconazole, both among *C. albicans* and non-*albicans* species, coming from materials collected from patients with chronic and recurrent vaginitis such as *Candida albicans* (13, 11.92%), *C. tropicalis* (1, 33.3%), *C. parapsilosis* (3, 60%), *C. glabrata* (5, 50%), and those obtained from asymptomatic women, such as *Candida albicans* (5, 6.94%), *C. tropicalis* (1, 10%), *C. parapsilosis* (1, 12.5%), *C. glabrata* (3.75%). We also recorded MIC > 16 µg/mL in relation to ketoconazole between *C. albicans* and non-*albicans* species. Resistance to antifungal treatments is also frequently observed in other studies [10] [25] [37] [38]. Non-*albicans* species, in addition to being frequent in complicated vulvovaginitis, have shown high levels of resistance to conventional antifungal agents, which was also verified in this research.

Our work has evidenced *C. glabrata* as an etiological agent resistant to antifungal agents ordinarily listed in the treatment of vaginitis, emphasizing that PDT reduced or even blocked *in vitro* the development of colonies of this agent, which is considered yeast associated with chronic vaginitis and/or recurrence of vaginitis and nowadays, it constitutes a considerable obstacle to the therapeutic efficacy of widely used antifungals, an event also registered in other studies [6] [10] [14] [25]. *Candida glabrata* is often associated with a chronic evolution, recurrence in vulvovaginitis and high resistance to antifungal agents. LED alone did not show homogeneous results for yeast in this study.

With respect to *C. albicans*, studies on photodynamic (PD) light and methylene blue also report similar results regarding the substantial blockage in the growth of colonies of this particular yeast [22] [39] [40]. In this study, the synergistic use of PD light and methylene blue with increased efficacy in blocking the growth of colonies of the tested yeast species such as *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. albicans*.

The PD light associated with methylene blue showed important reductions in the number of colonies resistant to fluconazole or ketoconazole (MICs > 16 µg/mL) and also in the reduction of resistant phenotypes, when compared to values recorded before treatment. A study with PD light and methylene blue found its use viable to inhibit *C. albicans* species from infection [41], which was also observed in this research, including in relation to other *Candida* species. These findings suggest PD light with methylene blue as a promising therapeutic alternative for fungal infections, including vulvovaginitis, especially in relation to non-*albicans* species, and further studies should be prospected to prove their *in vivo* efficacy. Other research on PD light and methylene blue treatments used for fluconazole resistant yeasts such as *C. glabrata* and *C. albicans* also describes similar results [21] [22] [25] [42]. Non-*albicans* species, many times, show resistance to conventional antifungal treatments [43] [44].

Investigations have shown the efficiency of gentian violet used alone, in terms of blocking the growth of *C. albicans* and non-*albicans* colonies. Gentian violet in 1% concentration was effective with regard to the total blockade in the growth of *C. albicans*, both in its use as a single agent and in association with light [26].

In our research, we also found the inhibitory activity *C. albicans* and non *albicans* species with PD light by LED and gentian violet solution 2% in both the separate use or as jointly.

Martins *et al.* in 2009 and Freitas in 2015 observed that curcumin, when compared to fluconazole, showed antifungal activity with greater inhibitory potential, on *C. albicans* and non-*albicans* species, such as *C. tropicalis*, *C. parapsilosis* and *C. glabrata* [27] [45]. In our work, however, it was only when we used curcumin together with LED light irradiation that we obtained antifungal efficacy, blocking the growth of *C. albicans* and non-*albicans* yeast colonies. We recorded only a mild antifungal effect of curcumin when used alone. However, its use associated with PD light revealed a good synergism *in vitro*, markedly inhibiting the growth of *C. albicans* and non-*albicans* colonies. Similar findings were also obtained in another investigation [24] [46].

We emphasize in our study, when using only LED or photosensitizers alone, we did not obtain reduction in the growth of *Candida* colonies, both *C. albicans* and non-*albicans*, resistant to fluconazole and with MIC > 16 µg/mL for ketoconazole, except for the gentian violet solution 2%, which actually showed antifungal activity, both in PDT and as a single agent. Other investigations have also found antifungal activity of gentian violet used in isolation [43] [47] [48] [49] [50].

We call attention that associations of photosensitizers with LED seem to be impactful with regard to blocking the growth of colonies of *C. albicans* or non-*albicans* species and the PDT procedure, after more studies and clinical trials, may constitute in future an important basis for the treatment of vulvovaginitis, particularly in chronic or recurrent cases, considering that it can represent efficient, low-cost treatment, without major side effects. In the specific case of gentian violet, we would still consider the practicality of representing possibly effective antifungal agent, potentially effective, having greater synergism when used with LED irradiation.

## 5. Conclusions

We emphasize that all 224 *Candida* spp isolates were submitted to photodynamic light with methylene blue, gentian violet and curcumin photosensitizers. We observed a reduction in the percentage of UFC/mL and of resistant and SDD phenotypes to fluconazole and to ketoconazole MICs > 16 µg/mL. Photodynamic therapy proved to be promising and effective *in vitro*, with regard to the significant reduction of fungal colonies of *C. albicans* and non-*albicans* species, including *C. tropicalis* and mainly *C. glabrata*, yeast species associated with severe cases of chronic recurrent vulvovaginitis. Our investigation was developed “*In Vitro*”, but its results may serve as parameters in future to *in vitro* and *in vivo* researches. New therapeutic solutions, more efficient, less aggressive, with minor or without organic toxicity, with treatments based on a short period of time, and that do not provoke the selection of new resistant and potentially aggravating

microbial strains, are required in fungal vulvovaginitis mainly for complicated cases. In these aspects, PDT could be an interesting tool, used as a single treatment or in addition to antifungal agents, for future studies involving *Candida* vulvovaginitis chronicity and recurrence especially to ways for gynecological clinic practice improvement.

### Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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