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Effects of dyes on *Candida spp*. viability

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Dissertação para obtenção de Grau Mestre em
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(ciclo de estudos integrado)

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Dedicatory

To my mother, Olga, and my father, Artur, because without them nothing would be possible.

To my family, for all the support.

To Renato, for the constant presence.

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Resumo Alargado

As infecções por *Candida* têm aumentado significativamente nos últimos anos, em larga medida como consequência do aumento dos casos de imunossupressão. *C. albicans* e *C. glabrata* são as espécies mais frequentemente isoladas. Estima-se que aproximadamente 75% de todas as mulheres terão pelo menos um episódio de candidose vulvovaginal durante toda a sua vida e que 5-10% destas irá experimentar episódios subsequentes.

Embora a *C. albicans* seja responsável por 80-90% de todas as infecções, as vaginites causadas por *Candida* não-*albicans* têm aumentado durante os últimos anos, estimando-se que apenas cerca de 50% dos casos de candidose vulvovaginal causada por *Candida* não-*albicans* respondem à terapia oral ou local convencional com azóis. Nos últimos anos têm surgido estudos que relatam a crescente resistência de espécies não-*albicans* aos antifúngicos clássicos. Este problema emergente salienta a necessidade do estudo de alternativas eficazes para tratar a candidose vulvovaginal. Neste contexto, os tratamentos tradicionais têm sido revistos e novas modalidades terapêuticas têm sido procuradas e propostas.

Vários corantes são reconhecidos pelas suas propriedades anti-microbianas. Contudo o seu perfil de actividade e de segurança não está, na maioria dos casos, definido. Da confirmação da sua actividade antimicrobiana e da segurança da sua aplicação *in vivo*, depende a confirmação do seu interesse no tratamento de infecções mucocutâneas, assim como no tingimento de roupa de uso hospitalar ou uso íntimo, com vista à prevenção da re-infecção e no revestimento de materiais médicos, ou outros produtos que se pretendam estéreis.

O violeta de genciana tem sido tradicionalmente usado no tratamento da candidose mucocutânea. Contudo, a informação existente sobre o seu espectro antimicrobiano não é suficiente para que se possa definir o seu valor clínico. O azul-de-metileno apresenta ampla aplicação na clínica. Porém, a sua actividade anti-séptica, apesar de referida na literatura, não é ainda completamente compreendida.

O objectivo deste estudo é avaliar a actividade *in vitro* do violeta de genciana e do azul-de-metileno em diferentes espécies de *Candida*, comparar a sua actividade com a de antifúngicos clássicos e contribuir para o conhecimento do mecanismo de acção destes produtos.

Foram estudadas dezanove estirpes de *Candida: C. tropicalis* (n=4), *C. albicans* (n=5), *C. parapsilosis* (n=4), *C. glabrata* (n=4) e *C. krusei* (n=2). As estirpes clínicas foram isoladas de casos graves de candidose vulvovaginal. O efeito antifúngico foi avaliado pela determinação da concentração mínima inibitória (CMI) com base no micrométodo descrito na referência CLSI M27-A3, após 48 horas de incubação a 37 ºC. O crescimento da levedura foi

visualmente comparado para cada concentração com a amostra controlo. O efeito fungicida foi verificado com base na determinação da concentração mínima letal (CML), adoptando-se o protocolo proposto por Canton (2003). Foram incluídos controlos de crescimento em etanol. Todas as determinações foram realizadas em duplicado e apenas resultados concordantes de três experiências independentes foram considerados. O protocolo do CLSI M27-A3 também foi utilizado para a avaliação do efeito da anfotericina B e fluconazol sobre as estirpes de *Candida* testadas. Por fim, o mecanismo de acção do corante sobre as leveduras foi avaliado por citometria de fluxo com base na metodologia descrita por Pina Vaz (2010).

No caso do violeta de genciana, as CMI e CML foram aproximadamente as mesmas para todas as estirpes estudadas, excepto para a *C. glabrata*. Os valores de CMI variaram entre 0,03 µg/mL e 0,24 µg/mL e de CML variam entre 0,03 µg/mL e 0,98 µg/mL. *C. albicans* e *C. tropicalis* foram as espécies mais sensíveis. O efeito antifúngico não foi influenciado pelo etanol a 20% e a 96%. O violeta de genciana mostrou uma potente actividade fungicida contra todas as estirpes de *Candida*.

Para o azul-de-metileno, as CMI variaram entre 0,039 mg/mL e 0,078 mg/mL e as CML entre 0,039 mg/mL e 0,3125 mg/mL para as estirpes sensíveis. Uma estirpe de *C. parapsilosis* foi apenas inibida pelo corante. As *C. glabrata* e *C. krusei* são resistentes às máximas concentrações utilizadas (5 mg/mL).

Os resultados da citometria de fluxo revelaram baixa marcação celular por iodeto de propídio, mostrando que o mecanismo principal de morte celular após exposição ao violeta de genciana não é a lesão primária da membrana. As leveduras expostas ao azul-de-metileno sofreram marcação pelo iodeto de propídio, mostrando que este corante provoca lesão primária da membrana.

O violeta de genciana apresenta actividade fungicida mesmo para as estirpes resistentes ao fluconazol. O azul-de-metileno apresenta um perfil de acção semelhante ao do fluconazol.

A candidose vulvovaginal recorrente que não é controlada com protocolos terapêuticos clássicos precisa de abordagens diferentes. O violeta de genciana e o azul-de-metileno parecem ser drogas com potencial para serem usadas nos casos de candidose vulvovaginal recorrentes.

Palavras-chave

Violeta de genciana; Azul-de-metileno; Actividade anti-*Candida*; Candidose vulvovaginal recorrente; Tratamento alternativo.

Abstract

Several dyes are widely recognized for their anti-microbial properties, although there spectrum of activity and security profiles are not always defined.

Gentian violet (GeV) has been traditionally used to treat mucocutaneous candidosis. However, information concerning its antimicrobial spectrum required to support its clinical value is scarce.

Methylene blue (MB) is widely used in clinic. Nevertheless, its antiseptic activity, although reported in the literature, is also not yet fully understood.

The aim of this study is to evaluate the *in vitro* activity of GeV and MB against different *Candida* species. Nineteen strains of *Candida* will be studied. Clinical strains were isolated from clinical resistant cases of *Candida* infections. The anti-*Candida* activity of GeV and MB was evaluated according to CLSI protocol M27-A3. The mechanism of action it was evaluated by flow cytometry.

About GeV, the minimal inhibitory concentrations (MIC) and minimal lethal concentrations (MLC) were approximately the same for all strains studied, except for *C. glabrata*. *C. albicans* and *C. tropicalis* were the most sensitive species. The antifungal effect was not influenced by alcohol at 20% and 96%. GeV showed a potent fungicidal activity against all strains of *Candida*. GeV doesn't cause primary lesion of cytoplasmic membrane.

Not all tested strains were susceptible to MB. The *C. glabrata* and *C. krusei* were resistant to the highest concentration used (5 mg/mL). In contrast to GeV, MB cause membrane lesion.

Recurrent vulvovaginal candidosis, especially if resistant to classical therapeutic protocols ask for new approaches. GeV and MB continue to stand up as potential drugs to be used topically isolated or in addition to oral antifungal drugs in clinical resistant vulvovaginal candidosis.

Keywords

Gentian violet; Methylene Blue; Anti-*Candida* activity; Vulvovaginal candidosis applicant; Alternative treatment.

Publications resulting from this work

- Oral presentation entitled "*In vitro* anti-*Candida* activity of gentian violet", on V Annual CICS Symposium (abstract and certificate: Annex 1);

- Oral presentation entitled "*In vitro* anti-*Candida* activity of gentian violet", on European College for the study of vulval disease Congress (abstract and certificate: Annex 2);

- Poster entitled "*In vitro* anti-*Candida* activity of gentian violet", on European College for the study of vulval disease Congress (abstract and certificate: Annex 2).

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List of Acronyms

AF	Autofluorescence
AIDS	Acquired immune deficiency syndrome
ATCC	American Type Culture Collection
CFU	Colony Forming Units
CMI	Concentração Mínima Inibitória
CML	Concentração Mínima Letal
CSLI	Clinical and Laboratory Standards Institute
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
GeV	Gentian Violet
GRP	Gabinete de Relações Públicas
HIV	Human immunodeficiency virus
MB	Methylene Blue
MIC	Minimal Inhibitory Concentration
MLC	Minimal Lethal Concentration
NADPH	Nicotinamide adenine dinucleotide phosphate
PI	Propidium Iodide
RPMI	Royal Park Memorial Institute
RVVC	Recurrent Vulvovaginal Candidosis
UBI	Universidade da Beira Interior
VVC	Vulvovaginal Candidosis

Chapter 1: Introduction

1.1 Candidosis

Candida infections, both mucocutaneous and systemic, have increased significantly in recent years in consequence of the increasing numbers of AIDS, chemotherapy and immunosuppressive drugs, extensive burns and the use of broad spectrum antibiotics (1, 2). *C. albicans* and *C. glabrata* are the most frequent isolated species (1, 3-5).

Vulvovaginal candidosis (VVC) is a mucocutaneous infection caused by *Candida spp.*, involving the vulva and vagina. This disease is frequently referred as vulvovaginal candidiasis, thrush or moniliasis and is a common worldwide problem, particularly in fertile women, representing about 20-30% of vaginal infections (6, 7). It has been estimated that approximately 75% of all women will have at least one episode of VVC throughout their lifetime and 5-10% of them will experience more than one episode (4).

Although, *C. albicans* is responsible for 80-90% of all infections, vaginitis caused by non-*albicans Candida* have increase in frequency during the last years being clinically indistinguishable from those caused by *C. albicans* (3, 6). This change in the pattern of *Candida* infection is thought to be related to single-dose treatments, low-dosage azole maintenance regimens and use of over-the-counter antimycotics (8). It has been estimated that only approximately 50% of all cases of VVC caused by non-*albicans Candida* spp. respond to conventional oral or local therapy with azoles (6). In a recent study a progressive decrease in sensitivity to fluconazole by *C. albicans* isolates in women with recurrent vulvovaginal candidosis (RVVC) has been reported, apparently as the result of long-term fluconazole therapy (9). Other studies reporting the resistance of non-*albicans* species to classical antifungals have been published (10-15). In fact, RVVC, defined as four or more episodes of VVC occurring in a 12-months period and frequently caused by non-*albicans* strains (especially *C. glabrata*), become more prevalent in the past few years (4, 16).

This emergent problem stresses the study of efficient alternatives to treat RVVC. In this context, conventional and traditional treatments have been reviewed (6, 17, 18) and new therapeutic modalities have been studied (19-23).

1.2 Dyes

A dye is a substance that can be applied in solution or dispersion to a substrate, giving them a colourful appearance. Generally, substrates are textile fibbers, but paper, hair, plastics, cosmetics, food, among many others, may also be stained (24, 25). Dyes are

characterized by their ability to absorb visible light and are used by humans since prehistoric times (26).

Natural dyes (animal, mineral or derived from plants) are known for a long time by their colouring properties and medicinal effect, namely its antimicrobial activity (27). These characteristics are shared with some synthetic dyes (28).

Their antimicrobial effect has been traditionally called for the treatment of mucocutaneous infections, although the security of its use has not been completely studied. The potential interest on the use of these products as pharmaceutical drugs for the management of mucocutaneous recurrent infections, or clothes coating, particularly intimate clothes, and hospital textiles, in order to prevent microorganisms' transmission, stresses the need for clarification of their effective antimicrobial activity against specific microorganisms and its mechanism of action.

In this work two dyes were studied, gentian violet (GeV) and methylene blue (MB). Both compounds are traditionally recognized for their antimicrobial effects. However, *in vitro* activity of both has been poorly investigated.

1.2.1 Gentian Violet

Gentian violet (GeV) is a triphenylmethane dye mixture used to paint hair, paper or textiles. It is derived from coal tar and has been widely used as an antiseptic. Its antimicrobial activity has been traditionally recognized and is recommended for candidosis treatment (4, 6, 29, 30). Despite being widely used, its *in vitro* antifungal activity has been poorly investigated. Recent studies reported the activity of GeV against *Candida* clinical isolates from the oral cavity of HIV-infected patients and its effect on virulent properties of *C. albicans*, namely inhibiting germ tube formation and enzymatic expression of yeasts (31, 32).

1.2.2 Methylene Blue

Methylene blue (MB) is an odourless composite of green salts, aromatic, heterocyclic, soluble in water, producing a blue solution. It has broad applications, such as bacteriological dye and vital indicator to identify cellular viability. It is also used to treat metehemoglobinemia and on the detection of precancerous lesions and cancer, as an *in vivo* epithelial marker (33). Its anti-*Candida* effect has been studied in association with lightherapy (34-37). Apparently, MB is a photosensitizer that absorbs energy from light generating an activated form of oxygen, that it is believed to be the main cytotoxic agent (38).

Although the well known antifungal properties of phenothiazine dyes, MB antifungal effect out of this association context is not full characterised (revised by Ohlow *et al*. (39)). Just as GeV, it is described as an antiseptic product (40).

The aim of this study was to evaluate the anti-*Candida* activity of GeV and MB against strains from collection and isolates from patients with clinical resistant *Candida* infections. The activity of fluconazole and amphotericin B, two classical antifungals, against selected yeasts strains was also determined and compared with GeV and MB susceptibility profile. In addition, studies were performed to clarify the action mechanism of both dyes.

Chapter 2: Material and Methods

2.1 Yeasts isolates

18 clinical strains of *Candida* were studied: 4 *C. tropicalis*; 4 *C. albicans*; 4 *C. parapsilosis;* 4 *C. glabrata;* 2 *C. krusei*. In addition strain 10231 from American Type Culture Collection (ATCC) was also used. Clinical strains were obtained from severe *Candida* infections, some with recurrent *Candida* infections mucocutaneous disease and showing variable resistance to fluconazole. Such isolates had been characterized to species level using API 32C (BioMérieux, Vercieux, France). The strains were kept frozen in Brain–Heart Broth (Difco Laboratories, Detroit, MI, USA) with 40% glycerol at -70 ºC until testing. For each experiment, the yeasts were subcultured twice from frozen stocks on Sabouraud agar (Difco) and incubated at 37ºC for 24h.

2.2 Drugs

A 1% (m/v) hydro-alcoholic GeV solution (Amresco, USA) was prepared in 20% ethanol. We also prepared a similar solution in 96% ethanol, according to the Portuguese Pharmacopeia recommendations (41). Both solutions were then passed through a 0.22 µm sterilizing filter.

A 50 mg/mL solution of MB was prepared on sterile water and subsequently diluted in RPMI 1640 (Sigma, Portugal), the culture medium, in order to obtain the work solution with 10 mg/mL. The solution was passed through a 0,22 µm sterilizing filter.

Serial dilutions were then performed on RPMI 1640 according to CLSI reference M27-A3 protocol (42).

Amphotericin B (Bristol-Myers Squibb, New York) and fluconazole (Pfizer, Groton, CT) were used as classical antifungal drugs for comparison.

2.3 Antifungal activity

Minimal inhibitory concentration (MIC) of GeV against *Candida* spp. was determined by the CLSI reference M27-A3 micromethod, after 48 hours of incubation at 37ºC (42). Yeast growth was visually compared for each concentration with the control sample. Only the 100% growth inhibition, visually evaluated, was taken as MIC.

The minimal lethal concentration (MLC) was determined based on the modified protocol proposed by Canton et al (43).

Growth controls in ethanol were included. All determinations were performed in duplicate and only concordant results from three independent experiments were considered.

CLSI protocol M27-A3 was also used for the evaluation of amphotericin B and fluconazole effect on *Candida* strains tested.

2.4 Mechanism of action

The effect of dyes on yeasts was enlightened by flow cytometry, as previously reported by Pina Vaz *et al*. (44). Briefly, after incubating 10^6 cells/mL for 1 hour at 37ºC with dye at half MLC, MLC and double MLC, the cells were stained with propidium iodide (PI, Sigma), 1 µg/mL, protected from light, at room temperature, during 15 minutes. PI is a fluorescent probe usually used to stain non-viable cells, because dead or dying cells with primary lesion of cytoplasmic membrane can incorporate PI (45). Following the staining step, the cells were analyzed on a FACS Calibur Cytometer (BD Biosciences, Sydney) at FL3 (620 nm-red).

Autofluorescence was detected by using non-treated and non-PI stained cells; non-treated and stained cells served as viability control and yeasts cells treated with 70% ethanol for 10 min were used as a death control.

For kinetic studies, fungal cells were incubated with the dye during 5, 10, 15, 30 e 45 minutes at MLC and then stained with PI using the protocol described above.

After treatment with each dye for 60 minutes, cells were platted on agar medium for evaluation of the number of Colony Forming Units (CFU).

The tests were performed on *C. albicans* ATCC10231.

Chapter 3: Results

3.1 Yeasts isolates

Table 1 shows the strains of Candida selected for the study and their source.

Table 1: Tested species and source.

Yeasts	Source
C. tropicalis ARTEMIS 41	Vagina
C. tropicalis ARTEMIS 35	Vagina
C. tropicalis MC 407	Vagina
C. tropicalis MC 374	Vagina
C. albicans ATCC10231	Collection
C. albicans 28	Vagina
C. albicans MC440	Vagina
C. albicans MC437	Vagina
C. albicans 030210A	Vagina
C. parapsilosis 030	Vagina
C. parapsilosis ARTEMIS 64	Vagina
C. parapsilosis MC 405	Vagina
C. parapsilosis MC409	Vagina
C. glabrata H16	Vagina
C. glabrata H30	Vagina
C. glabrata MC 426	Vagina
C. glabrata MC 370	Vagina
C. krusei OL099	Vagina
C. krusei OL103	Vagina

3.2 Determination of the anti-*Candida* activity

3.2.1 Susceptibility to Gentian Violet

The results show that GeV is active against all *Candida* species tested (Table 2). MIC values ranged from 0.03 µg/mL and 0.24 µg/mL. *C. albicans* and *C. tropicalis* yeasts proved to be more susceptible to GeV (MIC between 0.03 µg/mL and 0.12 µg/mL). *C. parapsilosis*, *C. glabrata* and *C. krusei* were less sensitive (MIC between 0.06 µg/mL and 0,12 µg/mL), but still highly susceptible with MIC values significantly lower than the concentration of 1% solution of GeV clinically used.

Table 2 also shows that MLC values range from 0.03 µg/mL and 0.98 µg/mL. MIC and MLC are approximately the same for all yeasts except for *C. glabrata*, which presents a MLC between two to four times higher than MIC (0.12 µg/mL - 0.98 µg/mL).

Table 2: MIC and MLC of a hydro-alcoholic solution of GeV.

Yeasts	Susceptibility to GeV	
	MIC 48h (µg/mL)	MLC 48h (µg/mL)
C. tropicalis ARTEMIS 41	0,03-0,06	0,03-0,06
C. tropicalis ARTEMIS 35	0,03-0,06	0,06
C. tropicalis MC 407	0,03-0,12	0,06-0,12
C. tropicalis MC 374	0,03-0,06	0,06
C. albicans ATCC10231	0,06	0,06-0,12
C. albicans 28	0,03	0,06
C. albicans MC440	0,06	0,06
C. albicans MC437	0,06	0,06
C. albicans 030210A	0,03-0,06	0,03-0,06
C. parapsilosis 030	0,12-0,24	0,12-0,24
C. parapsilosis ARTEMIS 64	0,06	0,06-0,12
C. parapsilosis MC 405	0,12	0,12-0,24
C. parapsilosis MC409	0,12	0,12
C. glabrata H16	0,06	0,12
C. glabrata H30	0,12	0,49
C. glabrata MC 426	0,12	0,49-0,98
C. glabrata MC 370	0,12	0,24
C. krusei OL099	0,12	0,12
C. krusei OL103	0,12	0,12

3.2.2 Susceptibility to Methylene Blue

The results concerning the susceptibility to MB (Table 3) show that not all strains are susceptible to this dye. *C. glabrata* and *C. krusei* are resistant to the highest concentration used. It is important to indicate that, for a concentration of 5 mg/mL the dye colour makes it impossible the visual evaluation of yeast growth. In contrast to GeV the MIC and the MLC are not coincident for all tested strains.

Table 3: MIC and MLC of an aqueous solution of MB.

Yeasts	Susceptibility to MB	
	MIC 48h (mg/mL)	MLC 48h (mg/mL)
C. tropicalis ARTEMIS 41	0,039	0,078
C. tropicalis ARTEMIS 35	0,078	0,078
C. tropicalis MC 407	0,039	0,039
C. tropicalis MC 374	0,039	0,078
C. albicans ATCC10231	0,078	0,3125
C. albicans 28	0,078	0,156
C. albicans MC440	0,078	0,156-0,3125
C. albicans MC437	0,078	0,078
C. albicans 030210A	0,078	0,078
C. parapsilosis 030	0,078	>5

C. parapsilosis ARTEMIS 64	0,039	0,078
C. parapsilosis MC 405	0,039	0,078
C. parapsilosis MC409	0,039	0,078
C. glabrata H16	≥5	>5
C. glabrata H30	≥5	>5
C. glabrata MC 426	≥5	>5
C. glabrata MC 370	≥5	>5
C. krusei OL099	≥5	>5
C. krusei OL103	≥5	>5

3.2.3 Susceptibility to Fluconazole

Table 4 shows the susceptibility to fluconazole, a classical antifungal used to treat candidosis. Some yeasts (*C. tropicalis* ARTEMIS 35, *C. albicans* 28, *C. glabrata* H30, *C. krusei* OL099, *C. krusei* OL103, *C. parapsilosis* ARTEMIS 64) are resistant to fluconazole (MIC> 64 µg/mL), and one strain of *C. tropicalis* and two of *C. glabrata* were susceptible dose dependent.

Table 4: Susceptibility to fluconazole classification of yeasts according to M27-A3 protocol on: Susceptible (S), Resistant (R), Depending-dose Susceptibility (S-DD) and Intermediate Susceptibility (I).

Yeasts	Susceptibility to fluconazole	
	MIC (µg/mL)	Phenotype
C. tropicalis ARTEMIS 41	16	S-DD
C. tropicalis ARTEMIS 35	>64	R
C. tropicalis MC 407	2	S
C. tropicalis MC 374	1	S
C. albicans ATCC10231	1	S
C. albicans 28	>64	R
C. albicans MC440	0,25	S
C. albicans MC437	2	S
C. albicans 030210A	<1	S
C. parapsilosis 030	0,5	S
C. parapsilosis ARTEMIS 64	>64	R
C. parapsilosis MC 405	0,5	S
C. parapsilosis MC409	0,25	S
C. glabrata H16	1	S
C. glabrata H30	>64	R
C. glabrata MC 426	32	S-DD
C. glabrata MC 370	16	S-DD
C. krusei OL099	>64	R
C. krusei OL103	>64	R

3.2.4 Susceptibility to Amphotericin B

In Table 5 we show the MIC values of amphotericin B. All strains presented a similar susceptibility profile, with MIC range between 0,125 µg/mL and 1 µg/mL.

Table 5: Susceptibility to amphotericin B.

Yeasts	MIC (µg/mL)
C. tropicalis ARTEMIS 41	0,5
C. tropicalis ARTEMIS 35	0,5
C. tropicalis MC 407	1
C. tropicalis MC 374	0,25
C. albicans ATCC10231	0,5
C. albicans 28	0,5
C. albicans MC440	0,25
C. albicans MC437	0,5
C. albicans 030210A	<0,25
C. parapsilosis 030	0,25
C. parapsilosis ARTEMIS 64	0,25
C. parapsilosis MC 405	0,125
C. parapsilosis MC409	0,125
C. glabrata H16	0,25
C. glabrata H30	0,5
C. glabrata MC 426	0,25
C. glabrata MC 370	0,25
C. krusei OL099	0,5
C. krusei OL103	0,5

3.3 Mechanism of action

Flow cytometry results showed that GeV fungicidal effect is not related with a primary lesion of the cytoplasmic membrane, since PI was not able to enter the cell (Figure1).

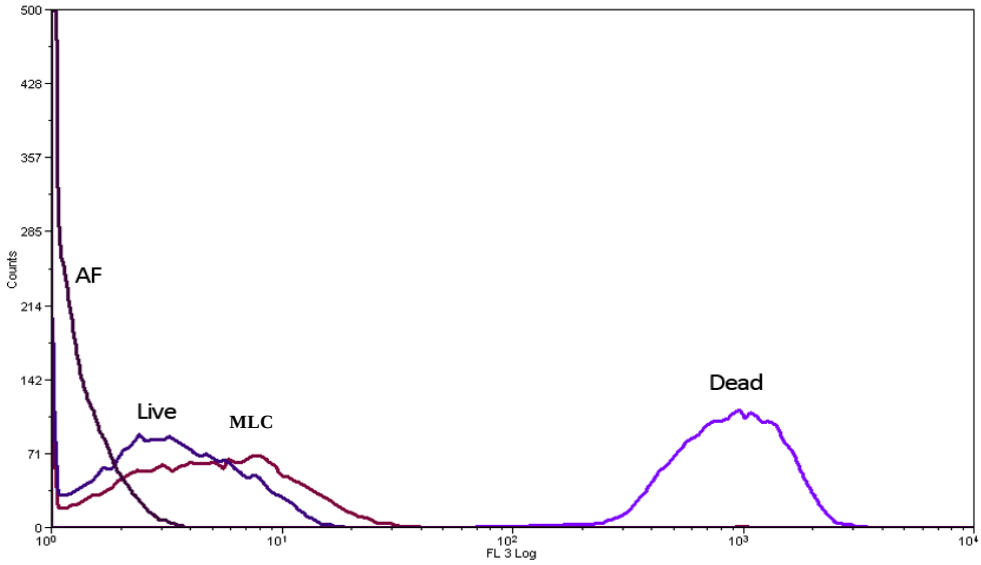

Figure 1: Histogram representing PI stained cells. **AF**: autofluorescence; **Live**: viable – non treated cells (viability control). **Dead**: death- cells treated with 70% ethanol (death control). **MLC**: cells treated with MLC concentration of GeV during 60 minutes.

This result was not influenced by the GeV concentration, since similar percentage of stained cells was obtained for MLC and double MLC determinations (Figure 2). Cell death was confirmed by CFU assessment.

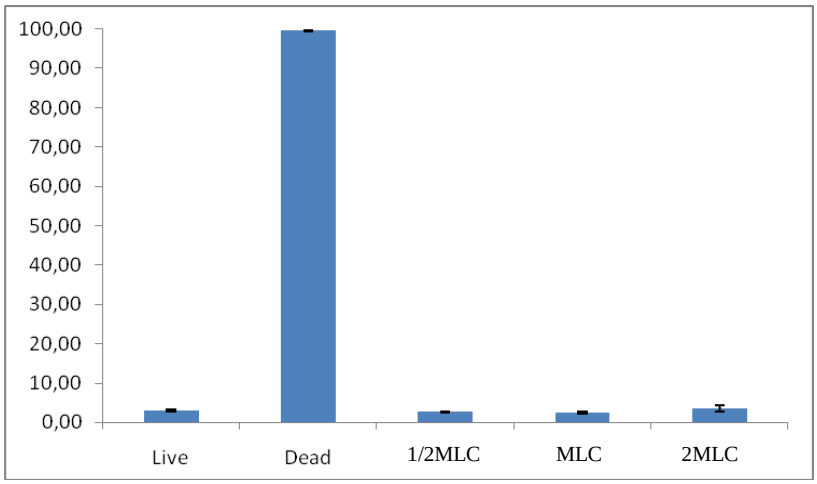

Figure 2: PI stained cells comparison between: **Live** – non treated cells (viability control); **Dead** – cells treated with 70% ethanol (death control); **1/2MLC** – cells treated during 1h with half MLC; **MLC** – cells treated during 1h with MLC; **2MLC** – cells treated during 1h with double MLC of GeV.

Cytometric study of MB reveals a fungicidal effect due to primary lesion of cytoplasmic membrane, as represented on Figure 3.

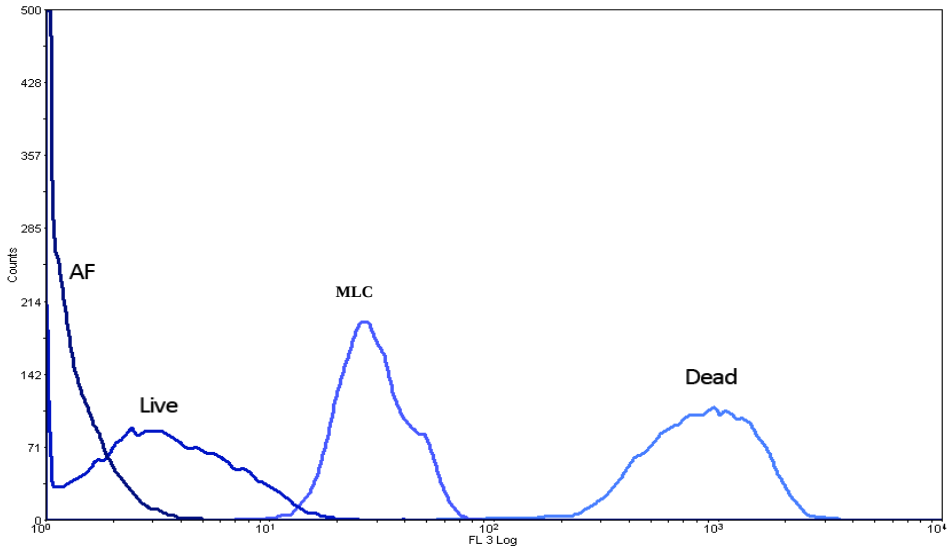

Figure 3: Histogram representing PI stained cells. **AF**: autofluorescence; **Live**: viable – none treated cells (viability control). **Dead**: death- cells treated with 70% ethanol (death control). **MLC**: cells treated with MLC of MB during 60 minutes.

The MB fungicidal effect is quick and effective as it is shown by the cytometric results: after 5 minutes of cells treatment with MB, the percentage of death cells was high (91,43%) and after 60 minutes it was about 98,28%. The kinetic study it is represented in Figure 4.

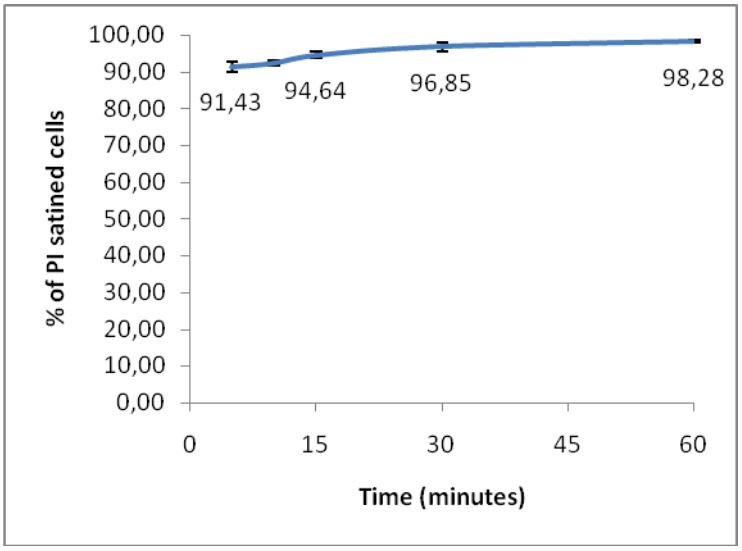

Figure 4: Kinetic study showing the percentage (%) of PI stained cells after treatment with MLC during 5, 10, 15, 30, 45 and 60 minutes, that is, dead cells.

The differences between dye concentrations are represented on the follow table.

Table 6: Death percentage with different MB concentrations.

MB concentration	Death %	Standard Deviation
½ MLC	96,18	0,652
MLC	98,28	0,225
2 MLC	98,82	0,070

Chapter 4: Discussion

The methodology used is in accordance with international accepted guidelines and allows for a comparison with other studies.

The results obtained with GeV are consistent with previously reported studies, although differences in methodology have been noted. Traboulsi *et al.* (2008) tested GeV in oral cavity isolates from individuals HIV seropositive. As the dye is considered water insoluble, they used dimethylsulfoxide (DMSO) as solvent (31). In this case, we chose to test the GeV solution as it is used on traditional therapies, say a hydro-alcoholic solution. However, MIC values obtained by them ranged from 0.03 µg/mL and 0.25 µg/mL, approximately the same values reported here (31).

An important fact that should be mentioned is that the GeV showed a fungicidal effect for the inhibitory concentration, that is, the MIC values and MLC were similar for most species tested. The inhibitory effect of the hydro-alcoholic solution of GeV against *Candida spp.* was not influenced by the presence of ethanol, since both solutions tested showed the same results and solvent controls had no effect on growth inhibition (data not shown).

The results emphasize the use of traditional GeV for the treatment of VVC, especially in recurrent cases resistant to classical treatment. Being effective, cheaper than other antifungal agents and easy to apply it is understandable that GeV is still being used.

Despite the fact that GeV is being widely used in clinical settings, its mode of action is not fully understood. Cytometric results showed that yeasts treated with GeV were not stained with PI, showing that GeV doesn't cause primary lesion of cytoplasmic membrane. However, it was not possible to determine the exactly mechanism of action of GeV because the required resources like metabolic markers were not available for the present study. Regardless this limitation, our results are in accordance with previous studies as discussed below.

Initial studies to elucidate the mechanism of action of GeV reported its reaction with the DNA of living cells by inhibiting its synthesis and causing damage to bacterial DNA (46, 47). The literature also mention a possible effect by producing free radicals through GeV microsomal reduction (48). More recent studies have shown that brief exposure of *C. albicans* to low concentrations of GeV (0.5 µg/mL and 2 µg/mL) led to a reduction of three virulence factors (phospholipase activity, proteinase activity and germination rate) (32). In fact, these possible mechanisms of action led to another contentious issue: the safety of GeV. The demonstration, *in vitro,* of the interaction of GeV with DNA (46) and carcinogenicity in mice points to a possible toxic effect (49). However, the carcinogenicity effect is dose-dependent

and GeV concentrations used in these studies were remarkably higher than those reported here, approximately one hundred times higher than MIC determined in our work. Tolba *et al.* revealed that GeV reduces or stops the absorption of nitrate nitrogen, inhibiting the synthesis of proteins and peptides, and at concentrations above 10 ppm (≈ 10 μg/mL) reduces the output of carbon dioxide (50). Despite these effects at the cellular level of GeV seem to indicate a level of toxicity, GeV has been used for a long time, even in children without complications (51). Moreover, its clinical use in treating acute episodes of VVC or recurrent cases unresponsive to conventional antifungal agents is limited to one or two applications (30). Besides, our results show that GeV is not able to disrupt the cytoplasmic membrane and is not probable to passively enter to the cell nucleus.

The clinical benefit of GeV use, especially in difficult cases to handle, seems to overcome some possible side effects (52). In fact, a recent study reported the safety and efficacy of using GeV in oral thrush, on a total of 17 patients, nine achieved clinical success, but purplish, cracked lips and dry mouth were reported as adverse effects (53). Oral irritation was also reported in another study (54). However, these effects can be overcome, as with the development of a pharmaceutical formulation suitable for reducing the effect of color and mucosal irritation is possible.

The results show that MB is a dye with antifungal activity. All *C. glabrata* and *C. krusei* tested strains were resistant at the maximum concentrations tested (MIC ≥ 5 mg/mL and MLC > 5 mg/mL). Due to the dye colour, we were not able to visually evaluate the yeasts growth for concentrations equal or higher than 5 mg/mL.

Although not extensively studied, the MB antimicrobial activity is being reported in the literature. Regarding its anti-*Candida* effect, Ofoegbu (2007) reported a success treatment of a sepsis by *C. albicans* in a premature newborn with MB continuous infusion during 8 weeks combined with systemic antifungal (flucytocine and liposomal amphotericin) (55). On the other hand, another study refereed the absence of MB fungicidal activity. These results could be explained by the low concentration tested and the insufficient exposure time (56).

The account of the routine use of MB with few toxic reactions described reveals the safety of the product and reinforces the possibility of its use as alternative therapy and/or complementary treatment of candidosis. This dye is frequently used for the evaluation of the digestive anastomosis integrity. It is also claimed to be neuroprotective and it is a promise drug in Alzheimer's disease treatment. Some reports also refer the use of MB to treat several conditions, namely ifosfamide-induced encephalopathy, and to early detection of cancer and precancerous lesions (33, 57-59).

Regarding its mechanism of action, MB seems to have anti-oxidant action properties, inhibiting the activity of NADPH oxidase and myeloperoxidase (60). Our results show that MB is a potent fungicidal compound that causes a primary lesion of the cytoplasmic membrane. PI was able to stain yeast cells treated with MB after 5 minutes of treatment. This shows that MB has a potent fungicidal effect right from the 5 minutes exposure. When cells were treated with half MLC after one hour about 96% were dead and about 98% were dead with MLC and twice MLC.

The mechanisms of action of classical antifungals are well characterised. Fluconazole acts by inhibiting ergosterol synthesis and so changing membrane permeability. On other hand, amphotericin B bounds to ergosterol, creating channels or pores, and then increasing cell permeability. By this, it is easily to understand that previously and/or simultaneously treatment with fluconazole reduces amphotericin B activity.

Tested strains represent the most common species isolated from clinic cases. The susceptibility results to classical antifungals are in accordance with previous studies and clinical observations (61, 62). All *Candida spp*. showed a low MIC value for amphotericin B, while for fluconazole, *C. glabrata* and *C. krusei* were the species with higher MIC value and six strains were resistant to it.

The study of new drugs target it has been focused on proposing new ways to inhibit or kill yeasts or to find a pathway to avoid pathogenic mechanisms, such as avoiding the biofilm formation.

In vitro GeV was highly fungicidal to all tested strains, even to those resistant to fluconazole. This is an important finding because it shows that this dye can be an important drug in treating infections due to emerging resistant strains.

MB shows an identical profile than fluconazole. It was not possible to determine MIC or MLC to *C. glabrata* and *C. krusei* showing that probably these are resistant species to MB.

So, this study reinforces the important role of these new molecules, especially GeV that presents a fungicidal effect at very low concentrations and also because of the extent clinical experience with these dyes.

4.1 Future Perspectives

It is imperative to continue studies on this area mostly because of the lack of drugs to control fungal infections and because of a crescent number of resistant cases.

It is important to study further on the mechanism of action of these dyes, to evaluate its activity *in vivo* and to develop a drug delivery system that both avoid the inconvenient of colour staining and allows for an easy application.

Ex vivo studies on epithelial cellular cells from vulva and vagina are also relevant to evaluate cytotoxic profile and systemic absorption.

Chapter 5: Conclusion

In conclusion, the GeV is fungicidal for all strains tested at a concentration significantly lower than that used in clinical practice. These results support the use of GeV as a complementary treatment for the management of clinical cases of mucocutaneous *Candida* infections, especially of those resistant to conventional treatment regimens.

This study shows that the effect of GeV is species dependent and not related to the antifungal susceptibility to classical therapy, that is, fluconazole and amphotericin B, making the GeV a potential alternative in the treatment of VVC resistant to classical antifungals.

It was observed that GeV does not cause primary lesion of the cytoplasmic membrane, but more studies are necessary to clarify its mode of action.

MB also showed an antifungal activity. It is fungicidal to the majority of tested strains. However, it was not possible to determine MIC values to *C. glabrata* and *C. krusei* showing that probably these are resistant species. This pattern is identical to that observed with fluconazole.

Cytometric results show that MB has potent and quick fungicidal effect on *C. albicans* ATCC10231. The cells were stained with PI which means that MB causes primary lesion of cytoplasmic membrane.

So, the tested dyes represent a potential group of drugs to be used on mucocutaneous candidosis.

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Annexes

Annex 1

IN VITRO ANTICANDIDA ACTIVITY OF GENTIAN VIOLET

Gomes-de-Elvas, A.R[1]; Palmeira-de-Oliveira, R.[1]; Gaspar, C.[1]; Gouveia, P[1,2]; Martinez-de-Oliveira, J.[1,3]; Palmeira-de-Oliveira, A.[1]

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Objective:

Gentian violet has been used traditionally to treat vulvovaginal candidosis. There is, however, scarce information concerning its antimicrobial spectrum, required to define its clinical value. This study was designed to evaluate *in vitro* activity of gentian violet against *Candida* spp.

Study design:

Ten strains of *Candida* were studied: 4 *C. albicans* (1 American Type Culture Collection strain, 3 clinical strains); 2 *C. krusei* clinical strains; 4 *C. glabrata* clinical strains. Clinical strains were isolated from recurrent vulvovaginal candidosis cases.

A 1% (m/v) hydro-alcoholic gentian violet (Amresco, USA) was prepared dissolving 1g of violet gentian on 9g of 20% ethanol, and adding purified sterile water up to 100 mL final volume. This solution was then passed through a 0,22 µm sterilizing filter.

The anticandida activity of gentian violet was studied according to CLSI reference M27-A3 protocol. Minimal inhibitory concentrations (MIC) were determined after 24 and 48 hours of incubation at 37ºC. Yeast growth was visually compared for each concentration with the control sample.

The minimal lethal concentration (MLC) was determined based on the modified protocol proposed by Canton et al (2003).

Controls were performed with RPMI medium containing ethanol 20% in the same concentration as the samples. All determinations were performed in duplicate.

Results:

Gentian violet showed to have a potent in vitro activity against *Candida* strains tested. MIC values achieved for *C. albicans* were lower (0,06 µg/ml) than for *C. krusei* and *C. glabrata (*0.12 µg/ml). For *C. albicans* and *C. krusei* MIC and MLC were the same. For *C. glabrata* MLC was higher than MIC (0.49-0.98 µg/ml).

This effect was not influenced by the 20% alcohol solvent as all tested strains and respective controls exhibited similar growth either when present or not.

Conclusion:

Gentian Violet has a fungicidal effect against *C. albicans* and *C. krusei.* Its activity against *C. glabrata* revealed a different pattern of susceptibility.

Gentian violet is considered a potential drug to be used in clinical resistant vulvovaginal candidosis.

Keywords: vulvovaginal candidosis; gentian violet; recurrence

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Centro de Investigação em Ciências da Saúde
Health Sciences Research Centre

V ANNUAL CICS SYMPOSIUM

6th JULY' 2010

CERTIFICATE

I herewith certify that Ana Rita Gomes de Elvas has participated as speaker in the V Annual CICS Symposium which was held in Covilhã, the 6th July 2010.

The Organizing Committee

Annex 2

IN VITRO ANTI-CANDIDA ACTIVITY OF GENTIAN VIOLET

Gomes-de-Elvas, A.R[1]; Palmeira-de-Oliveira, R.[1]; Gaspar, C.[1]; Gouveia, P[1,2]; Martinez-de-Oliveira, J.[1,3]; Palmeira-de-Oliveira, A.[1]

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Objective:

Gentian violet has been used traditionally to treat vulvovaginal candidosis. There is, however, scarce information concerning its antimicrobial spectrum, required to define its present clinical value. This study was designed to evaluate *in vitro* the activity of gentian violet against *Candida* spp.

Study design:

Six strains of *Candida* were studied: 4 *C. albicans* (1 American Type Culture Collection strain, 3 clinical strains); 2 *C. krusei* clinical strains. Clinical strains were isolated from recurrent vulvovaginal candidosis cases.

A 1% (m/v) hydro-alcoholic gentian violet (Amresco, USA) was prepared dissolving 1g of violet gentian in 9g of 20% ethanol, and adding purified sterile water up to 100mL final volume. This solution was then passed through a 0,22µm sterilizing filter.

The anti-Candida activity of gentian violet was studied according to CLSI reference M27-A3 protocol. Minimal inhibitory concentrations (MIC) were determined after 24 and 48 hours of incubation at 37ºC. Yeast growth was visually compared for each concentration with the control sample.

The minimal lethal concentrations (MLC) were determined based on the modified protocol proposed by Canton et al (2003).

Controls were performed with RPMI medium containing ethanol 20%. All determinations were performed in duplicate.

Results:

Gentian violet showed to have a potent *in vitro* activity against those *Candida* strains tested.

MICs and MLCs were the same. However, their values were higher for *C. krusei* (0.122µg/ml) than for *C. albicans* (0,061µg/ml).

This effect was not influenced by the alcohol solvent as all tested strains and respective controls exhibited similar growth in its presence and absence.

Conclusion:

Gentian violet showed, *in vitro*, a potent antifungal activity upon *Candida* spp. Our results explain the traditional use of gentian violet and support its use in refractory vulvovaginal candidosis.

certificate

This is to confirm that

José Martinez de Oliveira

attented
the 8th Congress and Postgraduate Course
Munich 2010, September 16th–18th
Frauenklinik LMU, Campus Innenstadt

Munich, 18th September 2010

Professor Rainer Kürzl
President
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