**UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL**
**FACULDADE DE FARMÁCIA**
**PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS**

# Desenvolvimento de novas entidades químicas e formulações para o tratamento de dermatomicoses

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# Desenvolvimento de novas entidades químicas e formulações para o tratamento de dermatomicoses

Tese apresentada por **Daiane Flores Dalla Lana** para obtenção do TÍTULO DE DOUTOR(A) em Ciências Farmacêuticas.

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Coorientador: Prof. Dr. Ricardo José Alves

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*“‘Lembrai do tempo que levastes para chegar aqui,*
*de todas as **vitórias** e lágrimas,*
*de todos os **sorrisos** e fracassos.*
*Lembrai dos **sonhos realizados**,*
*das frustrações, das decepções colhidas.*
*Lembrai de tudo o que passou.*
*Ganhastes **mais força**, **mais sabedoria***
*e finalmente podes olhar para o que há diante de ti*
*e perceber que apenas chegastes ao começo*.
- ***Que seja um lindo começo***!”

(Augusto Branco)

## RESUMO

**Desenvolvimento de novas entidades químicas e formulações para o tratamento de dermatomicoses**

As dermatomicoses são infecções fúngicas da pele, ocasionadas principalmente por dermatófitos e *Candida* spp. As dermatofitoses, denominação específica para dermatomicoses de origem dermatofítica, são as doenças cutâneas mais prevalentes da atualidade, possuindo como agentes etiológicos fungos filamentosos dermatofíticos de três gêneros anamórficos: *Microsporum*, *Trichophyton* e *Epidermophyton*. A resistência dos dermatófitos à terapêutica convencional vem sendo cada vez mais relatada, contribuindo para as comuns recidivas da micose. Infecções (muco) cutâneas e também sistêmicas podem ser causadas por leveduras oportunistas do gênero *Candida* - *C*. *albicans* e espécies de *C*. não-*albicans* (CNA; tais como *C*. *tropicalis*, *C*. *glabrata*, *C. parapsilosis*, *C*. *dubliniensis* e *C*. *krusei*). As espécies emergentes de CNA apresentam resistência mais pronunciada aos fármacos de escolha para o tratamento de candidíases, o que implica em falhas na terapia clínica e aumento considerável das taxas de morbimortalidade. Neste contexto, faz-se necessária a pesquisa por novos agentes antifúngicos com amplo espectro de ação e toxicidade seletiva para o patógeno, que superem as limitações da terapêutca atual. Assim, este trabalho teve como objetivo principal investigar, em uma ampla triagem, a atividade antifúngica de cento e oitenta e nove compostos sintéticos (de variadas classes químicas). Além disso, determinar as relações de estrutura-atividade, caracterizar o mecanismo de ação e perfil toxicológico das moléculas, a fim de prospectarem-se novas alternativas eficazes e seguras para o tratamento de infecções, primordialmente associadas a dermatófitos e *Candida* spp..

Em relação aos tiocianatos alílicos (TAs), 1ª classe investigada, o composto cloro-derivado apresentou um amplo espectro de atividade antifúngica e também atividade antiquimiotáxica, com 100% de redução da migração de leucócitos. A faixa de concentração inibitória mínima (CIM) dos compostos foi de 25 a 50 µg/mL, com mecanismo de ação relacionado à complexação com ergosterol fúngico. A nanoemulsão desenvolvida com o composto mais eficaz melhorou a atividade antifúngica *in vitro*, diminuindo os valores de CIM em até 64 vezes para dermatófitos e 4 vezes para *Candida* spp.. O composto cloro-derivado mais promissor não foi mutagênico, nem ocasionou inviabilidade celular em leucócitos humanos, embora tenha apresentado dano ao DNA dose-dependente. Além disso, não foi irritante (ensaio da membrana cório-alantoide - HET-CAM) e inibiu totalmente o crescimento fúngico em um modelo alternativo de dermatofitose.

Para as δ-lactonas (δ-Ls), 2ª classe, nenhum dos compostos foi mutagênico, genotóxico ou irritante, quando avaliados em concentrações mais elevadas que a CIM. Dois dos compostos da série apresentaram os menores valores de CIM (25-50 µg/mL) e um espectro mais amplo de atividade antifúnç

contra fungos filamentosos e leveduras. O mecanismo de ação foi relacionado ao dano na parede e membrana celular, com ação alvo específica dependente do tipo de halogênio presente na estrutura. O dano às células fúngicas foi corroborado por imagens de microscopia eletrônica de varredura, que destacaram células lisadas e completamente alteradas em sua morfologia, após tratamento *in vitro* com δ-Ls.

Para os 1,4-benzenodióis 2-substituídos (1,4-BZs), 3ª classe – com síntese inédita, seis moléculas (**2**, **5**, **6**, **8**, **11** e **12**) apresentaram ação antimicrobiana de amplo espectro, incluindo efeito contra espécies resistentes e multirresistentes de dermatófitos (*T*. *mentagrophytes*), CNA e bactérias. As faixas de CIMs destes compostos para fungos e bactérias foram 25 a 50 µg/mL e 8 a 128 µg/mL, respectivamente, sendo o mecanismo de ação antifúngico relacionado à parede e membrana celular. O composto **8**, mais promissor em relação ao efeito antifúngico, não ocasionou genotoxicidade e mutagenicidade em células leucocitárias humanas e nem hemólise. Complementarmente, os compostos não foram irritantes (HET-CAM).

Por fim, para os derivados substituídos de 1,3-bisariloxipropano (1,3-BXPs), representando o último grupo de moléculas investigadas, encontrou-se o melhor perfil de atividade antifúngica/toxicidade, dentre todas as classes químicas analisadas neste estudo, com ênfase para o composto **2j** (1,3-bis(3,4-diclorofenoxi)propan-2-amino-cloreto). **2j** foi o fungicida mais ativo contra dermatófitos e *Candida* spp., em concentrações fungicidas mínimas (CFMs) muito baixas (0,39 - 3,12 µg/mL), incluindo ação contra isolados clínicos resistentes e multirresistentes. O perfil de toxicidade de **2j** foi promissor, apresentando índice de seletividade > 10, em relação a linfócitos humanos. O composto foi classificado como não irritante pelo teste HET-CAM e não causou alterações histopatológicas na pele da orelha de porco, apresentando uma excelente perspectiva para aplicação tópica. **2j** tem como alvo a parede celular fúngica, o que foi confirmado por imagens de microscopia eletrônica de varredura. O composto foi incorporado em um hidrogel com potencial bioadesivo. Os resultados da permeação cutânea humana mostraram que **2j** permaneceu significativamente na epiderme, o que é o ideal para o tratamento de dermatomicoses. Adicionalmente, em relação à candidíase sistêmica em *Drosophila melanogaster* Toll-deficientes, **2j** apresentou eficácia estatisticamente comparável ao fluconazol no controle do processo infeccioso. Portanto, o composto **2j** demonstrou potencial para o desenvolvimento de fármacos antifúngicos, com um mecanismo de ação elucidado e já aplicado em uma formulação semissólida, visando uma nova opção terapêutica para infecções fúngicas da pele não responsivas ao tratamento convencional.

**Palavras-chave**: dermatomicoses; dermatófitos; *Candida* spp.; tiocianatos alílicos; δ-lactonas; 1,4-benzenodióis 2-substituídos; derivados substituídos de 1,3-bisariloxipropano; atividade antifúngica; mecanismo de ação; toxicidade.

## ABSTRACT

**Development of new chemical entities and formulations for the treatment of dermatomycoses**

Dermatomycoses are fungal infections of the skin, mainly caused by dermatophytes and *Candida* spp. Dermatophytoses, a specific name for dermatomycoses of dermatophytic origin, are the most prevalent skin diseases today, with dermatophytic filamentous fungi of three anamorphic genera as the etiological agents: *Microsporum*, *Trichophyton* and *Epidermophyton*. The resistance of dermatophytes to conventional therapy has been increasingly reported, contributing to the common recurrence of mycosis. (Mucus) cutaneous and also systemic infections may be caused by opportunistic yeasts of the genus *Candida* - *C. albicans* and species of non-*albicans Candida* (NAC; such as *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. dubliniensis* and *C. krusei*). Emerging NAC species show more pronounced resistance to the drugs of choice for the treatment of candidiasis, which implies failures in clinical therapy and a considerable increase in morbimortality rates. In this context, it is necessary to search for new antifungal agents with a broad spectrum of action and selective toxicity for the pathogen, which overcome the limitations of current therapy. Thus, the main objective of this work was to investigate, in a wide screening, the antifungal activity of one hundred and eighty-nine synthetic compounds (of various chemical classes). In addition, to determine the structure-activity relationships, characterize the mechanism of action and toxicological profile of the molecules, in order to prospect new effective and safe alternatives for the treatment of infections primarily associated with dermatophytes and *Candida* spp.

In relation to allylic thiocyanates (ATs), 1st class investigated, the chloro-derivative presented a broad spectrum of antifungal activity and also antichemotactic activity, with a 100% reduction in leukocyte migration. The minimum inhibitory concentration (MIC) range of the compounds was 25 to 50 µg/mL, with a mechanism of action related to complexation with fungal ergosterol. The nanoemulsion developed with the most effective compound improved the *in vitro* antifungal activity, reducing MIC values by up to 64-fold for dermatophytes and 4-fold for *Candida* spp. The most promising chloro derivative was not mutagenic, nor did it cause cellular inviability of human leukocytes, although it did present dose-dependent damage to the DNA. In addition, it was not irritant (chorioallantoic membrane assay - HET-CAM) and totally inhibited fungal growth in an alternative model of dermatophytosis.

For δ-lactones (δ-Ls), 2nd class, none of the compounds were mutagenic, genotoxic or irritant when evaluated at higher concentrations than MIC. Two of the compounds in the series had the lowest MIC values (25-50 µg/mL) and a broader spectrum of antifungal activity against filamentous fungi and yeasts. The mechanism of action was related to the damage in the cell wall and membrane, with specific target action dependent on the type of halogen present in the

structure. Damage to fungal cells was corroborated by scanning electron microscopy images, which highlighted lysed cells and completely altered their morphology after *in vitro* treatment with δ-Ls.

For the 2-substituted 1,4-benzenediols (1,4-BZs), six molecules (**2**, **5**, **6**, **8**, **11** and **12**) presented broad spectrum of antimicrobial action, including effect against resistant and multidrug-resistant species of dermatophytes (*T. mentagrophytes*), NAC and bacteria. The MIC ranges of these compounds for fungi and bacteria were 25 to 50 µg/mL and 8 to 128 µg/mL, respectively, and the mechanism of antifungal action was related to cell wall and membrane. Compound **8**, which is more promising regarding the antifungal effect, did not cause genotoxicity and mutagenicity in human leukocyte cells nor hemolysis. In addition, the compounds were non-irritating (HET-CAM).

Finally, for the substituted 1,3-bisaryloxypropane derivatives (1,3-BXPs), representing the last group of molecules investigated, the best profile of antifungal activity/toxicity was found among all the chemical classes analyzed in this study, with emphasis for the compound **2j** (1,3-bis(3,4-dichlorophenoxy)propan-2-aminium chloride). **2j** was the most active fungicide against dermatophytes and *Candida* spp., in very low minimal fungicidal concentrations (MFCs) (0.39 - 3.12 µg/mL), including action against resistant and multidrug-resistant clinical isolates. The toxicity profile of **2j** was promising, with a selectivity index > 10, in relation to human lymphocytes. The compound was classified as non-irritant by the HET-CAM test and did not cause histopathological changes in pig ear skin, presenting an excellent perspective for topical application. **2j** targets the fungal cell wall, which was confirmed by scanning electron microscopy images. The compound was incorporated into a hydrogel with bioadhesive potential. The results of human skin permeation showed that **2j** remained significantly in the epidermis, ideally for the treatment of dermatomycosis. In addition, in relation to systemic candidiasis in Toll-deficient *Drosophila melanogaster*, **2j** presented a statistically comparable efficacy to fluconazole in the control of the infectious process. Therefore, compound **2j** has demonstrated potential for the development of antifungal drugs with a mechanism of action elucidated and already applied in a semi-solid formulation aimed at a new therapeutic option for fungal skin infections not responsive to conventional treatment.

**Keywords**: dermatomycosis; dermatophytes; *Candida* spp.; allylic thiocyanates; δ-lactones; 2-substituted 1,4-benzenediols; substituted 1,3-bisaryloxypropane derivatives; antifungal activity; mechanism of action; toxicity.

## APRESENTAÇÃO

De acordo com as normas vigentes no Regimento Interno do Programa de Pós-Graduação em Ciências Farmacêuticas (PPGCF), a tese segue a seguinte sequência:

**1**) Introdução e relevância do tema
**2**) Objetivos
3) Mapa conceitual do trabalho
**4**) Revisão bibliográfica
**5**) Manuscritos - Capítulos I ao VII
**6**) Discussão geral
**7**) Conclusões e perspectivas
**8**) Referências Bibliográficas

Os tópicos **1**, **2**, **3**, **4**, **6**, **7** e **8** foram formatados conforme os padrões técnicos estabelecidos pela Associação Brasileira de Normas Técnicas (ABNT), através das NBR 6023/2002; NBR 10520/2002 e NBR 14724/2011. Os capítulos referentes aos manuscritos (artigos publicados, submetidos e a serem submetidos) foram formatados especificamente conforme as regras dos periódicos selecionados e mencionados na apresentação de cada capítulo.

# SUMÁRIO

# INTRODUÇÃO E RELEVÂNCIA DO TEMA

As doenças ocasionadas por fungos têm levado a óbito mais pessoas, por ano, do que a malária, e na agricultura, representam uma ameaça à produtividade dos cultivos e à segurança alimentar (PARKER et al., 2014; GAFFI, 2018; WHO, 2018). As micoses, pela elevada frequência com que acometem a população, são consideradas um problema de saúde pública (PEREIRA et al., 2014; CHEN et al., 2018a). As lesões decorrentes das infecções fúngicas manifestam-se, do ponto de vista clínico, nas mais diversas formas, podendo ser classificadas de acordo com as suas localizações no organismo. Quando se restringem às camadas mais superficiais e queratinizadas da pele e anexos (cabelos e unhas) são denominadas de dermatomicoses e onicomicoses (envolvimento ungueal), sendo ocasionadas primordialmente por dermatófitos e leveduras (CORRALO; HERAS-ALONSO; ACEBES, 2014; INDIRA et al., 2014; PEREIRA et al., 2014). Essas infecções micóticas mais superficiais são consideradas uma importante causa de morbidade e de consultas dermatológicas, afetando aproximadamente 20 – 25% da população em geral (HAVLICHOVA; CZAIKA; FRIEDRICH, 2008; CALADO et al., 2011; MAULINGKAR; PINTO; RODRIGUES, 2014).

As dermatofitoses, denominação dada às dermatomicoses de origem dermatofítica, são consideradas uma das primeiras micoses da humanidade e que comumentemente ocorrem no mundo todo (CLARK; FELDMAN; GERTLER, 2000; INDIRA et al., 2014; MAHALE et al., 2014; MAHALE et al., 2014). Estudos epidemiológicos indicam que as dermatofitoses figuram entre as doenças micóticas de maior ocorrência (WATANABE et al., 2001; VANDER; HOSSAIN; GHANNOUM, 2003; MARUYAMA et al., 2002; CORTEZ et al., 2012;). BRILHANTE et al. (2000) evidenciaram os dermatófitos como o grupo de fungos mais frequentemente isolados em laboratórios de micologia clínica. Porém por não figurarem entre as doenças de notificação obrigatória no Brasil, estima-se que mais casos de dermatofitose ocorram na realidade (BRILHANTE et al., 2000; MADRID; MATTEI, 2011). Além disso, essa infecção fúngica configura-se em um grave problema veterinário, por ser uma zoonose ou antropozoonose bem comum, cuja ocorrência é influenciada por fatores ambientais e de manejo (COPETTI et al., 2006; PEREIRA et al., 2006; CHERMETTE; FERREIRO; GUILLOT, 2008; MADRID; MATTEI, 2011).

As dermatofitoses compreendem uma extensa variedade de sintomas distintos. O quadro clínico mais comum inclui despigmentação, placas anulares, coceira e perda de cabelo, ou seja, lesões tipicamente cutâneas conhecidas pelo nome genérico de tinhas (do latim *tinea*) (MORAES et al., 2001; TEIXEIRA; GRIPP, 2014; DALLA LANA et al., 2016). Essas lesões impactantes na qualidade de vida dos pacientes, são ocasionadas por fungos filamentosos dermatofíticos de três gêneros anamórficos: *Microsporum*, *Trichophyton* e *Epidermophyton* (MORAES et al., 2001).

O tratamento das dermatofitoses faz-se, de modo geral, com o uso de antifúngicos tópicos e/ou sistêmicos, por vezes associados a substâncias queratolíticas (RESENDE, 2008). O tratamento é longo, logo a partir de observações clínicas, percebe-se que os antifúngicos disponíveis comercialmente são, por vezes, muito dispendiosos e, em alguns casos, desencadeiam sérias reações adversas (MARTINEZ, 2006; ROBINSON, 2014). Além disso, como agravante, as espécies dermatofíticas vêm desenvolvendo cada vez mais resistência e até multirresistência a terapêutica antifúngica convencional, o que ocasiona uma suscetibilidade muito variável *in vitro* e *in vivo*, aumento progressivo do número de recidivas e não responsividade aos fármacos antimicóticos usuais (MUKHERJEE et al., 2003; LAKSHMIPATHY; KANNABIRAN, 2010; ACHTERMAN; WHITE, 2013; TOUKABRI et al., 2018).

O desenvolvimento de novos agentes antifúngicos é contínuo, porém ainda não se encontrou um medicamento completamente ideal em relação à eficácia, segurança e curtos períodos de tratamento (KATHIRAVAN et al., 2012; PARKER et al., 2014). O avanço na terapêutica das dermatofitoses em termos clínicos reais está longe de ser totalmente satisfatório e continua sendo problemático o manejo farmacológico em pacientes transplantados, com alguma doença de base (hipertensão, diabetes, nefrite, entre outras) e/ou que apresentam algum tipo de imunodeficiência (SEYMOR, 2000; CHANG et al., 2007). A casuística das dermatofitoses em conjunto com os dados epidemiológicos, que ressaltam a importância dessa micose na clínica, e os problemas no tratamento das mesmas motivam novas pesquisas que forneçam alternativas terapêuticas potenciais (KATHIRAVAN et al., 2012; PARKER et al., 2014).

Além das dermatofitoses, as micoses causadas por leveduras do gênero *Candida* são de extrema importância clínica e estão entre aquelas que apresentam maior incidência, principalmente em relação a infecções nosocomiais (SILVA et al., 2012). Esse gênero é estudado em vários países, incluindo o Brasil (PONGSIRIWET et al., 2004; PASSOS et al., 2007; WANG et al., 2009; COSTA et al., 2010; NUCCI 2010; FRAISSE et al., 2011; ATAIDES et al., 2012; SARDI et al., 2013; CANELA et al., 2018).

Espécies do gênero *Candida* são microrganismos comensais, que fazem parte da microbiota de pessoas hígidas. Essas leveduras colonizam com alta frequência o hospedeiro humano, sendo que cerca de 20 a 80% da população adulta saudável apresenta colonização do trato gastrointestinal. A colonização da mucosa bucal ocorre entre 20 a 40% das pessoas e entre as mulheres, a colonização por *Candida* na região vaginal ocorre entre 20 a 30% (SOLL, 2002; BARBEDO; SGARBI, 2010). Digno de nota, existem alguns fatores que podem interferir na relação entre o microrganismo e o hospedeiro determinando a transição de leveduras comensais para patogênicas (COLOMBO et al., 2013; GULATI; NOBILE, 2016).

As infecções causadas por *Candida* spp. são denominadas candidíases ou candidoses, sendo consideradas doenças oportunísticas com características clínicas que variam desde superficiais até invasivas e podem ocorrer com evolução aguda ou crônica (BARBEDO; SGARBI, 2010; SARDI et al., 2013). As manifestações clínicas que acometem as mucosas e pele tendem a ser mais brandas, porém acontecem com alta frequência, prejudicando a qualidade de vida dos pacientes, ao passo que as manifestações da candidíase vulvovaginal aparecem em aproximadamente 75% das mulheres em idade reprodutiva (HOFS et al., 2016). A forma mais severa da doença é denominada candidemia - estabelecida devido a capacidade de disseminação da levedura através da corrente sanguínea para diferentes sítios anatômicos como fígado, coração, pulmão e rins, caracterizando uma infecção sistêmica, ocorrendo principalmente em pacientes imunodebilitados (LIM et al., 2012; GULATI; NOBILE, 2016; MCCARTY; PAPPAS, 2016). *Candida* spp. estão entre os principais agentes etiológicos de infecções fúngicas invasivas, as quais são responsáveis por elevados índices de morbi-mortalidade em todo o mundo (PFALLER; DIEKEMA,

2007; LASS-FLORL, 2009; KHAN et al., 2010; ARENDRUP, 2013; BITAR et al., 2014; MOHD et al., 2018).

*C*. *albicans* é a espécie predominantemente relacionada aos quadros clínicos de candidíase, tanto cutâneos quanto invasivos (KUMAR et al. 2009). A frequência de isolamento dessa espécie nos casos de infecções hematogênicas é de aproximadamente 40% em países da América Latina e aproximadamente 70% em alguns países da Europa (PFALLER; DIEKEMA, 2007; SILVA et al., 2012). Embora *C. albicans* seja a mais comum, espécies não-*albicans* como: *C*. *tropicalis*, *C*. *glabrata*, *C*. *krusei*, *C*. *parapsilosis* e *C*. *auris* têm emergido como patógenos clinicamente relevantes (SPOLIDORIO et al., 2009; BARBEDO; SGARBI, 2010; SILVA et al., 2012; COLOMBO et al., 2013; ISHIDA et al., 2013; QUINDOS, 2014; SARIGUZEL et al., 2015; ANEJA et al., 2016; MOHD et al., 2018).

Três classes principais de antifúngicos são utilizados no tratamento das candidíases, os quais atuam em componentes vitais para as células fúngicas. Duas dessas classes são os azólicos e os poliênicos, que possuem a membrana celular fúngica como alvo, especificamente interferindo no ergosterol (BAILLY et al., 2015). O esgosterol é o principal constituinte da membrana celular dos fungos, possuindo estrutura semelhante ao colesterol presente nas células humanas, fato que desfavorece o tratamento das candidíases por ocasionar recorrente prejuízo ao hospedeiro. Além disso, espécies de *Candida* podem desenvolver diferentes mecanismos de resistência aos principais antifúngicos disponíveis para uso clínico; essa resistência pode ser classificada em natural (intrínseca) ou secundária (adquirida) (SANGLARD et al., 2009; KHANDELWAL et al., 2018). O considerável aumento da resistência é uma das maiores preocupações entre médicos e cientistas (FUENTEFRIA et al., 2018), pois o arsenal de fármacos antifúngicos disponíveis para o tratamento apresenta limitações, sendo necessário o desenvolvimento de novas terapias antifúngicas que apresentem eficácia, toxicidade seletiva especificamente contra o patógeno e baixo custo (ORHAN et al., 2010; BOSTROM et al., 2012; ANEJA et al., 2016).

Diante do cenário apresentado, das dificuldades impostas pelo surgimento da resistência microbiana e os efeitos adversos relativos à toxicidade dos fármacos, pesquisas vêm sendo conduzidas buscando terapias alternativas para o tratamento de dermatofitoses e candidíases (FUENTEFRIA et al., 2018).

Os estudos de novas classes de substâncias vêm sendo impulsionados pela necessidade de novos fármacos para tratar infecções fúngicas (ROLLAS; KUÇUKGUZEL, 2007; FUENTEFRIA, 2018). Dessa forma, os compostos sintetizados laboratorialmente têm emergido como fator de interesse para a biotecnologia médica. Entre esses, as classes dos tiocianatos alílicos (TAs), δ-lactonas (δ-Ls), 1,4-benzenodióis 2-substituídos (1,4-BZs) e os derivados substituídos de 1,3-bisariloxipropano (1,3-BXPs) são promissores devido as suas propriedades biológicas (WANI et al., 1980; UMEZAWA et al., 1984; XU; LING, 1985; OHKUMA et al., 1992; HARBONE; BAXTER; MOSS, 1999; WHITTING, 2001; CAPON et al., 2004; KISHIMOTO et al., 2005; TANAKA et al., 2007; ROLDOS et al., 2008; DAI; MUMPER, 2010; ANDERSON; LONG 2010; YANG et al., 2011; SILVEIRA et al., 2012; OKSANA; MARIAN; MAHENDRA, 2012; DE ARIAS et al., 2012; CALEMAN et al., 2012; MODRANKA et al., 2012; SÁ et al., 2014; BERGAMO et al., 2014; BERGAMO et al., 2015; SERNA et al., 2015; DALLA LANA et al., 2015; FORTES et al., 2016; SOTO-HERNANDEZ; PALMA-TENANGO; GARCIA-MATEOS, 2017; VALENCIA-GALICIA et al., 2017; LAVORATO et al., 2015; LAVORATO, 2016; LAVORATO et al., 2017; LAVORATO; DUARTE; DE ANDRADE, 2017).

Propõe-se, portanto, nesse estudo, a investigação do potencial antifúngico, em uma ampla triagem, de 189 compostos pertencentes as classes acima mencionadas. Para os compostos mais promissores de cada classe, o perfil de toxicidade e o mecanismo de ação antifúngica foram determinados, além de outras análises complementares para uma prospecção mais efetiva dessas moléculas como candidatos a antifúngicos. Para o composto mais ativo do total dos 189 compostos avaliados, que apresentou os menores valores de CIM e com as melhores perspectivas em relação à citotoxicidade, desenvolveu-se e caracterizou-se uma formulação semissólida para tratamento tópico de dermatomicoses. Realizou-se também um estudo inicial do potencial da molécula **2j** para uso sistêmico, a partir de modelagem farmacocinética/farmacodinâmica com os dados de *time kill* e seu efeito protetor em modelo de infecção fúngica em mosca-das-frutas. Todas essas estratégias experimentais foram estabelecidas a fim de auxiliar na pesquisa e descoberta de novas opções terapêuticas para micoses reconhecidamente de difícil tratamento, como as associadas a *Candida* spp.e dermatófitos.

# OBJETIVOS

Diante da necessidade e importância da identificação de novas substâncias com atividade antifúngica, este estudo propõe a avaliação do potencial antifúngico de variadas classes de moléculas sintéticas (tiocianatos alílicos, δ-lactonas, 1,4-benzenodióis 2-substituídos e derivados substituídos de 1,3-bisariloxipropano), frente a fungos patogênicos filamentosos e leveduriformes (sensíveis e resistentes); bem como a caracterização da toxicidade e mecanismo de ação dos compostos mais promissores. Inseridos nesta proposta geral, os objetivos específicos incluem:

1. Revisar a literatura sobre os agentes etiológicos, formas clínicas, terapêutica convencional e novas perspectivas de tratamento das dermatofitoses (Capítulo I);
2. Para a classe dos TAs: determinar a suscetibilidade *in vitro* de dermatófitos e *Candida* spp., para moléculas da classe e nanoemulsão. Verificar a eficácia do composto mais promissor em um modelo alternativo de infecção fúngica *in vivo* e avaliar a atividade antiquimiotáxica, mecanismo de ação antifúngico e toxicidade (Capítulo II);
3. Para a classe das δ-Ls: avaliar a suscetibilidade de dermatófitos e leveduras a uma série de compostos, definir as relações de estrutura atividade por análise exploratória multivariada, e por fim, indicar o principal mecanismo de ação antifúngico envolvido e a toxicidade (Capítulo III).
4. Para a classe dos 1,4-BZs: analisar a atividade antimicrobiana, incluindo cepas bacterianas e fungos filamentosos não-dermatofíticos no estudo, bem como sugerir o mecanismo de ação antifúngico e analisar a toxicidade dos compostos da série, alguns de síntese inédita (Capítulo IV);
5. Investigar, por microscopia eletrônica de varredura, o processo de invasão ungueal (unhas humanas) por *Microsporum canis* (Capítulo V);
6. Para 1,3-BXPs: determinar as relações de estrutura-atividade de cento e vinte e uma moléculas, bem como o perfil de toxicidade para os compostos mais eficazes como antifúngicos. Selecionar o composto mais promissor (atividade antifúngica/citotoxicidade) para elucidar o mecanismo de ação, bem como desenvolver e caracterizar um hidrogel,

como forma farmacêutica de aplicação tópica contendo o composto de interesse, para tratamento de dermatomicoses (Capítulo VI). Adicionalmente, dar início aos estudos para um provável uso sistêmico desse composto, com modelagem farmacocinética/farmacodinâmica a partir dos dados de *time kill*, bem como avaliar o respectivo efeito protetor em modelo de candidíase sistêmica em mosca-das-frutas (Capítulo VII).

## MAPA CONCEITUAL DO ESTUDO

**Descoberta e desenvolvimento de novas entidades químicas e formulações com POTENCIAL ANTIFÚNGICO**

FONTE imagem: PORVIR.ORG (2018).

**Ampla triagem da atividade antifúngica de 189 compostos pertencentes às classes químicas descritas a seguir, frente a dermatófitos e *Candida* spp.**

**Suscetibilidade Relação estrutura-atividade**

**Toxicidade**

**Mecanismo de ação**

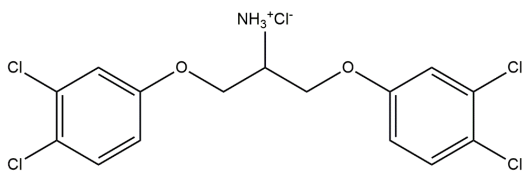
+ Revisão bibliográfica sobre dermatofitoses
+ Microscopia eletrônica de varredura sobre a invasão da lâmina ungueal humana por *Microsporum canis*

**Tiocianatos alílicos (15 compostos)** + Nanoformulação

**1,4-Benzenodióis 2-substituídos (42 compostos)** + Atividade antimicrobiana

**δ-lactonas (11 compostos)** + Análise multivariada

**Derivados substituídos de 1,3-bisariloxipropano (121 compostos)** + Hidrogel
*Time kill*
Modelagem farmacocinética/farmacodinâmica
Efeito protetor em modelo de infecção fúngica sistêmica em mosca-das-frutas




FONTE imagem: SANTANA (2017).

## 1. Dermatomicoses

Dermatomicoses são compreendidas como infecções superficiais e cutâneas de tecidos queratinosos e membranas mucosas causadas por uma variedade de agentes fúngicos, sendo os mais comuns dermatófitos e leveduras. No geral, o processo gradual da infecção do hospedeiro é semelhante entre as principais espécies fúngicas dermatomicóticas. Leveduras como *Candida* spp. provocam um nível relativamente mais ameno de danos e inflamação no tecido do hospedeiro durante a patogênese, enquanto os dermatófitos podem induzir um nível mais intenso de dano tecidual e reação inflamatória. Ambos os patógenos podem, no entanto, manipular a resposta imune do hospedeiro, garantindo a sobrevivência e tornando a infecção fúngica em um processo crônico. Sinais e sintomas comuns da maioria dos casos incluem eritema, ardor, prurido, dentre outros, que afetam a qualidade de vida e a socialização dos pacientes. A seguir, apresenta-se uma revisão mais detalhada dos principais agentes etiológicos de dermatomicose, o tratamento e a problemática associada a esse tipo de micose, altamente prevalente (HUBE et al., 2015; CHEON et al., 2018).

## 2. Dermatófitos

Os dermatófitos são um grupo de fungos filamentosos septados e hialinos com características morfológicas e fisiológicas especializadas, tidos como os agentes etiológicos de dermatofitose. Esses fungos possuem a capacidade de digerir a queratina da pele, cabelo e unhas, tanto em humanos quanto em animais, e utilizá-la como nutriente durante o progresso da infecção (ACHTERMAN; WHITE, 2013; SEGAL, 2015; PIHET; GOVIC, 2017). Por esse forte biotropismo por estruturas queratinizadas como pele, pelo e unha, que a habilidade dermatofítica de ocasionar a doença é estabelecida (ANDOH; TAKAYAMA; KURAISHI, 2014). Atualmente já se sabe que fungos dermatofíticos são capazes de formar biofilme, sendo esse um fator agravante para a virulência e patogenicidade (COSTA-ORLANDI et al., 2014; DANIELLI et al., 2017; TOUKABRI et al., 2018).

Em relação à ecologia, os dermatófitos podem ser antropofílicos, zoofílicos ou geofílicos, segundo seu habitat natural e preferência por hospedeiro (KECHIA et al., 2014). Os fungos antropofílicos encontram-se restritos aos

humanos e raramente infectam os animais (TAYLOR; GURR, 2014). Os zoofílicos são, primariamente, isolados de animais, mas podem causar a doença em humanos em contato com animais como gato, cão, cavalo, entre outros (SPIEWAK; SZOSTAK, 2000; TAYLOR; GURR, 2014). Os geofílicos têm como reservatório o solo e apenas ocasionalmente infectam o homem (GALLO et al., 2005). O estágio inicial de todos os dermatófitos consistia no estágio geofílico. Posteriormente, ocorreu a evolução de algumas espécies para o estágio zoofílico, atacando roedores e, posteriormente, animais domésticos. Finalmente, algumas espécies avançaram para o estágio antropofílico, ocasionando infecções fúngicas no ser humano (SPIEWAK; SZOSTAK, 2000). Evidencia-se que todos os três grupos incluem espécies que causam doenças em animais e seres humanos, sendo que transmissões de animais para outros animais, animais para humanos, de humanos para outros e do solo para animais e humanos são possíveis (TAYLOR; GURR, 2014).

Quanto à distribuição, os dermatófitos são cosmopolitas, embora a real ocorrência seja influenciada por condições sociais e geoclimáticas. Os perfis epidemiológicos tornam-se assim variados, segundo fatores regionais, estacionais, de faixa etária, frequência de contato com animais, condições higiênico-sanitárias e exposição a locais públicos (AQUINO; CONSTANTE; BAKOS, 2007). Clinicamente, os dermatófitos ocorrem em várias espécies animais e no homem, determinando de modo geral, lesões secas, arredondadas e, comumente, não pruriginosas, com áreas de alopecia, bordos eritematosos e vesiculares, que circunscrevem uma parte central descamativa, a qual se distribui focalmente na superfície cutânea (INDIRA et al., 2014). Os dermatófitos desenvolvem-se crescendo do centro da lesão para as bordas, ocasionando intensa descamação associada ou não à resposta inflamatória, resultante da atividade queratinolítica (SIDRIM; ROCHA, 2004).

Em cultura, os dermatófitos patogênicos produzem hifas septadas que se ramificam e formam o micélio com estruturas de reprodução assexuada, os conídios. Microscopicamente, esses conídios diferem entre as espécies dermatofíticas quanto à forma, ao tamanho, ao número e à disposição ao longo das hifas, sendo critérios essenciais para a identificação (SIDRIM; ROCHA, 2004; LEMSADDEK, 2008). A maioria das espécies de dermatófitos produz dois tipos de conídios: os grandes - macroconídios pluricelulares e os pequenos -

microconídios unicelulares. A presença/ausência desses tipos de estruturas reprodutivas e o aspecto da parede das mesmas (rugosa ou lisa, espessa ou fina) são importantes para o correto reconhecimento das espécies, sempre se correlacionando com a macromorfologia e com o caso específico de cada paciente (WOREK et al., 2014). Os dermatófitos crescem em temperatura ótima que varia entre 25 a 35 ºC, mas a maioria apresenta uma termotolerância que lhes permite um bom crescimento à 37 ºC (LEMSADDEK, 2008).

As espécies dermatofíticas são classificadas em três gêneros anamórficos (assexuados ou imperfeitos): *Microsporum*, *Trichophyton* e *Epidermophyton*, que juntos compreendem mais de quarenta espécies (CAFARCHIA et al., 2013; REFAI; EL-YAZID; EL-HARIRI, 2013), das quais vinte e sete são patogênicas, quinze ocorrem no Brasil e sete são comuns no Rio Grande do Sul (HAVLICHOVA; CZAIKA; FRIEDRICH, 2008). Esses gêneros anamórficos são os de grande relevância clínica, porém, a saber, os dermatófitos também apresentam estágio de reprodução sexuada (teleomórfico ou perfeito), nesse caso sendo agrupados no gênero *Arthroderma*. Data-se o início da ocorrência dos dermatófitos em meados do século XIX, quando foi isolado o *Achorion schoenleinii* (atualmente *Trichophyton schoenleinii*) de uma lesão no couro cabeludo de um paciente (CHABASSE, 2008). Dentre os dermatófitos de maior relevância clínica, ou seja, mais frequentemente relatados como causadores de infecção em humanos e animais citam-se *Microsporum canis, Microsporum gypseum, Trichophyton mentagrophytes* e *Trichophyton rubrum* (INDIRA et al., 2014), os quais apresentam distribuição geográfica bastante variável, dependendo das condições socioeconômicas, ambientais e higiênicas da população (SANTOS et al., 1997; FALAHATI et al., 2003; REZENDE et al., 2008, MAGAGNIN et al., 2011).

*M*. *canis*, quando cultivado, apresenta um crescimento com cerca de 6 a 10 dias. Macroscopicamente, a colônia apresenta textura cotonosa, discreto relevo umbilicado, radiado e brancacento (Fig. 1a). Já o reverso possui tonalidade amarelo-limão, que com o passar do tempo tende a tornar-se castanho por difusão do pigmento no meio (MARTINÉZ et al., 2014). O pleomorfismo é evidenciado com rapidez, apresentando-se como colônias cotonosas. Micromorfologicamente, há grande quantidade de macroconídios fusiformes (20 a 80 x 40 a 120 µm), de paredes grossas, rugosas, com um pico

terminal pontiagudo característico e numerosas septações (até 15 septos) (Fig. 1b) (QUINN et al., 2002). Quando presentes, os microconídios são sésseis e sem muito predicativo diagnóstico. Podem ainda ser observados clamidoconídios, órgãos nodulares e hifas pectinadas (MARTINÉZ et al., 2014).

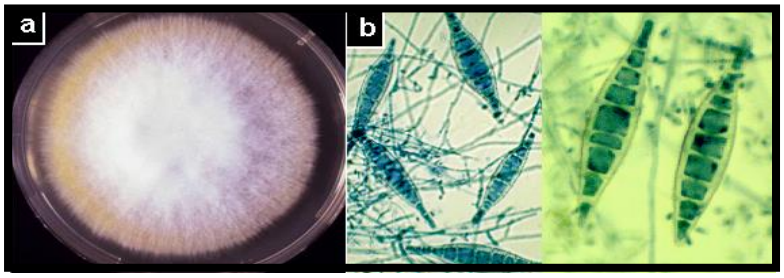



**Figura 1.** Imagens da macro (a) e micromorfologia (b) de *M*. *canis* (FONTE: MYCOLOGY ONLINE – National Mycology Reference Centre, 2018).

*M*. *gypseum* cresce bem em Agar Sabouraud com cloranfenicol e cicloheximida, incubado à temperatura ambiente ou a 37 ºC. Essa espécie tem um crescimento e maturação das colônias entre 3 a 7 dias. Macroscopicamente, as colônias caracterizam-se por serem planas, com bordas irregulares e extremamente pulverulentas, com pigmentação que varia a diversos tons de amarelo a acastanhado (Fig. 2a). Tem uma forte tendência ao pleomorfismo e nessas situações, observam-se colônias cotonosas e brancacentas, constituídas apenas por hifas estéreis (LACAZ et al., 2002; OLIVEIRA, 2014a). O reverso apresenta cores que variam do alaranjado ao marrom. Microscopicamente, observam-se para essa espécie, hifas hialinas septadas e macroconídios abundantes (7 a 15 x 25 a 60 µm), “em forma de canoa” e simétricos (Fig. 2b), de parede celular fina, com até seis septos e extremidades mais arredondadas e menos afiladas que as do *M. canis* (QUINN et al., 2002). Algumas linhagens ainda apresentam numerosos microconídios piriformes (OLIVEIRA, 2014a).


**Figura 2.** Imagens da macro (a) e micromorfologia (b) de *M*. *gypseum* (FONTE: MYCOLOGY ONLINE – National Mycology Reference Centre, 2018).

*T. mentagrophytes* apresenta rápido crescimento, com amadurecimento entre 3-6 dias. Numerosas variações na morfologia da colônia decorrem em virtude das diferenças que compõem o complexo *T. mentagrophytes*. As variações da espécie, *T. mentagrophytes* var. *interdigitale* e *T. mentagrophytes* var. *mentagrophytes* podem apresentarem-se nas formas antropofílicas e zoofílicas, respectivamente, acometendo o homem principalmente nas regiões do couro cabeludo, pés, mãos, unhas e regiões interdigitais, sendo diferenciadas por biologia molecular (SYMOENS et al., 2011). Quando essas lesões são provocadas por variações zoofílicas, apresentam maior intensidade inflamatória (OYECA, 2000). Em Agar Sabouraud Dextrose (ASD), a forma antropofílica mais comum, *T. mentagrophytes* var. *interdigitale*, cresce com colônias sobre-elevadas que se estendem rapidamente, de coloração branca a creme e superfície aveludada-algodonosa, mas também podem haver zonas mais lisas e granulares, sendo que o reverso é pigmentado de amarelo a marrom, escurecendo com o tempo (Fig. 3a) (KAUFMAN et al., 2005; OLIVEIRA, 2014a). Em Agar Batata Dextrose (ABD), o micélio aéreo é esparso com numerosos conídios. Para isolados clínicos zoofílicos (*T. mentagrophytes* var. *mentagrophytes*), no ASD, observam-se colônias geralmente planas, de coloração branca a creme e superfície mais granular; o reverso apresenta pigmento marrom-acastanhado (OLIVEIRA, 2014a). Microscopicamente, o fungo possui hifas hialinas septadas, raros macroconídios, que são longos (com dimensões em torno de 20-50 µm), chaturoides e de parede fina e lisa. A principal característica micromorfológica de *T. mentagrophytes* é a presença de microconídios globosos e agrupados nas ramificações das hifas, formando os denominados “acladium”, cujo arranjo lembra um “cacho de uvas” (Fig. 3b) (KONEMAN, 2006). Tipicamente esses microconídios possuem a forma de “lágrima”, por vezes são mais alargados e as hifas, de modo frequente, podem ficar em espiral (“zarcilos”) (Fig. 3) (KAUFMAN et al., 2005; COSTA 2008; OLIVEIRA, 2014a). Para diferenciação das variações do complexo faz-se uso de técnicas moleculares (SYMOENS et al., 2011).

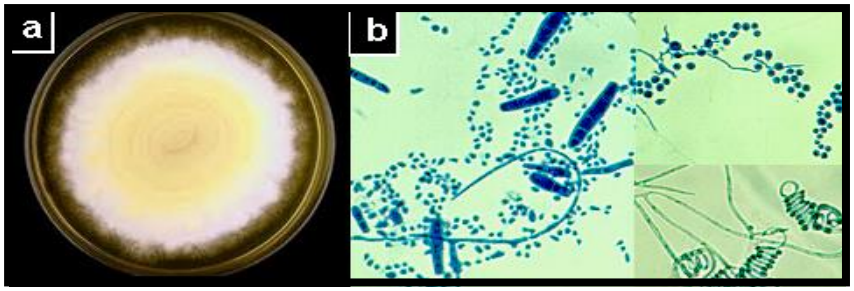



**Figura 3.** Imagens da macro (a) e micromorfologia (b) de *T. mentagrophytes* (FONTE: MYCOLOGY ONLINE – National Mycology Reference Centre, 2018).

*T. rubrum* possui uma taxa de crescimento relativamente lenta em Agar Sabouraud. As colônias, em seu crescimento primário, são geralmente cotonosas e brancas, tornando-se aveludadas posteriormente (Fig. 4a). O reverso apresenta pigmentação de coloração avermelhada ou vermelho-púrpura, que se difunde no meio de cultivo; melhor evidenciado em ABD (OLIVEIRA, 2014). Em certas ocasiões, a coloração é inicialmente amarelada, escurecendo gradativamente até tornar-se vermelha. As colônias possuem pregas radiais, formando uma pequena saliência central. Na micromorfologia, observam-se hifas hialinas, septadas, com microconídios em forma de lágrima ou gota, dispostos ao longo das mesmas ou em cachos (Fig. 4b) (OLIVEIRA, 2014a). Os macroconídios são raros, produzidos geralmente por amostras mais granulosas, esporulantes, formados no final das hifas e dispostos isoladamente ou em grupo. Eles são longos, estreitos, com bordas laterais bem paralelas e paredes finas (HARVEY; CHAMPE; FISHER, 2008). Muitas vezes a identificação é difícil de ser feita apenas com esses dados, mesmo para micologistas experientes, por isso em alguns casos faz-se o diagnóstico diferencial, como a capacidade que *T. rubrum* tem de produzir pigmento em Agar fubá a 1%, teste de perfuração do pelo e urease negativos (SAENZ, 2001; OLIVEIRA, 2014a).

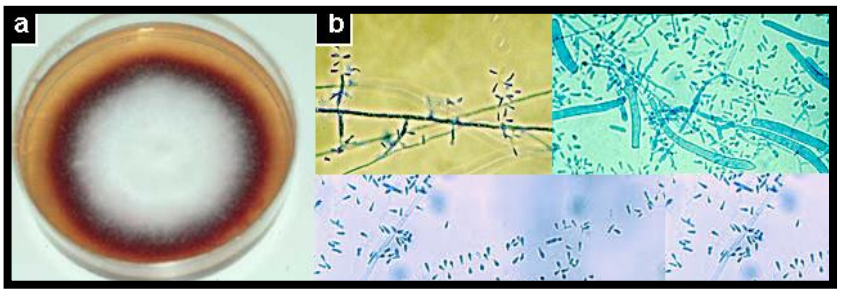



**Figura 4.** Imagens da macro (a) e micromorfologia (b) de *T. rubrum* (FONTE: MYCOLOGY ONLINE – National Mycology Reference Centre, 2018).

Os mecanismos de infecção por dermatófitos envolvem primordialmente três etapas (ESQUENAZI et al., 2004):

1) ruptura da camada intacta de queratina e a superação do mecanismo de defesa do hospedeiro;

2) adesão decorrente da expressão de glicoproteínas adesinas que se ligam a resíduos de manose e galactose na superfície das células;

3) início da germinação dos artroconídios favorecendo a penetração das hifas na pele.

Após a instalação do fungo no estrato córneo da epiderme, esse passa a utilizar as macromoléculas do hospedeiro como fonte de carbono, enxofre, nitrogênio e fósforo, através da secreção de enzimas hidrolíticas como, nucleases, lipases, proteases e queratinases. Esse processo enzimático é o maior fator de virulência e a atividade queratinolítica está intimamente ligada a patogenicidade dos dermatófitos (GIDDEY et al., 2007; ROUZAUD et al., 2018). Devido ao favorável pH ácido da pele, o agente patogênico expressa a síntese de queratinases e proteases não especificas. Entretanto, na sequência do processo, com a utilização do substrato e conseguinte produção de aminoácidos, ocorre a alcalinização do meio, favorecendo a atividade de queratinases com atividade ótima em pH alcalino, permitindo a manutenção da infecção (CERVELATTI et al., 2004).

A infecção é geralmente cutânea, restrita à camada córnea e epidérmica, devido à incapacidade do fungo de penetrar em tecidos profundos ou órgãos de indivíduos imunocompetentes. Porém, em indivíduos imunocomprometidos, existem inúmeros relatos de infecções invasivas e profundas (SATO; TAGAMI, 2003; LANTERNIER et al., 2013; ACHTERMAN; WHITE, 2013; ROUZAUD et al., 2015; SU, et al., 2017). A instalação de um processo patogênico em pele glabra inicia-se sempre pela inoculação de um artroconídio ou fragmento de hifa depositado sobre a pele, favorecido por uma lesão cutânea ou escoriação preexistente, mesmo que mínima (SIDRIM; ROCHA, 2004). O fungo se adere às células, mediado por adesinas que interagem com receptores do hospedeiro. A penetração no estrato córneo inicia-se com o desenvolvimento de tubos germinativos a partir dos artroconídios. Após sete dias de incubação, as hifas começam a formar artroconídios, completando assim, o ciclo vegetativo de

crescimento do fungo. O fungo secreta proteases que degradam a queratina, auxiliando na invasão (BALDO et al., 2012).

Os pelos são atacados secundariamente à evolução de uma lesão na pele, que apresenta em sua superfície uma grande quantidade de folículos pilosos. O dermatófito remove a cutícula e tem acesso aos pelos, e só cessa a sua progressão quando não encontra mais queratina no colete do bulbo pilar (SIDRIM; ROCHA, 2004). Por último, tem-se o comprometimento das unhas, que ocorre após a penetração do dermatófito no estrato córneo (BRISTOW, 2004).

Para o estabelecimento da infecção, o contato do artroconídio ou fragmento de hifa com a pele do hospedeiro é essencial. O fungo expressa adesinas carboidrato específicas na superfície dos microconídios que reconhecem a manose e a galactose. Espécies como *T. mentagrophytes* desenvolvem projeções fibrilares longas e curtas que ancoram e conectam os artroconídios aos queratinócitos e a outros artroconídios (MARTINEZ-ROSSI; PERES; ROSSI, 2008), além de proteases como as subtilisinas, dipeptidil peptidases e metaloproteinases, que estão diretamente envolvidas na adesão aos queratinócitos e na invasão ao estrato córneo. Essa adesão é tempo dependente e pode variar conforme a espécie de dermatófito (VERMOUT et al., 2008; BALDO et al., 2012).

Durante a degradação da queratina, os dermatófitos secretam sulfito (utilizando uma bomba de efluxo de sulfito, codificada pelo gene SSU1). O sulfito é um agente redutor que cliva as pontes dissulfito da queratina em cisteína e S-sulfocisteína, deixando as proteínas capazes de serem digeridas por muitas endo e exoproteases secretadas pelos fungos. A expressão elevada do gene SSU1 é característica dos dermatófitos e auxilia na degradação eficiente dos tecidos queratinizados pelos dermatófitos (BALDO et al., 2012). A maior atividade queratinolítica está diretamente relacionada à produção de infecções mais sintomáticas e à ativação da resposta imune. A infecção dermatofítica induz a hipersensibilidade do tipo tardia, que é caracterizada pela ação dos macrófagos como células efetoras e também pela ação de algumas citocinas, como interferon-γ (INF- γ) (VERMOUT et al., 2008). O padrão de secreção das proteases desempenha um papel importante nas respostas imune e inflamatória (ACHTERMAN; WHITE, 2012).

A intensidade da inflamação depende da profundidade da lesão na pele e o dano é dependente da maior ou menor secreção de proteases (VERMOUT et al., 2008). Em um estudo conduzido por YOUNGCHIM et al. (2011), relatou-se a produção de melanina por várias espécies de dermatófitos. Sabe-se que a melanina é considerada um fator de virulência em várias espécies de fungos, uma vez que protege os microrganismos dos mecanismos de defesa do hospedeiro e do meio ambiente. Contudo, ainda não há evidências suficientes para sugerir que a melanina exerça um papel crucial na patogênese desses fungos (YOUNGCHIM et al., 2011).

Os dermatófitos ocasionam infecções cutâneas bem características e definidas, sendo que uma espécie pode estar envolvida em diferentes formas clínicas, dependendo do sítio anatômico envolvido. Sendo assim, as infecções dermatofíticas podem ser classificadas clinicamente de acordo com as localizações anatômicas das lesões, utilizando-se a denominação *tinea* (do latim *tinea* = verme ou traça) para todas as dermatofitoses, seguida do sítio anatômico onde se localiza a infecção, também em latim (REFAI; EL-YAZID; EL-HARIRI, 2013). As modalidades dermatofíticas mais relevantes são: *tinea capitis* (couro cabeludo)*, tinea corporis* (corpo)*, tinea cruris* (grandes pregas)*, tinea unguium* (unha)*, tinea barbae* (barba)*, tinea manuum* (mãos) e *tinea pedis* (pés) (Fig. 5; WEITZMAN; SUMMERBELL, 1995; SEEBACHER; BOUCHARA; MIGNON, 2008; DIEGO, 2011; REFAI; EL-YAZID; EL-HARIRI, 2013; ALTER et al., 2018).

Maiores detalhes das formas clínicas acima mencionadas e epidemiologia são apresentados no Capítulo 1 (artigo de revisão específico sobre o tema – dermatófitos e dermatofitoses, publicado na Clinical & Biomedical Research). Por fim, o diagnóstico é realizado pela observação de três parâmetros em conjunto: 1) exame direto, 2) aspectos macro e micromorfológicos da cultura e 3) epidemiologia correlacionada (BRASCH, 2014).

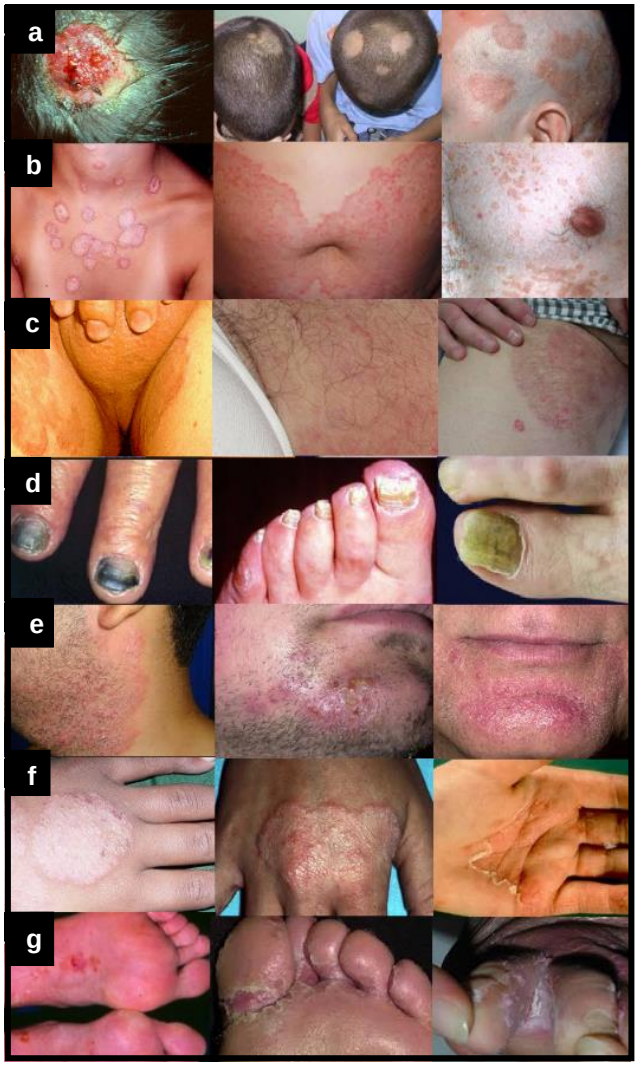



**Figura 5.** Imagens de lesões características das distintas formas clínicas de dermatofitose: (a) *tinea capitis* (couro cabeludo), (b) *tinea corporis* (corpo), (c) *tinea cruris* (grandes pregas), (d) *tinea unguium* (unha), (e) *tinea barbae* (barba), (f) *tinea manuum* (mãos) e (g) *tinea pedis* (pés) (FONTE: MYCOLOGY ONLINE – National Mycology Reference Centre; DOCTOR FUNGUS, 2018).

### 3. *Candida* spp.

O primeiro relato de *Candida* no ser humano ocorreu em 1839 após isolamento de *C. albicans* da cavidade oral de um paciente com afta, que posteriormente se tornaria a mais importante levedura de interesse médico. A espécie foi classificada somente anos depois, em 1923, por Berkhout (BARNETT; PAYNE; YARROW, 200; SIDRIM; ROCHA, 2004). *Candida* spp. apresentam células em brotamento, que podem formar pseudohifas. A coloração de suas colônias pode variar de branca à creme, ligeiramente acinzentadas. Sua

textura é cremosa ou membranosa com superfície rugosa, lisa ou sulcada e brilhante. Geralmente são circulares e as bordas podem ser regulares ou irregulares. Leveduras desse gênero possuem importante capacidade adaptativa e podem se desenvolver tanto em aerobiose, quanto em anaerobiose. Podem ser encontradas na maioria dos ecossistemas, estando presentes no solo, na água, em alimentos e colonizando animais e homens (comensais, fazem parte da microbiota normal do trato digestório, geniturinário, respiratório e pele de indivíduos sadios). A ocorrência da candidíase depende da predisposição do hospedeiro (imunodepressão), carga microbiana e virulência da espécie, logo, quando estes três fatores estão presentes, as leveduras tornam-se oportunistas e, portanto, patogênicas (BARBEDO; SGARBI, 2010; TRAGIANNIDIS et al., 2012; PEIXOTO et al., 2014; NAGLIK; RICHARDSSON; MOYES, 2014).

Sabe-se que mais de 200 espécies de *Candida* estão presentes na microbiota normal do corpo humano e de animais (SARDI et al., 2013; NAGLIK; RICHARDSSON; MOYES, 2014). Dentre essas, somente quinze espécies são comumente isoladas e identificadas como agentes infeciosos. São elas: *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. guilliermondii*, *C. lusitaniae*, *C. dubliniensis*, *C. pelliculosa*, *C. kefyr*, *C. lipolytica*, *C. famata*, *C. inconspicua*, *C. rugosa*, e *C. norvegensis*. Embora a frequência com que são isoladas varie bastante, diferentes estudos nos últimos 20 anos têm mostrado que em 95% dos casos de candidíases, os patógenos envolvidos são *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* e *C. krusei* (LEWIS, 2009; DIEKEMA et al., 2012). Apesar de *C. albicans* ser a espécie mais isolada nos casos de candidíases, espécies de CNA estão emergindo como a segunda ou terceira maior causa de candidemia principalmente em crianças (OLIVEIRA et al., 2014b). Manifestações clínicas relevantes associadas a algumas dessas espécies são apresentadas na Tabela 1. Há ainda a possibilidade de infecções mistas envolvendo a associação de mais de uma espécie de *Candida*, isoladas de um mesmo paciente e/ou sítio anatômico (LIMA et al., 2008; BOUZA et al., 2013).

**Tabela 1**. Manifestações clínicas relevantes associadas a espécies de *Candida* não-*albicans*.

| Espécie | Manifestações clínicas |
|---|---|
| ***C. krusei*** | Candidemia, endoftalmite, endocardite, osteomielite |
| ***C. glabrata*** | Candidemia, candidúria, vulvovaginite, esofagite, candidíase orofaríngea |
| ***C. tropicalis*** | Candidemia, candidíase disseminada, candidúria associada à cateter, candidíase orofaríngea, vulvovaginite |
| ***C. parapsilosis*** | Candidemia, endoftalmite, endocardite, artrite séptica, peritonite e outras infecções disseminadas associadas a dispositivos protéticos, candidíase orofaríngea |
| ***C. dubliniensis*** | Candidíase orofaríngea em pacientes com síndrome da imunodeficiência adquirida (SIDA). Raramente desencadeia candidíase invasiva. |
| ***C. guilliermondii*** | Candidemia em doentes previamente sujeitos a cirurgias cardiovasculares ou gastrointestinais, endocardite em indivíduos toxicodependentes |
| ***C. lusitaniae*** | Candidemia e outras formas de candidíase sistêmica |

FONTE: Adaptado de PEIXOTO et al. (2014); DEORUKHKAR; SAINI (2015); KOLACZKOWSKA; KOLACZKOWSKI (2016).

As espécies de *Candida* têm a capacidade de colonizar diversas regiões anatômicas, causando infecções superficiais da pele e mucosas – candidíases cutâneas e mucocutâneas (Quadro 1) – ou infecções disseminadas e potencialmente fatais – candidemia e candidíases invasivas (CI) (Quadro 1) (SARDI et al., 2013). O tipo e dimensão da infecção são determinados pelo estado imunológico do hospedeiro (PEIXOTO et al., 2014). Durante as últimas décadas, as infecções por *Candida* têm aumentado não somente em ocorrência, mas também na gravidade da doença (ZAUGG et al., 2001; PAPPAS et al., 2004). O aumento das infecções, com lesões graves, tem levado a um alto índice de mortalidade, principalmente em pacientes imunocomprometidos (GARNACHO-MONTERO et al., 2018).

Conforme Williams e Lewis (2011), as candidíases mucocutâneas ocorrem com maior regularidade. Nesse sentido, os locais os quais são mais frequentes o isolamento de *Candida* correspondem ao trato geniturinário e cavidade oral, sendo diagnosticadas infecções em cerca de 31-35% de indivíduos saudáveis (SILVA et al., 2012). *Candida* spp. são consideradas a segunda causa de infecções vaginais, sendo que aproximadamente 75% das

mulheres sofrem um episódio durante a idade fértil, e 40-50% poderão ter recidivas. Apesar de 80-90% dos casos serem causados por *C. albicans*, assiste-se à emergência de espécies de CNA, sendo 10-20% dos casos atribuídos a *C. tropicalis*, *C. glabrata*, *C. krusei*, *C. parapsilosis* e *C. guilliermondii* (PAPON et al., 2013; HOSPENTHAL**;** RINALDI, 2015; BHAWNA; SANGEETA; UDAYAN, 2015).

No estudo de Sadeghi et al. (2018), 79 casos de infecção por CNA foram confirmados em infecções cutâneas e sistêmicas, sendo que *C. parapsilosis* (36,8%) foi a espécie mais prevalente, seguida por *C. glabrata* (32,9%), *C. orthopsilosis* (11,4%), *C. tropicalis* (8,9%), *C. krusei* (5%) e *C. guilliermondii* (5%). Esses isolados foram identificados por técnicas morfológicas, bioquímicas e moleculares (sequenciamento ITS). Os isolados clínicos de *C. glabrata* e *C. krusei* não foram suscetíveis ao fluconazol, avaliados pela metodologia de microdiluição em caldo.

Em pacientes com a imunidade debilitada, poderão desenvolver-se infecções sistêmicas, as quais se revestem de grande importância clínica dado as elevadas taxas de mortalidade associadas – 71 a 79% (PATIL et al., 2015). A incidência anual de CI é de 2.53-11/100.000 indivíduos nos países Europeus e 6-23/100.000 indivíduos nos EUA. Num relatório global, verificou-se um incremento de 10-11% nos casos de candidemia, num período de aproximadamente 7 anos (PATIL et al., 2015).

*Candida* spp. são indicadas como responsáveis pela crescente incidência de casos de septicemia nas unidades hospitalares (GIOLO; SVIDZINSKI, 2010; OREN; PAUL, 2014; SANTANA et al., 2018), estando classificadas nos EUA entre a terceira ou quarta causa de infecções nosocomiais (DEORUKHKAR; SAINI, 2016). Essas infecções são mais comuns nas unidades de cuidados intensivos (UCI), visto ser onde se encontram pacientes mais vulneráveis e sujeitos a um maior número de processos terapêuticos invasivos (VIRIATO, 2014). Na UCI, a taxa de mortalidade associada a CI é cerca de 30-50% (ZARRIN; MAHMOUDABADI, 2009; VAZQUEZ, 2010). Segundo Hospenthal e Rinaldi (2015), 10-12% de todas as infecções nosocomiais são provocadas por *Candida*, e 8-15% das infecções nosocomiais que afetam a corrente sanguínea são provocadas por espécies desse gênero.

**Quadro 1**. Caracterização das candidíases cutâneas/mucocutâneas e invasivas.

| Candidíases cutâneas e mucocutâneas | Características |
|---|---|
| **Candidíase orofaríngea**<br>Mucosa bucal, palato, língua, cantos da boca (queilite angular), amígdalas ou faringe | Candidíase pseudomembranosa aguda ("sapinhos): nódulos brancos, aderentes e aspecto cremoso. Facilmente removíveis.<br>Candidíase atrófica crônica ("estomatite pelo uso de dentaduras"): eritema doloroso, com ausência de placas |
| **Esofagite**<br>Esôfago | Geralmente assintomática, associada a náuseas e vômitos;<br>Placas brancas eritematosas, disfagia e odinofagia, hemametese e dor epigástrica |
| **Balanite**<br>Glande do pênis, podendo estender-se a virilhas e zona perianal | Eritema pruriginoso pustular e placas pseudomembranosas |
| **Vulvovaginite**<br>Mucosa vaginal | Eritema vulvar, prurido intenso, corrimento vaginal, disúria e dispareunia |
| **Mastite**<br>Sulco inframamário | Lesão eritematosa e pruriginosa |
| **Candidíases anais**<br>Ânus | Lesões pruriginosas bem delimitadas, sensação de queimadura e maceração da pele |
| **Candidíases das unhas**<br>Unha ou pele na sua periferia | Paroníquia (pele na periferia da unha): lesão inflamatória, eritematosa e dolorosa<br>Oníquia (unha): unhas espessas, opacas e friáveis |
| **Candidíases mucocutâneas crônicas (CMC)**<br>Mucosa oral, pele, unhas, couro cabeludo, tronco, mãos e dedos | Lesões vermelhas com hiperqueratinização, geralmente indolores |
| **Intertrigo**<br>Zonas quentes e úmidas da pele (espaços interdigitais das mãos e pés, pregas submamárias ou supra púbica, virilhas e axilas) | Lesão eritematosa, descamativa, exsudativa e pruriginosa, geralmente com bordas bem definidas, rodeadas por pequenas vesículas ou pústulas |
| **Candidíases invasivas** | **Características** |
| **Infecções disseminadas** | |
| **Candidemia**<br>Sangue | -Manifestação mais comum de candidíase disseminada<br>-Poderá originar choque séptico ou candidíase disseminada, apesar da ausência de positividade nas hemoculturas<br>-Histopatologicamente: microabcessos em diversos órgãos<br>-Aparecimento de lesões cutâneas e retinianas |
| **Endocardite** | -Complicação da candidemia<br>-Normalmente fatal |
| **Candidíase disseminada crônica**<br>(Candidíase hepatoesplênica) | Após contagem normalizada de neutrófilos:<br>-Febre alta<br>-Dor e sensibilidade no hipocôndrio direito<br>-Lesões no fígado e baço |
| **Infecções focais invasivas** | |
| **Infecções do trato urinário**<br>(Candidúria) | -Possibilidade de febre<br>-Dor na região lombar<br>-Náuseas e vômitos |
| **Infecções osteoarticulares** | Febre e dores nas costas poderão ocorrer semanas após episódio de fungemia |
| **Endoftalmite**<br>Normalmente por *C. parapsilosis* | Lesões brancas na retina que podem atingir humor vítreo e conduzir a cegueira |
| **Peritonite** | -Dor abdominal<br>-Febre |
| **Meningite** | -Febre, rigidez no pescoço, dores de cabeça, alteração do estado mental |

FONTE: Adaptado de SILVA et al. (2012); PEIXOTO et al. (2014); PATIL et al. (2015); PAPPAS et al. (2016).

As espécies distribuem-se de acordo com a população em estudo (idade) e respectiva região geográfica, bem como doenças subjacentes ao hospedeiro e terapêuticas instituídas, podendo ainda ocorrer variações entre hospitais de uma mesma região e entre as diversas unidades hospitalares (DEORUKHKAR; SAINI, 2013; GUINEA, 2014; DEORUKHKAR; SAINI, 2015). Esse fato foi demonstrado em um estudo de prevalência de *C. albicans* e das espécies de CNA em amostras sanguíneas, de pacientes em internamento, que foi variada de acordo com as regiões geográficas. Enquanto *C. albicans* foi predominantemente isolada do Centro e Norte dos EUA, as espécies de CNA foram principalmente isoladas na Ásia, no Sul da Europa e na América do Sul (FALAGAS; ROUSSOS; VARDAKAS (2010).

Em relação à patogênese, a capacidade que o microrganismo tem de aderir à superfície das células do hospedeiro, representa o primeiro estágio deste processo. Os mecanismos usados por espécies de *Candida* para aderir são múltiplos e não têm sido definidos precisamente (VIDOTTO et al., 2003). A hidrofobicidade da superfície da mucosa, a presença de açúcar no meio e formação de tubo germinativo parecem interferir no processo de adesão (MANFREDI et al., 2007). Doenças infecciosas, como a candidíase são conhecidas por começar com a fixação do patógeno a um alvo particular no hospedeiro (CALDERONE; FRONZI, 2001).

As espécies de *Candida* podem também aderir a superfícies de dispositivos médicos e formar biofilmes. A adesão de *Candida* em dispositivos como o cateter facilita a ocorrência de candidemia e a formação de uma massa de microrganismos - denominada de biofilme, que torna a levedura mais resistente aos antifúngicos (AULER et al., 2009). Espécies como *C. parapsilosis* e *C. albicans* têm sido constantemente descobertas como causadoras de candidemia, acreditando-se que essa elevada frequência, principalmente quando se refere a *C. parapsilosis* pode ser explicada pela alta capacidade desta espécie de aderir a superfícies plásticas tais como o cateter (DAGDEVIREN; CERIKCIOGLU; KARAVUS, 2005).

Por fim, o diagnóstico das candídiases cutâneas/mucocutâneas baseia-se na realização de exame direto de esfregaços de pele, unhas, mucosa oral e vaginal, com o auxílio de hidróxido de potássio (KOH) ou de corantes como o azul de metileno ou coloração de Gram, que permitem a observação ao

microscópio das diferentes estruturas: leveduras, hifas e pseudohifas (HOSPENTHAL; RINALDI, 2015). A observação e estudo macro e microscópico das amostras clínicas, a sua inoculação em meios que permitem o isolamento do agente causal e a avaliação da resposta imunitária do hospedeiro destacam-se como métodos convencionais de diagnóstico geral de uma infecção por *Candida* (QUINDÓS et al., 2012; OREN; PAUL, 2014). Nos casos de infecção invasiva, o diagnóstico é bastante complexo e apresenta algumas limitações, entre elas a baixa sensibilidade e morosidade dos métodos convencionais (GÓMEZ et al., 2010; QUINDÓS et al., 2012).

## 4. Tratamento das infecções fúngicas (dermatofitoses e candidíases) e problemática associada

O tratamento das dermatofitoses e candidíases faz-se, de modo geral, com o uso de antifúngicos tópicos e/ou sistêmicos (PIRES et al., 2014; BASSETTI; PEGHIN, M; TIMSIT, 2016). Na terapêutica das candidíases cutâneas é comum o uso de pomadas e cremes de nistatina, de clotrimazol, de terbinafina e de cetoconazol (BARBEDO; SGARBI, 2010; BASSETTI; PEGHIN; M; TIMSIT, 2016). Para infecções na pele mais severas associadas a essas leveduras, ou ainda se houver o agravamento para um caso mais disseminado, opta-se pela terapia oral, com a utilização de antifúngicos de três principais classes: azólicos (ex.: fluconazol, itraconazol, voriconazol), poliênicos (ex.: anfotericina B) e equinocandinas (ex.: micafungina) (BARBEDO; SGARBI, 2010; ROTTA et al., 2013; DATTA et al., 2018). Em relação a dermatofitose, nos quadros mais simples da infecção, usa-se geralmente creme de ciclopirox olamina, de terbinafina, de butenafina e de naftifina (GINTER-HANSELMAYER, 2012; KAUL; YADAV; DOGRA, 2017; KHURANA, 2018).

Adicionalmente, o esmalte de ciclopirox olamina é muito utilizado para os casos de onicomicoses, tanto as de origem dermatofítica como as também ocasionadas por *Candida* spp., apesar de que nas infecções das unhas indica-se normalmente a associação da terapia tópica e sistêmica, pois apenas com a monoterapia as chances de recidivas são bem maiores (SHEMER, et al., 2010; KHANNA; MANCHANDA; AGARWAL, 2014). Além desse caso, a terapia sistêmica das dermatofitoses é indicada quando as lesões são generalizadas, recorrentes, de caráter crônico, ou quando não há resposta à terapia tópica. O

tratamento sistêmico oral é realizado principalmente pelo uso de derivados azólicos como o cetoconazol, terbinafina e griseofulvina, os quais possuem importante atividade antidermatofítica (LAKSHMIPATHY; KANNABIRAN, 2010; PIRES et al., 2014; KAUL; YADAV; DOGRA, 2017).

A escolha do tratamento mais apropriado para cada caso é determinada pela extensão da infecção, pelo sítio afetado e pela espécie envolvida, tão bem quanto pela eficácia, perfil de segurança e biodisponibilidade dos antifúngicos disponíveis (PIRES et al., 2014). A terapêutica pode ser conduzida topicamente, sistematicamente ou associando-se ambas as formas, combinadas ou não a antibacterianos e substâncias queratolíticas (PIRES et al., 2014; LAWTON, 2017).

### 4.1. Derivados azólicos

Os antifúngicos azólicos, de administração oral ou intravenosa, são comumente utilizados no tratamento das micoses inibindo a produção do ergosterol (KLEPSER et al., 2000; SILVA, 2005; BROWN, 2007). Os compostos dessa classe são classificados como imidazólicos ou triazólicos, diferindo-se nas suas constituições químicas, com base no número de nitrogênios (N) presentes no anel azólico - composto de cinco membros, sendo que os imidazólicos possuem dois “N” e os triazólicos três “N” no heterociclo, respectivamente (LACAZ et al., 2002; ODSS; BROWN; GOW, 2003). Os parâmetros essenciais para a ação antifúngica são a presença de, pelo menos, um anel imidazólico e a união desse anel ao restante da molécula por ligações “C-N” (LACAZ et al., 2002).

Apesar das diversidades químico-estruturais entre as classes de azólicos, que de algum modo interferem no espectro de ação e toxicidade, o mecanismo é o mesmo (HAMDAN; HAHN, 2006). Cetoconazol (imidazólico) e fluconazol, itraconazol e voriconazol (triazólicos) atuam bloqueando a enzima 14-α-desmetilase, presente no citocromo P-450 da célula fúngica, impedindo a desmetilação do precursor lanosterol em ergosterol (VANDEN-BOSSCHE, 1997).

Cetoconazol (CTZ) foi o primeiro imidazólico oral biodisponível, com amplo espectro de ação, e a primeira alternativa sistêmica à griseofulvina no tratamento das dermatofitoses, também se apresentando na opção tópica

(MOOSSAVI; BAGHERI; SCHER, 2001). CTZ é eficaz no tratamento da blastomicose, histoplasmose, coccidioidomicose, paracoccidioidomicose, *tinea versicolo*r, candidíase mucocutânea crônica, oral e esofagiana, vulvovaginite por *Candida* e infecções dermatofíticas cutâneas, não sendo muito recomendado na terapêutica de aspergilose e mucormicose (PEI-LAN; LI-MIN; PO-REN, 2007).

Especificamente em relação às dermatofitoses, estudos demonstram que o creme de cetoconazol 2% tem sido eficaz e seguro no tratamento de *tinea pedis*, *tinea cruris* e *tinea corporis*, atuando em infecções ocasionadas pelas espécies dos três gêneros – *Microsporum*, *Trichophyton* e *Epidermophyton* (LESTER, 1995). As taxas de cura com CTZ referentes ao tratamento das tinhas da pele glabra muito se assemelham com as taxas de resposta a terapêutica com a griseofulvina (REFAI; EL-YAZID; EL-HARIRI, 2013). Em 2003, em um estudo para avaliação *in vitro* do CTZ e outros agentes antifúngicos contra *Trichophyton* spp. e *Microsporum* spp., constatou-se valores de concentração inibitória mínima (CIM) baixos para a maioria dos isolados clínicos, ou seja, as espécies mostraram-se bastante sensíveis (GUPTA; KOHLI, 2003). Em 2013, relatou-se o uso tópico do xampu de CTZ em combinação com terbinafina oral, durante três meses, em uma criança com *tinea capitis*, com melhora progressiva da paciente (ANAHORY; SANTOS; BORGES, 2013).

Fluconazol (FLZ) é eficaz na maioria das infecções por *Candida*, sendo capaz inclusive de penetrar no sistema nervoso central (NUCCI; COLOMBO, 2002). FLZ é usado como profilático em pacientes neutropênicos com risco de desenvolverem infecção fúngica (BURGESS et al., 2000). A introdução deste antifúngico como profilaxia possibilitou a redução de candidíase invasiva de 10-25% para pacientes transplantados alogênicos de células estaminais hematopoiéticas (STABER et al., 2007). Embora a profilaxia com FLZ em transplantados alogênicos tenha resultado na redução de infecções por *Candida*, essencialmente por *C. albicans*, este procedimento estimulou uma mudança de etiologia. Espécies de *Candida* não-*albicans*, como *C. krusei* e *C. glabrata* resistentes ao FLZ, tornaram-se emergentes, dificultando o tratamento para estas infecções (RICHARDSON; LASS-FLÖRL, 2008).

O itraconazol (ITZ) é um antifúngico de amplo espectro, disponível em duas formulações: oral e intravenosa, sendo que esta última é a mais utilizada. Este fármaco, entretanto, deve ser administrado com cuidado, pois pode produzir

efeitos tóxicos ao organismo do hospedeiro, como hipocalemia e edema (KONTOYIANNIS et al., 2000). A absorção de ITZ em solução apresenta-se com maior eficácia do que quando formulado em cápsulas. Bons resultados desta formulação líquida em pacientes neutropênicos e submetidos a transplantes de medula foram verificados (PRENTICE et al., 2000).

O voriconazol (VRZ) é um triazólico que está estruturalmente relacionado ao FLZ, porém possui atividade tanto para *Candida* quanto para *Aspergillus*. Efeitos adversos como rash cutâneo, toxicidade hepática, e distúrbios visuais podem ser vistos com o uso de VRZ. Maior eficácia de VRZ sobre o FLZ tem sido observada, sendo que em casos de candidíase esôfágica, observou-se uma cura em 94,8% dos pacientes tratados com VCZ sobre 90,1% dos tratados com FLZ (MARTINEZ, 2006). Corroborando para estes dados, Hegener et al. (1998) verificaram a eficácia de VRZ em 12 pacientes com SIDA refratários ao FLZ. Testes de suscetibilidade *in vitro* mostraram que este antifúngico apresenta valores muito baixos de CIM, sendo que *in vitro*, VRZ é efetivo para várias espécies de *Candida* inclusive *C. glabrata*, *C. krusei* e *C. tropicalis* (GERZENSHTEIN et al., 2005).

Isolados clínicos de *Candida* resistentes aos azólicos e dificuldades no tratamento têm emergido em pacientes com infecções fúngicas oportunísticas (FLECK; HOF, 2008; KHANDELWAL et al., 2018). *C. glabrata* e *C. krusei* têm se mostrado comumente resistentes ao FLZ devido ao uso prolongado deste agente antifúngico (COLOMBO et al. 2002). Segundo Barchiesi et al. (2002), pacientes infectados pelo vírus da imunodeficiência humana (HIV), que fazem uso de terapia antirretroviral altamente ativa (HAART), frequentemente apresentam leveduras na cavidade bucal resistentes ao FLZ. Além disso, a prevalência de resistência em cepas isoladas de infecção da corrente sanguínea está cada vez maior. Outra observação interessante com relação à resistência aos azólicos, refere-se a aderência e colonização de dispositivos médico-hospitalares, como o cateter, que devido ao favorecimento da formação de biofilmes, induz a uma menor suscetibilidade ao FLZ (KHANDELWAL et al., 2018).

São conhecidos três mecanismos de resistência aos azólicos: 1- Redução do acúmulo intracelular do fármaco resultante da utilização reduzida deste agente antifúngico ou do aumento da excreção do fármaco devido à ação de produtos de genes de resistência aos antifúngicos, 2- Alteração estrutural da

enzima 14-α-desmetilase, resultando em uma diminuição na sua ligação aos azólicos, 3- Síntese aumentada de 14- α-desmetilase, devido à amplificação do gene ERG11 (transformação de lanosterol em ergosterol não é totalmente impedida quando sob a ação do derivado azólico) (HEILMANN et al., 2010).

### 4.2. Poliênicos

A anfotericina B (AFB) pertencente à ampla família dos macrolídeos poliênicos, atua ligando-se ao ergosterol da membrana celular fúngica, alterando sua permeabilidade, o que acarreta desequilíbrio osmótico pela perda de íons intracelulares e consequentemente lise e morte das células (VANDEN-BOSSCHE, 1997). AFB pode ser usada para o tratamento de doenças endêmicas causadas por diferentes agentes como *Paracoccidioides brasiliensis*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, que são causadores de lesões sistêmicas graves, e também tem sido amplamente utilizada no tratamento de CI (CARRILLO-MUÑOZ et al., 2001). Os efeitos adversos mais comuns de AFB são instabilidade cardiovascular, febre, hipoxemia e nefrotoxicidade. Os riscos de nefrotoxicidade aumentam com a administração concomitante de outros agentes nefrotóxicos. Formulações com AFB, podem diminuir a toxicidade desta substância como as preparações associadas a lípidios, ou seja, AFB lipossomal (AmBisome®), dispersão coloidal de AFB (Amphotec®) e complexo lipídico de AFB (Abelcet®). Embora AFB e suas formulações não sejam muito toleradas pelos pacientes, este fármaco tem sido usado em casos de transplantados que não respondem a demais tratamentos (BROWN, 2007).

Poucos relatos sobre resistência aos poliênicos têm sido verificados. Isolados de *Candida*, tais como, *C. lusitaniae* e *C. tropicalis* já foram detectados demonstrando resistência a poliênicos (BOFF et al., 2008). O mecanismo de resistência a AFB pode ocorrer devido a uma alteração dos lipídios na membrana celular do fungo, onde o ergosterol dá lugar à formação de outros lipídios, não reconhecidos pelo fármaco (SANGLARD, 2002).

### 4.3. Equinocandinas

É uma classe de fármacos, cujo mecanismo de ação consiste na capacidade que estas substâncias têm de interferir na síntese da parede celular

do microrganismo, inibindo a β-(1,3)-glucana-sintase, por consequência impedindo a síntese de glucanas. O bloqueio da produção de glucanas resulta em desequilíbrio osmótico, o que interfere na viabilidade da célula fúngica (MARTINEZ, 2006). A inibição da síntese de glucanas também apresenta efeitos secundários sobre outros componentes da célula, como redução de lanosterol e ergosterol e o aumento das concentrações de quitina (DOUGLAS et al., 1997). Entre as equinocandinas são conhecidas: micafungina (MCF), caspofungina (CSF) e anidulafungina (ANF).

A MCF possui uma atividade fungicida natural para espécies de *Candida* e é a única equinocandina aceita para tratamento de candidíase de neonatos (THEURETZBACHER, 2004). MCF é também indicada nos casos de candidíase esofágica, candidemia, peritonites e abscessos e como profilático nas infecções por *Candida* em pacientes submetidos a transplante de células progenitoras hematopoiéticas (THEURETZBACHER, 2004). A CSF assim como o VRZ tem demonstrado uma atividade antifúngica *in vitro* contra *Aspergillus* spp. e espécies de *Candida*, incluindo isolados resistentes ao FLZ (KARTSONIS et al., 2002; HOLLENBACH, 2008).

A classe das equinocandinas é a menos tóxica, comparando-se com as demais já citadas, e o desenvolvimento de resistência é raro (PARK et al., 2005; PERLIN, 2015). O mecanismo de resistência de microrganismos a estes agentes antifúngicos pode ser decorrente de um aumento na síntese da proteína Sbep do complexo de Golgi, controlada pelo gene GAL1, que regula os mecanismos de transporte de componentes celulares para a parede da célula fúngica. Moudgal et al. (2005) relataram o desenvolvimento de resistência de *Candida* a múltiplas equinocandinas durante a exposição a esses agentes antifúngicos. Dados preliminares de um estudo *in vitro* utilizando equinocandinas sugerem CIMs desses fármacos maiores para isolados de *C. parapsilosis* do que para outras espécies de *Candida* (PFALLER et al., 2002).

Em consequência da similaridade bioquímica e fisiológica existente entre as células fúngicas e células humanas, o arsenal terapêutico antifúngico disponível para tratamento da candidíase torna-se bastante limitado. A maioria dos agentes antifúngicos disponíveis, como os derivados azólicos e AFB acima mencionados, tem como alvo o ergosterol que é um esterol da membrana celular fúngica, que possui estrutura semelhante ao colesterol presente na célula

humana (ODDS et al., 2003). Medicamentos como as equinocandinas, representam hoje em dia uma das poucas alternativas para o tratamento de candidíase não responsiva as demais classes de antifúngicos. Equinocandinas atuam sobre a parede celular fúngica, que apresenta constituição diferenciada das células dos mamíferos (LARKIN et al., 2018). Desse modo, com as tendências atuais de aumento da resistência aos azólicos e a emergência das CNAs, diretrizes recomendam as equinocandinas como tratamento de primeira linha para candidemia (LARKIN et al., 2018).

### 4.4. Ciclopirox olamina

Ciclopirox olamina (CPO) é um antimicótico empregado muito comumentemente por via tópica, pertencente à classe das hidroxipiridonas, com atividade antifúngica e antibacteriana, através de múltiplos mecanismos de ação. De modo geral, CPO liga-se a cátions trivalentes (por exemplo, $Fe^{3+}$) e tem capacidade de inibir várias enzimas fúngicas citocromais metalo-dependentes (BOHN; KRAEMER, 2000; SUBISSI et al., 2010; LECERF; ANDRÉ; RICHERT, 2014). Relata-se ainda que o CPO afeta a produção energética mitocondrial, reduz a ação de peroxidases e catalases, as quais degradam peróxidos prejudiciais e tóxicos; e ainda afeta a síntese proteica e de ácidos nucleicos da célula fúngica. Agindo por tantas vias, CPO tem, na maioria dos casos efeito fungicida, dependendo sempre da concentração utilizada e do tempo correto de tratamento (SUBISSI et al., 2010). Recomenda-se uma aplicação diária do esmalte de ciplopirox 8% por 24 a 48 semanas, para as unhas das mãos e pés, respectivamente (BOHN; KRAEMER, 2000; SUBISSI et al., 2010; LECERF; ANDRÉ; RICHERT, 2014). Existem formulações do esmalte a base de quitosana hidroxipropil, que facilitam mais a permeabilidade através da lâmina ungueal. Considera-se o uso do CPO nas onicomicoses subungueais (distal e lateral) mínimas, quando não houver mais de 50% da área total da unha comprometida e/ou quando o paciente não é um bom candidato para a terapêutica antifúngica por via oral (BOHN; KRAEMER, 2000; SUBISSI et al., 2010; LECERF; ANDRÉ; RICHERT, 2014).

A partir de 1976, numerosos estudos foram conduzidos com o CPO em pacientes com infecções fúngicas da pele, sendo que uma série de trabalhos foi publicada desde então (SUBISSI et al., 2010). Em um levantamento de 20

estudos, com 991 casos de doenças de pele, principalmente dermatofitoses (71%), realizado com creme de CPO 1% e solução de CPO 1%, observou-se que após um período de tratamento de três semanas, a taxa de sucesso terapêutico (cura clínica e micológica) foi de 96% (DITTMAR, 1981). Em 2005, avaliou-se a eficácia do gel de CPO 0,77% no tratamento da *tinea pedis interdigitalis*, com infecção bacteriana secundária, em um estudo prospectivo duplo-cego, randomizado e controlado por placebo. Verificou-se, que o gel aplicado uma ou duas vezes ao dia reduziu significativamente os sinais e sintomas das lesões, na oitava semana de tratamento. As taxas de cura completa foram muito mais elevadas para CPO do que para o veículo, e redução da contagem bacteriana foi também observada pós-tratamento com CPO (GUPTA; SKINNER; COOPER, 2005). Em 2014, em um estudo da eficácia do CPO 1% e cloridrato de terbinafina 1%, em um modelo de onicomicose dermatofítica *in vitro*, notou-se alta taxa de permeação para ambos os compostos analisados, que mostraram eficácia muito semelhante (TAUBER; MULLER-GOYMANN, 2014). Além da atividade contra fungos e bactérias, recentemente descobriram-se propriedades anticâncer para o CPO (SHEN; ZHOU; HUANG, 2013).

### 4.5. Terbinafina

A terbinafina (TBF) é um antimicótico empregado, por via tópica e/ou oral, no tratamento de infecções fúngicas cutâneas e até mesmo subcutâneas. TBF estabeleceu-se no mercado farmacêutico entre as décadas de 80-90 (PETRANYI; RYDER; STUTZ, 1984) e é considerada o principal antifúngico mais eficaz para dermatofitoses, visto que os dermatófitos, em geral, apresentam uma elevada suscetibilidade, sendo especialmente empregada nos casos em que há condições crônicas da micose (SOARES et al., 2013). TBF pertence à classe das alilaminas (alilamina terciária), fármacos sintéticos que atuam de forma distinta das demais classes de antifúngicos e cuja atividade, *in vitro* e *in vivo*, é direcionada especialmente contra dermatófitos e algumas leveduras, tendo também ação contra *Sporothrix schenckii*, *Scopulariopsis brevicaulis* e *Acremonium* spp. (LACAZ et al., 2002). A ação desse antifúngico dá-se pela inibição não-competitiva, específica e seletiva da esqualeno epoxidase, uma enzima envolvida nas etapas iniciais da síntese do ergosterol, o principal esterol

componente da membrana celular fúngica, acarretando tanto o acúmulo de esqualeno, como a deficiência do ergosterol (MOOSSAVI; BAGHERI; SCHER, 2001; ODSS; BROWN; GOW, 2003). Elevadas concentrações de esqualeno tóxico no citoplasma fúngico podem interferir na função da membrana e na síntese da parede celular dos fungos, bem como níveis deficitários de ergosterol influenciam na manutenção da célula, envolvendo tanto a membrana quanto a parede dos fungos, com consequências fungicidas em espécies suscetíveis (MOOSSAVI; BAGHERI; SCHER, 2001). TBF não tem uma elevada afinidade de ligação pela esqualeno epoxidase humana, e assim a produção de colesterol não é fortemente afetada (WAKELIN; MAIBACH, 2003).

Em relação à farmacocinética, a TBF é um fármaco muito bem absorvido, após a sua administração oral e liga-se fortemente às proteínas plasmáticas (MOOSSAVI; BAGHERI; SCHER, 2001). Essa ligação não é específica e a eliminação plasmática é lenta. A afinidade da TBF pelo citocromo P450 é baixa, em contraste com os azóis, sendo essa uma das razões para uma menor taxa de interações da mesma com comediadores ou demais medicamentos, em comparação, por exemplo, com os triazólicos e imidazólicos (REFAI; EL-YAZID; EL-HARIRI, 2013). Além disso, trata-se de um antifúngico lipo e queratinofílico, que é extensivamente distribuído pelo tecido adiposo, derme, epiderme e unhas. Devido a isso e à eficácia comprovada, considera-se a TBF um dos antimicóticos mais apropriados para o tratamento das dermatofitoses disseminadas de pele, pelos e unhas, bem como para casos de candidíase cutânea (DARKES; SCOTT; GOA, 2003).

Relata-se para o uso específico da TBF no tratamento das dermatofitoses, que a mesma apresenta uma maior eficácia contra espécies do gênero *Trichophyton* (KAKOUROU; UKSAI, 2010). Logo, após o início do uso da TBF, alguns estudos passaram a recomendá-la topicamente para lesões dermatofíticas não muito extensas, tendo um custo-benefício melhor que miconazol e CTZ tópicos (MCCLELLAN; WISEMAN; MARKHAM, 1999). Em um estudo de metanálise de ensaios clínicos randomizados, observou-se que particularmente para a espécie *T*. *tonsurans*, a TBF e a griseofulvina tiveram uma eficácia semelhante, porém o tempo necessário de tratamento com TBF foi menor (4 semanas), do que com a griseofulvina (8 semanas) (GUPTA; DRUMMOND-MAIN, 2013). A dose habitual da TBF por via oral é 250 mg/dia.

Para *tinea corporis* e *tinea cruris*, administra-se a mesma durante 2 a 4 semanas; em *tinea pedis*, durante 2 a 6 semanas e em onicomicoses dermatofíticas, durante 4 a 12 semanas (REFAI; EL-YAZID; EL-HARIRI, 2013). A TBF demonstrou superioridade, em eficácia e segurança, em relação ao esmalte de CPO 8%, para o tratamento de onicomicoses em pacientes diabéticos (MATRICCIANI; TALBOT; JONES, 2011). Mais recentemente, estuda-se a administração intralesional de injetáveis contendo microesferas de TBF, de liberação controlada, para o tratamento de onicomicoses, como uma forma de aplicação mais localizada e direcionada do antifúngico (ANGAMUTHU, 2014).

### 4.6. Griseofulvina

A griseofulvina (GSF) foi descoberta por Oxford et al. (1939), tendo origem a partir do fungo *Penicilium griseofulvum* – foi o primeiro agente terapêutico específico para espécies fúngicas (ODSS; BROWN; GOW, 2003). É um derivado benzofurano, somente de administração via oral e com eficácia comprovada, principalmente para o tratamento de micoses cutâneas e onicomicoses dermatofíticas (LACAZ et al., 2002). O mecanismo de ação da GSF consiste na inibição da completa formação do fuso mitótico, por meio da interação com microtúbulos polimerizados, inibindo, desse modo, a mitose e o consequente desenvolvimento e crescimento fúngico, tendo ação fungistática (MOOSSAVI; BAGHERI; SCHER, 2001).

Comercialmente, a GSF está disponível em várias formas farmacêuticas (comprimidos, suspensão), porém a suspensão não é uma formulação licenciada, por exemplo, no Reino Unido, e os comprimidos já não estão disponíveis em alguns países europeus, tendo sido substituídos por outros agentes antifúngicos (KAKOUROU; UKSAI, 2010). As dosagens recomendadas variam muito com o tipo de formulação e o quão facilmente a mesma é absorvida (FULLER et al., 2014).

A GSF se distribui largamente por variados tecidos queratinizados (pele, pelos e unhas), sendo por isso, antes do surgimento da terbinafina e imidazólicos, um dos primeiros antifúngicos de escolha para a terapêutica das tinhas. Sua indicação é direcionada especialmente aos casos de *tinea corporis*, *tinea capitis* e *tinea unguium*. GSF apresenta uma maior aplicabilidade para o tratamento das dermatofitoses ocasionadas por espécies do gênero

*Microsporum*, especialmente *M*. *canis* (CHADEGANIPOUR; NILIPOUR; HAVAEI, 2004). A partir de sete estudos de metanálise, observaram-se taxas de resposta terapêutica com o uso da GSF diferenciadas, em relação às espécies dermatofíticas, evidenciando-se para espécies do gênero *Microsporum*, uma taxa de aproximadamente 90% e para *Trichophyton* cerca de 70%, corroborando com a afirmação de efetividade espécie-específica desse antifúngico (GUPTA; COOPER; BOWEN, 2008). Em concordância a isso, outro estudo de metanálise sugere que um tratamento de 8 semanas com GSF é bem mais efetivo que um tratamento de 4 semanas com TBF em tinhas do couro cabeludo, envolvendo espécies microspóricas, o que não se verifica para as espécies tricofíticas (GUPTA; DRUMMOND-MAIN, 2013). Por ser um antifúngico de baixa solubilidade, envolvendo problemas de precipitação, há pesquisas no desenvolvimento e modulação da liberação *in vitro* e melhora da solubilidade da GSF, utilizando, por exemplo, nanopartículas de sílica na formulação desse composto (JAMBHRUNKAR et al., 2014).

### 4.7. Tratamento combinado

A associação de antimicóticos tem produzido cada vez mais avanços, por exemplo, no tratamento das dermatofitoses, principalmente quando se trata de casos mais graves e disseminados. Já foram descritas na literatura combinações entre TBF e ITZ oral administrados de forma consecutiva; tioconazol tópico e GSF oral; TBF e CPO; GSF e amorolfina, entre outras (MAGAGNIN, 2010). Algumas dessas combinações ainda não possuem ação total sobre dermatófitos resistentes, porém, todas apresentam evidências de efetiva melhora dos quadros clínicos infecciosos, quando comparadas com a monoterapia oral ou tópica tradicional (GROVER et al., 2007; HAVLICKOVA; FRIEDRICH, 2008).

Pires et al. (2014) apontam que em relação ao tratamento de escolha para a maioria das dermatofitoses, há uma predominância da terapia combinada com antifúngicos tópicos e sistêmicos (62,8%), sendo os azólicos (33,1%) e CPO (49%) os agentes sistêmicos e tópicos mais comumente prescritos, respectivamente (PIRES et al., 2014). A TBF vem sendo cada vez mais utilizada em combinação com outros agentes antifúngicos no tratamento das micoses refratárias, como onicomicoses, devido à sua capacidade, observada *in vitro*, de sinergismo com demais compostos. Usando-se modelos de farmacocinética e

farmacodinâmica, nota-se uma potente sinergia entre a TBF e antifúngicos azólicos, o que aumenta consideravelmente a chance de sucesso terapêutico. Os mecanismos de ação complementares da TBF e azólicos em diferentes pontos da via de biossíntese do ergosterol fúngico, teoricamente têm um impacto maior na inibição e até mesmo morte do microrganismo (DOLTON et al., 2014). Para os casos de *tinea pedis* com hiperqueratose, observou-se que a utilização da combinação de pomada de ureia a 10%, juntamente com TBF tópica e oral, otimiza o tempo de tratamento, fazendo com que o paciente apresente maior adesão, além de ser segura e eficaz (SHI et al., 2014). No Brasil, são comumente comercializadas associações dos antifúngicos mais recomendados para as tinhas com anti-inflamatórios e antibióticos, por exemplo, CTZ e betametasona, CTZ, betametasona e neomicina, dentre outras formulações, a fim de amenizar os sinais inflamatórios locais decorrentes da infecção fúngica, além de auxiliar no processo de cicatrização das lesões (PERARO, 2001).

Por fim, apesar do arsenal disponível de fármacos antifúngicos agir por diferentes mecanismos e vias, os alvos celulares são limitados em função da similaridade que existe entre as células eucarióticas fúngicas e humanas, em vários aspectos (MARTINEZ-ROSSI; PERES; ROSSI, 2008). Os relatos de recorrentes falhas na terapêutica são normalmente associados com a descontinuação da terapia em decorrência do tratamento prolongado, efeitos adversos de alguns antifúngicos e com a cada vez mais comum problemática de resistência fúngica (MUKHERJEE et al., 2003; MARTINEZ-ROSSI; PERES; ROSSI, 2008; PERES et al., 2010; VENA et al., 2012; TSAY et al., 2018). A crescente incidência de infecções fúngicas, aliado à SIDA e ao aumento da utilização de imunossupressores, foi seguido por uma utilização consequentemente mais ampla e frequente de antifúngicos, com intenções profiláticas e terapêuticas. A utilização demasiada ou em doses abusivas e inadequadas dos antimicóticos faz com que as espécies fúngicas desenvolvam resistência, por evoluções epigenéticas e em resposta ao estresse, fortemente dificultando o tratamento (CALO et al., 2014). Essa resistência pode ser intrínseca (todos os membros da espécie são resistentes) ou extrínseco-adquirida (quando as espécies se tornam resistentes mediante uma prévia exposição ao agente antifúngico) (MARTINEZ-ROSSI; PERES; ROSSI, 2008).

Em 2003, foi reportado o primeiro caso confirmado de resistência dermatofítica *in vivo* (clínica) e *in vitro* à TBF (MUKHERJEE et al., 2003). Tratavam-se de seis isolados clínicos de *T*. *rubrum*, originários de um mesmo paciente com onicomicose dermatofítica, cuja terapia oral com TBF (250 mg/dia por 24 semanas) não foi eficaz. Os isolados apresentaram *in vitro* um valor de CIM para TBF 4.000 vezes maior do que o valor encontrado para cepas–referência (sensíveis), com suscetibilidade também reduzida para outros inibidores da esqualeno-epoxidase, incluindo naftidina, butenafina, tolnaftato e tolciclato, sugerindo-se um mecanismo de resistência alvo-específico (MUKHERJEE et al., 2003). Em 2005, um estudo objetivou caracterizar as possíveis modificações moleculares para a resistência dos isolados clínicos de *T*. *rubrum*, relatados por Mukherjee e colaboradores (2003). Posterior ao sequenciamento dos isolados e análise, relataram-se substituições únicas de aminoácidos na enzima esqualeno epoxidase, o que explicaria a resistência, já que modificações no alvo implicam dificuldades no reconhecimento e consequente ação antifúngica das alilaminas, no caso específico dessa enzima envolvida (OSBORNE et al., 2005).

Adicionalmente, dermatófitos mutantes resistentes à GSF são relatados desde a década de 60 (LENHART, 1969). Em um estudo realizado no México, em 2007, com 36 dermatófitos, detectou-se sete espécies (19,4%) – três *T*. *rubrum*, três *T*. *mentagrophytes* e um *T*. *tonsurans* resistentes a um ou mais antifúngicos (MANZANO-GAYOSSO et al., 2008). Pesquisas mais recentes constataram resistência múltipla em cepas de *T*. *rubrum* em relação à TBF, ao ITZ e à amorolfina (GHELARDI et al., 2014). Resistência aos azólicos também já foi relatada para dermatófitos (GHANNOUM, 2015) e *Candida* spp. (ACCOCEBERRY et al., 2018).

Agregando-se a toda essa problemática apresentada, infelizmente, o arsenal terapêutico dos antifúngicos apresenta espectro de atividade muito variável, podendo levar a falhas no tratamento *in vivo*, possivelmente devido aos multifatores já mencionados, como baixa adesão dos pacientes à terapia, eventual baixa penetração do fármaco, problemas na biodisponibilidade do medicamento, interações medicamentosas ou (multi) resistência (MANZANO-GAYOSSO et al., 2008). Desse modo, há uma real necessidade de novos

compostos mais eficazes e menos tóxicos, o que torna a pesquisa por novos agentes antifúngicos muito pertinente e relevante (SOARES et al., 2013).

## 5. Prospecção de compostos sintéticos e formulações como novos agentes antifúngicos

A procura por novos agentes antimicrobianos é objeto de contínua pesquisa na Química Medicinal. O arsenal terapêutico necessita de novos representantes, em decorrência da progressiva ineficácia dos agentes farmacológicos atuais frente à resistência dos microrganismos e limitações inerentes aos próprios fármacos (biodisponibilidade, toxicidade, farmacocinética, farmacodinâmica, etc.) (TEGOS; MYLONAKIS, 2012; DHANASEKARAN; THAJUDDIN; PANNEERSELVAM, 2015).

Grandes vantagens são verificadas durante o planejamento e desenvolvimento racional de novos compostos, por via sintética. Essas decorrem, entre outros fatores, do prévio conhecimento de suas propriedades, rendimento e prováveis futuras aplicações, com base nas características químicas. A síntese é um dos processos mais utilizados na obtenção de protótipos para o desenvolvimento de novas moléculas com potencial terapêutico, cuja compreensão das características físico-químicas interferentes na ação antimicrobiana pode direcionar a pesquisa de novos agentes anti-infecciosos (TEGOS; MYLONAKIS, 2012; DHANASEKARAN; THAJUDDIN; PANNEERSELVAM, 2015; CAMPOY; ADRIO, 2017).

O aprimoramento da Química Orgânica tem proporcionado um aumento considerável de compostos sintéticos para uso medicinal, os quais têm sido empregados no combate a várias doenças. O delineamento e a síntese de novas substâncias e a avaliação de suas possíveis atividades biológicas compõem a primeira etapa para o surgimento de um novo composto potencialmente ativo, que pode conduzir ao lançamento de um novo fármaco (TEGOS; MYLONAKIS, 2012; DHANASEKARAN; THAJUDDIN; PANNEERSELVAM, 2015; CAMPOY; ADRIO, 2017).

A abordagem mais comum em pesquisas sintéticas e semissintéticas é a correlação entre a estrutura e propriedade. Diferentemente dos compostos naturais, as moléculas de origem sintética e semissintética apresentam propriedades mais homogêneas e reprodutíveis, permitindo uma clara

compreensão da correlação entre propriedade e estrutura. Assim, a maioria dos antifúngicos comumentemente usados oferece pelo menos uma etapa sintética, produzindo moléculas que permitem avaliar seu mecanismo de ação (CAMPOY; ADRIO, 2017). Além disso, a síntese ainda oportuniza a otimização estrutural, com a produção de análogos daquelas moléculas que se mostrarem mais ativas frente a patógenos (TEGOS; MYLONAKIS, 2012; DHANASEKARAN; THAJUDDIN; PANNEERSELVAM, 2015).

Em vista do exposto, a pesquisa e desenvolvimento de novos agentes antifúngicos, vem cada vez mais incluindo a triagem de algumas classes de compostos sintéticos, como novos candidatos a tratamentos alternativos para infecções fúngicas (FUENTEFRIA et al., 2018), tais como: SCY-078/MK-3118/Enfumafungin – contra *Candida* spp. e *Aspergillus fumigatus* (JIMÉNEZ-ORTIGOSA et al., 2014), piridazinonas – contra *Candida* spp., *Aspergillus* spp., C*. neoformans*, *Fusarium* spp. e *Trychophyton* spp. (ROEMER; KRYSAN, 2014), luliconazol – contra dermatófitos (SCHER NAKAMURA; TAVAKKOL, 2014), sais imidazólicos – contra *Trichosporon asahii*, CNA, dermatófitos e *Fusarium graminearum* (BERGAMO et al., 2014, DALLA LANA et al., 2015, RIBAS et al., 2016), derivados de hidrazonas – contra *Candida* spp. e *T. asahii* (CASANOVA et al., 2015), VT1161 – contra *C. albicans* (GARVEY et al., 2015), novos azólicos alquilados – contra *Candida* spp. e *Aspergillus* spp. (SHRESTHA et al., 2017), VT1129 – contra *Cryptococcus* spp. (CAMPOY; ADRIO, 2017), biafungina (CD101/SP3025) – contra *Aspergillus* spp. e *Candida* spp. (CAMPOY; ADRIO, 2017) e derivados cumarínicos contra *Sporothrix* spp. (FOREZI et al.; 2018).

Além desses, sabe-se que novos compostos estão sendo sintetizados e posteriormente serão avaliados quanto a atividade biológica, podendo exibir mecanismos de ação inéditos, em alvos microbianos anteriormente não explorados, ou usarem estratégias diferenciadas (pró-fármaco), o que pode consolidar as tentativas de combate à emergência e resistência microbiana (TEGOS; MYLONAKIS, 2012; DHANASEKARAN; THAJUDDIN; PANNEERSELVAM, 2015).

Dentre as moléculas deste estudo, TAs são compostos orgânicos contendo o núcleo S-C-N (SILVEIRA et al., 2012). Recentemente atividades biológicas começaram a ser relatadas para essa classe, tais como:

antituberculostática (SILVEIRA et al., 2012), antimicrobiana (SÁ et al., 2014; CAPON et al., 2014) e antitumoral (FORTES et al., 2016).

δ-Ls são importantes constituintes de sabor e aroma em muitos produtos naturais (ROMERO-GUIDO et al. 2011), mas também podem ter origem sintética e vêm sendo muito estudadas pelo seu potencial anticâncer e antimicrobiano (WANI et al., 1980; UMEZAWA et al., 1984; XU; LING, 1985; OHKUMA et al., 1992; KISHIMOTO et al. 2005; TANAKA et al. 2007; YANG et al. 2011; MODRANKA et al., 2012; VALENCIA–GALIZA et al., 2017).

Benzenodióis ou dihidroxibenzenos são compostos químicos orgânicos nos quais dois grupos hidroxila são substituídos em um anel de benzeno. Estes compostos aromáticos são classificados como (poli)fenóis. Existem três isômeros do benzenodiol: 1,2-benzenodiol (o isômero orto) é comumente conhecido como catecol, 1,3-benzenodiol (o isômero meta) é comumente conhecido como resorcinol e 1,4-benzenodiol (o isômero para) é comumente conhecido como hidroquinona. Todos os três isômeros possuem a fórmula química $C_6H_6O_2$. Semelhante a outros fenóis, os grupos hidroxilas no anel aromático de um benzenodiol são fracamente ácidos (LIMA; GRAZIANO; PANDOLFI, 2016). Os polifenóis são amplamente distribuídos na natureza e exibem uma gama notável de atividades biológicas e farmacológicas (HARBONE; BAXTER; MOSS, 1999; WHITTING, 2001; DAI; MUMPER, 2010; OKSANA et al., 2012; DE ARIAS ET AL., 2012; SERNA et al., 2015; SOTO-HERNANDEZ; PALMA-TENANGO; GARCIA-MATEOS, 2017). Especificamente para as hidroquinonas, atividade tumoral também já foi relatada (SILESS et al., 2012).

1,3-BXPs são compostos sintéticos cujas atividades biológicas (leishmanicida - *Leishmania amazonenses* e tripanocida - *Trypanosoma cruzi*) foram descobertas a partir de 2015 em amplas triagens, (LAVORATO et al., 2015; LAVORATO, 2016; LAVORATO et al., 2017; LAVORATO; DUARTE; DE ANDRADE, 2017). Essa prévia ação antiprotozoários despertou nosso interesse para investigação da atividade antifúngica desse grupo de compostos, visto que não há nada na literatura até então relacionado a ação antifúngica de seus intermediários e derivados sintéticos. O processo de síntese relativamente simples e já bem elucidado é muito vantajoso para a pesquisa e desenvolvimento de 1,3-BXPs como candidatos a antifúngicos.

Além da síntese de novos compostos, a proposta de estudo das formulações antifúngicas está em progresso. Tem-se de ter novas moléculas, mas posteriormente é necessário pensar no modo de aplicação em alguma forma farmacêutica e nas melhorias desse processo para um resultado ainda mais vantajoso de eficácia/toxicidade (TEGOS; MYLONAKIS, 2012; AIAD et al., 2017; STEIMBACH et al., 2017).

As nanoformulações possuem qualidades únicas que as tornam veículos ideais para o tratamento de muitas doenças infecciosas (ROLLER, 2014). Nanopartículas têm a capacidade de melhorar a solubilidade de antimicrobianos, reduzir a citotoxicidade para o hospedeiro, alcançar maiores concentrações intracelulares e proporcionar terapia medicamentosa combinada (AIAD et al., 2017). Além disso, permitem o direcionamento de ação contra patógenos específicos (ROLLER, 2014). O desenvolvimento contínuo de novos antimicrobianos associados à nanotecnologia tem sido alvo de grande interesse (YUEN-KI et al., 2017). Quanto aos antifúngicos em particular, também cada vez mais estudos são reportados (KHALIL et al., 2018; SYLVIA et al., 2018). No contexto farmacêutico, a nanotecnologia mostra ainda muito interesse na resolução de problemas farmacotécnicos e farmacocinéticos, associados à utilização de alguns fármacos. Paralelamente, o uso de biomarcadores possibilita uma aplicação conjunta de um nanossistema como meio terapêutico e/ou de diagnóstico (YUEN-KI et al., 2017).

Além disso, formulações semissólidas, tais como hidrogéis, vêm sendo cada vez mais utilizadas para incorporação de agentes antifúngicos visando o tratamento tópico de dermatomicoses (WINNICKA et al., 2012; SAHOO et al., 2014; MAHATA et al., 2014; ALDAWSARI et al., 2015; SHU et al., 2018). Hidrogéis são sistemas simples e vantajosos, uma vez que quando aplicados sobre a pele, espalham-se facilmente; sendo que alguns ainda possuem propriedades bioadesivas, o que favorece a ação-local do antifúngico e a aderência do paciente ao tratamento (WINNICKA et al., 2012; SAHOO et al., 2014; MAHATA et al., 2014; ALDAWSARI et al., 2015; SHU et al., 2018).

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**CAPÍTULO I – Dermatofitoses: agentes etiológicos, formas clínicas, terapêutica e novas perspectivas de tratamento**

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DERMATOFITOSES: AGENTES ETIOLÓGICOS, FORMAS CLÍNICAS, TERAPÊUTICA E NOVAS PERSPECTIVAS DE TRATAMENTO

*DERMATOPHYTOSES: ETIOLOGIC AGENTS, CLINICAL FORMS, THERAPY AND NEW PERSPECTIVES OF TREATMENT*

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**Dermatofitoses: agentes etiológicos, formas clínicas, terapêutica e novas perspectivas de tratamento**

**Dermatophytoses: etiologic agents, clinical forms, therapy and new perspectives of treatment**

**Título resumido do artigo - Dermatofitoses: epidemiologia e tratamento**

**Summary title - Dermatophytosis: epidemiology and treatment**

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**RESUMO**

As dermatofitoses têm ocorrência mundial, sendo mais prevalentes em países de clima tropical e subtropical. Dados epidemiológicos indicam que essas micoses figuram entre as infecções fúngicas de maior ocorrência. O quadro clínico mais comum de dermatofitose inclui despigmentação, placas anulares, prurido e perda de cabelo, com lesões tipicamente conhecidas como *tineas*, ocasionadas por fungos filamentosos dermatofíticos de três gêneros anamórficos: *Microsporum*, *Trichophyton* e *Epidermophyton*. O tratamento das dermatofitoses, em geral, está relacionado ao uso de antifúngicos tópicos e/ou sistêmicos, apresentando como problemática o surgimento de espécies multirresistentes. Esta revisão aborda as dermatofitoses e seus agentes etiológicos de forma aprofundada em aspectos epidemiológicos, apresentando a importância clínica do tema, com ênfase na causa, prevenção, tratamento e prognóstico dessa micose cutânea.

**Palavras-chave**: dermatofitoses, dermatófitos, epidemiologia, tratamento.

**ABSTRACT**

Dermatophytoses have worldwide occurrence with higher prevalence in tropical and subtropical countries. Epidemiological data show that these mycoses are among the most frequent fungal infections. The most common symptoms of dermatophytoses include depigmentation, annular plaques, itching and hair loss, with lesions such as *tinea*, caused by dermatophytic filamentous fungi of three anamorphic genera: *Microsporum*, *Trichophyton* and *Epidermophyton*. Topical and/or systemic antifungal medications are used in the treatment of dermatophytoses in general, resulting in problems such as the emergence of multidrug-resistant species. This review discusses dermatophytoses and their etiological agents with a focus on epidemiological aspects, presenting the clinical

importance of the issue, with emphasis on cause, prevention, treatment and prognosis of this skin mycosis.

**Keywords**: dermatophytoses, dermatophytes, epidemiology, treatment.

As dermatofitoses têm ocorrência mundial, sendo prevalentes em países de clima tropical e subtropical. São consideradas o terceiro distúrbio dermatológico mais frequente em crianças menores de 12 anos e o segundo mais frequente em adultos[1]. Em 2001, no Japão, constatou-se mais de 12 milhões de indivíduos com dermatofitose, sendo que nesse mesmo ano, nos EUA, a porcentagem de pacientes com essa micose cutânea chegava a 20%[2].

No Brasil, a prevalência das dermatofitoses entre as lesões cutâneas varia de 18 a 23%[3]. Em Porto Alegre, no Rio Grande do Sul (RS), realizou-se uma análise retrospectiva dos exames micológicos do serviço de dermatologia do Hospital de Clínicas de Porto Alegre (HCPA), durante o período de 1998 a 2006, e constatou-se que das 5.077 amostras coletadas, 2.033 (40%) foram positivas para dermatofitose, o que representa um elevado percentual quando comparado a outros tipos de micose[4].

O quadro clínico mais comum de dermatofitose inclui despigmentação, placas anulares, prurido e perda de cabelo, lesões tipicamente cutâneas conhecidas como *tineas*. Essas lesões, infectocontagiosas e extremamente impactantes na qualidade de vida dos pacientes, são ocasionadas por fungos filamentosos dermatofíticos de três gêneros anamórficos: *Microsporum*, *Trichophyton* e *Epidermophyton*[5].

O tratamento das dermatofitoses é feito com uso de antifúngicos tópicos e/ou sistêmicos[6]. Uma das problemáticas da terapêutica é que as espécies dermatofíticas vêm desenvolvendo resistência à terapêutica antifúngica convencional, o que ocasiona uma

suscetibilidade muito variável *in vitro* e *in vivo*, aumento progressivo do número de recidivas e não responsividade aos fármacos antimicóticos usuais[7].

Esta revisão tem o propósito de abordar as dermatofitoses e seus agentes etiológicos, elucidando a importância clínica do tema, com ênfase na causa e prevenção dessa micose, bem como no diagnóstico, tratamento e prognóstico. Serão abordadas questões como a prevalência de algumas espécies em determinadas regiões, a epidemiologia da micose, a relação com a queratina da pele e demais fatores de patogenicidade, bem como o impacto da terapia antifúngica em idosos e pacientes com coinfecções associadas. Por fim, será discutida a relação do uso de antifúngicos agronômicos com a predominância ou ausência de algumas espécies fúngicas nas variadas regiões do Brasil.

**Metodologia**

Realizou-se um estudo de revisão bibliográfica sistemática com pesquisas em bancos de dados e coleções *online* da Elsevier Editora (ScienceDirect), da biblioteca virtual de saúde do National Institutes of Health (PubMed) e do Portal de Periódicos da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior do Ministério da Educação (CAPES/MEC), no período de novembro de 2015 a novembro de 2016. Os termos utilizados nas buscas eletrônicas foram “dermatofitoses/dermatophytoses”, “*tineas*” e “dermatófitos/dermatophytes”. A revisão teve como objetivo abordar os aspectos mais atuais que contemplam agentes etiológicos, formas clínicas, terapêutica convencional e novas perspectivas de tratamento das dermatofitoses. A pesquisa foi efetuada independentemente por dois pesquisadores (DFD e BGB) a partir da leitura de título e resumo e, posteriormente, das obras completas que continham as características específicas dos descritores de busca. Os critérios de inclusão foram artigos originais que

se enquadrassem na temática proposta, publicados entre os anos de 1990-2016. Foram excluídos os estudos que não possuíam referências bibliográficas adequadas, que se apresentavam incompletos ou com fontes terciárias. Além disso, optou-se por não incluir teses, dissertações, monografias e livros, visto que a realização de uma busca sistemática dos mesmos é inviável logisticamente. Ao final, 202 artigos atenderam a todos os critérios acima mencionados e foram detalhadamente analisados para integrar este estudo.

## Dermatofitoses

### Agentes etiológicos – fungos dermatófitos

Os dermatófitos, pertencentes à família *Arthrodermataceae*, são fungos filamentosos, hialinos, septados, queratinofílicos e queratinolíticos[8]. Esses fungos possuem um forte biotropismo por estruturas queratinizadas, como pele, pelos e unhas, sendo que a habilidade de ocasionar a micose está diretamente relacionada a essa dependência da queratina[9].

Esses fungos apresentam perfis epidemiológicos variados com relação a fatores regionais, estacionais, de faixa etária, frequência de contato com animais, condições higiênico-sanitárias e exposição a locais públicos[4]. Desenvolvem-se do centro da lesão para as bordas, ocasionando intensa descamação associada ou não à resposta inflamatória resultante da atividade queratinolítica[10]. Seus metabólitos difundem-se pelas células da epiderme, causando reações de hipersensibilidade. As manifestações das lesões são decorrentes da resposta imune do hospedeiro aos metabólitos do fungo, da virulência da espécie infectante e da localização anatômica da infecção fúngica[11].

Em cultura, observam-se hifas septadas que se ramificam e formam o micélio com estruturas de reprodução assexuada, os conídios. Microscopicamente, esses conídios diferem entre as espécies quanto a forma, tamanho, número e disposição ao longo das

hifas, sendo esses critérios essenciais para a identificação[12]. A maioria das espécies de dermatófitos produz dois tipos de conídios: os macroconídios pluricelulares e os microconídios unicelulares[8].

Entre os dermatófitos de maior relevância clínica estão *Microsporum canis, M. gypseum, M. nanum, Trichophyton mentagrophytes, T. rubrum, T. tonsurans, T. violaceum, T. shoenlini* e *Epidermophyton flocosum*[11].

*M. canis* é tido como um dos principais agentes etiológicos de dermatofitose, sendo o responsável por cerca de 15% das infecções dermatofíticas em seres humanos[13]. Estudos epidemiológicos realizados no Rio de Janeiro, Rio Grande do Sul, Espírito Santo, São Paulo, África e continente europeu indicam que *M. canis* é o agente mais isolado nos casos de infecções dermatofíticas do couro cabeludo, sendo responsável por 67% dos casos de *tinea capitis*[14]. Corroborando esse achado, em um estudo realizado em Goiânia, foram examinadas 353 amostras de lesões do couro cabeludo, das quais 164 (46,4%) foram positivas para dermatófitos e destas, 117 (71,3%) para *M. canis*[15].

*M. gypseum* infecta o homem com lesões clínicas de intensa reação inflamatória[16]. Sabe-se que pode provocar dermatites graves e crônicas em pacientes com imunocomprometimento, como lúpus eritematoso sistêmico[17]. Também já se relatou na literatura infecções em recém-nascidos[18].

*M. nanum* raramente causa micose em seres humanos, acometendo principalmente gatos. Devido ao contato próximo desses animais com humanos e à possível ausência de sintomas por parte dos animais, observamos um potencial de infecção em humanos por esse dermatófito[12].

*T. mentagrophytes* tem ocorrência em infecções em humanos e animais e ocasiona lesões dermatofíticas com intermediária a intensa reação inflamatória. A espécie parasita

um número elevado de animais (coelhos, cavalos, porcos, galinhas, etc.), razão pela qual a infecção em meio rural é até mais frequente que em meio urbano[19].

*T. mentagrophytes* é a segunda espécie dermatofítica mais isolada nos casos de dermatofitose em humanos[20]. No RS, entre os meses de novembro de 2010 e agosto de 2011, foram analisados laudos de pacientes submetidos a exames micológicos, com suspeita de micose superficial, cutânea e profunda, encaminhados a um laboratório no município de Novo Hamburgo, da Região do Vale dos Sinos. Dos resultados positivos para micoses superficiais e cutâneas, envolvendo pele e unha, a maioria (38,70%) foi devido a *T. mentagrophytes*, sendo que as demais espécies relacionadas com esse tipo de micose, em ordem de ocorrência, foram *Candida* spp., *M. cani*s, *T. rubrum* e *T. interdigitale*[21].

*T. rubrum* é responsável por cerca de 70% de todas as infecções por dermatófitos, sendo o agente mais isolado, especialmente de onicomicoses[22]. A altíssima prevalência está correlacionada com o fato de essa espécie ser geralmente refratária ao tratamento usual devido à sua grande adaptação ao hospedeiro humano – daí sua maior facilidade em burlar as defesas inatas do paciente e permanecer como uma infecção residual, com eventuais complicações clínicas e sintomas mais severos[10].

Na Alemanha, *T. rubrum* já foi a espécie isolada em 85% dos casos de dermatofitose[23], na Finlândia em 66% [24], na Rússia em 70%[25], e na China em 44%[26]. No Brasil, em um estudo em Florianópolis, constatou-se que as infecções fúngicas de maior ocorrência foram as dermatofitoses (22,1% dos casos), sendo que *T. rubrum* foi o isolado mais frequentemente (52,3%), seguido de *T. mentagrophytes*, observado em 31,8% dos casos[27]. Essas altas porcentagens evidenciadas nos mais variados trabalhos corroboram o documentado para os agentes etiológicos das dermatofitoses, de que *T. rubrum* é de fato

o principal dermatófito envolvido na maioria dos casos, no passado e também atualmente[28].

Outras espécies comumente relatadas como agentes etiológicos são *T. tonsurans*, *T. schoenleinii* e *T. concentricum*[29]. No Brasil, o *T. tonsurans* apresenta uma acentuada predominância nas regiões Norte/Nordeste, sendo um importante patógeno emergente de dermatofitose, mais especificamente no couro cabeludo[14].

*T. violaceum* está envolvido principalmente nos casos de *tinea capitis*, *tinea corporis* e *tinea ungueal*. Smriti et al. (2015) relataram dois casos de *tinea corporis* e *tinea ungueal* em crianças da Índia. Visto que infecções por essa espécie são raras e a incidência varia geograficamente, é importante o relato das mesmas para que se evite a transmissão por contato direto[30]. Magill et al. (2007) relataram um aumento na incidência de *T. violaceum* e *T. soudanense* isolados de pele, cabelo e unhas na região de Baltimore, EUA, onde antes havia relatos esporádicos. Esse aumento está relacionado à imigração africana. Um estudo realizado na Finlândia reportou isolamento de *T. violaceum* onde antes não havia relatos, também relacionado à imigração africana. Na Líbia, *T. violaceum* é o agente etiológico mais comum encontrado em *tinea corporis*, responsável por 44% do total de dermatofitoses[30].

*Epidermophyton floccosum* é um dermatófito patógeno que se oportuniza especialmente de pacientes imunocomprometidos, iniciando a infecção na pele do hospedeiro. É descrito como um fungo que têm preferência por queratina em tecido morto, sendo ele da virilha, pés e unhas, demonstrando também uma relação com *tinea ungueal* e *tinea manuum*[12].

Com relação à epidemiologia geral das espécies de dermatófitos em nível de Brasil, as condições ambientais da Amazônia, como alta temperatura e umidade relativa do ar, proporcionam circunstâncias favoráveis à dispersão e ao desenvolvimento de

fungos, elevando a ocorrência e propagação de infecções micóticas, de acordo com as características climáticas de cada área. A epidemiologia das espécies dermatofíticas e, consequentemente, das infecções ocasionadas por esses fungos vem mudando no decorrer dos anos devido às condições próprias do ambiente, às características socioeconômicas da população e ao turismo, que proporciona uma maior migração dos microrganismos de sua região de origem para regiões onde antes essas espécies não eram relatadas como causadoras de dermatofitoses. Como resultado da migração, por exemplo, estão sendo relatados casos de infecção por *T. violaceum* e *M. audouinii* na Europa e América do Norte, enquanto antes só existiam na Ásia e na África do Sul[31]. Desse modo, o perfil de prevalência das espécies dermatofíticas é bem variado[29], como se pode observar na Tabela 1.

As mudanças epidemiológicas com relação à ocorrência de dermatofitose torna difícil determinar a incidência e prevalência desses fungos especificamente como patógenos, já que muitas vezes estão dispersos no meio e até presentes no hospedeiro, sem necessariamente causar a infecção. Corroborando esse fato, a incidência de determinadas espécies é claramente específica a cada região, visto que, no sul do Brasil, *M. canis* é o principal causador de dermatofitoses, correspondendo a 60% dos agentes etiológicos. Já na região Norte do país, encontramos relatos de *T. tonsurans* acometendo 74% dos pacientes infectados e *M. canis* representando a causa do desenvolvimento da micose em apenas uma pequena parcela dos casos[31].

Adicionalmente, um outro exemplo clássico das variações epidemiológicas dos dermatófitos ocorre também para *E. floccosum*. Essa espécie já foi muito relatada em algumas regiões, como no Brasil e Alemanha, onde atualmente não tem mais uma prevalência considerável[29]. As razões pelas quais há essa mudança de perfil da presença de determinados dermatófitos ou dermatofitoses em diferentes regiões são múltiplas:

(a) Aumento da expectativa de vida da população, que eleva o índice de prevalência de dermatofitoses, já que essas são comumente relatadas em idosos acima dos 65 anos[32]. Nessa população, é importante avaliar os fatores que afetam a resposta terapêutica[33], como anomalias de unha, comorbidades (doença vascular periférica, diabetes, imunossupressão e traumas físicos)[32], impacto farmacológico, patógeno causador e risco de interações medicamentosas[34];

(b) Aumento gradativo dos processos de migração e miscigenação, que afetam a distribuição das espécies dermatofíticas[29];

(c) Maior contato das pessoas com animais domésticos, que podem ser a fonte principal de disseminação de alguns dermatófitos[35];

(d) Impacto dos antifúngicos ambientais (utilizados na área agronômica), evidenciando que, para os fungos, há uma seleção de determinadas espécies em meios nos quais faz-se a utilização de pesticidas ou agrotóxicos[36]. Tais produtos exercem efeito na ocorrência ou no desaparecimento de alguns fungos por região. Espera-se, dessa forma, esse mesmo comportamento de pressão seletiva sobre as espécies dermatofíticas, o que corrobora a epidemiologia variada desse tipo de fungo[37].

**Formas clínicas**

As infecções podem ser classificadas de acordo com a localização anatômica da lesão, utilizando a denominação *tinea* para todas as dermatofitoses, seguida do sítio anatômico onde se localiza a infecção. As modalidades dermatofíticas mais relevantes são: *tinea capitis, tinea corporis, tinea cruris, tinea unguium, tinea barbae, tinea manuum* e *tinea pedis*[38].

*Tinea capitis* acomete couro cabeludo, sobrancelhas e cílios, afetando principalmente crianças e sendo pouco frequente em adultos[39]. É causada principalmente

por fungos dos gêneros *Microsporum* e *Trichophyton*, caracterizando-se por lesões que variam de uma forma branda e descamativa a uma forma mais eritematosa, acompanhada de alopecia, e que podem tornar-se severamente inflamadas, formando lesões ulceradas profundas[40]. Podem apresentar-se sob três formas: (a) favosa, com crostas compostas de restos celulares epiteliais e massas densas de hifas; (b) tonsurante, mais comum, caracterizada pela presença de uma ou mais lesões circulares e descamativas, podendo ainda se diferenciar em dois tipos – microspórica (lesões únicas, grandes e arredondadas) e tricofícia (lesões múltiplas com intensa descamação); e (c) quérion, com quadro inflamatório intenso[40]. Em função dos agentes etiológicos, as manifestações clínicas das lesões também podem variar. *M. gypseum, T. mentagrophytes* e *T. verrucosum* provocam quadros agudos com intensa reação inflamatória e lesões na forma de quérion com microabscessos. *T. schoenleinii* causa escútula fávica, com crostas de coloração amarelada extremamente aderentes ao couro cabeludo[39]. Além disso, os dermatófitos podem parasitar os pelos em três padrões principais: (a) endotrix (artroconídios localizados no interior dos fios, comumente ocasionado pelas espécies *T. tonsurans e T. violaceum*); (b) ectotrix (artroconídios em torno do eixo do cabelo, como observado em *M. canis* e *T. mentagrophytes*); e (c) favus (hifas e espaços de ar no interior da haste capilar, como ocorre para *T. schoenleinii*)[39].

*Tinea capitis* tem ocorrência mundial, sendo considerado um problema de saúde pública na África[41]. Em um estudo realizado em Camarões, país da região ocidental da África Central, com 4.601 crianças entre 10 a 16 anos, 377 (≈ 8,2%) apresentavam lesões típicas dessa patologia no couro cabeludo. O isolado mais prevalente foi *T. soudanense* (56,8%), seguido por *T. rubrum* (29,2%)[41]. Na Tunísia, 378 pacientes foram diagnosticados com *tinea capitis*; desses, 367 eram crianças e 11 eram adultos. Um percentual de ≈ 64% estava relacionado com lesões microspóricas, sendo *M. canis* o

agente mais isolado nesses casos (99,14%), e ≈ 36% manifestavam lesões do tipo tricofícias, com *T. violaceum* como principal responsável por praticamente todos os casos (98,48%)[42]. Na Argélia, foi realizado um estudo retrospectivo dos casos dessa micose do couro cabeludo durante 3 anos (2010 a 2013), sendo verificado que para um total de 213 amostras de cabelo, 133 foram positivas (exame direto ou cultura). A média de incidência foi de 44 casos por ano, e 91% dos pacientes tinham menos de 12 anos. Três espécies de dermatófitos foram isoladas nesses casos: *T. violaceum* (66%), *M. canis* (32,5%) e *T. mentagrophytes* (1,5%)[43]. Embora a incidência de *tinea capitis* seja evidentemente maior em crianças e adolescentes, não só em regiões africanas como em outros países também[44], já se relataram pequenos surtos em pacientes idosos, com grave acometimento do couro cabeludo[45]. Por consequência, é considerada uma das dermatofitoses de maior relevância clínica internacionalmente, tanto que, em 2014, a Associação Britânica de Dermatologistas estabeleceu diretrizes padrão para a detecção e principais tratamentos desse tipo de micose[46]. No Brasil, *T. tonsurans* e *M. canis* são os principais agentes de *tinea* do couro cabeludo, e as crianças pré-púberes representam o grupo demográfico mais acometido[44].

*Tinea corporis* acomete principalmente ombros, tronco, braços e, de modo mais ocasional, face, sendo frequentemente relatada em crianças[47]. As lesões se manifestam geralmente com aspecto anelar, sob a forma de pequenos eritemas de contorno delimitado[39]. Os principais fungos envolvidos pertencem às várias espécies de *Microsporum* e *Trichophyton*[48]. O dermatófito mais comumente isolado em todo o mundo nesses casos é o *T. rubrum,* seguido por *T. mentagrophytes*[47]. Há variantes crônicos de *tinea corporis*, como granuloma de Majocchi, com vesículas pápulo-eritematosas, e *tinea imbricata*, caracterizada por anéis concêntricos por todo o corpo, a qual ocorre endemicamente entre os indígenas de certas ilhas do Oceano Pacífico, das Américas do

Sul e Central e sudeste da Ásia, tendo *T. concentricum* como principal agente etiológico antropofílico[49].

Na Índia, a *tinea corporis* é a mais comum dermatofitose observada, sendo mais frequente em pacientes do sexo masculino[11]. O principal agente etiológico envolvido é *T. rubrum*, e as lesões mais observadas incluem placas anulares eritematosas[11]. Na França, identificou-se um caso em um paciente adulto com lesões extensas no tronco e nos membros inferiores[50], bem como na Espanha em uma paciente de 21 anos, com lesão na região interescapular, isolando-se neste caso *M. canis*[51]. Na Alemanha, já se relatou um surto por *T. tonsurans* em 46 crianças e adolescentes, com idade entre 7 a 17 anos. Nesse mesmo país, em 2007, evidenciou-se a ocorrência de cinco casos com lesões atípicas (bolhas na pele)[52].

No Brasil, 137 crianças menores de 12 anos clinicamente diagnosticadas com *tineas* foram submetidas ao exame micológico do raspado de pele e unhas, pelos e pus das lesões. Meninos na faixa etária de 2 a 12 anos foram os mais acometidos; *tinea capitis* por *M. canis* foi a mais frequente (78 casos) e *tinea corporis* por *T. rubrum* foi a segunda forma clínica mais observada neste estudo (43 casos)[53]. Em São Paulo, entre os anos de 1992 e 2002, *tinea corporis* foi a forma clínica dermatofítica de maior ocorrência nesse estado, tendo como principal agente etiológico *T. rubrum*[54]. No RS, *T. mentagrophytes* foi o principal dermatófito envolvido na maioria dos casos[22].

*Tinea cruris* envolve as regiões perineais, inguinais e perianais, de forma aguda ou crônica, sendo mais frequente em adultos[39]. As lesões se manifestam sob a forma de placas avermelhadas, descamativas, marginadas, bilaterais ou unilaterais com sensação de queimação e prurido intenso nas áreas afetadas, chamadas de “eczema marginado de hebra”[55]. As principais espécies envolvidas são *T. mentagrophytes*, *T. rubrum* e *E.*

*floccosum*. Encontra-se em todas as partes do mundo, mas é mais prevalente nas regiões tropicais, onde a umidade favorece a colonização do dermatófito[39].

Na Índia, *tinea cruris* é a segunda forma clínica de maior prevalência, sendo que *T. rubrum* é o dermatófito isolado da maioria dos casos[11]. Na Espanha, em 2008, descreveu-se casos em dois pacientes oriundos da Nigéria com lesões nos glúteos, ocasionadas por uma nova variedade de *T. rubrum* – *T. rubrum* var. *raubitschekii*[56]. No Brasil, um estudo cujo objetivo foi determinar a epidemiologia em São Paulo foi conduzido de abril de 1995 a março de 1997. Um total de 2.000 pacientes foram investigados, dos quais 105 foram selecionados por apresentarem algum sintoma condizente. A cultura foi positiva para os dermatófitos em ≈ 63% dos casos, com *T. rubrum* sendo a espécie mais prevalente (90% dos casos), seguido por *T. tonsurans* (6%) e *T. mentagrophytes* (4%)[57].

*Tinea unguium*, *tinea* das unhas ou onicomicose dermatofítica é uma invasão da lâmina ungueal ocasionada por dermatófitos, considerada uma das micoses de diagnóstico e tratamento mais difíceis[58]. As unhas ficam extremamente frágeis, podendo ocorrer o aparecimento de sulcos, fraturas e calos, com aspecto geralmente amarelado, também podendo ficar arroxeadas ou esbranquiçadas. As unhas dos pés são mais frequentemente infectadas por dermatófitos do que as unhas das mãos, sendo isso resultado de uma possível coinfecção dermatofítica nos pés[55].

O comprometimento das unhas pode ser subungueal (distal e/ou proximal) ou superficial (leuconíquia micótica/tricofítica/branca), sendo *T. rubrum* e *T. mentagrophytes* var. *interdigitale* as espécies mais frequentemente implicadas[55]. Digno de nota, as onicomicoses em geral (infecções fúngicas das unhas) podem ser causadas por agentes outros que não somente dermatófitos, tais como *Candida albicans*, *Scytalidium* spp., *Geotrichum candidum*, *Aspergillus* spp., *Trichosporon* spp. e *Scopulariopsis*

*brevicaulis*. Embora haja essa variedade de microrganismos como potenciais envolvidos nos casos de micoses de unha, vários estudos citam os fungos dermatofíticos como os principais envolvidos na totalidade das onicopatias micóticas (80% dos casos), seguidos por leveduras (5 a 17%) e fungos filamentosos não dermatofíticos (2 a 12%)[59]. As onicomicoses dermatofíticas figuram entre as mais prevalentes formas clínicas de dermatofitose, tendo inclusive testes específicos, mais recentemente, para detecção de dermatófitos nas unhas[60].

Dados epidemiológicos indicam que a incidência de *tinea unguium* aumenta com a idade, chegando a atingir 30% de indivíduos com mais de 60 anos[61] e sendo pouquíssimo frequente em crianças[53]. Quanto aos dermatófitos mais envolvidos estão *T. rubrum* (60%), *T. mentagrophytes* (20%) e *E. floccosum* (10%)[60]. No Irã, realizou-se um estudo, entre 1994 e 2004, com 590 pacientes com alguma onicopatia (354 do sexo feminino e 236 do sexo masculino), constatando-se *tinea unguium* em 41 casos (7%); entre esses, 66% foram ocasionados por dermatófitos zoofílicos, 31% por antropofílicos e 3% por geofílicos[62].

*Tinea barbae* ou *tinea* da barba afeta regiões pilosas do pescoço e da face e, por consequência, é mais restrita a homens adultos[55]. Apresenta dois quadros clínicos distintos: (a) o tipo leve/superficial, com lesões vesículo-pustulares e reação do hospedeiro mais amena; e (b) o tipo mais profundo e pustuloso, com formação de pústulas foliculares e crostas que podem resultar na formação de nódulos, semelhante clinicamente à foliculite bacteriana[55]. Na África, *tinea barbae* é relativamente comum[63]. Nos EUA, em 2002, foi diagnosticado um caso em um paciente de 22 anos, ocasionado por uma espécie do gênero *Trichophyton*[64]. No Brasil, em um estudo na Paraíba, verificou-se a incidência em 1% da população[65].

*Tinea manuum* refere-se às infecções por dermatófitos nas superfícies planares das mãos e regiões interdigitais. Quanto às manifestações clínicas, há amplo comprometimento palmar, geralmente com hiperqueratose unilateral difusa, lesões esfoliativas, eritematosas ou papulosas discretas. As espécies mais frequentemente relacionadas são antropofílicas – *T. mentagrophytes* e *T. rubrum*[39].

Na Itália, em 2003, relatou-se um caso de *tinea manuum* bolhosa em um agricultor de 36 anos. A lesão, na mão direita, assemelhava-se à dermatite de contato, isolando-se, neste caso, *T. verrucosum*[66]. Em 2004, na Espanha, descreveu-se dois casos de intensa inflamação em crianças[67]. Na China, é considerada uma micose muito comum, sendo *T. rubrum* seguida por *E. floccosum*, *T. violaceum* e *T. mentagrophytes* as espécies mais relacionadas[68]. No Brasil, em SP, entre 1992 e 2002, *tinea manuum* teve um percentual de ocorrência de ≈ 2%, sendo *T. rubrum* o dermatófito mais prevalente[54].

*Tinea pedis* é uma infecção dermatofítica que envolve particularmente as solas e os dedos dos pés. Considera-se uma das doenças micóticas que mais acometem o homem, figurando entre as de maior ocorrência em todo o mundo[39]. Estima-se que 30 a 70% da população mundial apresentam ou apresentaram t*inea pedis*, muitas vezes com evolução clínica oculta ou subclínica[69]. É ocasionada particularmente por dermatófitos antropofílicos – *T. rubrum*, *T. mentagrophytes* var. *interdigitale* e *E. floccosum*[39].

Na Espanha, em 2009, procedeu-se um estudo epidemiológico em crianças da cidade de Barcelona, verificando-se *tinea pedis* como a micose mais frequente, com *T. mentagrophytes* sendo a principal espécie relacionada aos casos. Em 2002, em Hong Kong, foram investigados 1.014 pacientes, constatando-se pé de atleta em 20,4% dos indivíduos analisados[70]. Em 2011, na Bélgica, relatou-se um caso interdigital bem grave em uma paciente diabética[71]. No Brasil, já foram reportados alguns casos com lesões mais

localizadas na região plantar do que interdigital e tendo *T. rubrum* como principal agente etiológico[72].

Além de todas as manifestações clínicas das formas de dermatofitose isoladas em cada paciente, cabe ressaltar a ocorrência de coinfecções dermatofíticas[11]. Já foram relatadas infecções mistas envolvendo mais de um tipo de *tinea* em um mesmo paciente, ou um tipo de *tinea* ocasionada por vários agentes etiológicos dermatófitos, ou ainda a manifestação simultânea da *tinea* e outra patologia (de origem micótica ou não) [73]. Na Índia, de 400 casos de dermatofitose, 14 (3,5%) eram infecções dermatofíticas simultâneas em um mesmo indivíduo. Nesse mesmo estudo, verificou-se que o mais ocorrente é a combinação de *tinea corporis* com *tinea cruris*, e de *tinea unguium* com *tinea manuum*[11]. Já foi relatado também um *mix* de parasitismo em infecções micóticas, relacionado a coinfecções por dermatófitos e *Aspergillus fumigatus*, *Candida albicans* e *Mucor* spp.[74].

Uma combinação de fungos dermatófitos, fungos saprófitas, leveduras e bactérias Gram-positivas e Gram-negativas pode ser evidenciada a partir de indivíduos com *tinea interdigital* crônica[75]. Na erisipela, também pode ocorrer essa associação entre fungos e bactérias, acometendo gravemente os pacientes. Problemas decorrentes disso, assim como a morbidade e o desconforto para o paciente, têm sido um fator importante para novas investigações sobre o assunto, tendo em vista que muitos aspectos clínicos, patogenicidade e tratamento dessas infecções ainda não estão totalmente esclarecidos. Um estudo analisou 200 pacientes com infecções de pele a fim de identificar erisipela; dessas amostras, 93 (46,5%) foram diagnosticadas como tal, o que evidencia a importância clínica dessa doença, principalmente em pacientes idosos[76].

O amplo espectro dos tratamentos tópicos e sistêmicos utilizados com antifúngicos, e até mesmo a presença de bactérias capazes de fornecer efeito fungistático

ou fungicida, acabam por inibir os dermatófitos que possam estar causando determinada infecção na pele, dificultando o isolamento e identificação do real agente causador. Desse modo, os dermatófitos desempenham um papel importante na patogênese de várias doenças que não só exclusivamente micoses, podendo ocorrer em associação com outros microrganismos. Em decorrência desse contexto, a dificuldade de demonstrar a sua presença não significa sua ausência propriamente dita, e as dificuldades de tratamento nesses casos de coinfecções simultâneas tornam-se ainda maiores[75].

Na Bulgária e Grécia, há prevalência de infecções fúngicas em unhas de pacientes com psoríase vulgar. Dos 228 pacientes com alterações nas unhas e psoríase vulgar, 62% tinham uma cultura de fungos positiva e, em 67% desses dermatófitos foram isolados, concluindo-se que a incidência de onicomicose de origem dermatofítica é maior em pacientes com psoríase[77].

Outras patologias que servem como fator de predisposição ao desenvolvimento de dermatofitose são: distúrbios circulatórios, linfodema de membros inferiores e microtraumas. Pode-se destacar diabetes melito, imunossupressão, insuficiência venosa crônica, perturbações da imunidade celular e predisposição genética como importantes fatores de risco para o desenvolvimento de onicomicose[29].

## Terapêutica das dermatofitoses

### Tratamento convencional

A escolha do tratamento mais apropriado é determinada pela extensão da infecção, pelo sítio anatômico afetado e pela espécie dermatofítica envolvida. Pode ser tópico ou sistêmico[3].

O tratamento com antifúngicos tópicos pode servir como apoio à terapêutica oral ou profilaxia. Mais comumente são utilizados cremes com cetoconazol, isoconazol,

miconazol, clotrimazol, bifonazol, terbinafina, butenafina e ciclopirox olamina. O ciclopirox olamina é popular na prática clínica para o tratamento tópico de onicomicoses[3].

A terapia sistêmica é indicada para lesões generalizadas, recorrentes, de caráter crônico, ou quando não há resposta à terapia tópica. O tratamento sistêmico oral é realizado principalmente com uso de terbinafina, griseofulvina e derivados azólicos como cetoconazol e fluconazol[3].

**Ciclopirox olamina:** mostrou-se eficiente em estudos realizados para tratamento de dermatofitoses com creme e gel[78].

**Terbinafina:** apresenta eficácia contra espécies do gênero *Trichophyton*[79], sendo recomendado seu uso para lesões não muito extensas[80]. O tempo de tratamento com terbinafina é menor se comparado a outros antifúngicos[81].

**Butenafina:** Possui atividade fungicida primária contra dermatófitos, tais como *T. mentagrophytes*, *M. canis* e *T. rubrum*. Relata-se que é eficaz mais especificamente no tratamento de *tinea pedis*, *tinea cruris* e *tinea corporis*[82, 83].

**Griseofulvina:** distribui-se largamente por variados tecidos queratinizados, caracteriza-se como um dos primeiros antifúngicos de escolha para a terapêutica das *tineas* e apresenta uma maior aplicabilidade para o tratamento das dermatofitoses ocasionadas por espécies do gênero *Microsporum*, especialmente *M. canis*[84].

**Cetoconazol:** é eficaz no tratamento da blastomicose, histoplasmose, coccidioidomicose, paracoccidioidomicose, *tinea versicolor*, candidíase mucocutânea crônica, oral e esofagiana, vulvovaginite por *Candida* e infecções dermatofíticas cutâneas[85]. Estudos demonstram que o creme de cetoconazol a 2% tem sido eficaz e seguro no tratamento de *tinea pedis*, *tinea cruris* e *tinea corporis*, atuando em infecções ocasionadas pelas espécies dos três gêneros – *Microsporum*, *Trichophyton* e *Epidermophyton*.

**Fluconazol:** é uma opção terapêutica eficaz no tratamento da *tinea capitis* em crianças, com aprovação pelo Infarmed – Autoridade Nacional do Medicamento e Produtos de Saúde, de Portugal, para uso em idade pediátrica[86]. Apesar de o fluconazol ser um dos agentes antifúngicos mais utilizados para o tratamento sistêmico de dermatofitose, constitui-se como o menos eficaz na prática clínica, pois sua atividade é bastante variada em relação ao agente causador da infecção[3].

**Tratamento combinado:** a associação de antifúngicos tem produzido cada vez mais avanços no tratamento das dermatofitoses, principalmente quando se trata de casos mais graves e disseminados. Já foram descritas na literatura combinações entre terbinafina e itraconazol oral administrados de forma consecutiva, tioconazol tópico e griseofulvina oral, terbinafina e ciclopirox olamina, griseofulvina e amorolfina, entre outras[87]. Algumas dessas combinações ainda não possuem ação total sobre dermatófitos resistentes; porém, todas apresentam evidências de efetiva melhora dos quadros clínicos infecciosos quando comparadas com a monoterapia oral ou tópica tradicional[48].

Nota-se uma potente sinergia entre a terbinafina e antifúngicos azólicos, o que aumenta consideravelmente a chance de sucesso terapêutico. Os mecanismos de ação complementares da terbinafina e azóis em diferentes pontos da via de biossíntese do ergosterol fúngico, teoricamente, têm um impacto maior na inibição e até mesmo morte do microrganismo[88]. No Brasil, são comumente comercializadas associações dos antifúngicos mais recomendados para as *tineas* com anti-inflamatórios e antibióticos, por exemplo, cetoconazol e betametasona, ou cetoconazol, betametasona e neomicina, entre outras formulações, a fim de amenizar os sinais inflamatórios locais e gerais decorrentes da infecção fúngica, além de auxiliar no processo de cicatrização das lesões[89].

**Novos compostos com potencial anti-dermatofítico**

Com relação aos produtos de síntese, em 2001, a atividade antifúngica de cinco novos compostos sintéticos foi avaliada contra *T. rubrum* e *E. floccosum*, sendo que os dois pirazóis-tiocianatos testados se mostraram altamente ativos contra ambas as espécies dermatofíticas[90]. Em 2014, um estudo avaliou as atividades antifúngicas de naftoquinonas sintéticas contra dermatófitos, constatando que todas apresentaram atividade contra *M. canis*, *M. gypseum*, *T. rubrum* e *T. tonsurans*, relacionando-se o mecanismo de ação à lise na membrana celular dermatofítica[91].

**Problemas relacionados à terapêutica e resistência dermatofítica aos antifúngicos**

Apesar dos fármacos antifúngicos atuarem por diferentes mecanismos e vias, os alvos celulares são limitados em função da similaridade que existe entre as células eucarióticas fúngicas e humanas[92]. Os relatos de recorrentes falhas na terapêutica das dermatofitoses são normalmente associados à descontinuação da terapia, aos efeitos adversos de alguns antifúngicos e à problemática de resistência dermatofítica[7].

Além dos inconvenientes da terapia anti-dermatofítica convencional prolongada e dos efeitos adversos em alguns casos, comuns a qualquer medicamento, não só antifúngicos, observa-se cada vez mais o surgimento de cepas resistentes ou multirresistentes ao tratamento[93]. O crescente aumento da incidência de infecções fúngicas, aliado à síndrome da imunodeficiência adquirida (SIDA) e ao aumento da utilização de imunossupressores, foi seguido por uma utilização consequentemente mais ampla e frequente de antifúngicos, com intenções profiláticas e terapêuticas. A utilização demasiada ou em doses abusivas e inadequadas dos antimicóticos faz com que as espécies fúngicas desenvolvam resistência por evoluções epigenéticas e em resposta ao estresse, dificultando fortemente o tratamento. Essa resistência pode ser intrínseca ou adquirida[92].

Antifúngicos azóis (por exemplo, fluconazol e itraconazol), que têm sido amplamente utilizados no tratamento de infecções fúngicas superficiais causadas por dermatófitos, são igualmente associados ao desenvolvimento de resistência[94]. Citam-se como prováveis mecanismos de resistência ao fluconazol, por exemplo, o aumento do efluxo do medicamento e a adaptação ao estresse – resposta não específica à exposição do fungo ao antifúngico[95].

Agregando-se à toda essa problemática apresentada, infelizmente, o arsenal terapêutico dos antifúngicos apresenta espectro de atividade muito variável, podendo levar a falha no tratamento *in vivo*, possivelmente devido aos multifatores já mencionados[96]. Desse modo, há uma real necessidade de novos agentes antifúngicos mais eficazes e menos tóxicos, o que torna a pesquisa com novos compostos anti-dermatofíticos muito pertinente e relevante[97].

**Conflito de interesses**

Nenhum conflito de interesse declarado.

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**Tabela 1**. Principais dermatófitos antropofílicos e epidemiologia relacionada.

| | |
|---|---|
| *Epidermophyton floccosum* | Cada vez mais raro, causa infecções nos pés, unhas dos pés e virilha. |
| *Microsporum audouinii* | Sua incidência está principalmente na África Subsaariana. |
| *Microsporum ferrugineum* | Encontrado principalmente na Ásia e raramente na África ou no Leste europeu. |
| *Trichophyton concentricum* | Presença limitada ao sudeste da Ásia, causador de *tinea imbricata*. |
| *Trichophyton interdigitale* | Segundo dermatófito mais comum na Alemanha. |
| *Trichophyton megninii* | Muito raro, causador de *tinea pedis*, *tinea manuum*, *tinea ungueal* e *tinea barbae*. |
| *Trichophyton rubrum* | Dermatófito mais comum de incidência global, causa *tinea ungueal*, *tinea pedis* e *tinea corporis*, com rara invasão aos cabelos. |
| *Trichophyton rubrum* var. *raubischekii* | Variante do *T. rubrum*, presente basicamente na África, com relatos isolados de infecção na Alemanha, Turquia, Espanha e Ásia. |
| *Trichophyton schoenleinii* | Raramente relatado na Europa. |
| *Trichophyton tonsurans* | Dermatófito muito comum na atualidade, é causador de *tinea capitis* na América. Na Alemanha, acomete praticantes de artes marciais. |
| *Trichophyton violaceum* | Principal dermatófito da África. |
| *Trichophyton soudanense* | Encontrado mais especificamente na África, é genotipicamente idêntico ao *T. violaceum*, mas os dois se diferem fenotipicamente. |
| *Trichophyton vanbreuseghemii* | Muito raramente isolado a partir de seres humanos (pele) ou do solo. |

**CAPÍTULO II – Nanoemulsion improves the antifungal activity of allylic thiocyantes against yeasts and filamentous pathogenic fungi**

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Medicinal Chemistry & Drug Discovery

# Nanoemulsion Improves the Antifungal Activity of Allylic Thiocyanates against Yeasts and Filamentous Pathogenic Fungi

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**Nanoemulsion improves the antifungal activity of allylic thiocyanates against yeasts and filamentous pathogenic fungi**

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**ABSTRACT**

We report the antifungal and antichemotactic activities of a series of allylic thiocyanates with low toxicity. We also show improved antifungal activity of the most promising compound when used in a nanoemulsion (NE). The 4-chlorophenyl-substituted allylic thiocyanate (compound 11) exhibited a broad spectrum of antifungal activity and showed antichemotactic effects with 100% reduction in leukocyte migration. Minimal inhibitory concentrations ranged from 25 to 50 μg $mL^{-1}$, and the mechanism of action was related to complexation with fungal ergosterol. The NE containing compound **11** enhanced the antifungal activity approximately 64-fold for dermatophytes and 4-fold for *Candida* spp.. Compound **11** was not mutagenic and did not cause cell death or significant haemoloysis, although it exhibited mild dose-dependent DNA damage. It was not an irritant for chorioallantoic membrane of fertile white eggs and exhibited 100% inhibition of fungal growth in an *in vivo* model of dermatophytosis. Our data indicate that allylic thiocyanates are very promising for the antifungal potential in nanostructured systems, with associated anti-inflammatory effect.

**Keywords**: Allylic thiocyanates, Antifungal activity, Nanoemulsion, Toxicity.

## Introduction

Fungal infections produce high rates of morbidity and mortality, especially in severely ill or immunocompromised patients.[1,2] *Candida albicans* is a prominent fungal pathogen in humans. It is responsible for a wide spectrum of clinical presentations, and infection can lead to death.[3] In the last 20 years, *C. albicans* has been the most common strain isolated from hospitalised patients. However, non–*albicans Candida* (NAC) infections are rapidly growing. *C. albicans*, *C. glabrata*, *C. tropicalis* and *C. krusei* account for 95–97% of all invasive fungal infections caused by yeast of this genus.[4] In addition to yeast infections, fungal infections caused by dermatophytes have increased during the last decades.[5] These conditions also lead to morbidity-associated cutaneous mycoses that are frequently ineffectively treated.[6]

Illnesses caused by *Candida* spp. are associated with inflammatory processes (also observed for dermatophytoses)[7,8] that are possibly exacerbated by enzymes secreted by the fungus during invasion.[9] Therefore, uncontrolled inflammation can compromise treatment and lead to other associated diseases.[10,11] Chemical compounds have been investigated for their ability to inhibit leucocyte migration through an anti-inflammatory mechanism (antichemotactic activity).[12] Thus, it is interesting to investigate if new libraries of molecules exert antifungal and anti-inflammatory effects.

Although numerous effective antifungal agents are available, their therapeutic outcome is less than optimal due to limitations associated with toxicity and physicochemical characteristics. Nanoparticles hold the promise to overcome these problems due to their ability to improve bioavailability, antifungal efficacy and aqueous solubility. Further, drug incorporation into a nanoemulsion (NE) could greatly minimise its toxicity. Despite these potential advantages, there are few marketed nanoparticle-based antifungal drug formulations, and thus research into antifungal therapy with nanostructured systems is needed.[13]

Allylic thiocyanates showed moderate-to-high activity against methicillin-resistant *Staphylococcus aureus* (MRSA),[14] *Mycobacterium tuberculosis* (Mtb),[15] human cancer cells[16] and other targets.[17] Therefore, we present the antifungal activity of functionalised allylic thiocyanates derived from the Morita-Baylis-Hillman reaction,[18] as well as the toxicity and anti-inflammatory properties of this collection of sulphur-containing derivatives. The antifungal activity of the most promising compound was also evaluated in NE and in an alternative fungal infection model.

## Results

### Nanoemulsion

NE was prepared as a white and milky liquid with a macroscopically homogeneous appearance, bluish reflection and a droplet size characteristic of colloidal systems. The pH of the thiocyanate-containing NE (**NE-C**, 7.45 ± 0.06) was slightly higher compared to the placebo formulation (prepared without the compound **11** - **NE-WC**, 7.29 ± 0.09). Granulometric analysis of the formulations revealed that the droplets were 190.42 ± 8.64 nm for the NE composed of the active thiocyanate **11** – **NE-C** and 225.3 ± 29.67 nm for **NE-WC**. Polydispersion index (PDI) values were 0.120 ± 0.04 and 0.200 ± 0.07 for **NE-C** and **NE-WC**, respectively.

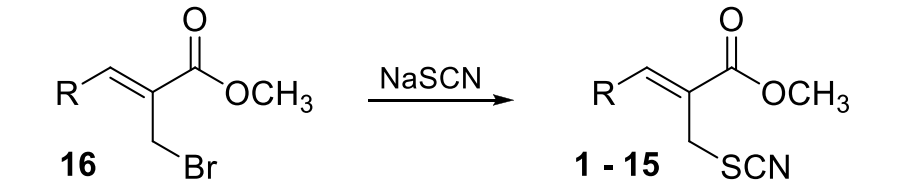
The zeta potential (ZP) values were -8.68 ± 0.31 mV and -7.32 ± 1.41 mV for **NE-C** and **NE-WC**, respectively. ZP increased slightly for **NE-C**, which can be attributed to greater physicochemical stability of the developed colloidal system. In addition, the compound showed high values of total content and encapsulation efficiency in the NEs being 96% ± 7.84 and 92%, respectively, which is attributed to the affinity of compound **11** with the oil phase of the NE.

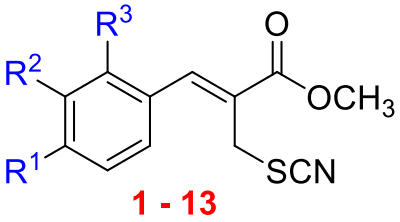
### *In vitro* antifungal susceptibility tests

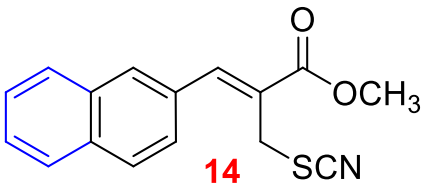
Eight fungal strains were initially used to evaluate the antifungal activity of allylic thiocyanates **1**–**15** (Scheme 1; Supporting Information, Table S1). Compounds **1**, **4**, **6**, **7**, **10**, **12**, **13** and **15** (Scheme 1) failed to inhibit fungal growth up to the maximum evaluated concentration (50 μg $mL^{-1}$). Thiocyanates **2**, **3**, **5**, **8**, **9** and **14** (Scheme 1) exhibited fungicide activity against *Candida* spp., with minimal inhibitory concentrations (MICs) of 50 μg $mL^{-1}$, but

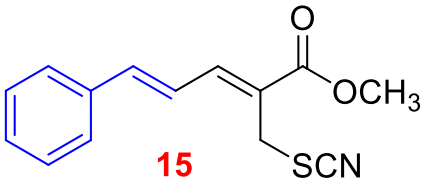
were not effective against filamentous fungi. The 4-chloro-substituted allylic derivative **11** demonstrated a broad fungistatic spectrum against dermatophytes and yeasts, with an MIC of 50 µg $mL^{-1}$ (Supporting Information, Table S1). It is noteworthy that **11** inhibited the growth of drug-resistant *T. mentagrophytes*, *C. krusei*, *C. glabrata* and *C. tropicalis* (MICs and breakpoints of commercial antifungal agents are presented in Supporting Information, Table S1). Based on these findings, compound **11** was chosen for the development of a NE.

The MICs of compound **11** in the free form and in a NE for 25 clinical strains of dermatophytes and *Candida* spp. are presented in Table 1. For some species, such as *T. rubrum*, *T. schoenleinii*, *C. parapsilosis* and others, thiocyanate **11** showed a MIC of 25 µg $mL^{-1}$. With the comparison of the MIC values that inhibited approximately 50% of the clinical strains ($MIC_{50}$) for the free compound **11** and thiocyanate-containing NE (**NE-C**) was possible to observed that **NE-C** was much more active than the free compound (decrease of MIC values up to 64-fold for dermatophytes and 4-fold for *Candida* spp.). In addition, **NE-C** was fungicidal at the MIC concentration for all fungal species analysed. This is an important advantage, since free compound **11** was only fungistatic. **NE-WC** showed no antifungal activity; thus, the other components of the NE do not exert antifungal activity.




| | $R^1$ | $R^2$ | $R^3$ |
|---|---|---|---|
| **1** | H | H | $NO_2$ |
| **2** | H | $NO_2$ | H |
| **3** | $NO_2$ | H | H |
| **4** | $CH_3O$ | H | H |
| **5** | H | H | H |
| **6** | $CH_3$ | H | H |
| **7** | F | H | H |
| **8** | H | H | Br |
| **9** | Br | H | H |
| **10** | H | H | Cl |
| **11** | Cl | H | H |
| **12** | Cl | H | Cl |
| **13** | $OCH_2O$ | | H |

**Scheme 1**. Chemical structure of allylic thiocyanates **1**–**15**.

**Table 1**. MIC/MFCs (µg $mL^{-1}$) of free thiocyanate **11** and nanoemulsion containing compound **11 (NE-C)**.

| **Fungal strains** | **Compound 11** | **NE-C** |
|---|---|---|
| **Dermatophytes** (n=15) | | |
| *Microsporum canis* (MCA 01) | 50/>50 | 1.56/1.56 |
| *Microsporum canis* (MCA 33) | 25/>25 | 0.78/0.78 |
| *Microsporum canis* (MCA 38) | 50/>50 | 0.78/0.78 |
| *Microsporum gypseum* (MGY5 HCPA) | 25/>25 | 1.56/1.56 |
| *Microsporum gypseum* (MGY 42) | 50/>50 | 1.56/1.56 |
| *Microsporum gypseum* (MGY 50) | 50/>50 | 1.56/1.56 |
| *Microsporum gypseum* (MGY 58) | 50/>50 | 1.56/1.56 |
| *Trichophyton mentagrophytes* (TME 16*) | 50/>50 | 0.78/0.78 |
| *Trichophyton mentagrophytes* (TME 40) | 25/>25 | 0.78/0.78 |
| *Trichophyton mentagrophytes* (TME ) | 50/>50 | 1.56/1.56 |
| *Trichophyton rubrum* (TRU 2 HCPA) | 25/>25 | 0.78/0.78 |
| *Trichophyton rubrum* (TRU 3 HCPA) | 25/>25 | 0.78/0.78 |
| *Trichophyton rubrum* (TRU 45) | 50/>50 | 0.78/0.78 |
| *Trichophyton rubrum* (TRU 48) | 50/>50 | 1.56/1.56 |
| *Trichophyton schoenleinii* (TSHO 3 HCPA) | 25/>25 | 0.78/0.78 |
| **$MIC_{50}$** | 50 | 0.78 |
| **MIC range** | 25 – 50 | 0.78 – 1.56 |
| **Yeasts** (n=10) | | |
| *Candida albicans* (CA ATCC 18804) | 50/>50 | 12.5/12.5 |
| *Candida albicans* (CA 01) | 25/>25 | 25/25 |
| *Candida krusei* (CK 02) | 50/>50 | 50/50 |
| *Candida krusei* (CK 03) | 25/>25 | 12.5/12.5 |
| *Candida glabrata* (CG 05) | 50/>50 | 50/50 |
| *Candida glabrata* (CG 09) | 50/>50 | 50/50 |
| *Candida tropicalis* (CT ATCC 750) | 25/>25 | 12.5/12.5 |
| *Candida tropicalis* (CT 72A*) | 50/>50 | 50/50 |
| *Candida parapsilosis* (CP 06) | 50/>50 | 12.5/12.5 |
| *Candida parapsilosis* (CP 07) | 25/>25 | 12.5/12.5 |
| **$MIC_{50}$** | 50 | 12.5 |
| **MIC range** | 25 – 50 | 12.5 – 50 |

*Multidrug-resistant and resistant fungal isolates; MIC, minimal inhibitory concentration; MFC, minimal fungicidal concentration; $MIC_{50}$, minimal inhibitory concentration that inhibits approximately 50% of the clinical strains analyzed; MIC range, minimum and maximum limits of MIC values; n, number of clinical strains.

***In vivo* antifungal efficacy in Infected Egg Test-Chorioallantoic Membrane (IET-CAM)**

For dermatophytes, infected CAM treated with thiocyanate **11** showed eggs without microbial growth in which the embryos were still alive at the time of incubation (Supporting Information, Table S2). We initially counted 1 x $10^2$ – 1 x $10^3$ conidia $mL^{-1}$. After the incubation period, the eggs treated with thiocyanate **11** showed the absence of fungal growth (Supporting Information, Fig. S1). Thus, compound **11** presented 100% efficiency in *in vivo* antifungal activity, considering that the infection did not develop and no embryos died after treatment with the compound. While untreated eggs scored between 1 x $10^2$ – 1 x $10^5$ conidia $mL^{-1}$ (measured in triplicate), all eggs showed fungal growth, and all embryos died.

For the examined yeast strains (Supporting Information, Table S2), fungal growth occurred on some embryos (1 x $10^4$ – 1 x $10^5$ colony forming units [CFU] $mL^{-1}$) and consequently led to death. For all clinical *Candida* strains analysed, only one out of the 3 eggs showed fungal growth after treatment with compound **11**; this embryo subsequently died. Thus, thiocyanate **11** was 66.6% effective in the yeast infection model (Supporting Information, Table S2).

Embryonic death was evaluated both by egg transluminescence and embryo heartbeat cessation. After treatment with compound **11** at a concentration 4 times higher than the MIC (200 µg $mL^{-1}$), we observed perfectly formed vessels just below the chorioallantoic membrane (Supporting Information, Fig. S1A), while these vessels were absent in untreated controls (Supporting Information, Fig. S1B). This finding suggested overall disrupted embryonic development and consequent non-viability.

**Antichemotactic assay**

Antichemotactic activity was expressed as a percentage of neutrophil migration inhibition relative to controls. Compound **11**, for all tested concentrations, significantly inhibited the leukocytes migration. Lipopolysaccharide from *Escherichia coli* (LPS; Table 2) was used as chemoattractant. Complete leucocyte migration inhibition occurred at the maximum evaluated concentration (5 μg $mL^{-1}$), while the positive control, indomethacin, inhibited approximately 60% of migration at the same concentration. Compound **11** demonstrated the potential for antichemotactic action because at a concentration 10 times lower than the MIC it was able to inhibit leucocyte migration completely.

**Table 2.** *In vitro* effect of allylic thiocyanate **11** and indomethacin compared to negative control.

| Samples | Concentration (μg $mL^{-1}$) | Migration (μm) | Migration inhibition (%) |
|---|---|---|---|
| **11** | 5 | 0.0 ± 0.0 | 100.0* |
| | 1 | 16.0 ± 2.8 | 78.0* |
| | 0.1 | 28.8 ± 8.2 | 58.6* |
| | 0.01 | 51.2 ± 8.0 | 26.2* |
| | 0.001 | 51.2 ± 4.8 | 26.2* |
| **Indomethacin** | 5 | 32.6 ± 7.8 | 59.7* |
| | 1 | 34.0 ± 5.7 | 57.9* |
| | 0.1 | 40.8 ± 14.5 | 49.5* |
| | 0.01 | 95.6 ± 7.7 | 0 |
| **Negative Control** | - | 80.8 ± 8.4 | 0 |

Mean ± standard deviation. *$p < 0.05$ indicates a significant difference compared to negative control (reference chemoattractant - lipopolysaccharide from *Escherichia coli* (LPS)) (ANOVA–Tukey's test).

**Antifungal mechanism of action**

**Sorbitol assay**

The MIC of allylic derivative **11** against *Candida* spp. and dermatophytes was evaluated in the presence and absence of sorbitol at different times using anidulafungin (AND) as an antifungal control (Supporting Information, Table S3). As expected, the MIC of **11** increased after 48, 96 and 168 h due to its fungistatic effect. However, the MICs were the same regardless of being administrated with sorbitol. Meanwhile, the minimal effective concentration (MEC) of AND changed abruptly (more than 8 times) in the presence of sorbitol (Supporting Information, Table S3).

**Ergosterol assay**

The MIC of **11** increased after addition of ergosterol to all strains of *Candida* spp. and dermatophytes. For yeast (*C. albicans*, *C. tropicalis*, *C. krusei* and *C. glabrata*), MICs increased 4-8-fold after addition of a total of 200 μg $mL^{-1}$ (maximum concentration) of ergosterol during the 5 days of the experiment (Supporting Information, Table S4). As expected, AmB demonstrated an ergosterol-dependent effect. For instance, after addition of 200 μg $mL^{-1}$ of ergosterol, the MIC of amphotericin B (AmB) against *C. tropicalis* increased from 0.5 to >128 μg $mL^{-1}$ (more than 256 times in this case; see Table S4). The same trend was observed for the dermatophytes studied. However, MICs increased more discretely in the case of filamentous fungi (for example, 2-fold for **11**; see Table S4).

**Scanning electron microscopy (SEM) analysis**

*C. albicans* was treated with either the antifungal itraconazole (ITZ) or the compound **11**. Before addition of the drugs, *C. albicans* cells appeared oval (as expected) without apparent alteration (Figure 1A-B). After treatment with ITZ, changes in cell shape and size were remarkable (Figure 1C-D; yellow arrows point to damaged cells), and damage was also observed from compound **11** treatment (Figure 1E-F; green arrows point to damaged cells). For compound **11**, the fungal cell appeared to rupture, and it was possible to visualise extravasation of intracellular material (Figure 1E; green arrows).

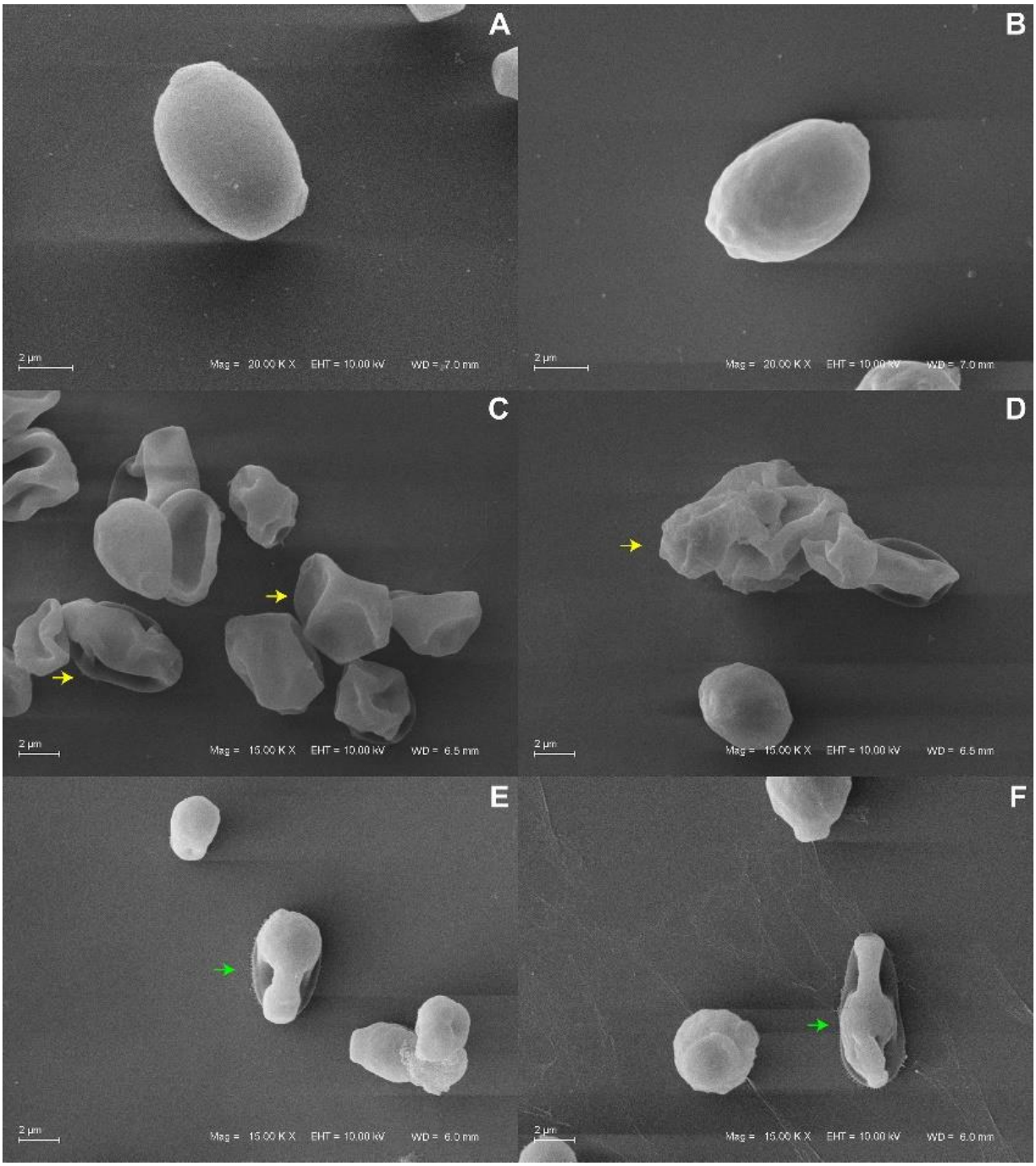



**Figure 1.** SEM images: (A and B) *C. albicans* ATCC 18804 without treatment (control); (C and D) treated with itraconazole and (E and F) treated with thiocyanate **11**.

### Toxicity evaluation

#### Cytotoxicity, genotoxicity and mutagenicity assays

Compound **11** (50 µg $mL^{-1}$) caused DNA damage similar to the hydrogen peroxide ($H_2O_2$; 100 µM) control (Figure 2-A). However, at 25 µg $mL^{-1}$, the cell damage was not significant and was comparable to phosphate-buffered saline (PBS, negative control; Figure 2-A). Approximately 70% and 90% of leucocytes were viable after treatment with compound **11** at 50 and 25 µg $mL^{-1}$, respectively (Figure 2-B). Finally, micronucleus was not significantly observed after addition of 25-50 µg $mL^{-1}$of compound **11** (Figure 2-C).

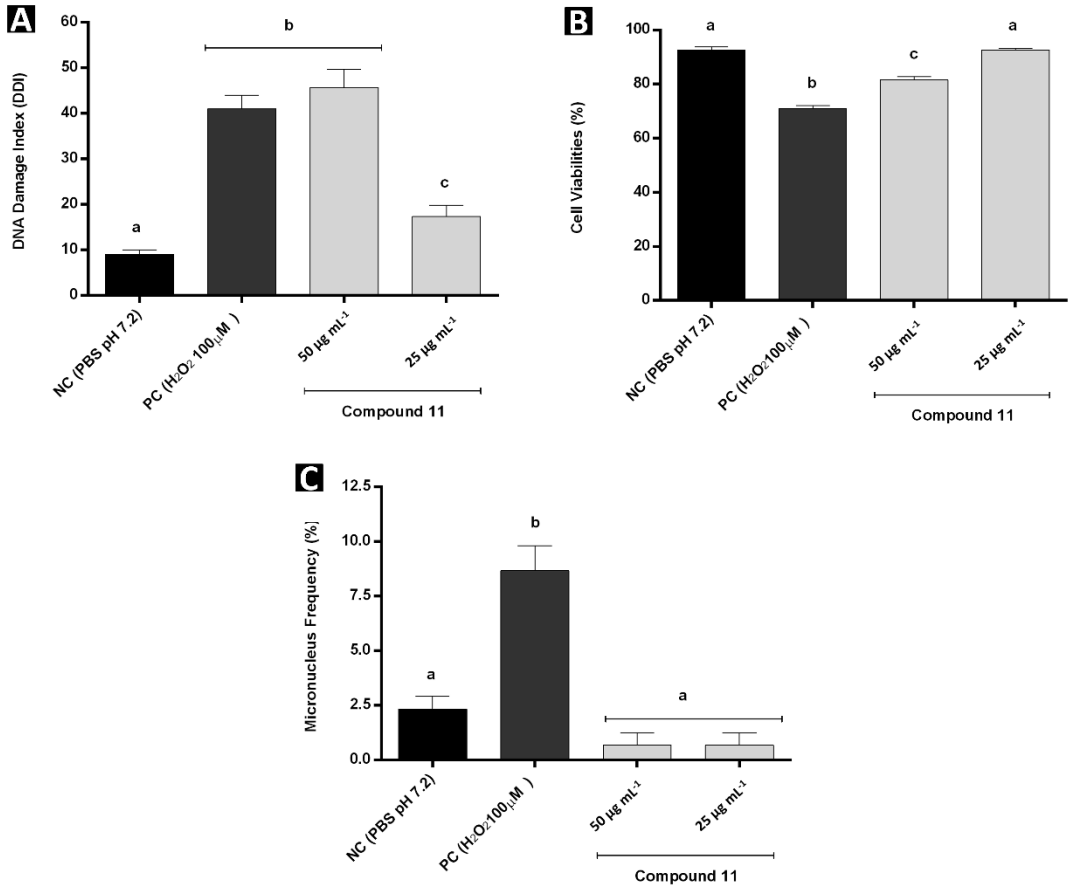



**Figure 2.** Effect of compound **11** (25 and 50 μg $mL^{-1}$) in DNA damage (A), cell viability (B) and micronucleus frequency (C). $^{a,b,c}p$ <0.05 indicates significant difference between the controls and the compound **11** (ANOVA followed by Tukey's test).

**Hemolysis**

The mean percentage of haemoloysis (± SD) induced by compound **11** at 50 μg $mL^{-1}$ and 100 μg $mL^{-1}$ (concentration 2 times higher than MIC) was 4.20% ± 0.0028 and 4.35% ± 0.0032, respectively. These values are considered very low when compared to water, which causes 100% erythrocyte lysis. PBS did not cause significant haemoloysis. These results corroborated the aforementioned cytotoxicity evaluation in human leucocytes.

**Hen's Egg Test-Chorioallantoic Membrane (HET-CAM)**

The HET-CAM analysis showed the irritation score (IS) of 3.06 ± 0.50 for the allylic thiocyanate **11**. This value classifies the compound as a nonirritant, and suggests it is not allergenic to membranes and thus suitable for future topical use.

**Discussion**

Previous data showed that thiocyanate derivative **11** exhibited moderate-to-good activity against *C. albicans* (12.5 μmol $L^{-1}$) and *C. tropicalis* (25 μmol $L^{-1}$);[14] these findings corroborate to our results. Furthermore, compounds **8**, **9**, **11** and **14** inhibited MRSA growth,[14] and thus demonstrated a broad spectrum of action against fungi and bacteria. 4-chloro-substituted thiocyanate **11** and bromo-substituted analogues **8** and **9** exhibited promising antitubercular activity against replicating and non-replicating forms of Mtb $H_{37}Rv$ (MIC 0.25 μmol $L^{-1}$) with relatively low toxicity toward VERO cells.[15] Contrarily, 2-chloro-substituted analogues **10** and **12** were inactive against Mtb (MICs >128 μmol $L^{-1}$).[15] The same trend was observed in the current study for adducts **10** and **12**, since both were

not active at concentrations up to 50 µg $mL^{-1}$ (Supporting Information, Table S1). Thus, compound **11** is a potential antimicrobial agent that exhibits broad-spectrum activity against MRSA, *Candida* spp., dermatophytes and Mtb. This profile was previously verified for imidazolium salts, with chloro-derived compounds being the most effective *in vitro* antifungal agents when compared to other analogues.[19]

Lipophilicity at the 2- and 3-position of the aromatic ring seems to play a pivotal role for activity (Scheme 1; Supporting Information, Table S1). The best results were achieved for thiocyanates **8** and **14**, both of which contained more lipophilic groups, such as 2-bromophenyl and 2-naphthyl, respectively. Other less lipophilic analogues, such as the 2-nitro- and 2-chloro-substituted thiocyanates **1** and **10**, were not active up to 50 µg $mL^{-1}$. The presence of an electron-withdrawing substituent at the aryl group, including nitro (**2** and **3**), bromo (**9**) and chloro (**11**), seemed to improve activity. The only exception to this trend was the 4-fluoro-substituted thiocyanate **7** that was also inactive to the endpoint chosen in this study. Meanwhile, thiocyanates containing electron-donating groups, including methoxy (**4**) and methyl (**6**), were not active up to 50 µg $mL^{-1}$. Chain elongation was also deleterious to activity (see the cinnamoyl derivative **15**; Supporting Information, Table S1) as well as the simultaneous presence of two substituents at the aromatic ring, such as in **12** and **13**. Hence, our screening of the thiocyanates **1**–**15** against the present panel of fungi correlated with previous results for these compounds towards Mtb[15] and bacteria,[14] allowed us to select compound **11** as the best lead for further evaluation to develop a new broad spectrum drug and NEs to treat microbial skin infections. Therefore, its mechanism of antifungal action, anti-inflammatory capacity and toxicity were determined.

Our NE displayed all characteristics expected to a nanostructure.[20-22] Droplet size and PDI may vary according to the composition of the formulations and the method employed in the preparation.[23] The presence of thiocyanate in the NE reduced the droplet size and the PDI values, and these observations indicate that this compound possess some surface activity that contributes to the formation of smaller and uniform droplets. Additionally, the **NE-C** showed a slightly higher pH value attributed to the weakly basic character of the thiocyano group. Incorporation of compound **11** in the NE (**NE-C**) potentiated its *in vitro* antifungal activity, as shown by the considerable decrease of the MIC values for dermatophytes and yeast (Table 1). AmB is a classic example of an antifungal agent that is associated with improved performance, including the control of drug delivery, lower toxicity and improved effectiveness, when it is administered in nanostructured formulations.[24,25] In addition to the lipophilic nature of the NE, high surface area due to reduced particle size improved drug permeation across biological membranes, and resulted in better drug efficiency, and bioavailability.[13,26]

With regards to *in vivo* testing, an alternative model of fungal infection in embryonated chicken eggs was utilised. Thiocyanate **11** reversed the infectious process by dermatophytic clinical strains in 100% of the analyzed eggs. For *Candida* spp., the efficiency of compound **11** was 66.6%. Thus, in addition to broad spectrum *in vitro* action, thiocyanate **11** was also effective *in vivo* by greatly reducing the microbial load associated with the infectious process, including for drug-resistant species. It is important to emphasise that our study represents for the first time, an alternative model of fungal infection in chicken eggs that has been applied for the evaluation of antifungal activity of new small molecules. Embryonic death in the treated eggs may be linked to infection by *Candida* spp. or filamentous fungi strains, but there were also eggs treated with thiocyanate **11** that resulted in dead embryos. These results, and the deaths of the embryos in the controls without inoculum, can be explained by manipulation of the eggs, but may also be related to genetic defects or embryonic development; the latter two factors are independent of the experimental procedure. The infection dose of $1 \times 10^3$ CFU $mL^{-1}$ was considered low and was likely not the determining factor for the embryo inviability.[27]

The Boyden chamber method (antichemotactic assay) was used to evaluate whether allylic thiocyanate **11** would inhibit polymorphonuclear neutrophil migration and to analyze the anti-inflammatory properties of the compound. Leukocytes migration to the site of injury is considered one of the first major steps for inflammation.[28] Our results suggested that compound **11** acted in response to an acute inflammatory process (Table 2). Mechanisms that promote inflammation and impair the antifungal immune response are continually discovered. It is known, for example, that *C. albicans* and *Aspergillus fumigatus* colonizations are associated with elevated levels of pro-inflammatory cytokines (IL-17, IL-23 and Th17).[11,29] Fungal colonization, however, does not necessarily

imply infection and disease development. The stability of the host–fungus relationship is maintained by a complex balance of pro- and anti-inflammatory intracellular signals.[30,31] Consequently, control of the inflammatory response may represent a strategy to combat fungal infections.[11] Dermatophyte metabolites generally induce inflammation at the site of infection.[32] Occasionally, accentuated inflammatory responses are also associated with increased severity and chronicity of mycoses.[31] Therefore, an antifungal substance with related anti-inflammatory properties will likely more effectively ameliorate a fungal infection.[33] Compound **11** significantly reduced neutrophil migration (part of the acute phase of inflammation) at 0.1 to 5 μg $mL^{-1}$ (10 times lower than the MIC), and these results suggest that this compound would work as a drug to treat fungal infections associated with inflammatory disorders.

Sorbitol exerts osmotic protection on the fungal cell wall by blocking chemicals from acting on this target,[34] and antifungal activity will decrease in the presence of sorbitol if a drug acts on the cell wall. Our results indicated that the antifungal effect of **11** was not related to the cell wall, since the MIC values did not vary with the addition of sorbitol.

Some antifungal drugs act by interacting with ergosterol in the cell membrane; ergosterol is an important target since it is not present in mammalian cells. The addition of ergosterol in growth medium will increase the concentration of this substance outside of the membrane and allow the drug to more easily interact with it. Consequently, if the drug's mechanism of action involves the cell membrane, it would become less active (higher MICs).[35,36] Thus, MICs of the allylic thiocyanate **11** against all fungi strains were determined in the presence of exogenous ergosterol. AmB, a commercial antifungal, was used as control (Supporting Information, Table S4). MICs of **11** increased after addition of ergosterol to all *Candida* spp. and dermatophytes, and these findings suggested a mechanism of action related to complexation with ergosterol in the cell membrane, as similarly observed for AmB. The loss of activity in the presence of exogenous ergosterol was time- and dose-dependent for *Candida* spp. (Supporting Information, Table S4). Since our results indicated that thiocyanate **11** acts on the fungal cell membrane, we next evaluated the effect of **11** on the fungal cellular structure by SEM (Fig. 1). *C. albicans* was treated with either the antifungal itraconazole or the compound **11**. In both cases, treated cells lost their internal contents in a process characteristic of plasmolysis. This observation corroborated the proposed mechanism of action of **11** against all *Candida* spp. and dermatophytes strains presented in this study.

Next, cytotoxicity studies demonstrated that the cell damage was directly related to the concentration of **11**. The mutagenic effect of **11** evaluated by the micronucleus assay indicated that this compound does not generate mutations at the concentration necessary for *in vitro* antifungal activity (Fig. 2). Besides, **11** did not cause significant leucocyte death at the evaluated concentrations (Fig. 2). While **11** did not cause significant haemoloysis, we observed a genotoxic effect at 50 μg $mL^{-1}$.

Overall, a future topical formulation that contains the chloro-substituted allylic thiocyanate **11** would be safe. This supposition is corroborated by the lack of allergenicity by HET-CAM. The HET-CAM, an alternative to the Draize test, mimics vascular changes in the chorioallantoic membrane as a model for the conjunctival ocular surface and can be a qualitative method of assessing the potential irritancy of chemicals.[37] Our results demonstrated that embryonated eggs are highly susceptible to yeast and dermatophytes infection via the CAM since these fungi proliferate radially on tissue and blood vessels. The compound **11** was classified as nonirritant by the HET-CAM assay. This result is a good indication of general low membrane toxicity.

**Conclusions**

Fifteen allylic thiocyanates were screened against a panel of *Candida* spp. and filamentous fungi. Six compounds exhibited fungicide activity against *Candida* spp. at 50 μg $mL^{-1}$. The 4-chlorophenyl-substituted allylic thiocyanate **11** demonstrated a fungistatic effect (50 μg $mL^{-1}$) against the entire fungal panel, and exhibited anti-inflammatory capability by reducing neutrophil migration. These results designate compound **11** as a possible complement to conventional antifungal therapy with the advantage of an anti-inflammatory effect, which can accelerate the relief of symptoms, facilitate healing and prevent infection dissemination. The incorporation of the compound in a NE greatly potentiated the *in vitro* antifungal activity as denoted by reduced MIC values ($MIC_{50}$ = 0.78 μg $mL^{-1}$ for dermathophytes and 12.5 μg $mL^{-1}$ for *Candida* spp.). In the *in vivo* assay, **11** completely eliminated

the dermatophytosis of infected egg chorioallantoic membrane. The mechanism of action of **11** was not related to the fungal cell wall since MICs were not altered in the presence of sorbitol. However, compound **11** formed an ergosterol complex similar to that observed for AmB, and this complex is possibly related to its broad-spectrum activity. SEM images suggested cell damage through plasmolysis and modifications of the regular yeast cell shape. Compound **11** also caused dose-dependent DNA damage in human leukocytes. Micronucleus did not occur after treatment with compound **11** at 25–50 μg $mL^{-1}$, and this finding indicated that the compound does not induce mutations in human leucocytes at concentrations that produce *in vitro* antifungal activity. In addition, 70% and 90% of leukocytes became viable after treatment with **11** at 50 μg $mL^{-1}$ and 25 μg $mL^{-1}$, respectively; the compound did not cause haemolysis. The results of HET-CAM classified this compound as nonirritant. In addition to its antifungal, antibacterial[14] and antituberculosis activities,[15] compound **11** can be easily prepared from inexpensive and readily available chemicals. NEs that use **11** as the active ingredient can be a future alternative or a complement to conventional treatments for cutaneous mycoses caused by yeast and filamentous fungal pathogens.

## Declarations

### Support material

Details of the experimental method can be found in the electronic Supporting Information, as well as complementary results such as complete tables of minimal inhibitory concentration and figures that support the main results reported in this article.

### Conflict of interest

The author(s) declare(s) that they have no conflicts of interest to disclose.

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**SUPPORTING INFORMATION**

**Nanoemulsion improves the antifungal activity of allylic thiocyanates against yeasts and filamentous pathogenic fungi**

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**Experimental**

**Chemical Synthesis**

Fifteen allylic thiocyanates **1**–**15** (Scheme 1) were readily synthesized from the corresponding allylic bromides **16** according to previously described methods.[1–4] The typical procedure for the synthesis of allylic thiocyanates **1**-**15**, consists of a stirred solution of allylic bromide **16** (1.0 mmol) in 4.0 mL of acetone/$H_2O$ (3:1 v/v) at 25 °C was added 2.0 mmol of NaSCN. After stirring for 1 h, the final mixture was diluted with $CH_2Cl_2$ and washed with $H_2O$ and brine. The organic extract was dried over $Na_2SO_4$, filtered and concentrated under reduced pressure. The resulting residue was purified by chromatography (hexane/ethyl acetate 9:1) to give the corresponding (*Z*)-2-(thiocyanomethyl)alkenoates. Spectral and analytical data for the novel compound 2: 2 Methyl (*Z*)-3-(3-nitrophenyl)-2-(thiocyanomethyl)-2-propenoate. Yield 98%; white solid, mp 71.0-72.0 °C. IR (KBr): $\nu_{max}$/cm$^{-1}$ 3085, 2952, 2155, 1716, 1532, 1351, 1270. $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 3.90 (s, 3H), 4.04 (s, 2H), 7.67 (t, *J* = 8.0 Hz, 1H), 7.75-7.78 (m, 1H), 8.00 (s, 1H), 8.22-8.27 (m, 2H). $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ 30.8, 53.2, 111.7, 124.1, 124.5, 129.1, 130.5, 135.0, 135.5, 141.9, 148.7, 165.8. Anal. Calcd for $C_{12}H_{10}N_2O_4S$ (%): C, 51.79; H, 3.62; N, 10.07. Found: C, 51.78; H, 3.55; N, 9.98.

Each compound **1**-**15** was solved in DMSO and diluted using sterile ultrapure water to give solutions having DMSO in concentrations lower than 1% for subsequent investigation of antifungal/antichemotactic activities and toxicity determinations.

**Preparation and physicochemical characterization of nanoemulsion (NE)**

The NE were prepared in triplicate by the spontaneous emulsification solvent diffusion method, where an organic phase preheated to 40 °C, composed of the active thiocyanate **11** (**NE–C**, 5 mg), the oil (medium chain triglycerides – MCT, 0.15 g), the SPAN® 80 (0.077 g) and the solvent (ethanol, 27 mL), was injected under magnetic stirring into an aqueous phase (distilled water, 53 mL) containing Tween® 80 (0.077 g). The emulsion formed was kept under magnetic stirring for 10 min and then the organic solvent and part of the aqueous solvent were removed on a rotary evaporator to a final volume of 10 mL. For comparison purposes, a NE was prepared without the compound **11** (**NE–WC**).

The NEs were submitted to a physicochemical characterization, where all the parameters were evaluated in triplicate. The pH of the NEs was determined directly on the samples by the use of a potentiometer. The NEs diameter and polydispersion index (PDI) evaluation was performed by photon correlation spectroscopy, after adequate dilution of an aliquot of the samples in ultrapure water (1:500) (Zetasizer Nanoseries, Malvern Instruments, UK). The zeta potential (ZP) values were determined by micro electrophoresis after dilution of the formulations into 10 mM NaCl solution.

The compound content in the formulations as well as the encapsulation efficiency was evaluated by high performance liquid chromatography (HPLC). For this, an aliquot of the samples was diluted in 10 mL of methanol and subsequently sonicated for 10 min to compound extraction. After, samples were filtered through a 0.45 µm membrane and injected into the HPLC system. Chromatographic instruments and conditions were the following: LC-10A HPLC system (Shimadzu, Japan) equipped with a LC-20AT pump, an UV-VIS SPD-M20A detector, a CBM-20A system controller and a SIL-20A HT valve sample automatic injector. Separation was achieved at room

temperature using an Inertsil ODS-3 $C_{18}$ Gel Sciences column (150 mm × 4.6 mm, 5 µm) coupled with a $C_{18}$ guard column. The isocratic mobile phase consisted of methanol and water (80:20, v/v) at 1.2 mL $min^{-1}$ flow rate. The compound was detected at 284 nm with a retention time of about 4.03 min. The analytical methodology was previously validated. The method was found to be linear (r =0.995) at the concentration range of 2.5-12.5 µg $mL^{-1}$ and specific.

The encapsulation efficiency was determined by ultrafiltration/centrifugation technique. An aliquot of the samples was placed in a 10.000 MW centrifugal device (Amicon®Ultra, Millipore) and free compound was separated at 2200 × g for 10 min. The ultrafiltrate was analyzed by HPLC method. The encapsulation efficiency (%) was calculated from the difference between the total and free drug concentrations. The results of this analysis were expressed as averages followed by standard deviations.

### *In vitro* antifungal susceptibility test

#### Fungal strains

Yeast species of the genus *Candida* (*C. albicans* - CA ATCC 18804, *C. krusei* - CK 02*, *C. glabrata* - CG 09*, and *C. tropicalis* - CT 72A*) and dermatophytic filamentous fungi (*Microsporum canis* - MCA 01, *Microsporum gypseum* - MGY 42, *Trichophyton mentagrophytes* - TME 16*, and *Trichophyton rubrum* - TRU 45) were selected for the screening of antifungal activity. Subsequently, for the comparison of the free compound and incorporated in the nanoformulation, more strains were included in this study. All fungal species are deposited in the Mycology Collection of the research group in Applied Mycology, Faculty of Pharmacy (Universidade Federal do Rio Grande do Sul, Brazil). For *Candida* spp. the resistance (*) was defined based on Kuriyama et al. (2005)[5] and CLSI breakpoints,[6,7] considering in this case the clinical isolates CK 02* resistant to: itraconazole (MIC =1 µg $mL^{-1}$) and fluconazole (MIC ≥64 µg $mL^{-1}$), GC09* itraconazole (MIC >4 µg $mL^{-1}$) and miconazole (MIC =8 µg $mL^{-1}$), and CT 72A* resistant to itraconazole (MIC =1 µg $mL^{-1}$), miconazole (MIC >8 µg $mL^{-1}$), and voriconazole (MIC =2 µg $mL^{-1}$). As for dermatophytes, the resistance (in the sense of reduced susceptibility) was established according to the increase in minimal inhibitory concentration (MIC) values for some clinical strains in relation to the majority, considering the following resistance threshold concentrations: terbinafine - MIC ≥1 µg $mL^{-1}$, griseofulvin - MIC ≥4 µg $mL^{-1}$, and ketoconazole - MIC ≥8 µg $mL^{-1}$. Consequently, the clinical isolate TME 16* was considered multidrug-resistant by the considerable elevation of MICs of three antifungal agents of different classes (MIC terbinafine =4 µg $mL^{-1}$, MIC griseofulvin >32 µg $mL^{-1}$ and MIC ketoconazole =16 µg $mL^{-1}$). The preparation, the origin and purity of the antifungal drugs are available in supporting information.

#### Antifungal agents

Terbinafine (TBF), amphotericin B (AmB) and anidulafungin (AND), purity ≥97%, were supplied by Cristalia (Sao Paulo, Brazil), griseofulvin (GSF), purity ≥97%, was acquired from Wallace Pharmaceuticals (Mumbai, India), ketoconazole (KTZ), purity ≥96%, was obtained from All Chemistry (Sao Paulo, Brazil), fluconazole (FCZ) purity ≥98% was obtained from Sigma-Aldrich (Sao Paulo, Brazil), itraconazole (ITZ) purity ≥97%, was supplied by Cassará laboratory (Dist. PHARMOS; Buenos Aires, Argentina), miconazole (MCZ) purity ≥97%, was supplied by Valdequimica Chemical Products (Sao Paulo, Brazil), and voriconazole (VRZ); purity ≥98%, was supplied by Pfizer (Sao Paulo, Brazil). The preparation of stock and work solutions followed the recommendations of the CLSI.[8] The commercial antifungals were used as reference substances for comparison with the synthetic compounds in the tests conducted.

#### Determination of minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC)

The series of synthetic compounds and the NE containing the compound **11** were evaluated for MIC determination, through the broth microdilution technique.[6–8] The inocula of yeasts ($0.5x10^3$ to $2.5x10^3$ CFU $mL^{-1}$) and dermatophytes ($1.0x10^3$ to $3.0x10^3$ CFU $mL^{-1}$) were prepared from cultures grown on sabouraud dextrose agar

*(SDA; Kasvi, Brazil)* and potato dextrose agar (PDA; Neogen, USA), respectively. [6–8] Posteriorly, aliquots of each serial microdilution (corresponding to MIC, 2xMIC, and 4xMIC) were spread on SDA (*Candida*) and PDA (dermatophytes), incubated at 35 °C,[6–8] and analyzed to determine the MFC, which was defined as the lowest concentration that yielded up to three colonies.[9]

***In vivo* antifungal efficacy in Infected Egg Test-Chorioallantoic Membrane (IET-CAM)**

Fresh and fertile white eggs were kept under optimized incubation conditions (38–39 °C, 55–60% humidity, 12 days). On the 4$^{th}$ day, the CAM of eggs were infected with 0.1 mL inoculum 4x/day until the occurrence of the infectious process was confirmed by the visualization of colonies onto the CAM. The preparation of the yeast ($4x10^3$ CFU $mL^{-1}$) and filamentous fungi ($1.0x10^3$ CFU $mL^{-1}$) inocula followed the CLSI.[6–8] On the 8$^{th}$ day of incubation, the treatment was started. An aliquot of 0.1 mL of **11** (200 µg $mL^{-1}$, 0.5% DMSO solution) and the negative control (0.9% NaCl) was added onto the CAM. On the 12$^{th}$ day, eggs were reopened. 0.1 mL of the embryonic contents were removed, spread on sabouraud agar plates, incubated and the viability of the embryo verified.[10] Subsequently, counting of colony forming units was performed.[10] The study was submitted and approved by the Committee on Ethics in the Use of Animals (CEUA nº 4/2016 - Instituto Federal Catarinense, Concordia, Brazil). The experiment was carried out in triplicate.

**Antichemotactic assay**

The evaluation of antichemotactic activity was performed according to the method of the modified Boyden chamber as described by Suyenaga et al. (2011).[11] Prior to assay, neutrophils were treated with the allylic thiocyanate **11** dissolved in Hank's balanced salt solution (HBSS pH 7.4) in concentrations of 0.001 to 10 µg $mL^{-1}$, at 37 °C for 30 min. As negative control was used a neutrophils solution with no addition of antichemotactic agent. Indomethacin was used as positive control. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Federal University of Rio Grande do Sul (Permission no. 32226, approved on April 24, 2017).

**Investigation of antifungal mechanism of action**

**Sorbitol assay**

The sorbitol solution (0.8 M, Sigma-Aldrich) was prepared and diluted in the culture medium (RPMI 1640; Sigma-Aldrich). Then, microplates were incubated at 35 °C for 168 h. The MIC was visually determined in the assay conducted in the presence and absence of sorbitol.[12] The minimal effective concentration (MEC), which is the lowest concentration of antifungal agent that leads to the growth of small, round and compact hyphal forms, was determined in triplicate only for the antifungal anidulafungin (AND; drug control).[6–8]

**Ergosterol assay**

The test was performed in triplicate according to the CLSI[6–8] in the presence and absence of the exogenous ergosterol (Sigma-Aldrich), at concentrations of 50 to 200 µg $mL^{-1}$ and using amphotericin B (AmB) as drug control.[13] The microplates were incubated (35 °C, 168 h) and MICs were determined visually in the presence and absence of exogenous ergosterol.

**Scanning electron microscopy (SEM) analysis**

After the incubation period defined by the susceptibility test, wells containing the coverslips were washed three times with phosphate-buffered saline (PBS). After washing, adhered cells received 500 µL of glutaraldehyde (2.5%, type 1, Sigma-Aldrich), diluted with sodium cacodylate (0.1 mol $L^{-1}$, pH 7.2, Sigma-Aldrich), and kept for 1 h at room temperature. Then, the wells were washed three times with sodium cacodylate (0.1 mol $L^{-1}$, pH 7.2) containing sucrose (0.2 mol $L^{-1}$) and $MgCl_2$ (2 mmol $L^{-1}$). Adhered cells were dehydrated in a series of freshly prepared solutions of ethanol (30, 50, and 70%, for 5 min/step, 95% and 2x100%, for 10 min/step). Samples were

subjected to critical point drying (EM CPD 300, Leica), mounted on metallic stubs, sputter-coated with a 15–20 nm gold-palladium layer, and visualized in a scanning electron microscope (Carl Zeiss EVO® MA10, Germany) operating at 10 kV. The images were performed with a strain of *C. albicans* (CA ATCC 18804) without treatment (control) and treated with thiocyanate **11** (25 µg $mL^{-1}$ - subinhibitory concentration) and ITZ (1 µg $mL^{-1}$, drug control).

**Toxicity evaluation**

**Cell culture, cytotoxicity, genotoxicity and mutagenicity**

Cell cultures of human leukocytes were prepared using venous blood collected by venipuncture from a male volunteer (protocol #23.081.005770/009-38). Aliquots (1 mL) of whole blood were immediately transferred to 10 mL of RPMI 1640 medium supplemented with 1% phytohemagglutinin, 10% fetal bovine serum and 1% streptomycin/penicillin.[14] Subsequently, cell culture treatments were performed with compound **11** (25 µg $mL^{-1}$ and 50 µg $mL^{-1}$, in 0.5% DMSO), hydrogen peroxide solution ($H_2O_2$, 100 µmol $L^{-1}$, positive control), and PBS (negative control). Cell cultures were incubated ($CO_2$ incubator for cell culture, 5% $CO_2$, Model MCO-19AIC, Sanyo) at 37 °C for 72 h.[15] Cytotoxic, genotoxic, and mutagenic parameters were established, in triplicate. Cell viability was assessed with 0.2% trypan blue (Sigma-Aldrich), according to Burow et al. (1998).[15] Genotoxicity was performed by the comet assay. Cells were classified according varying from 0 (no visible damage) to 4 (maximum damage) to provide a unique damage index (ID) from 0 to 400.[16] The assessment of mutagenicity was carried out by Panótico Rápido® (Laborclin), wherein all particles within the cells separated from the nucleus and counted as micronuclei (MN).[14,17]

**Hemolysis assay**

The hemolysis assay was performed using rabbit blood. After collection, the blood was mixed with the anticoagulant K2-EDTA. Rabbit erythrocytes were harvested by centrifugation for 5 min at 400 rpm and washed three times in PBS. A suspension of the 1% erythrocytes was prepared in PBS. The compound **11** solution was prepared and incubated with the erythrocyte suspension for 15 min h at 37°C. After incubation, the cells were spun down by centrifugation, the supernatant was transferred to a 96-well plate and the absorbance (650 nm) measured using a microplate spectrophotometer (BioTek Instruments). Two controls were used in this assay: (i) PBS was used as a negative control (0% hemolysis), and (ii) water was used as positive control (100% hemolysis).

**Hen's Egg Test-Chorioallantoic Membrane (HET-CAM)**

Fresh and fertile white eggs (Lohmann selected Leghorn, LSL) were kept under optimized incubation conditions (38–39 °C, 55–60% humidity, 10 days). On the $10^{th}$ day, the eggshell, around the airspace, was removed with a rotary tool (Dremel, WI). Subsequently, 0.3 mL of compound **11** (200 µg $mL^{-1}$, 0.5% DMSO solution) and controls (negative control: 0.9% NaCl; positive control: 0.1 mol $L^{-1}$ NaOH) were added to the CAM of the eggs.[18] The irritant effect was observed at three times: 30 sec, 2 min and 5 min after application of compound **11** and controls. The result of the irritation score (IS) was calculated according to the [Eq. (1)][18] and presents a maximum value of 21. The eggs were analyzed in relation to the appearance of hemorrhaging, lysis, and coagulation. Classification criterion used: 0 to 4.9 nonirritant (or practically no irritation); 5.0 to 21 irritant (moderate to severe or extreme irritation). The assay was performed in triplicate.

**Equation 1**. Formula for determination of irritation score (IS)

$$\mathrm{IS} = \left(\left(\frac{(301 - \text{hemorrhage time})}{300}\right)\mathrm{x5}\right) + \left(\left(\frac{(301 - \text{lysis time})}{300}\right)\mathrm{x7}\right) + \left(\left(\frac{(301 - \text{coagulation time})}{300}\right)\mathrm{x9}\right)$$

**Statistical analysis**

Differences between the control and treatments were statistically analyzed by ANOVA followed by Tukey's test ($p$ <0.05 was considered statistically significant). Data analysis was performed using the GraphPad Prism 5.0 software and expressed as mean ± SD.

**Results**

**Tables**

**Table S1.** MIC/MFCs (μg $mL^{-1}$) for allylic thiocyanates **1**–**15.**

**Table S2**. Number of eggs with and without fungal growth and number of eggs with live and dead embryos after treatment with compound **11** and controls.

**Table S3.** MICs (μg $mL^{-1}$) for the allylic thiocyanate **11** and MECs (μg $mL^{-1}$) for AND in the presence and absence of sorbitol.

**Table S4.** MICs (μg $mL^{-1}$) for the allylic thiocyanate **11** and AmB in the presence and absence of ergosterol.

**Figures**

**Figure S1.** Macroscopic changes in infected embryonated eggs; (A) Treatment with thiocyanate **11** (200 μg $mL^{-1}$) (B) Control, without treatment.

**Table S1.** MIC/MFCs (μg $mL^{-1}$) for allylic thiocyanates **1**–**15.**

| Compounds and antifungal drugs | Dermatophytes | | | | *Candida* spp. | | | |
|---|---|---|---|---|---|---|---|---|
| | **MCA 01** | **MGY 42** | **TME 16*** | **TRU 45** | **CA ATCC 18804** | **CK 02*** | **CG 09*** | **CT 72A*** |
| **1** | >50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 |
| **2** | >50/>50 | >50/>50 | >50/>50 | >50/>50 | 50/50 | 50/50 | 50/50 | 50/50 |
| **3** | >50/>50 | >50/>50 | >50/>50 | >50/>50 | 50/50 | 50/50 | 50/50 | 50/50 |
| **4** | >50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 |
| **5** | >50/>50 | >50/>50 | >50/>50 | >50/>50 | 50/50 | 50/50 | 50/50 | 50/50 |
| **6** | >50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 |
| **7** | >50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 |
| **8** | >50/>50 | >50/>50 | >50/>50 | >50/>50 | 50/50 | 50/50 | 50/50 | 50/50 |
| **9** | >50/>50 | >50/>50 | >50/>50 | >50/>50 | 50/50 | 50/50 | 50/50 | 50/50 |
| **10** | >50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 |
| **11** | 50/>200 | 50/>200 | 50/>200 | 50/>200 | 50/>200 | 50/>200 | 50/>200 | 50/>200 |
| **12** | >50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 |
| **13** | >50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 |
| **14** | >50/>50 | >50/>50 | >50/>50 | >50/>50 | 50/50 | 50/50 | 50/50 | 50/50 |
| **15** | >50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 |
| **TBF** | 0.03 (S) | 0.03 (S) | 4 (R*) | 0.06 (S) | - | - | - | - |
| **GSF** | 1 (S) | 1 (S) | >32 (R*) | 1 (S) | - | - | - | - |
| **KTZ** | 0.5 (S) | 1 (S) | 16 (R*) | 1 (S) | 0.25 (S) | 1 (S) | 0.5 (S) | 1 (S) |
| **FCZ** | - | - | - | - | 1 (S) | ≥ 64 (R) | 0.25 (DDS) | 2 (S) |
| **ITZ** | - | - | - | - | - | 1 (R) | >4 (R) | 1 (R) |
| **MCZ** | - | - | - | - | - | 0.5 (S) | >8 (R) | >8 (R) |
| **VRZ** | - | - | - | - | - | - | - | 2 (R) |

*Multidrug-resistant and resistant fungal isolates; MIC, minimal inhibitory concentration; MFC, minimal fungicidal concentration; MCA, *Microsporum canis*; MGY, *Microsporum gypseum*; TME, *Trichophyton mentagrophytes*; TRU, *Trichophyton rubrum*; CA, *Candida albicans*; CK, *Candida krusei*; CG, *Candida glabrata*; CT, *Candida tropicalis*. TBF, terbinafine; GSF, griseofulvin; KTZ, ketoconazole; FCZ, fluconazole; ITZ, itraconazole; MCZ, miconazole; VRZ, voriconazole; R, resistance; R*, resistance in the sense of reduced susceptibility compared to other strains; S, susceptible; IR, intermediary resistance; DDS, dose-dependent susceptibility.[6–8,19]

**Table S2**. Number of eggs with and without fungal growth and number of eggs with live and dead embryos after treatment with compound **11** and controls.

| Fungi | Compound 11 | | | | Efficiency (%) | Control 1 | | | | Control 2 | | | | Control 3 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Eggs | | Embryos | | | Eggs | | Embryos | | Eggs | | Embryos | | Eggs | | Embryos | |
| | With microbial growth | Without microbial growth | Dead | Live | | With microbial growth | Without microbial growth | Dead | Live | With microbial growth | Without microbial growth | Dead | Live | With microbial growth | Without microbial growth | Dead | Live |
| **MCA 01** | 0 | 3 | 0 | 3 | 100 | 3 | 0 | 3 | 0 | 2 | 1 | 2 | 1 | 1 | 2 | 1 | 2 |
| **MGY 42** | 0 | 3 | 0 | 3 | 100 | 3 | 0 | 3 | 0 | 3 | 0 | 3 | 0 | 2 | 1 | 2 | 1 |
| **TME 16*** | 0 | 3 | 0 | 3 | 100 | 3 | 0 | 3 | 0 | 3 | 0 | 3 | 0 | 1 | 2 | 1 | 2 |
| **TRU 45** | 0 | 3 | 0 | 3 | 100 | 3 | 0 | 3 | 0 | 3 | 0 | 1 | 2 | 3 | 0 | 3 | 0 |
| **CA ATCC 18804** | 1 | 2 | 1 | 2 | 66.6 | 3 | 0 | 2 | 1 | 1 | 2 | 0 | 3 | 0 | 3 | 0 | 3 |
| **CK 02*** | 1 | 2 | 1 | 2 | 66.6 | 3 | 0 | 2 | 1 | 1 | 2 | 0 | 3 | 0 | 3 | 0 | 3 |
| **CG 09*** | 1 | 2 | 1 | 2 | 66.6 | 3 | 0 | 3 | 0 | 3 | 0 | 1 | 2 | 2 | 1 | 0 | 3 |
| **CT 72A*** | 1 | 2 | 1 | 2 | 66.6 | 3 | 0 | 2 | 1 | 3 | 0 | 1 | 2 | 1 | 2 | 0 | 3 |

*Multidrug-resistant and resistant fungal isolates; MCA, *Microsporum canis*; MGY, *Microsporum gypseum*; TME, *Trichophyton mentagrophytes*; TRU, *Trichophyton rubrum*; CA, *Candida albicans*; CK, *Candida krusei*; CG, *Candida glabrata*; CT, *Candida tropicalis*. **Control 1** consists of chorioallantoic membrane of egg inoculated with the clinical strains; **Control 2** normal eggs without inoculation and without treatment; and **Control 3** normal eggs without inoculation and with compound **11**.

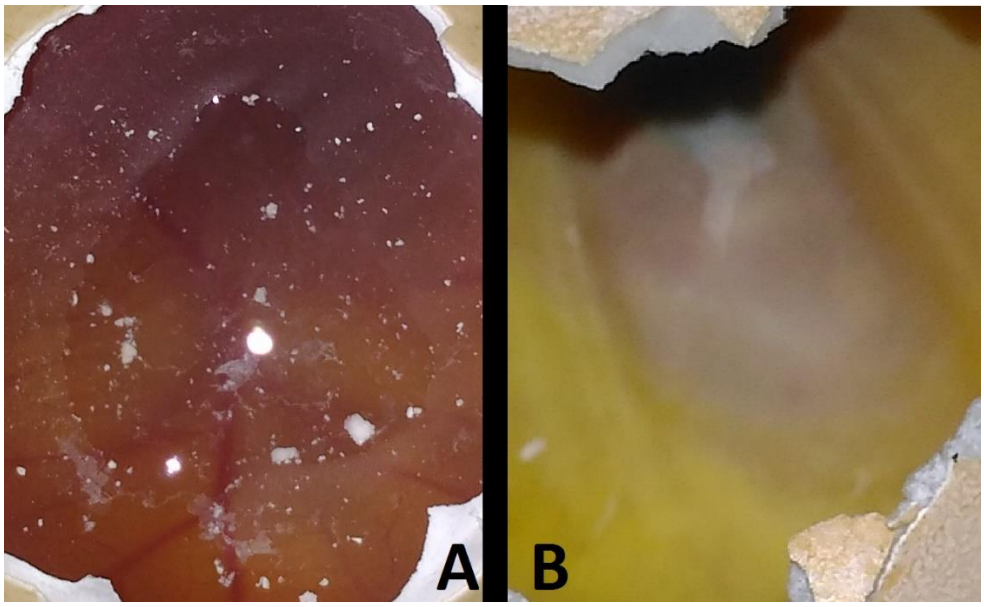


**Figure S1.** Macroscopic changes in infected embryonated eggs; (A) Treatment with thiocyanate **11** (200 µg $mL^{-1}$) (B) Control, without treatment.

**Table S3.** MICs (μg mL$^{-1}$) for the allylic thiocyanate **11** and MECs (μg mL$^{-1}$) for AND, in the presence and absence of sorbitol.

| Time | 48 h | | | | 96 h | | | | 168 h | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Dermatophytes** | **11** | | **AND** | | **11** | | **AND** | | **11** | | **AND** | |
| **and *Candida* spp.** | **AS** | **PS** | **AS** | **PS** | **AS** | **PS** | **AS** | **PS** | **AS** | **PS** | **AS** | **PS** |
| **MCA 01** | - | - | - | - | 50 | 50 | 32 | 128 | >800 | >800 | 64 | >256 |
| **MGY 42** | - | - | - | - | 50 | 50 | 32 | 128 | >800 | >800 | 64 | >256 |
| **TME 16*** | - | - | - | - | 50 | 50 | 32 | 128 | >800 | >800 | 64 | >256 |
| **TRU 45** | - | - | - | - | 50 | 50 | 32 | 128 | >800 | >800 | 64 | >256 |
| **CA ATCC 18804** | 50 | 50 | 0.12 | 32 | 100 | 100 | 0.12 | >64 | >800 | >800 | 0.12 | >64 |
| **CK 02*** | 50 | 50 | 0.12 | 32 | 100 | 100 | 0.12 | >64 | >800 | >800 | 0.12 | >64 |
| **CG 09*** | 50 | 50 | 0.12 | 32 | 100 | 100 | 0.12 | >64 | >800 | >800 | 0.12 | >64 |
| **CT 72A*** | 50 | 50 | 0.12 | 32 | 100 | 100 | 0.12 | >64 | >800 | >800 | 0.12 | >64 |

*Multidrug-resistant and resistant fungal isolates. Abbreviations: MIC, minimal inhibitory concentration; MEC, minimal effective concentration; AND, anidulafungin; AS, absence of sorbitol; PS, presence of sorbitol; MCA, *Microsporum canis*; MGY, *Microsporum gypseum*; TME, *Trichophyton mentagrophytes*; TRU, *Trichophyton rubrum*; CA, *Candida albicans*; CK, *Candida krusei*; CG, *Candida glabrata*; CT, *Candida tropicalis*.

**Table S4.** MICs (μg mL$^{-1}$) for the allylic thiocyanate **11** and AmB, in the presence and absence of ergosterol.

| **Fungi strains** | **Reading 1** (μg mL$^{-1}$) | | | | | **Reading 2** (μg mL$^{-1}$) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **11** | **MIC$^{1}$** | **MIC$^{2}$** | **MIC$^{3}$** | **MIC$^{4}$** | **MIC$^{5}$** | **MIC$^{1}$** | **MIC$^{2}$** | **MIC$^{3}$** | **MIC$^{4}$** | **MIC$^{5}$** |
| **MCA 01** | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 100 |
| **MGY 42** | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 100 |
| **TME 16*** | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 100 |
| **TRU 45** | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 100 |
| **CA ATCC 18804** | 50 | 50 | 100 | 100 | 200 | 50 | 100 | 100 | 100 | 200 |
| **CK 02*** | 50 | 50 | 50 | 100 | 100 | 50 | 50 | 100 | 200 | 200 |
| **CG 09*** | 50 | 50 | 50 | 200 | 200 | 50 | 50 | 100 | 400 | 400 |
| **CT 72A*** | 50 | 50 | 100 | 200 | 400 | 50 | 100 | 100 | 400 | 400 |
| **AmB** | **MIC$^{1}$** | **MIC$^{2}$** | **MIC$^{3}$** | **MIC$^{4}$** | **MIC$^{5}$** | **MIC$^{1}$** | **MIC$^{2}$** | **MIC$^{3}$** | **MIC$^{4}$** | **MIC$^{5}$** |
| **MCA 01** | 0.5 | 2 | 4 | 4 | 8 | 0.5 | 2 | 4 | 4 | 8 |
| **MGY 42** | 4 | 4 | 4 | 8 | 16 | 4 | 4 | 4 | 8 | 16 |
| **TME 16*** | 2 | 8 | 8 | 8 | 16 | 2 | 8 | 16 | 16 | 32 |
| **TRU 45** | 2 | 2 | 4 | 4 | 8 | 2 | 8 | 16 | 16 | 32 |
| **CA ATCC 18804** | 1 | 2 | 4 | 16 | 16 | 1 | 2 | 16 | 128 | 128 |
| **CK 02*** | 1 | 2 | 4 | 16 | 16 | 1 | 2 | 128 | 128 | 128 |
| **CG 09*** | 2 | 2 | 4 | 8 | 16 | 2 | 2 | 128 | 128 | 128 |
| **CT 72A*** | 0.5 | 2 | 4 | 32 | 128 | 0.5 | 2 | >128 | > 128 | > 128 |

*Multidrug-resistant and resistant fungal isolates. AmB = Amphotericin B; CA = *C. albicans* (CA ATCC 18804); CT = *C. tropicalis* (CT 72A*); CK= *C. krusei* (CK 02); CG= *C. glabrata* (CG 09); MCA= *M. canis* (MCA 01); MGY= *M. gypseum* (MGY 42); TME = *T. mentagrophytes* (TME 16*); TRU= *T. rubrum* (TRU 50). MIC$^{1}$ corresponds to MIC without addition of commercial ergosterol; MIC$^{2}$, MIC$^{3}$, MIC$^{4}$, and MIC$^{5}$, correspond to MIC with addition of ergosterol at the concentration of 50 μg mL$^{-1}$, 100 μg mL$^{-1}$, 150 μg mL$^{-1}$, and 200 μg mL$^{-1}$, respectively. For *Candidas*, readings 1 and 2 were performed after 2 and 5 days of incubation; for dermatophytes, readings 1 and 2 were performed after 4 and 7 days of incubation.

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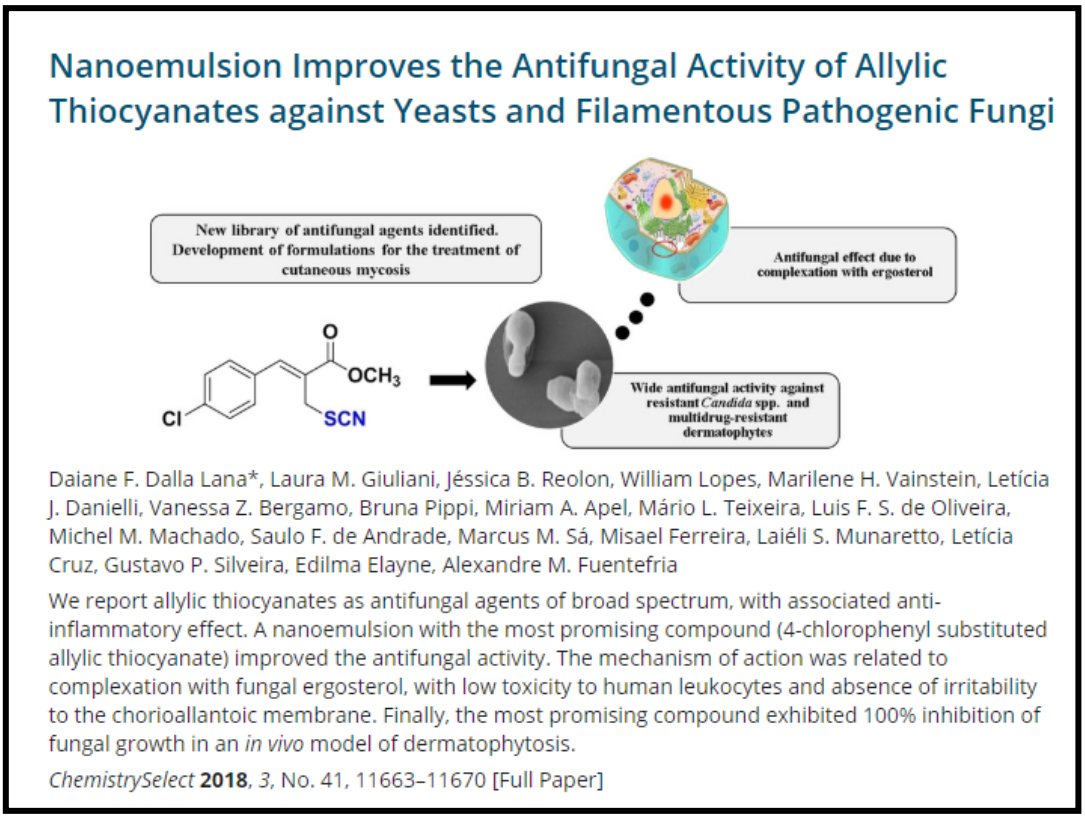
## ANEXOS

## Graphical abstract



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**CAPÍTULO III – Structure-based of δ-lactones for new antifungal drugs development: susceptibility, mechanism of action, and toxicity**

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**ABSTRACT**

Dermatophytes are the etiological agents of cutaneous mycoses, including the prevalent nail infections and athlete's foot. *Candida* spp. are opportunistic and emerging pathogens, causing superficial to deeper infections related to high mortality rates. As a consequence of prolonged application of antifungal drugs, the treatment failures combined with multidrug-resistance have become a serious problem in clinical practice. Therefore, novel alternative antifungals are required urgently. δ-lactones have attracted great interest owing to their wide range of biological activity. This article describes the antifungal activity of synthetic δ-lactones against yeasts of the genus *Candida* spp. and dermatophytes (through the broth microdilution method), discusses the pathways by which the compounds exert this action (toward the fungal cell wall and/or membrane), and evaluates the toxicity to human leukocytes and chorioallantoic membrane (by the Hen's Egg Test-Chorioallantoic Membrane). Two of the compounds in the series presented broader spectrum of antifungal activity, including against resistant fungal species. The mechanism of action was related to damage in the fungal cell wall and membrane, with specific target action dependent on the type of substituent present in the δ-lactone structure. The damage in the fungal cell was corroborated by electron microscopy images, which evidenced lysed and completely altered cells after *in vitro* treatment with δ-lactones. Toxicity was dose-dependent for the viability of human leukocytes, but none of the compounds was mutagenic, genotoxic, or membrane irritant when evaluated at higher concentrations than MIC. In this way, δ-lactones constitute a class with excellent perspectives regarding their potential applications as antifungals.

**Keywords**: Dermatophytes, *Candida* spp., δ-lactones, Antifungal activity, Antifungal mechanism of action, Toxicity.

**Introduction**

*Candida* spp. are typically opportunistic pathogens, which are prone to attack immunocompromised hosts or those with debilities to cause the infection of mucosa, skin, and deep-tissues, including the life-threatening candidemia (Vieira et al. 2018). With the disseminated use of antimicrobial drugs, immunosuppressive-cytotoxic agents, and catheters, as well as long periods in intensive care unit stays, the risk of *Candida*-associated nosocomial infections is increasing remarkably (Guinea 2014; Fuentefria et al. 2018). *C. albicans* is the main species involved in most infections, although, in recent years, diseases caused by non-*albicans Candida* species (NACS), including *C. tropicalis*, *C. glabrata*, *C. dubliniensis*, and *C. krusei*, are very prevalent (Eggimann et al. 2003; Naglik et al. 2014; Pfaller et al. 2015). Azoles, such as fluconazole, are the most frequently prescribed antifungals in candidiasis therapy, which destroy the cellular structures of fungi by inhibiting the biosynthesis of membranous ergosterol. However, long-term or repeated exposure to azoles in refractory infection can induce the emergence of resistant strains (Berkow et al. 2017; Rocha et al. 2018).

Dermatophytes are the etiological agents of cutaneous mycoses, including the frequent nail infections and athlete's foot. Contamination by this type of filamentous fungus is the most common cause of fungal infection worldwide (Dahdah et al. 2008; Dalla Lana et al. 2016). Developed countries have a higher prevalence of onychomycosis and athlete's foot, while developing countries have higher occurrences of *tinea capitis* and *tinea corporis* (body). As an aggravating factor, drug resistance related to dermatophytes has gained importance recently (Martinez-Rossi et al. 2008; Ghannoum 2015; Yamada et al. 2017).

The symptomatology and recurrence of candidiasis and dermatophytosis, together with epidemiological data, highlight the importance of these types of mycosis in clinical practice (Parker et al. 2014). In view of the scenario of fungal resistance and failures in current treatments of fungal infections, novel and alternative agents are needed against a broad range of fungi, without toxicity to the host (*i.e.*, selective action solely against the pathogen; Fuentefria et al. 2018).

δ-Lactones are important flavor and aroma constituents in many natural products (Romero-Guido et al. 2011). For many years, δ-lactones produced from plants or fungi, such as camptothecin (Wani et al. 1980), kazusamycin A (Umezawa et al. 1984), and sultriecin (Ohkuma et al. 1992), as well as synthetic analogues, including δ-alkyllactones (Kishimoto et al. 2005; Tanaka et al. 2007; Yang et al. 2011) and α-methylidene-δ-lactones (Janecka et al. 2012; Modranka et al. 2012), have been studied for their antitumor activity and antimicrobial properties. Therefore, this class of molecules have attracted great interest owing to their wide range of biological activity.

Based on our ongoing program seeking new molecules with antimicrobial properties (Silveira et al. 2012; Batista et al. 2017; Pippi et al. 2018) that can be developed as new leads for future *in vivo* studies, this article describes the screening of a series of synthetic δ-lactones for their antifungal activity against yeasts of the genus *Candida* spp. (*C. albicans* and NACS) and dermatophytes (*Microsporum canis*, *M. gypseum*, *Trichophyton mentagrophytes*, and *T. rubrum*), discusses the pathways by which the compounds exert this action, and assesses the toxicity to human leukocytes and chorioallantoic membrane.

**Materials and methods**

**Synthetic compounds**

Eleven δ-lactones **1–11** (Table 1) were readily obtained from functionalized allylic bromides in a water–isopropanol medium through a microwave-assisted one-pot synthesis according to previously described methods (Ferreira et al. 2017). For the subsequent investigation of antifungal activity and determination of toxicity, each compound **1–11** was solved in dimethyl sulfoxide (DMSO) and diluted using sterile ultrapure water to give aqueous solutions with DMSO concentrations lower than 1%.

***In vitro* antifungal susceptibility test**

*Antifungal agents*

Terbinafine (TBF), amphotericin B (AmB), and anidulafungin (AFG), purity ≥ 97%, were supplied by Cristalia (Sao Paulo, Brazil), griseofulvin (GSF), purity ≥ 97%, was acquired from Wallace Pharmaceuticals (Mumbai, India), ketoconazole (KTZ), purity ≥ 96%, was obtained from All Chemistry (Sao Paulo, Brazil), fluconazole (FCZ), purity ≥ 98%, was obtained from Sigma-Aldrich (Sao Paulo, Brazil), itraconazole (ITZ), purity ≥ 97%, was supplied by Cassará laboratory (Dist. PHARMOS; Buenos Aires, Argentina), miconazole (MCZ), purity ≥ 97%, was supplied by Valdequimica Chemical Products (Sao Paulo, Brazil), and voriconazole (VCZ), purity ≥ 98%, was supplied by Pfizer (Sao Paulo, Brazil). The preparation of stock and work solutions followed the recommendations of the M38-A2 document (CLSI, 2008). The commercial antifungals were used as reference substances for comparison with the synthetic compounds screened.

*Fungal strains*

Yeast species of the genus *Candida* (*C. albicans* - CA ATCC 18804, *C. krusei* - CK 02*, *C. glabrata* - CG 09*, and *C. tropicalis* - CT 72A*) and dermatophytic filamentous fungi (*M. canis* - MCA 01, *M. gypseum* - MGY 42, *T. mentagrophytes* - TME 16*, and *T. rubrum* - TRU 45) were selected for the screening of antifungal activity. All fungal species are deposited in the Mycology Collection of the Research Group in Applied Mycology, Faculty of Pharmacy (Universidade Federal do Rio Grande do Sul, Brazil). For *Candida* spp., the resistance (*) was defined based on Kuriyama et al. (2005) and CLSI breakpoints M27-A3 (2008) and M27-S4 (2012), considering in this case the clinical isolates CK 02* resistant to ITZ (MIC = 1 µg/mL) and FCZ (MIC ≥ 64 µg/mL); CG 09* resistant to ITZ (MIC > 4 µg/mL) and MCZ (MIC = 8 µg/mL); and CT 72A* resistant to ITZ (MIC = 1 µg/mL), MCZ (MIC > 8 µg/mL), and VCZ (MIC = 2 µg/mL). As for dermatophytes, the resistance (in the sense of reduced susceptibility) was established according to the increase in MIC values for some clinical strains in relation to the majority, considering the following resistance threshold concentrations: TBF - MIC ≥ 1 µg/mL, GSF - MIC ≥ 4 µg/mL, and KTZ - MIC ≥ 8 µg/mL. Consequently, the clinical isolate TME 16* was considered multidrug-resistant due to the considerable elevation of MICs of three antifungal agents of different classes (TBF - MIC = 4 µg/mL, GSF - MIC > 32 µg/mL, and KTZ - MIC = 16 µg/mL).

*Determination of minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC)*

The series of synthetic compounds were evaluated for MIC determination according to the M27-A3 and M38-A2 documents (CLSI, 2008). The inocula of yeasts ($0.5\times10^3$ to $2.5\times10^3$ colony forming units (CFU)/mL) and dermatophytes ($1.0\times10^3$ to $3.0\times10^3$ CFU/mL) were prepared from cultures grown on sabouraud dextrose agar (SDA; Kasvi, Brazil) and potato dextrose agar (PDA; Neogen, USA), respectively. The assays were conducted in duplicate, with RPMI medium containing L-glutamine (without $NaHCO_3$; USA), buffered to pH 7.0 with 3-(*N*-morpholino)propanesulfonic acid (MOPS; 0.165 mol/L, Neon, Brazil), and incubation at 35 ºC. The tested concentration of synthetic compounds ranged from 0.09 to 100 μg/mL. Readings were performed in 24-48 h for *Candida* spp. and in 96 h for dermatophytes.

Aliquots of each serial microdilution (corresponding to MIC, 2×MIC, and 4×MIC) were spread on SDA (*Candida*) and PDA (dermatophytes), incubated at 35 °C (M27-A3 and M38-A2 documents CLSI, 2008), and analyzed to determine the MFC, which was defined as the lowest concentration that yielded up to three colonies (Espinel-Ingroff et al. 2002). This assay was performed in triplicate.

*Multivariate exploratory analysis – structure-activity relationship*

Two multivariate exploratory analyzes were performed: principal component analysis (PCA) and hierarchical cluster analyses (HCA), both with the purpose of correlating the explained total variance. PCA is a mathematical methodology that reduces the dimensionality of the data, thus allowing data sets to be visualized in two or three dimensions (Jackson 2003). From the physicochemical data obtained through the Chemdraw Ultra 12.0 software, the following parameters were determined for each compound: molecular weight, melting point, boiling point, critical temperature, critical pressure, critical volume, Gibbs energy, octanol/water partition coefficient (LogP), molar refraction (MR), Henry's law, heat of formation, topological polar surface area (tPSA), calculated octanol/water partition coefficient (ClogP), calculated molar refractivity (CMR) (Supplementary Information, Table S1). From this software, we selected the 14 previously described properties, thus associating these 14 physicochemical dimensions to each compound. Consequently, PCA rotates these vectors to a new set of orthogonal axes called principal components, where the variance retained from the original data is maximized in each successive major component (Wenderski et al. 2015). In addition to the PCA, the HCA was performed, which is a two-dimensional representation of the profile of similarity of the compounds, showing how similar are each of the molecules studied based on their physicochemical parameters. ChemoStat - desktop software (http://www.chemostat.com.br/) was also used to perform the multivariate analysis.

**Antifungal mechanism of action**

*Sorbitol and ergosterol assay*

The sorbitol solution (0.8 M, Sigma-Aldrich) was prepared and diluted in the culture medium (RPMI 1640; Sigma-Aldrich). Then, microplates were incubated at 35 °C for 168 h. The MIC was visually determined in the assay conducted with δ-lactones **3** and **10** in the presence and absence of external sorbitol (Frost et al. 1995). The minimal effective concentration (MEC), which is the lowest concentration of

antifungal agent that leads to the growth of small, round and compact hyphal forms, was determined in triplicate only for the antifungal AFG (M27-A3 and M38-A2 documents– CLSI, 2008).

The assays determining the complexing effect with ergosterol were performed in triplicate for δ-lactones **3** and **10** and for AmB, used as the drug control, according to the M27-A3 and M38-A2 documents (CLSI, 2008) in the presence and absence of the exogenous ergosterol (Sigma-Aldrich) (Carrasco et al. 2012). The microplates were incubated (35 °C, 168 h), and MICs were determined visually in the presence and absence of exogenous ergosterol.

*Scanning electron microscopy (SEM) analysis*

After the incubation period defined by the susceptibility test, wells containing the coverslips were washed three times with phosphate-buffered saline (PBS). After washing, adhered cells received 500 µL of glutaraldehyde (2.5%, type 1, Sigma-Aldrich), diluted with sodium cacodylate (0.1 mol/L, pH 7.2, Sigma-Aldrich), and kept for 1 h at room temperature. Then, the wells were washed three times with sodium cacodylate (0.1 mol/L, pH 7.2) containing sucrose (0.2 mol/L) and $MgCl_2$ (2 mmol/L). Adhered cells were dehydrated in a series of freshly prepared solutions of ethanol (30, 50, and 70%, for 5 min/step, 95% and 2×100%, for 10 min/step). Samples were subjected to critical point drying (EM CPD 300, Leica), mounted on metallic stubs, sputter-coated with a 15–20 nm gold-palladium layer, and visualized in a scanning electron microscope (Carl Zeiss EVO® MA10, Germany) operating at 10 kV. The images were performed with a strain of *C. albicans* (CA ATCC 18804) without treatment (control) and treated with selected δ-lactone **10** (25 µg/mL - subinhibitory concentrations), ITZ (1 µg/mL, drug control), and TBF (0.016 µg/mL, drug control).

**Toxicity evaluation**

*Cell culture and genotoxicity profile*

Cell cultures of human leukocytes were prepared using venous blood collected by venipuncture from a male volunteer. This study was approved by the Research Ethics Committee of the Federal University of Pampa (n°. 27045614.0.0000.5323). Aliquots (1 mL) of whole blood were immediately transferred to 10 mL of RPMI 1640 medium supplemented with 1% phytohemagglutinin, 10% fetal bovine serum, and 1% streptomycin/penicillin (Montagner et al. 2014). Subsequently, cell culture treatments were performed with selected δ-lactones **3** and **10** (25 µg/mL, 50 µg/mL, and 100 µg/mL, in 0.5% DMSO), hydrogen peroxide solution ($H_2O_2$, 100 µmol/L, positive control), and PBS (negative control). Cell cultures were incubated ($CO_2$ incubator for cell culture, 5% $CO_2$, Model MCO-19AIC, Sanyo) at 37 °C for 72 h (Montagner et al. 2014). Cytotoxic, genotoxic, and mutagenic parameters were established in triplicate. Cell viability was assessed with 0.2% trypan blue (Sigma-Aldrich), according to Burow et al. (1998). Genotoxicity was performed by the comet assay. Cells were classified according to a variation from 0 (no visible damage) to 4 (maximum damage) to provide a unique damage index (ID) from 0 to 400 (Singh et al. 1988). The assessment of mutagenicity was carried out by Panotic Fast® (Laborclin), wherein all particles within the cells separated from the nucleus and counted as micronuclei (MN) (Thomas et al. 2008; Montagner et al. 2014).

*Hen's Egg Test-Chorioallantoic Membrane* (HET-CAM)

Fresh and fertile white eggs (Lohmann selected Leghorn, LSL) were kept under optimized incubation conditions (38–39 °C, 55–60% humidity, 10 days). On the 10$^{th}$ day, the eggshell, around the airspace, was removed with a rotary tool (Dremel, WI). Subsequently, 0.3 mL of selected δ-lactones **3** and **10** (100 µg/mL, 0.5% DMSO solution) and controls (negative control: 0.9% NaCl; positive control: 0.1 mol/L NaOH) were added to the CAM of the eggs (ICCVMA, 2010). The irritant effect was observed at three times: 0.5 min, 2 min, and 5 min after application of compounds **3**, **10**, or controls. The result of the irritation score (IS) was calculated according to the equation 1 (ICCVMA, 2010) and presents a maximum value of 21. The eggs were analyzed in relation to the appearance of hemorrhage, lysis, and coagulation. Classification criterion used: 0 to 4.9 nonirritant (or practically no irritation); 5.0 to 21 irritant (moderate to severe or extreme irritation). The time of bleeding, lysis, and coagulation are expressed in seconds, considering the first occurrence of blood hemorrhage, vessel lysis and protein coagulation, respectively. The assay was performed in triplicate. The experiment was approved by the Animal Use Ethics Committee of the Federal Catarinense Institute - Campus Concordia (nº. 11.794), that establishes procedures for the scientific utilization of animals in researches.

**Equation 1**. Formula for determination of irritation score (IS)

$$\mathrm{IS} = \left(\left(\frac{(301 - \text{hemorrhage time})}{300}\right)\mathrm{x5}\right) + \left(\left(\frac{(301 - \text{lysis time})}{300}\right)\mathrm{x7}\right) + \left(\left(\frac{(301 - \text{coagulation time})}{300}\right)\mathrm{x9}\right)$$

*Statistical analysis*

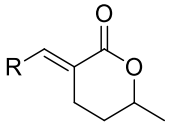
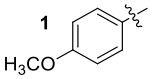
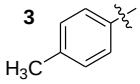
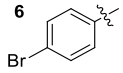
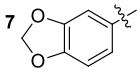
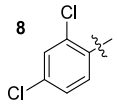
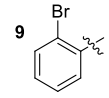
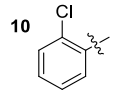
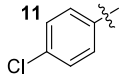
Differences between the control and treatments were statistically analyzed by ANOVA followed by Tukey's test ($p < 0.05$ was considered statistically significant). Data analysis was performed using the GraphPad Prism 5.0 software and expressed as mean ± SD.

**Results and discussion**

***In vitro* antifungal susceptibility test**

Eleven δ-lactones were synthesized and *in vitro* evaluated for the first time regarding the antifungal potential against filamentous fungi and yeasts. Of all molecules analyzed, six showed satisfactory performance with MICs ≤ 50 µg/mL (Table 1) for *Candida* spp. and/or dermathophytes (compounds **3**, **4**, **6**, **9**, **10**, and **11**). In particular, δ-lactones **3** and **10** exhibited the broadest spectrum against *C. albicans*, NACS, *M. canis*, *M. gypseum*, *T. mentagrophytes*, and *T. rubrum* (Table 1).

**Table 1** Chemical structures and MICs (µg/mL) of δ-lactones (**1**-**11**) for dermatophytes and *Candida* spp.

|  **R** | **Dermatophytes** | | | | ***Candida* spp.** | | | |
|---|---|---|---|---|---|---|---|---|
| | **MCA 01** | **MGY 42** | **TME 16*** | **TRU 45** | **CA ATCC 18804** | **CK 02*** | **CG 09*** | **CT 72A*** |
| **1**  | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| **2**  | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| **3**  | 50 | 25 | 50 | 25 | 50 | 50 | 50 | 50 |
| **4** $CH_3CH_2CH_2$- | 50 | 50 | 50 | 50 | 100 | 100 | 100 | 100 |
| **5** $CH_3$- | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| **6**  | 50 | 50 | 50 | 50 | 100 | 100 | 100 | 100 |
| **7**  | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| **8**  | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| **9**  | 100 | 50 | 100 | 50 | 100 | 100 | 100 | 100 |
| **10**  | 50 | 50 | 50 | 25 | 25 | 25 | 25 | 25 |
| **11**  | 100 | 100 | 100 | 100 | 50 | 50 | 100 | 100 |
| **TBF** | 0.03 (S) | 0.03 (S) | 4 (R*) | 0.06 (S) | - | - | - | - |
| **GSF** | 1 (S) | 1 (S) | > 32 (R*) | 1 (S) | - | - | - | - |
| **KTZ** | 0.5 (S) | 1 (S) | 16 (R*) | 1 (S) | 0.25 (S) | 1 (S) | 0.5 (S) | 1 (S) |
| **FCZ** | - | - | - | - | 1 (S) | ≥ 64 (R) | 0.25 (DDS) | 2 (S) |
| **ITZ** | - | - | - | - | - | 1 (R) | > 4 (R) | 1 (R) |
| **MCZ** | - | - | - | - | - | 0.5 (S) | > 8 (R) | > 8 (R) |
| **VCZ** | - | - | - | - | - | - | - | 2 (R) |

Asterisk (*) in clinical strains indicates multidrug-resistance and resistance; MIC, minimal inhibitory concentration; MFC, minimal fungicidal concentration; MCA, *Microsporum canis*; MGY, *Microsporum gypseum*; TME, *Trichophyton mentagrophytes*; TRU, *Trichophyton rubrum*; CA, *Candida albicans*; CK, *Candida krusei*; CG, *Candida glabrata*; CT, *Candida tropicalis*. TBF, terbinafine; GSF, griseofulvin; KTZ, ketoconazole; FCZ, fluconazole; ITZ, itraconazole; MCZ, miconazole; VCZ, voriconazole; R, resistance; R*, resistance in the sense of reduced susceptibility compared to other strains; S, susceptible; DDS, dose-dependent susceptibility.

The 2-chlorophenyl substituted derivative **10** presented the lower values of geometric mean (GM), minimal concentration that inhibits approximately 50% of the strains ($MIC_{50}$), and minimal concentration that inhibits approximately 90% of the strains ($MIC_{90}$) in µg/mL and nmol/mL (see Supplementary Information, Table S2). In addition, **10** was the only δ-lactone with fungicide activity at the MFC value of 4×MIC, for all fungal species studied. Remarkably, this compound was also active against resistant species of *Candida* (*C. krusei*, *C. glabrata*, and *C. tropicalis*) and a multidrug-resistant dermatophyte (*T.*

*mentagrophytes*). Other compounds of the series were fungistatic at the three concentrations assessed (MIC, 2×MIC, and 4×MIC).

Antifungal activity for other natural and synthetic δ-lactones have already been reported (Kishimoto et al. 2005; Ohkuma et al. 1992; Yang et al. 2011). The MIC values previously reported for this class are of the same order as those displayed in Table 1: sultriecin (metabolite of *Streptomyces roseiscleroticus*) - 25 µg/mL for *Candida* spp. and dermatophytes (species of the genus *Trichophyton*) (Ohkuma et al. 1992); δ-decalactones - 62 to 250 µg/mL for *Aspergillus niger* and *C. albicans* (Kishimoto et al. 2005). However, Yang et al. (2011) reported much higher MICs of δ-dodecalactone against various fungi and bacteria, ranging from 350 to 6250 mg/mL. Strong antifungal activity of δ-dodecalactone was only observed against molds, such as *A. flavus*, *A. fumigatus*, *A. petrakii*, *A. ochraceus*, *A. nidulans*, and *Penicillium roqueforti*. Antibacterial activity was evident but less potent than the antifungal activity (Yang et al. 2011). Interestingly, the three tested yeast strains of *C. albicans* reported by Yang et al. (2011) were more resistant than the molds, which is the same profile observed in our studies, since the δ-lactones had the lowest MIC values for dermatophytes (Table 1; Table S2).

**Multivariate exploratory analysis – structure-activity relationship**

A PCA (Figure S1-A) was performed to evaluate the common characteristics of the compounds (Table S1) that presented antifungal activity in relation to those that had no activity or were less active (higher values of inhibitory concentration - MIC and/or lower activity spectrum). The physicochemical properties were evaluated for each δ-lactone, so we can anticipate the most important properties for the occurrence of antifungal activity. δ-Lactone **10** showed the highest antifungal efficacy (lower MIC values for yeasts and filamentous fungi), followed by compound **3**. Both compounds are very closely situated at the PCA analysis, with similarity of more than 90% between the compounds in the analysis of HCA (Fig. 1).

PCA and HCA are useful tool in the design and planning of chemical libraries and can be used to reveal differences in structural and physicochemical parameters among various compounds and display them in a convenient graphical format (Jackson, 2003). Here, we demonstrate the use of PCA to obtain information on the structural characteristics and properties of each compound that would explain the biological activity in question.

Although the isomeric δ-lactones **10** and **11** have the same predicted physicochemical parameters (Table S1; data from ChemDraw Ultra 12.0), and consequently the same profile in multivariate tests (Fig. 1), distinct MIC values were observed for them (Table 1), with a higher potential of antifungal activity observed for compound **10**. Therefore, it is evident that the aromatic position of the chloro group (*ortho*- in **10** and *para*- in **11**) is important for the activity under evaluation. A similar trend occurs with isomeric compounds **6** and **9**, but in this particular case they exhibit relative similarity when fungal activity is compared, with compound **6** being slightly more active against filamentous species. The same selectivity did not occur for the chloro-substituted lactones **10** and **11**, nor for the dichloro derivative **8**, which was not active against any fungi, at concentrations ≤ 50 µg/mL. For the latter case, it is interesting to note that the simultaneous presence of chloro atoms in both the *ortho*- and *para*- positions in **8** led to total suppression of activity, possibly due to the large electronic delocalization that occurred in the aromatic ring. According

to El Hage et al. (2011), it is important that the molecule has an appropriate distribution of the electrostatic potential, which seems indispensable for the antifungal potential. Two-dimensional PCA interpretation did not resolve between compounds **10** and **11** due to their high similarity, requiring three-dimensional PCA (Fig. S1-B) to separate them. The same happens with the predicted properties for isomeric compounds **6** and **9**, which are also overlapped due to the equivalence of the physicochemical parameters. The vector distance between the points becomes more evident with one more vector component, this third PC being essential to distance the compounds with interesting antifungal activity.

Nevertheless, if we visualize Fig. S1-A, in which the PCA is placed with the biplot, where the loadings are willing together with the scores, we see that the most prevailing physicochemical parameters for the separation seen between two main components were CLogP, LogP, and Gibbs Free Energy (Fig. S1-A). Correlating the data obtained in the PCA analysis together with the MIC data, we hypothesized that the most important physicochemical data for the antifungal potential were those previously described. Among the parameters cited above one of the most important is the CLogP (lipophilicity), as it identifies the ability of the molecule to cross biological barriers activity as the fungal cells in this case (Topliss, 1996; Tavares, 2004; Santos et al. 2009).

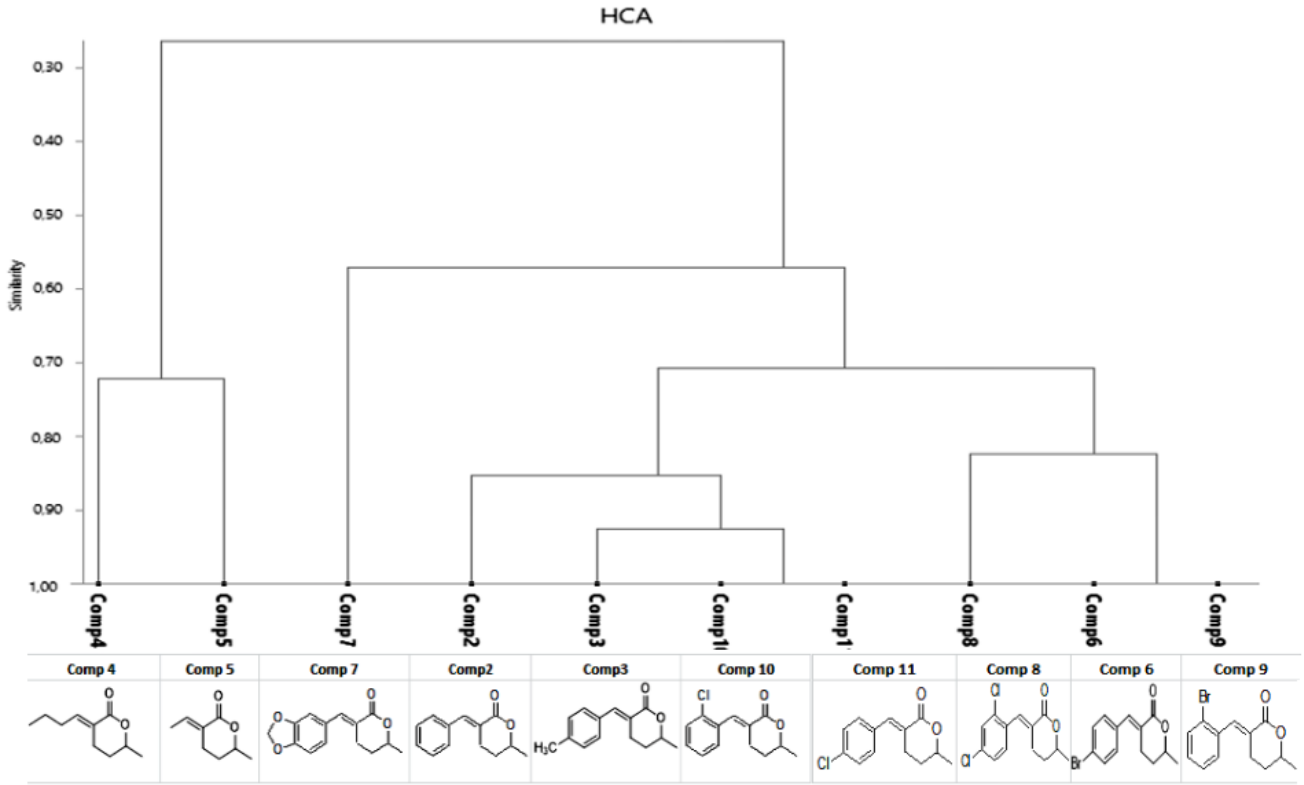



**Fig. 1** Hierarchical analysis of clusters (HCA) of δ-lactones

**Antifungal mechanism of action**

*Sorbitol and ergosterol assays*

MICs of the δ-lactones **3** and **10** for eight dermatophytes and *Candida* were evaluated in the presence and absence of external sorbitol, at different times, using ANF as antifungal control (MEC of the antifungal agent – Table S3). For δ-lactone **3**, the addition of sorbitol in the assay caused an expressive variation of the MIC for *Candida* spp., where an 8-fold increase was observed in the last reading (Table S3); for dermatophytes, the increase in MIC was 4-fold for this molecule (Table S3). On the other hand, the presence of external sorbitol did not affect the activity of compound **10**, with the MIC values being unchanged for both *Candida* and dermatophytes. In contrast, the MICs of ANF increased sharply after the addition of sorbitol, both for *Candida* spp. as for dermatophytes (Table S3).

A certain osmotic protection is exerted by sorbitol to the fungi cell wall, chasing chemicals to act on this target (Frost et al. 1995). Consequently, antifungal activity decreases if the drug acts on the cell wall. Hence, loss of activity in the presence of sorbitol is a strong indication that a specific molecule is acting on this target. Thus, δ-lactone **10** does not seem to be related with fungi cell wall, since the MIC values did not vary according to the presence or absence of sorbitol, while for **3**, the increase in the MICs observed in the sorbitol assays is suggestive of a mechanism of action involving the cell wall of filamentous and yeast species.

Regarding the addition of ergosterol in the medium, substantial enhancement of the MIC (4-to-8 fold) of δ-lactone **3** for both dermatophytes and *Candida* spp. were observed in the first and last reading (Table S4). For compound **10**, a pronounced increase of the MIC was observed in both readings of the tests (up to 32-fold for yeasts and 16-fold for dermatophytes; Table S4). For AmB – drug control, MICs increased sharply in the presence of ergosterol (Table S4).

Some antifungals act by interacting with ergosterol in the cell membrane, which is an important target since mammalian cells lack this sterol. Thus, the addition of ergosterol at the medium increases the concentration of this substance outside of the membrane allowing the drug to interact more easily with this free ergosterol. Consequently, the drug becomes less activated (higher MICs) indicating a mechanism of action on the cell membrane due to the competitive binding with ergosterol, such as AmB (Carrasco et al. 2012; Montagner et al. 2014). AmB is one of the most effective drugs in fungal infections (Fernández-García et al. 2017), so having a new prototype with similar mechanism of action is very advantageous.

Interestingly, although the substituted δ-lactones **3** and **10** pertain to the same class, each one generates distinct effects. While the methyl-substituted δ-lactone **3** acts in the fungal cell wall (higher MICs in the presence of sorbitol) and by complexation with ergosterol at the membrane, the chloro-substituted compound **10** exerts a more specific effect toward the fungal cell membrane, as observed previously for AmB. These effects can also occur by the lipophilicity of the compounds, evidenced in the chemometric analysis, which facilitates the interaction of both molecules with the fungal cell membrane. Therefore, it becomes evident that the substitution pattern on the δ-lactone is directly related to the type of mechanism of action against the pathogens under study.

Two structurally similar compounds, differentiating only by an atom or position that it occupies in the molecule, may present differences in their physicochemical properties and, consequently, in

biological action, both quantitatively and qualitatively. The therapeutic action of drugs occurs through interactions of these with biological systems and is dependent on factors related to their chemical structure and physicochemical characteristics. These factors, whether electronic, steric or hydrophobic, influence the relationship of the drug with biophase and its distribution in the compartments that make up the biological system or target. Understanding this process is fundamental for planning new substances that have a therapeutic profile more suited to current needs (Tavares, 2004; Fuentefria, 2018).

*SEM analysis*

The yeast *C. albicans* was treated separately with the antifungal ITZ and the compound **10**. It was noticed that before the addition of the substances, *C. albicans* cells presented the expected oval shape without alteration (Fig. 2A). After treatment with ITZ, changes in cell shape and size were remarkable (Fig. 2B; arrows point to damaged cells), as observed for compound **10**, which causes complete yeast cell lysis - presence of pores in blastoconidia (Fig. 2C; arrows point to damaged cells). For *M. canis*, the intact hyphae were observed in the untreated control, apparently without structural damage (Fig. 2D). After treatment with terbinafine (TBF, Fig. 2E; arrows point to damaged cells), the hyphae collapsed to a very different appearance from the untreated control (Fig. 2D). Similar trends were observed for compound **10**, which causes ruptures of the tubular structures of the molds (Fig. 2F; arrows point to damaged cells).

**Toxicity evaluation**

*Cell viability and genotoxicity profile*

For δ-lactones **3** and **10**, at the highest concentration evaluated, the viability of human leukocytes was approximately 75% and 85%, respectively, differing statistically from the negative control (approximately 90% cell viability; Fig. 3A). However, both compounds at 100 µg/mL (2×MIC) did not cause significant DNA damage (Fig. 3B) and were not mutagenic, since no significant micronucleus frequency was observed after *in vitro* treatment with these compounds (Fig. 3C).

The comet assay relates the genotoxicity potential of substances to affect the cell integrity and its genetic material (Batista et al. 2017), while the micronuclei test indicates a possible mutagenicity of the compounds (Batista et al. 2017). The evaluation of the toxicological profile of δ-lactones **3** and **10** over human leucocyte cells provided important input on their dose-dependent safety in biological applications. Both compounds were shown to be safe for genotoxicity and mutagenicity when evaluated at concentrations 2-fold higher than MIC (Fig. 3 B-C).

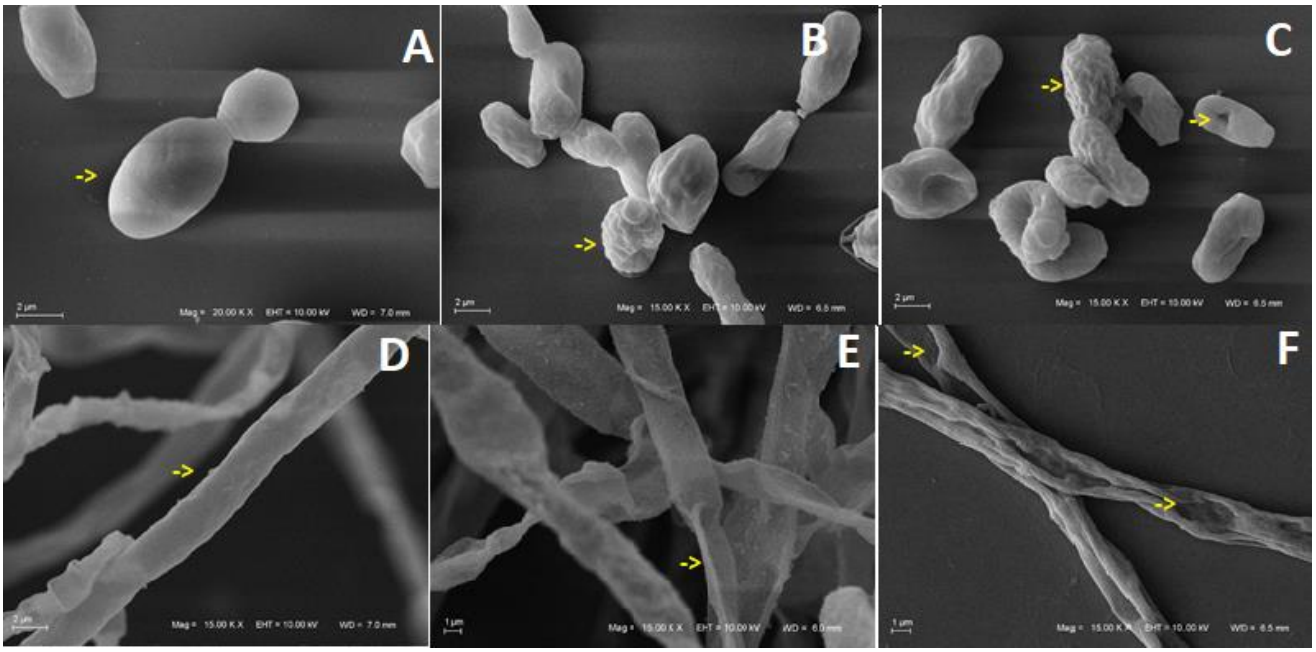



**Fig. 2** SEM images; (A) *C. albicans* without treatment - arrows indicating blastoconidium without change, (B) *C. albicans* treated with itraconazole (1 µg/mL) - arrows indicating blastoconidium with irregular surface and size and dehydrated appearance, (C) *C. albicans* treated with δ-lactone **10** (12.5 µg/mL) - arrows indicating blastoconidium with irregular surface and size and dehydrated appearance with pores, (D) *M. canis* without treatment - arrows indicating characteristic hyphae of the species with no apparent change in shape and surface, (E) *M. canis* treated with terbinafine (0.016 µg/mL) - arrows indicating superficially altered and collapsed hyphae, and (F) *M. canis* treated with **10** (25 µg/mL) - arrows indicating superficially altered and collapsed hyphae, with signs of rupture and pores

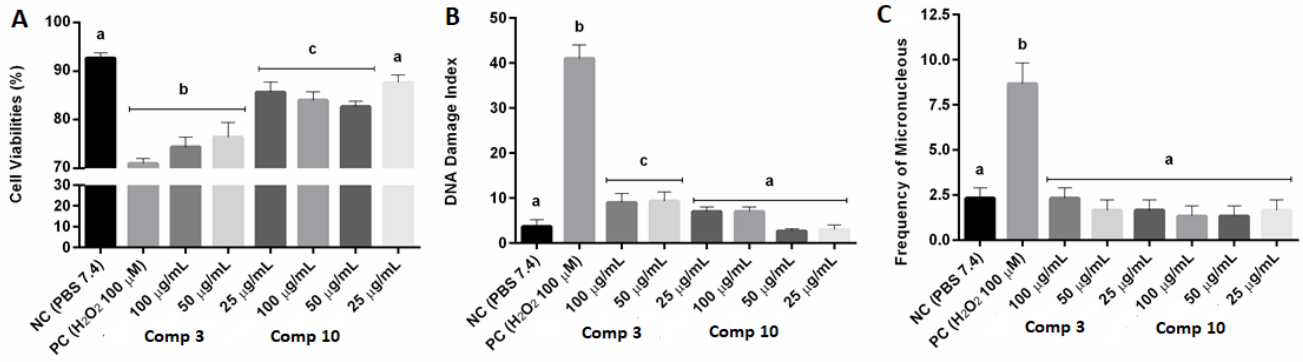



**Fig. 3** Toxicological analysis; (A) cell viability, (B) DNA damage, and (C) micronucleous frequency effects of compounds **3** and **10**, in three different concentrations (25, 50, and 100 µg/mL). Bars with the same letters indicate statistical similarity (ANOVA–Tukey's test)

*Evaluation of the effect on chorioallantoic membrane of the chicken eggs*

Results of the HET-CAM tests show the relationship between IS and the logarithms of the concentrations of δ-lactones **3** and **10**. This relationship is represented by equation 1 as a function of the log concentration for each tested molecule. The IS for **3** was 3.96 and for **10** was 3.34 (Figure S2), both being classified as nonirritant according to the methodology. Toxicological studies using alternative models such as the *ex vivo* porcine skin model and HET-CAM allow determining the permeation profile of new organic compounds and discriminate levels of potential irritancy by means of the IS calculation (Batista et

al. 2017). The HET-CAM is an *ex vivo* method alternative to the Draize Rabbit Eye test which measures vascular changes in the CAM. The membrane is analog of the ocular conjunctive and responds to injuries caused by processes such as inflammation similarly to what is observed in the conjunctival tissue of rabbit's eyes. Since substances are directly applied over the CAM, the results obtained are generally more sensible than animal models (ICCVMA, 2010). Results from HET-CAM using embryonated eggs demonstrated that δ-lactones **3** and **10** are nonirritant of the CAM (ICCVMA, 2010). Therefore, according to these assays, these molecules would be safely applied over the human mucosa, at the analyzed concentration (2×MIC).

## Conclusions

Finally, the findings of this study showed that δ-lactones exhibited a broad spectrum of antifungal activity against *Candida* spp. and dermatophytes. The mechanism of antifungal action was related to damages in the fungal cell wall and membrane. The preference for compounds target cell wall or membrane is influenced by the type of substituent of the basic chemical structure. Regarding the toxicity to human leukocytes, δ-lactones are not mutagenic, and genotoxic. In addition, the molecules tested were not allergenic to chorioallantoic membrane of the chicken eggs. Thus, compounds of this class are very promising as hits for the design of new antifungal drugs and topical formulations for the treatment of dermatophytoses and candidiasis.

## Declarations

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### Authors' contribution

Daiane F. Dalla Lana, Ânderson R. Carvalho, William Lopes conceived, planned, and carried out the experiments. Marcus M. Sá, Theo V. C. Russo, and Gustavo P. Silveira synthesized and characterized the samples. Marilene H. Vainstein, Luciano S. P. Guimarães, Mário L. Teixeira, Luis F.S. de Oliveira, and Michel M. Machado assisted in the toxicological and statistical analyzes and in obtaining the images of scanning electron microscopy. Alexandre M. Fuentefria and Saulo F. de Andrade contributed to the interpretation of the results and supervision of all the work. Daiane F. Dalla Lana took the lead in writing the manuscript and performed the general research. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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**Conflict of interest**

The authors declare that they have no conflicts of interest.

**Compliance with ethical standards**

**Ethical approval**

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. For this type of study formal consent is not required. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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**Supplementary information**

# Structure-based design of δ-lactones for new antifungal drugs development: susceptibility, mechanism of action, and toxicity

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Table S1 Chemical data* for δ-lactones 1-11

| **Parameters** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Formula** | $C_{14}H_{16}O_3$ | $C_{13}H_{14}O_2$ | $C_{14}H_{16}O_2$ | $C_{10}H_{16}O_2$ | $C_8H_{12}O_2$ | $C_{13}H_{13}BrO_2$ | $C_{14}H_{14}O_4$ | $C_{13}H_{12}Cl_2O_2$ | $C_{13}H_{13}BrO_2$ | $C_{13}H_{13}ClO_2$ | $C_{13}H_{13}ClO_2$ |
| **Molecular weight (g/mol)** | 232.28 | 202.25 | 216.28 | 168.23 | 140.18 | 281.14 | 246.26 | 271.14 | 281.14 | 236.69 | 236.69 |
| **Solubility** | DMSO | DMSO | DMSO | DMSO | DMSO | DMSO | DMSO | DMSO | DMSO | DMSO | DMSO |
| **Boiling point (K)** | - | 599.59 | 627.45 | 504.27 | 458.51 | 670.73 | 697.74 | 684.41 | 670.73 | 642 | 642 |
| **Melting point (K)** | - | 329.3 | 353.09 | 269.7 | 246.53 | 401.62 | 440.93 | 414.18 | 401.62 | 371.74 | 371.74 |
| **Critical Temperature (K)** | - | 852.13 | 854.17 | 774.17 | 754.43 | 880.69 | 887.19 | 880.56 | 880.69 | 866.26 | 866.26 |
| **Critical Pressure (Bar)** | - | 26.41 | 23.63 | 25.28 | 30.97 | 27.94 | 26.93 | 23.80 | 27.94 | 25.05 | 25.05 |
| **Critical volume ($cm^3$/mol)** | - | 613.5 | 669.5 | 553.5 | 441.5 | 675.5 | 669.5 | 711.5 | 675.5 | 662.5 | 662.5 |
| **Gibbs energy (KJ/mol)** | - | -45.27 | -46.48 | -182.94 | -199.78 | -40.58 | -159.89 | -88.39 | -40.58 | -66.83 | -66.83 |
| **LogP** | - | 2.77 | 3.26 | 2.42 | 1.58 | 3.60 | 2.55 | 3.89 | 3.60 | 3.33 | 3.33 |
| **MR ($cm^3$/mol)** | - | 59.53 | 65.43 | 49.03 | 39.84 | 67.23 | 67.71 | 68.74 | 67.23 | 64.14 | 64.14 |
| **Henry`s Law** | - | 3.69 | 3.64 | 1.90 | 2.14 | 4.09 | 6.62 | 3.95 | 4.09 | 3.82 | 3.82 |
| **Heat of Formation (KJ/mol)** | - | -249.73 | -281.84 | -424.34 | -383.06 | -234.87 | -464.17 | -304.15 | -234.87 | -276.94 | -276.94 |
| **tPSA** | 50.7 | 26.3 | 26.3 | 26.3 | 26.3 | 26.3 | 44.76 | 26.3 | 26.3 | 26.3 | 26.3 |
| **CLogP** | 1.912 | 3.488 | 3.987 | 2.517 | 1.459 | 4.351 | 3.453 | 4.914 | 4.351 | 4.201 | 4.201 |
| **CMR** | 6.555 | 6.225 | 6.689 | 4.877 | 3.949 | 7.002 | 6.817 | 7.208 | 7.002 | 6.7166 | 6.7166 |

*(ChemDraw Ultra 12.0); LogP, *octanol*/*water partition coefficient*; MR, molar refraction; tPSA, topological polar surface area; CLogP, calculated octanol/water partition coefficient; CMR, calculated molar refractivity; *DMSO, dimethyl sulfoxide*.

**Table S2** MICs, individual and general GM, $MIC_{50}$, and $MIC_{90}$ of δ-lactones **1**-**11**. Values in µg/mL (nmol/mL)

| Clinical strains | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **MCA 01** | 100 (430.5) | 100 (494.4) | 50 (231.2) | 50 (297.2) | 100 (713.4) | 50 (177.8) | 100 (406.1) | 100 (368.8) | 100 (355.7) | 50 (211.2) | 100 (422.5) |
| **MGY 42** | 100 (430.5) | 100 (494.4) | 25 (115.6) | 50 (297.2) | 100 (713.4) | 50 (177.8) | 100 (406.1) | 100 (368.8) | 50 (177.8) | 50 (211.2) | 100 (422.5) |
| **TME 16*** | 100 (430.5) | 100 (494.4) | 50 (231.2) | 50 (297.2) | 100 (713.4) | 50 (177.8) | 100 (406.1) | 100 (368.8) | 100 (355.7) | 50 (211.2) | 100 (422.5) |
| **TRU 45** | 100 (430.5) | 100 (494.4) | 25 (115.6) | 50 (297.2) | 100 (713.4) | 50 (177.8) | 100 (406.1) | 100 (368.8) | 50 (177.8) | 25 (105.6) | 100 (422.5) |
| **GM** | **100** (430.5) | **100** (494.4) | **35.4** (163.7) | **50** (297.2) | **100** (713.4) | **50** (177.8) | **100** (406.1) | **100** (368.8) | **70.7** (251.5) | **42** (177.4) | **100** (422.5) |
| **$MIC_{50}$** | **100** (430.5) | **100** (494.4) | **25** (115.6) | **50** (297.2) | **100** (713.4) | **50** (177.8) | **100** (406.1) | **100** (368.8) | **50** (177.8) | **50** (211.2) | **100** (422.5) |
| **CA ATCC 18804** | 100 (430.5) | 100 (494.4) | 50 (231.2) | 100 (594.4) | 100 (713.4) | 100 (355.7) | 100 (406.1) | 100 (368.8) | 100 (355.7) | 25 (105.6) | 50 (211.2) |
| **CK 02** | 100 (430.5) | 100 (494.4) | 50 (231.2) | 100 (594.4) | 100 (713.4) | 100 (355.7) | 100 (406.1) | 100 (368.8) | 100 (355.7) | 25 (105.6) | 50 (211.2) |
| **CG 09** | 100 (430.5) | 100 (494.4) | 50 (231.2) | 100 (594.4) | 100 (713.4) | 100 (355.7) | 100 (406.1) | 100 (368.8) | 100 (355.7) | 25 (105.6) | 100 (422.5) |
| **CT 72A*** | 100 (430.5) | 100 (494.4) | 50 (231.2) | 100 (594.4) | 100 (713.4) | 100 (355.7) | 100 (406.1) | 100 (368.8) | 100 (355.7) | 25 (105.6) | 100 (422.5) |
| **GM** | **100** (430.5) | **100** (494.4) | **50** (231.2) | **100** (594.4) | **100** (713.4) | **100** (355.7) | **100** (406.1) | **100** (368.8) | **100** (355.7) | **25** (105.6) | **70.7** (298.7) |
| **$MIC_{50}$** | **100** (430.5) | **100** (494.4) | **50** (231.2) | **100** (594.4) | **100** (713.4) | **100** (355.7) | **100** (406.1) | **100** (368.8) | **100** (355.7) | **25** (105.6) | **50** (211.2) |
| **GM general** | **100** (430.5) | **100** (494.4) | **42** (194.2) | **70.7** (420.3) | **100** (713.4) | **70.7** (251.5) | **100** (406.1) | **100** (368.8) | **84** (298.8) | **32.4** (136.9) | **84** (354.9) |
| **$MIC_{90}$** | **100** (430.5) | **100** (494.4) | **50** (231.2) | **50** (297.2) | **100** (713.4) | **50** (177.8) | **100** (406.1) | **100** (368.8) | **100** (355.7) | **25** (105.6) | **100** (422.5) |

Asterisk (*) in clinical strains indicates multidrug- resistance and resistance; MICs, minimal inhibitory concentration; GM, geometric mean; $MIC_{50}$, minimal concentration that inhibits approximately 50% of the strains; $MIC_{90}$, minimal concentration that inhibits approximately 90% of the strains, considering dermatophytes and *Candida* spp.. MCA, *Microsporum canis*; MGY, *Microsporum gypseum*; TME, *Trichophyton mentagrophytes*; TRU, *Trichophyton rubrum*; CA, *Candida albicans*; CK, *Candida krusei*; CG, *Candida glabrata*; CT, *Candida tropicalis*.

**Table S3** MICs (µg/mL) for the δ-lactones **3** and **10** and MECs (µg/mL) for ANF in the presence and absence of sorbitol, with readings performed at different times (48 h, 96 h, and 168 h)

| | **48 h** | | | | | | **96 h** | | | | | | **168 h** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Dermatophytes** | **3** | | **10** | | **ANF** | | **3** | | **10** | | **ANF** | | **3** | | **10** | | **ANF** | |
| **and *Candida* spp.** | S - | S + | S - | S + | S - | S + | S - | S + | S - | S + | S - | S + | S - | S + | S - | S + | S - | S + |
| **MCA 01** | - | - | - | - | - | - | 50 | 50 | 50 | 50 | 32 | 128 | 50 | 400 | 50 | 50 | 64 | >256 |
| **MGY 42** | - | - | - | - | - | - | 25 | 25 | 50 | 50 | 32 | 128 | 25 | 200 | 50 | 50 | 64 | >256 |
| **TME 16*** | - | - | - | - | - | - | 50 | 50 | 50 | 50 | 32 | 128 | 50 | 400 | 50 | 50 | 64 | >256 |
| **TRU 45** | - | - | - | - | - | - | 25 | 25 | 25 | 25 | 32 | 128 | 25 | 200 | 25 | 25 | 64 | >256 |
| **CA ATCC 18804** | 50 | 50 | 25 | 25 | 0.12 | 32 | 50 | 50 | 25 | 25 | 0.12 | >64 | 50 | 400 | 25 | 25 | 0.12 | >64 |
| **CK 02*** | 50 | 50 | 25 | 25 | 0.12 | 32 | 50 | 50 | 25 | 25 | 0.12 | >64 | 50 | 400 | 25 | 25 | 0.12 | >64 |
| **CG 09*** | 50 | 50 | 25 | 25 | 0.12 | 32 | 50 | 50 | 25 | 25 | 0.12 | >64 | 50 | 400 | 25 | 25 | 0.12 | >64 |
| **CT 72A*** | 50 | 50 | 25 | 25 | 0.12 | 32 | 50 | 50 | 25 | 25 | 0.12 | >64 | 50 | 400 | 25 | 25 | 0.12 | >64 |

*Multidrug-resistant and resistant fungal isolates. Abbreviations: MIC, minimal inhibitory concentration; MEC, minimal effective concentration; ANF, anidulafungin; S -, absence of sorbitol; S +, presence of sorbitol; MCA, *Microsporum canis*; MGY, *Microsporum gypseum*; TME, *Trichophyton mentagrophytes*; TRU, *Trichophyton rubrum*; CA, *Candida albicans*; CK, *Candida krusei*; CG, *Candida glabrata*; CT, *Candida tropicalis*.

**Table S4** MICs (μg/mL) for the δ-lactones **3**, **10** and AmB in the presence and absence of ergosterol

| **Fungi strains** | **Reading 1** | | | | | **Reading 2** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **3** | **MIC[1]** | **MIC[2]** | **MIC[3]** | **MIC[4]** | **MIC[5]** | **MIC[1]** | **MIC[2]** | **MIC[3]** | **MIC[4]** | **MIC[5]** |
| MCA 01 | 50 | 50 | 50 | 50 | 100 | 50 | 50 | 50 | 100 | 400 |
| MGY 42 | 25 | 25 | 25 | 50 | 100 | 25 | 25 | 25 | 100 | 100 |
| TME 16* | 50 | 50 | 50 | 50 | 100 | 50 | 50 | 50 | 100 | 400 |
| TRU 45 | 25 | 25 | 25 | 50 | 100 | 25 | 25 | 25 | 100 | 100 |
| CA ATCC 18804 | 50 | 50 | 50 | 100 | 200 | 50 | 50 | 50 | 200 | 400 |
| CK 02* | 50 | 50 | 50 | 100 | 200 | 50 | 50 | 50 | 200 | 400 |
| CG 09* | 50 | 50 | 50 | 200 | 200 | 50 | 50 | 50 | 200 | 400 |
| CT 72A* | 50 | 50 | 50 | 200 | 200 | 50 | 50 | 50 | 200 | 400 |
| **10** | **MIC[1]** | **MIC[2]** | **MIC[3]** | **MIC[4]** | **MIC[5]** | **MIC[1]** | **MIC[2]** | **MIC[3]** | **MIC[4]** | **MIC[5]** |
| MCA 01 | 50 | 50 | 100 | 100 | 800 | 50 | 50 | 100 | 200 | 800 |
| MGY 42 | 50 | 50 | 50 | 50 | 400 | 50 | 50 | 50 | 200 | 800 |
| TME 16* | 50 | 50 | 50 | 50 | 400 | 50 | 50 | 50 | 200 | 800 |
| TRU 45 | 25 | 25 | 50 | 50 | 200 | 25 | 25 | 50 | 200 | 400 |
| CA ATCC 18804 | 25 | 25 | 200 | 400 | 800 | 25 | 25 | 200 | 400 | 800 |
| CK 02* | 25 | 25 | 200 | 400 | 800 | 25 | 25 | 200 | 400 | 800 |
| CG 09* | 25 | 25 | 200 | 400 | 800 | 25 | 25 | 200 | 400 | 800 |
| CT 72A* | 25 | 25 | 200 | 400 | 800 | 25 | 25 | 200 | 400 | 800 |
| **AmB** | **MIC[1]** | **MIC[2]** | **MIC[3]** | **MIC[4]** | **MIC[5]** | **MIC[1]** | **MIC[2]** | **MIC[3]** | **MIC[4]** | **MIC[5]** |
| MCA 01 | 0,5 | 2 | 4 | 4 | 8 | 0,5 | 2 | 4 | 4 | 8 |
| MGY 42 | 4 | 4 | 4 | 8 | 16 | 4 | 4 | 4 | 8 | 16 |
| TME 16* | 2 | 8 | 8 | 8 | 16 | 2 | 8 | 16 | 16 | 32 |
| TRU 45 | 2 | 2 | 4 | 4 | 8 | 2 | 8 | 16 | 16 | 32 |
| CA ATCC 18804 | 1 | 2 | 4 | 16 | 16 | 1 | 2 | 16 | 128 | 128 |
| CK 02* | 0,5 | 2 | 4 | 32 | 128 | 0,5 | 2 | >128 | > 128 | > 128 |
| CG 09* | 1 | 2 | 4 | 16 | 16 | 1 | 2 | 128 | 128 | 128 |
| CT 72A* | 2 | 2 | 4 | 8 | 16 | 2 | 2 | 128 | 128 | 128 |

*Multidrug-resistant and resistant fungal isolates. AmB = Amphotericin B; CA = *C. albicans*; CT = *C. tropicalis*; CK= *C. krusei*; CG= *C. glabrata*; MCA= *M. canis*; MGY= *M. gypseum*; TME = *T. mentagrophytes*; TRU= *T. rubrum*. MIC[1] corresponds to MIC without addition of commercial ergosterol; MIC[2], MIC[3], MIC[4], and MIC[5], correspond to MIC with addition of ergosterol at the concentration of 50 μg/mL, 100 μg/mL, 150 μg/mL, and 200 μg/mL, respectively. For *Candidas*, readings 1 and 2 were performed after 2 and 4 days of incubation; for dermatophytes, readings 1 and 2 were performed after 4 and 7 days of incubation.

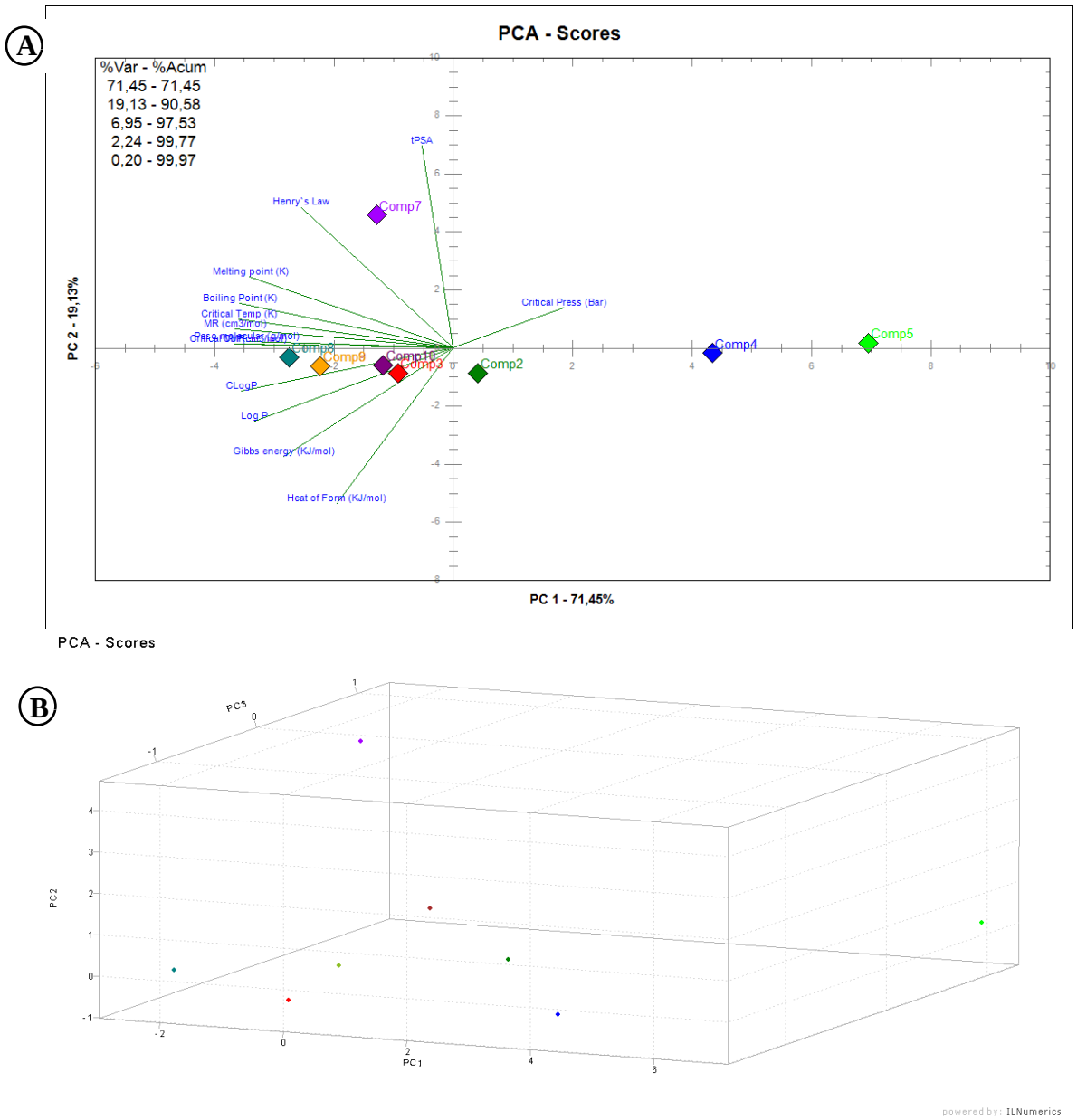


**Fig. S1** (A) Principal component analysis (PCA) with two main components - A (PC1xPC2). (B) PCA with the three main components representing 97.53% of the total variance of the model with the 14 original physico-chemical characteristics. The colors identify the same points of bidimensional PCA representation

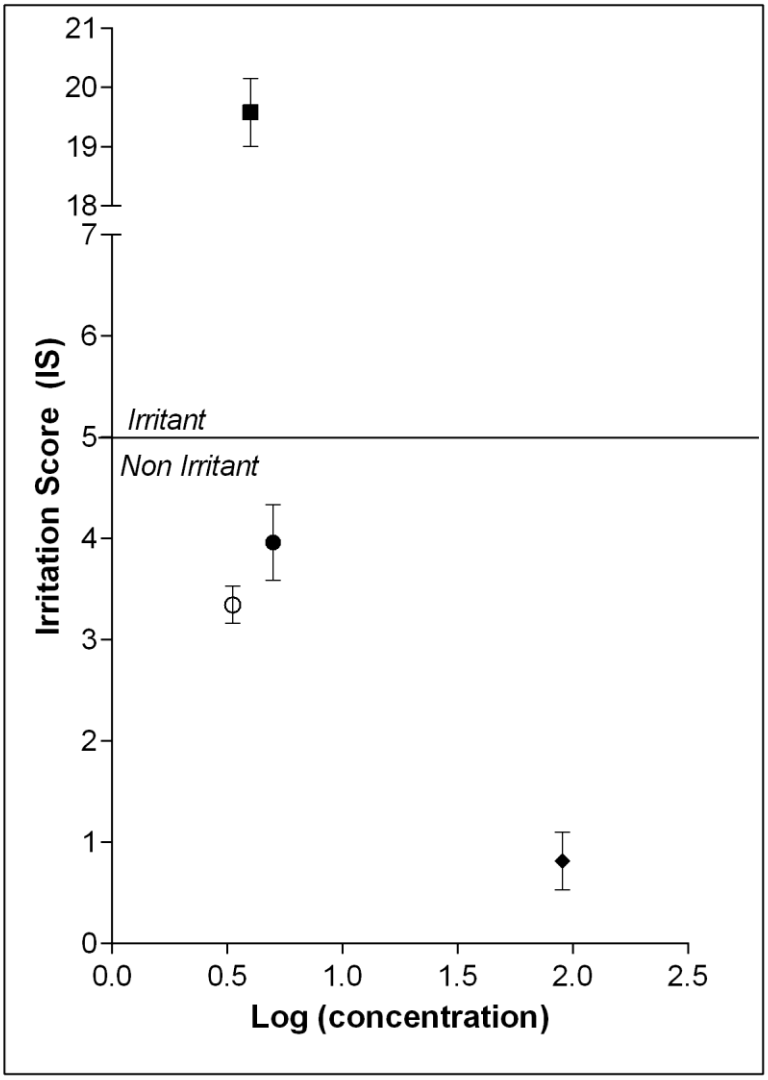


**Fig. S2** Dose-response relationship for compounds **3** (●) and **10** (o), negative control (0.9% NaCl) (♦) and positive control (0.1 NaOH) (■). Each point represents one experiment (n = three eggs). Concentrations were transformed logarithmically: 0.05 to 2

**CAPÍTULO IV – Design, synthesis, and avaluation of novel 2-substituted 1,4-benzenediol library as antimicrobial agents against clinically relevant pathogens**

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Nota: Manuscrito submetido ao periódico New Journal of Chemistry.

**Design, synthesis, and evaluation of novel 2-substituted 1,4-benzenediol library as antimicrobial agents against clinically relevant pathogens**

**Running Head**: 2-substituted-1,4-benzenediol as antimicrobial agents

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## ABSTRACT

Development of new antimicrobial agents, capable of combating resistant and multidrug-resistant fungal and bacterial clinical strains, is necessary. This study presents the synthesis and antimicrobial screening of 42 2-substituted-1,4-benzenediols, being 11 novel compounds. In total, 23 compounds showed activity against fungi and/or bacteria. Benzenediol compounds **2**, **5**, **6**, **8**, **11**, and **12** demonstrated broad spectrum antimicrobial actions, including resistant and multidrug-resistant species of dermatophytes (*Trichophyton mentagrophytes*), *Candida* spp. and the ESKAPE panel of bacteria. Minimum inhibitory concentrations of these compounds for fungi and bacterial strains

ranged from 25–50 µg/ml and 8–128 µg/ml, respectively. The antifungal mechanism of action is related to the fungal cell wall of dermatophytes and membrane disruption to dermatophytes and yeasts, in the presence of compound **8**. Specific structural changes, such as widespread thinning along the hyphae and yeast lysis, were observed by scanning electron microscopy. The effects of compound **8** on cell viability are dose-dependent; however they did not cause genotoxicity and mutagenicity in human leukocyte cells nor haemolysis. Moreover, the compounds were identified as nonirritant by the *ex-vivo* Hen's egg test-chorioallantoic membrane (HET-CAM). The furan-1,4-benzenediol compound **5** showed *in vivo* efficacy to combat *S. aureus* infection using embryonated chicken eggs. Therefore, the compounds **8**, and **5** are promising as hits for the development of new antimicrobial drugs with reduced toxicity.

**KEYWORDS:** Fungi, ESKAPE pathogens, 1,4-benzenediol derivatives, Antimicrobial activity, Mechanism of action, Toxicity.

## 1. Introduction

Fungal infections occur on the skin and mucous membranes causing invasive and systemic diseases in humans (Kim, 2016; Fuentefria et al., 2018). Dermatomycoses, or cutaneous mycoses, are the most prevalent fungal infections worldwide. Although these diseases are rarely life-threatening, they reduce the quality of life and are the main reason for patients to see a dermatologist (Pfaller et al., 2006; Nenoff et al., 2014; Dalla Lana et al., 2016). Between 2001 and 2011 in France, a retrospective study surveying human skin and nail infections showed that dermatophytes such as *M. canis*, *M. gypseum*, *T. mentagrophytes*, and *T. rubrum* were responsible for approximately 70% of cases of superficial infections (Chermette et al., 2008; Faure-Cognet et al., 2015). In addition, cats and dogs can become infected and also represent a source of dermatophytosis for humans (Chermette et al., 2008). *Fusarium*, another genus of filamentous fungi, is an emerging opportunistic fungal pathogen that causes local or systemic infections. Reports of multidrug-resistance are frequent. Hence, the successful use of a therapeutic drug, or combination of antifungal therapies against these species, is compromised (Batista et al., 2017).

*C. albicans* is the most frequently isolated fungus in humans (Li et al., 2013). However, an increase in infections caused by other *Candida* spp. has been observed, such

as: *C. krusei*, *C. tropicalis*, *C. glabrata*, *C. guilliermondii*, and *C. parapsilosis* (Kaur et al., 2016). Fungal diseases caused by *Candida* spp. are not routinely diagnosed. Consequently, hospitalized patients, especially in intensive care units, are often inappropriately treated with broad-spectrum antibiotics. Bloodstream infection and invasive candidiasis are substantially more common than realized and probably result from multiple factors, including: unrestrained antibiotic use, indwelling devices, immunocompromised patients, and renal support (Denning et al., 2017).

The Infectious Diseases Society of America classified a panel of six pathogens capable of escaping the biocidal action of drugs and responsible for a large part of the nosocomial infectious around the world as ESKAPE. This acronym is used as reference to the group of bacteria: *E. faecium, S. aureus, K. pneumonia, A. baumannii, P. aeruginosa*, and *Enterobacter* spp. (Pendleton et al., 2013). Most of these strains are multidrug-resistant which makes the development of new drugs to combat infections caused by this panel of pathogens imperative (Santajit and Indrawattana, 2016).

Polyphenols are commonly found in nature and demonstrate a remarkable range of biological activities ( Harborne et al., 1999; Whitting, 2001; Dai and Mumper, 2010; Oksana et al., 2012; De Arias et al., 2012; Soto-Hernandez et al., 2017). The potent *in vitro* and *in vivo* pharmacological activity of the 2,5,4´-trihydroxybibenzyl against *Leishmania* spp. was reported (Roldos et al., 2008; Serna et al., 2015). This result motivated us to synthesize a library of 2-substituted-1,4-benzenediols and investigate their antimicrobial effect. Therefore, this study presents the antimicrobial activity of new 2-substituted-1,4-benzenediol derivatives against a panel of clinical fungal and bacteria strains, as part of our ongoing program for the identification of new leads for the development of new antimicrobials (Silveira et al., 2012; Batista et al., 2017). In addition, the target and mechanism of action of the 1,4-benzenediols on fungal cells and the toxicity of these hits were investigated. Finally, an alternative *in vivo S. aureus* (ATCC 25923) model of infection followed by the treatment using a 1,4-benzenediol was demonstrated.

## 2. Materials and methods

### 2.1. Chemicals

In total, 42 1,4-benzenediol derivatives were evaluated for antifungal and antibacterial activity. The synthesis of compounds **1-4, 9-18**, **22**, **23**, **25-31**, **36**, and **39** (Lima et al., 2016), **5**, **19**, **32**, **33**, and **38** (Rolón et al., 2018), **20**, and **40** (Ozaki et al.,

1997 (a); Ozaki et al., 1997 (b)) were previously demonstrated (Ozaki et al., 1997 (a); Ozaki et al., 1997 (b), Lima et al., 2016; Rolón et al., 2018). General procedures for preparation of novel compounds **6**-**8**, **21**, **24**, **34**, **35**, **37**, **41**, and **42**, and the hydrogen characterization data of other compounds that not yet been presented (**20**, and **40**) are shown in the supp. info. All compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted using sterile ultrapure water to give solutions with DMSO concentrations lower than 1% for subsequent investigation of biological activities, mechanism of action and toxicity.

### 2.2. *In vitro* susceptibility tests

#### 2.2.1. Antifungal agents

Terbinafine (TBF), amphotericin B (AMB), and anidulafungin (AFG), purity ≥ 97%, were supplied by Cristalia (Sao Paulo, Brazil), griseofulvin (GSF), purity ≥ 97%, was acquired from Wallace Pharmaceuticals (Mumbai, India), ketoconazole (KTC), purity ≥ 96%, was obtained from All Chemistry (Sao Paulo, Brazil), fluconazole (FLC) purity ≥ 98% was obtained from Sigma-Aldrich (Sao Paulo, Brazil), itraconazole (ITC) purity ≥ 97%, was supplied by Cassará laboratory (Dist. PHARMOS; Buenos Aires, Argentina), miconazole (MCZ) purity ≥ 97%, was supplied by Valdequimica Chemical Products (Sao Paulo, Brazil), and voriconazole (VRC); purity ≥ 98%, was supplied by Pfizer (Sao Paulo, Brazil). The preparation of stock and work solutions followed the recommendations of the CLSI (M38-A2, 2008). Commercial antifungals were used as references.

#### 2.2.2. Fungal strains

*Candida* spp. (*C. albicans* - CA ATCC 18804, *C. krusei* - CK 02*, *C. glabrata* - CG 09*, and *C. tropicalis* - CT 72A*), dermatophytic filamentous fungi (*M. canis* - MCA 01, *M. gypseum* - MGY 42, *T. mentagrophytes* - TME 16*, and *T. rubrum* - TRU 45), and other filamentous fungi (*G. fujikuroi* – F2 and *F. solani* – F9 were selected to evaluate the antifungal activity of the 1,4-benzenediols. Some clinical strains are resistant or multidrug-resistant (*). For *Candida* spp. the resistance was defined based on Kuriyama et al. (2005) and the Clinical and Laboratory Standards Institute (CLSI) breakpoints (M27-A3, 2008; M27-S4, 2012) considering, in this case, the minimum inhibitory concentration (MIC) values: CK 02* resistant to: ITC (MIC = 1 µg/ml) and FLC (MIC ≥

64 µg/mL), GC09* ITC (MIC > 4 µg/ml) as well as MCZ (MIC = 8 µg/ml) resistant, and CT 72A* resistant to ITC (MIC = 1 µg/ml), MCZ (MIC > 8 µg/ml), and VRC (MIC = 2 µg/ml). As for dermatophytes, the resistance (in the sense of reduced susceptibility) was established considering the following resistance threshold concentrations (MICs): terbinafine (≥ 1.0 µg/ml), griseofulvin (≥ 4.0 µg/ml), and ketoconazole (≥ 8.0 µg/ml). Clinical isolate TME 16* was considered multidrug-resistant (MIC terbinafine = 4 µg/ml, MIC griseofulvin > 32 µg/ml and MIC ketoconazole = 16 µg/ml). *G. fujikuroi* and *F. solani* are resistant to itraconazole and fluconazole (MICs >128 µg/ml). All the clinical and reference strains used in this study are deposited in the Mycology Collection of the Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil.

Susceptibility tests were performed for determining the MIC and the minimum fungicidal concentration (MFC). The MIC was determined by the broth microdilution method, according to the protocol established for yeasts - M27-A3 (CLSI, 2008) and filamentous fungi - M38-A2 (CLSI, 2008). The inoculum of yeasts ($0.5 \times 10^3$ to $2.5 \times 10^3$ CFU/ml) and filamentous fungi ($1.0 \times 10^3$ to $3.0 \times 10^3$ CFU/ml) were prepared from cultures grown on sabouraud dextrose agar (SDA; Kasvi, Curitiba, Brazil) and potato dextrose agar (PDA; Neogen, Lansing, USA), respectively. The assays were conducted in duplicate, with RPMI medium, containing L-glutamine (without sodium bicarbonate; Gibco, USA), buffered to pH 7.0 with 0.165 M of 3-(*N*-morpholino)propanesulfonicacid (MOPS; Neon, São Paulo, Brazil), with subsequent incubation at 35 ºC. The analysed concentrations of the synthetic compounds ranged from 0.03–50 µg/ml. The reading of the results for *Candida* spp. and *Fusarium* spp. was performed in 24–48 h, and for dermatophytes after 96 h of incubation. MIC was defined as the lowest concentration of the substance capable of inhibiting the visible fungal growth. Sterility control (drug-free medium only) and positive control (inoculum and culture medium only, for evaluation of the fungal cell viability) were used.

To determine the MFC, aliquots of each serial microdilution (corresponding to MIC, 2×MIC and 4×MIC) were spread on SDA (*Candida*) and PDA (filamentous fungi), which were incubated at 35 °C and analysed. The MFC was defined as the lowest drug concentration that yielded up to three colonies (*i.e.*, ≥ 99% of the inoculum was killed) (Espinel-Ingroff et al., 2002). The assay was performed in triplicate.

### 2.2.3. Antibacterial agents

Oxacillin, ampicillin, gentamicin and imipenem purity ≥ 97% were purchased from Sigma-Aldrich (Sao Paulo, Brazil).

### 2.2.4. Bacterial strains

*E. coli* (ATCC 25922) and ESKAPE panel of pathogens, including strains of *S. aureus* methicillin-resistant (ATCC 33591) and methicillin-susceptible (ATCC 25923), *E. faecalis* (ATCC 29212) and (ATCC 51299), *K. pneumoniae* (ATCC700603), *P. aeruginosa* (ATCC 27853), *A. baumanni* (ATCC 19606), *A. baumanni* (IOC 3174), and *E. aerogenes* (ATCC 13048) were obtained by donation from Instituto Oswaldo Cruz, RJ, Brazil. Evaluation of MICs followed the CLSI microdilution method using BBL™ Mueller Hinton II broth (Interlab, Brazil) as described previously - M100 (CLSI, 2018). Briefly, two-fold serial dilutions of each compound were prepared in triplicate in 96-well plates and inoculated with $5 \times 10^5$ CFU/ml of the bacterial suspension. Plates were incubated at 37 °C for 16–20 h (CLSI, 2018). Antibiotics abovementioned were used as controls.

## 2.3. *In vivo* antifungal efficacy in Infected Egg Test-Chorioallantoic Membrane (IET-CAM)

Fresh and fertile white eggs (Lohmann selected Leghorn, LSL) were kept under optimized incubation conditions (38–39 °C, 55–60% humidity, 12 days). The preparation of *S. aureus* strain (ATCC 25923) inoculum followed the M100 document recommendations (CLSI, 2018). On the 4$^{th}$ day, the CAM of eggs were infected with 0.1 ml inoculum 4x/day and the infectious process confirmed by the visualization of colonies onto the CAM (holes sealed with parafilm). The treatment started on the 8$^{th}$ day of incubation. An aliquot of 0.1 ml of the compound **5** (64 µg/ml, 0.5% DMSO solution) or 0.1 ml 0.9% NaCl (negative control) was added onto the CAM. On the 12$^{th}$ day, eggs were opened and 0.1 ml aliquot removed and spread into Blood agar. Then, the plates were incubated at 35 °C for 48 h, for subsequent counting of CFU/ml (Jacobsen et al., 2010). This study is approved by the Animal Ethics Committee (CEUA), protocol number 4/2016 (Instituto Federal Catarinense, Concordia, Brazil). The experiment was carried out in triplicate.

## 2.4. Investigation of antifungal mechanism of action

### 2.4.1. Sorbitol assay

The effect of the benzenediol derivatives on the integrity of the fungal cell wall was evaluated by sorbitol protection assay (Frost et al., 1995, Escalante et al., 2008). MICs were determined by the standard broth microdilution (M27-A3 (CLSI, 2008) for *Candida* spp. and M38-A2 (CLSI, 2008) for dermatophytes) in the absence and presence of 0.8 M sorbitol (Sigma-Aldrich) added to the RPMI 1640 growth medium (Gibco) as an osmoprotectant. The minimal effective concentration (MEC), which is the lowest concentration of antifungal agent that leads to the growth of small, round and compact hyphal forms, was determined only for the antifungal AFG (M38-A2 document– CLSI, 2008). Microplates were incubated at 35 °C for 168 h. AFG (Pfizer, New York, USA) was used as positive control. MICs were measured after 48, 96, and 168 h for *Candida* spp.; and after 96 and 168 h for dermatophytes (Frost et al., 1995, Escalante et al., 2008). Experiments were carried out in triplicate.

### 2.4.2. Ergosterol assay

The ability of the benzenediol derivatives to complex with ergosterol in the fungal membrane was evaluated by ergosterol binding assay. The MICs were determined by the standard broth microdilution following the documents M27-A3 and M38-A2 (CLSI, 2008) in the absence and presence of different concentrations (50–200 mg/ml) of external ergosterol (Sigma-Aldrich, St. Louis, MO, USA) added to the RPMI 1640 growth medium. The plates were incubated at were incubated at 35 °C for 168 h. AMB was used as positive control. MICs were measured after 2 and 5 days for *Candida* spp.; and after 4 and 7 days for dermatophytes (Escalante et al., 2008; Carrasco et al., 2012). Experiments were carried out in triplicate.

### 2.4.3. SEM analysis

The experiment followed the M27-A3 and M38-A2 guidelines (CLSI, 2008): *C. albicans* and *M. canis* adhered cells obtained from the susceptibility test were washed with phosphate buffer saline (PBS). Then, 500 µl glutaraldehyde (2.5%, type 1, Sigma Aldrich) was poured into the cells and the mixture diluted with 50 ml sodium cacodylate (0.1 M, pH 7.2, Sigma Aldrich) and kept for 1 h at room temperature. Next, the cells were washed three times with sodium cacodylate (0.1 M, pH 7.2, 10 ml) containing sucrose (0.2 M) and $MgCl_2$ (2 mM). Adhered cells were dehydrated using solutions of ethanol (30, 50 and 70%, for 5 min, then 95% and 100% (2×) for 10 min). Samples were subjected

to critical point drying (EM CPD 300, Leica) immediately after dehydration, mounted on metallic stubs, sputter-coated with a 15–20 nm gold-palladium layer, and visualized in a scanning electron microscope (Carl Zeiss EVO® MA10, Oberkochen, Germany) operating at 10 kV. Concentration evaluated **8** (25 µg/ml). Drug controls: TBF (0.016 µg/ml for *M. canis*) and ITC (1 µg/ml for *C. albicans*).

### 2.4.4. Epifluorescence microscopy

*C. albicans* and *M. canis* samples (approximately 200 µl) treated with the compound **24** (50 µg/ml) from the susceptibility test were used to epifluorescence microscopy analysis (Silveira et al., 2017; Soo et al., 2017). This mixture was placed over a slide and analysed by the epifluorescent microscope (Bel Photonics®) (B–green filter, C–UV filter) equipped with a CCD blacklight camera. Photos were taken with a digital camera (NIKON model D600, macro mode, 60 mm, f/3.2D) after 60 s (dermatophytes) and 5 s (*Candida* spp.) (Barros et al., 2016).

## 2.6. Toxicity evaluation

### 2.6.1. Cell culture, cytotoxicity, genotoxicity and mutagenicity

Cell cultures of human leukocytes were prepared using venous blood collected by venepuncture from a male volunteer (number of the approval protocol of the Ethics Committee: 23.081.005770/009-38). Aliquots (1 ml) of whole blood were immediately transferred to 10 ml of RPMI 1640 medium supplemented with 1% phytohaemagglutinin, 10% foetal bovine serum and 1% streptomycin/penicillin (Montagner et al., 2010). Subsequently, cell culture treatments were performed with compound **8** (25 µg/ml and 50 µg/ml, in 0.5% DMSO), hydrogen peroxide solution ($H_2O_2$, 100 µM, positive control), and PBS (negative control). Cell cultures were incubated ($CO_2$ incubator for cell culture, 5% $CO_2$, Model MCO-19AIC, Sanyo) at 37 °C for 72 h (Montagner et al., 2010). Cytotoxic, genotoxic, and mutagenic parameters were established in triplicate. Cell viability was assessed with 0.2% trypan blue (Sigma-Aldrich), according Burow et al. (1998). Genotoxicity was performed by the comet assay. Cells were classified from 0 (no visible damage) to 4 (maximum damage) to provide the damage index (DI) from 0 to 400 (Singh et al., 1988). The assessment of mutagenicity was by Panótico Rápido® (Laborclin). In total, 100 cells were counted per slide. All particles within the cells were

separated from the nucleus and counted as micronuclei (MN) (Thomas et al., 2008; Montagner et al., 2010).

### 2.6.2. Haemolysis assay

Fresh rabbit blood was mixed with the anticoagulant K2-EDTA. Rabbit erythrocytes were harvested by centrifugation for 5 min at 400 rpm and washed three times with PBS buffer. Serial dilutions of 1,4-benzenediol derivatives **2**, **5**, **8**, and **12** (25 to 100 μg/ml) were prepared and incubated with 1% erythrocyte suspension (PBS) for 15 min at 37 °C. Then, the cells were spun down by centrifugation and the supernatant transferred to a 96-well plate. Next, the absorbance (650 nm) was measured using a microplate spectrophotometer (BioTek Instruments). PBS (negative, 0% haemolysis) and water (positive, 100% haemolysis) were used as controls.

### 2.6.3. Hen's egg test-chorioallantoic membrane (HET-CAM)

Fresh and fertile white eggs (Lohmann selected Leghorn, LSL) were kept under optimized incubation conditions (38–39 °C, 55–60% humidity, 10 days). On the $10^{th}$ day, the eggshell, around the airspace, was removed with a rotary tool (Dremel, WI). Subsequently, 0.3 ml of the compounds **2**, **5**, and **12** (64 μg/ml, 0.5% DMSO solution), **8** (50 μg/ml, 0.5% DMSO solution) and controls (negative control: 0.9% NaCl; positive control: 0.1 M NaOH) were added to the CAM of the eggs (Pammi et al., 2013). The irritant effect was observed at three times: 30 sec, 2 min, and 5 min after application of substances and controls. The result of the irritation score (IS) was calculated according to the equation 1 (Pammi et al., 2013) and presents a maximum value of 21. The eggs were analysed in relation to the appearance of haemorrhaging, lysis and coagulation. Classification criterion used: 0 to 4.9 nonirritant (or practically no irritation); 5.0 to 21 irritant (moderate to severe or extreme irritation). The time of bleeding, lysis, and coagulation are expressed in seconds, considering the first occurrence of blood haemorrhage, vessel lysis and protein coagulation, respectively. The assay was performed in triplicate.

**Equation 1**. Formula for the determination of irritation score (IS)

$$\mathrm{IS} = \left(\left(\frac{(301 - \text{hemorrhage time})}{300}\right)\mathrm{x5}\right) + \left(\left(\frac{(301 - \text{lysis time})}{300}\right)\mathrm{x7}\right) + \left(\left(\frac{(301 - \text{coagulation time})}{300}\right)\mathrm{x9}\right)$$

### 2.7. Statistical analysis

Differences between controls and treatments were statistically analysed by ANOVA followed by the Tukey's test ($p < 0.05$ was considered statistically significant). Data analysis was performed using the GraphPad Prism 5.0 software.

## 3. Results

### 3.1. Chemicals

#### 3.1.1 Synthesis of 2-substituted 1,4-benzenediols

We developed a synthetic method for the synthesis of 2-substituted 1,4-benzenediols (Lima et al., 2016; Rolón et al., 2018) based on green chemistry principles (Anastas and Warner, 1998). This strategy involves a tandem aldolic condensation/isomerization/aromatization between 1,4-cyclohexanedione and the selected aldehyde, to give 2-substituted-1,4-benzenediol derivatives with moderate to excellent yields (see supp. info; Scheme S1 and S2). Unfortunately, the nitro group could not be introduced because of synthetic unsuitability. Our groups are focusing in preparing the $CF_3$ derivative to support SAR observations and the results will be reported in the due course (see supp. info.). All spectral and chemical data for the new molecules are available at the supp. info. (FIG. S1-S30).

#### 3.1.2 General procedure for the synthesis of (*E*)-2-(2,5-dihydroxystyryl) quinoline:

Diacetoxystyryl quinolines were prepared based on Polansky´s method (Polanski et al., 2002). Methylquinaldine derivatives were treated with 2,5-dihydroxybenzaldehyde in acetic anhydride to obtain the desired diacetoxystyryl analogues after an aldolic condensation reaction. This procedure takes advantage of using the acidity of the methyl group of methylquinaldines. Then, the diacetoxy derivatives obtained were hydrolysed in sulfuric acid (12 M) under reflux until the reaction was completed by thin layer chromatography (TLC) analysis (see supp. info.; Scheme S4).

#### 3.1.3 General procedure for the synthesis of 2-(2,5-dihydroxyphenethyl) quinoline derivatives:

2,5-Dihydroxyphenethyl quinoline derivatives were synthetized from the respective unsaturated starting material under usual catalytic hydrogenation conditions

(see supp. info.; Scheme S5). Surprisingly, low yields were obtained, which might be associated with the quinolone moiety absorption over the charcoal used as a support for the metal catalyst during catalysis.

### 3.2. *In vitro* susceptibility tests

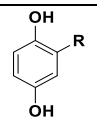
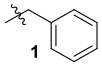
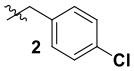
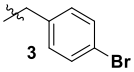
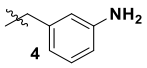
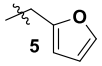
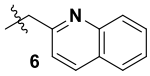
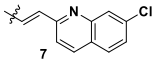
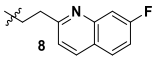
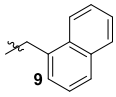
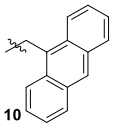
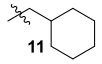
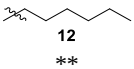
#### 3.2.1. Fungal and bacterial strains

Eight strains were used to determine the MICs and the MFCs of 42 1,4-benzenediol derivatives. In total, 12 compounds showed antifungal activity: **1-12** (Table 1). 1,4-Benzenediols **1** and **10** were fungicide for all *Candida* spp. analysed at 50 µg/ml. Derivative **7** was fungistatic at 25 µg/ml for the whole panel of yeasts, which was the lowest MIC observed for the library. Compounds **2**, **4**, and **6** exhibited action only against dermatophytes, being fungistatic at 50 µg/ml. Meanwhile, 1,4-benzenediols **9** and **11** proved to be fungicides at 50 µg/ml, Table 1. The furan derivative **5** was active against just one *Candida* strain (CA ATCC 18804) and the whole panel of dermatophytes tested. Compounds **3** and **12** inhibited the growth of *G. fujikuroi* and *F. solani* (50 µg/ml). These two strains are resistant to ITC and FLC (MICs >128 µg/ml). Finally, the fluoroquinoline derivative **8** (fungicide, MICs = MFCs of 50 µg/ml, Table 1) demonstrated broad spectrum antifungal activity against the entire panel of dermatophytes and yeasts tested. This panel includes the multidrug-resistant and resistant species of *T. mentagrophytes*, *C. krusei*, *C. glabrata*, and *C. tropicalis* (MICs and breakpoints of commercial antifungal agents are presented in Table 1).

Next, 17 compounds were found to be active (MICs ≤ 128 µg/ml) against the Gram-positive bacteria (*S. aureus* methicillin sensitive (ATCC 25923), *S. aureus* methicillin resistant (ATCC 33591), *E. faecalis* (ATCC 29212), and *E. faecalis* (ATCC 51299)) from the ESKAPE panel of pathogens: **2**, **5**, **6**, **8**, **11-23**. The furan derivative **5** demonstrated the highest activity of the library of 1,4-benzodiols (MICs 8–64 µg/ml, Table 2).

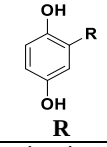
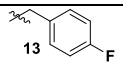
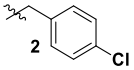
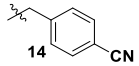
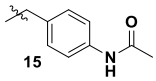
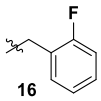
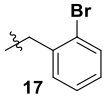
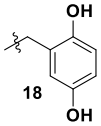
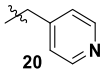
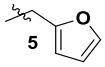
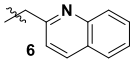
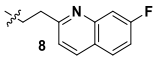
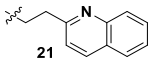
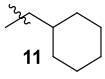
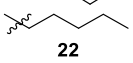
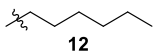
1,4-Benzenediols **2**, **5**, **19**, and **20** were also active (MICs ≤ 64 µg/ml) against *E. coli* (ATCC 25922), which is used as a model for identification of molecules that could inhibit the growth of Gram-negative bacteria, Table 2.

**TABLE 1**. MICs/MFCs (µg/ml) of 1,4-benzenediols derivatives against a panel of eight filamentous fungi and yeasts.

|  R | **Dermatophytes** | | | | ***Candida* spp.** | | | |
|---|---|---|---|---|---|---|---|---|
| | **MCA 01** | **MGY 42** | **TME 16*** | **TRU 45** | **CA ATCC 18804** | **CK 02*** | **CG 09*** | **CT 72A*** |
|  1 | >50/>50 | >50/>50 | >50/>50 | >50/>50 | 50/50 | 50/50 | 50/50 | 50/50 |
|  2 | 50/>50 | 50/>50 | 50/>50 | 50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 |
|  3 ** | 50/>50 | 50/>50 | 50/>50 | 50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 |
|  4 | 50/>50 | 50/>50 | 50/>50 | 50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 |
|  5 | 50/>50 | 50/>50 | 50/>50 | 50/>50 | 50/>50 | >50/>50 | >50/>50 | >50/>50 |
|  6 | 50/>50 | 50/>50 | 50/>50 | 50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 |
|  7 | >50/>50 | >50/>50 | >50/>50 | >50/>50 | 25/>50 | 25/>50 | 25/>50 | 25/>50 |
|  8 | 50/50 | 50/50 | 50/50 | 50/50 | 50/50 | 50/50 | 50/50 | 50/50 |
|  9 | 50/50 | 50/50 | 50/50 | 50/50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 |
|  10 | >50/>50 | >50/>50 | >50/>50 | >50/>50 | 50/50 | 50/50 | 50/50 | 50/50 |
|  11 | 50/50 | 50/50 | 50/50 | 50/50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 |
|  12 ** | 50/>50 | 50/>50 | 50/>50 | 50/>50 | >50/>50 | >50/>50 | >50/>50 | >50/>50 |
| **TBF** | 0.03 (S) | 0.03 (S) | 4 (R*) | 0.06 (S) | - | - | - | - |
| **GSF** | 1 (S) | 1 (S) | >32 (R*) | 1 (S) | - | - | - | - |
| **KTC** | 0.5 (S) | 1 (S) | 16 (R*) | 1 (S) | 0.25 (S) | 1 (S) | 0.5 (S) | 1 (S) |
| **FLC** | - | - | - | - | 1 (S) | ≥ 64 (R) | 0.25 (DDS) | 2 (S) |
| **ITC** | - | - | - | - | - | 1 (R) | >4 (R) | 1 (R) |
| **MCZ** | - | - | - | - | - | 0.5 (S) | >8 (R) | >8 (R) |
| **VRC** | - | - | - | - | - | - | - | 2 (R) |

*Multidrug-resistant and resistant fungal isolates; MIC, minimal inhibitory concentration; MFC, minimal fungicidal concentration; MCA, *Microsporum canis*; MGY, *Microsporumgypseum*; TME, *Trichophyton mentagrophytes*; TRU, *Trichophyton rubrum*; CA, *Candida albicans*; CK, *Candida krusei*; CG, *Candida glabrata*; CT, *Candida tropicalis*. TBF, terbinafine; GSF, griseofulvin; KTC, ketoconazole; FLC, fluconazole; ITC, itraconazole; MCZ, miconazole; VRC, voriconazole; R, resistance; R*, resistance in the sense of reduced susceptibility compared to other strains; S, susceptible; DDS, dose-dependent susceptibility. **1,4-benzenodiols **3** and **12** showed antifungal activity at 50 µg/ml for *Gibberella fujikuroi* (F2) and *Fusarium solani* (F9), which are resistant to ITC and FLC (MICs >128 µg/ml).

**TABLE 2**. MICs (µg/ml) of 1,4-benzenediols derivatives against the Gram-positive strains *S. aureus*, *E. faecalis* and the Gram-negative strain *E. coli*.

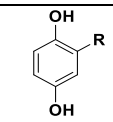
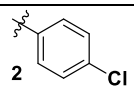
|  **R** | **Bacteria** | | | | |
|---|---|---|---|---|---|
| | ***S. aureus*** **ATCC 25923**[a] | ***S. aureus*** **ATCC 33591** | ***E. faecalis*** **ATCC 51299** | ***E. faecalis*** **ATCC 29212** | ***E. coli*** **ATCC 25922** |
|  **13** | >128 | 8 | 32 | 16 | >128 |
|  **2** | 64 | 8 | >128 | 16 | 32 |
|  **14** | >128 | 32 | >128 | >128 | >128 |
|  **15** | >128 | 32 | 8 | 32 | >128 |
|  **16** | >128 | 64 | 64 | 128 | >128 |
|  **17** | >128 | 64 | 32 | 8 | >128 |
|  **18** | >128 | >128 | 64 | 64 | >128 |
|  **19** | 128 | 64 | >128 | >128 | 64 |
|  **20** | 128 | 64 | 16 | 32 | 32 |
|  **5** | 8 | 8 | 64 | 16 | 8 |
|  **6** | 64 | 16 | 64 | 128 | >128 |
|  **8** | >128 | 64 | >128 | >128 | >128 |
|  **21** | >128 | 64 | 32 | >128 | >128 |
|  **11** | >128 | >128 | 8 | >128 | >128 |
|  **22** | >128 | 32 | 128 | 16 | 128 |
|  **12** | 16 | 64 | 8 | 8 | >128 |
|  **23** | >128 | 32 | 16 | 32 | >128 |
| **oxacilin** | 0.5 | 2 | 16 | - | - |
| **ampicilin** | 1 | 1 | 1 | 1 | 4 |

[a]resistant to methicillin.

1,4-Benzenediols **2**, **5**, **19**, and **20** were screened against the entire Gram-negative ESKAPE panel. The 4-chlorophenyl derivative **2** presented moderate activity (32–128

µg/ml) against the strains tested. This substance showed a broad spectrum of action inhibiting the growth of Gram-negative and Gram-positive bacteria from the ESKAPE panel. Meanwhile, the furan benzenediol **5** demonstrated an MIC of 8 µg/ml to *K. pneumoniae* (ATCC 700603) being the most active molecule of the library on this strain, Table 3.

**TABLE 3**. MICs (µg/ml) of 1,4-benzenediol derivatives against Gram-negative ESKAPE panel bacteria.

|  R | Bacteria | | | | |
|---|---|---|---|---|---|
| | ***K. pneumoniae* ATCC700603** | ***P. aeruginosa* ATCC 27853** | ***A. baumannii* ATCC 19606** | ***A. baumanni* IOC 3174** | ***E.aerogenes* ATCC 13048** |
|  2 | 64 | 32 | 64 | 64 | 128 |
|  5 | 8 | >128 | >128 | >128 | >128 |
|  19 | >128 | >128 | >128 | >128 | >128 |
|  20 | 128 | 128 | 64 | 128 | 128 |
| **Antibiotic** | >128[a] | 1[b] | 2[c] | 2[c] | 1[c] |

[a]ampicilin; [b]gentamicin; [c]imipinem; IOC: Instituto Oswaldo Cruz

Another 19 1,4-benzenediols synthesized neither showed *in vitro* antifungal nor antibacterial *in vitro* activities, up to the highest concentration tested for fungi and bacteria, 50 µg/ml and 128 µg/ml, respectively (see supp. info; FIG S31).

### 3.3. Antifungal mechanism of action

#### 3.3.1. Sorbitol assay

The influence of sorbitol on the MICs of the vinyl-fluoroquinoline-1,4-benzenediol **8** against the whole panel of dermatophytes and *Candida* spp. were evaluated, using the AFG as antifungal control. For dermatophytes, after 168 h of incubation, MICs (Table 1) to **8** folded once in the presence of sorbitol, whereas for AFG the MICs (Table 1) increased more than four times (Table S1). MICs (Table 1) changed more abruptly when sorbitol was added to the yeast medium in the presence of compound **8**: twice at 96 h and 16-fold after 168 h. MICs to *Candida* spp., using AFG as control, changed over 500-fold for the whole period of analysis (data are present in the supp. info., Table S1).

### 3.3.2. Ergosterol assay

After addition of ergosterol to the culture medium of the susceptibility test with *Candida* spp. and dermatophytes, MICs of compound **8** rose. For yeasts (*C. albicans*, *C. tropicalis*, *C. krusei*, and *C. glabrata*), MICs increased up to 4–8-fold after adding a total of 200 μg/ml of ergosterol, during five days of the experiment. As expected, the AMB drug control demonstrated an ergosterol dependent effect. For instance, after adding 200 μg/ml of ergosterol, MIC of AMB against *C. tropicalis* jumped from 0.5 to > 128 μg/ml. The same trend was observed for the dermatophytes. However, MICs increased more discretely in the case of filamentous fungi (twice for compound **8** and up to 16 times for AMB, after 7 days and maximum concentration of ergosterol). Data are present in the supp. info., Table S2.

### 3.3.3. SEM analysis

*C. albicans* was treated separately with ITC (drug control) and compound **8**. It was noticed that before the addition of the substances, *C. albicans* cells presented the expected oval shape without alteration (FIG 1A). After treatment with ITC, changes in cell shape and size were remarkable (FIG 1B; arrows point to damaged cells). The same was observed in the cells in the presence of **8**. This compound appeared to lyse yeast cells (FIG 1C; arrows point to damaged cells). Intact hyphae of *M. canis* (dermatophyte) were observed, and are indicated in FIG 1D. After treatment with TBF, hyphae collapsed (FIG 1E; arrows point to damaged cells). The same was observed for the vinyl fluoroquinoline-benzenediol **8** (FIG 1F; arrows point to damaged cells).

### 3.3.4. Epifluorescence microscopy

*C. albicans* and *M. canis* were treated with the fluorescent vinyl-fluoroquinoline-1,4-benzenediol **24** which was inactive against both fungi (MICs > 50 μg/ml, Table 1). After 5 min, fungi cells were observed under white light using an epifluorescence microscope equipped with a digital camera. The characteristic oval and filamentous shapes of yeasts and filamentous fungi cells were noted, respectively. Then, fungi cells were exposed to an ultraviolet light source with a 365 nm wavelength. Pictures were taken successively after 5 s for *C. albicans* and 60 s for *M. canis* using the confocal microscope (FIG 2, B-E and G-I, respectively). The last photo was taken of *M. canis* after 300 s from the beginning of the experiment (FIG 2J). The quinoline derivative **24** was able to

penetrate into both fungi cells (FIG 2, B-D and G-I) and deteriorated much faster inside *C. albicans* than *M. canis* due to the UV-light (FIG 2).

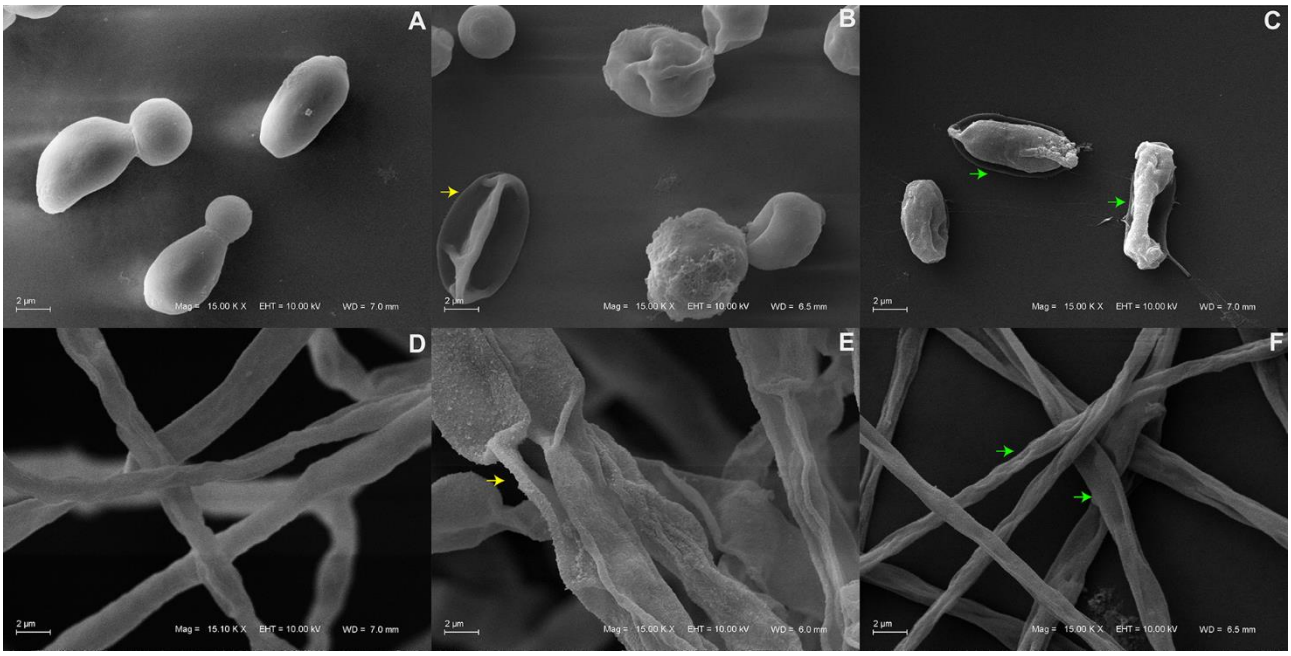



**FIG 1** SEM images; (A) *C. albicans* without treatment, (B) *C. albicans* treated with ITC, (C) *C. albicans* treated with compound **8**, (D) *M. canis* without treatment, (E) *M. canis* treated with TBF, and (F) *M. canis* treated with compound **8**.

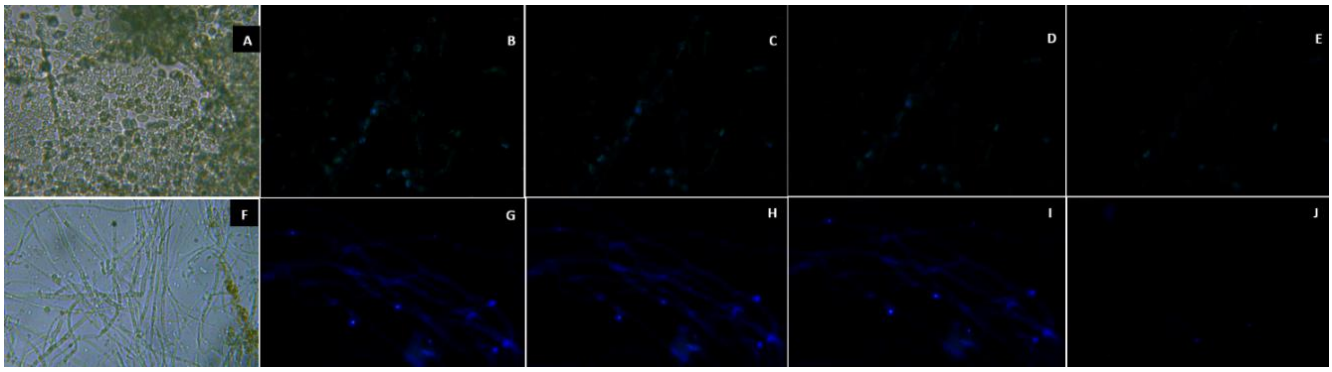



**FIG 2** Epifluorescence microscopy. (A) *C. albicans* before adding the fluorescent probe the vinylfluoroquinoline-1,4-benzenediol **24**; (B, C, D and E): *C. albicans* treated with **24**. Picture taken after 5 s; (F) *M. canis* before adding **24**; (G, H and I): *M. canis* treated with **24**. Pictures were taken after 60 s**,** and (J) after 300 s.

### 3.4. Toxicity evaluation

#### 3.4.1. Cytotoxicity, genotoxicity and mutagenicity

The viability of human leukocytes was approximately 70% in the presence of the vinyl-fluoroquinoline **8** (50 μg/ml), FIG 3(a). Meanwhile, PBS used as negative control demonstrated approximately 100% cell viability, FIG 3(a-c). However, at this

concentration (50 μg/ml), compound **8** did not cause significant DNA damage (FIG 3(c)) and was not mutagenic (since there was no significant micronucleus frequency after *in vitro* treatment with this compound (FIG 3(b)).

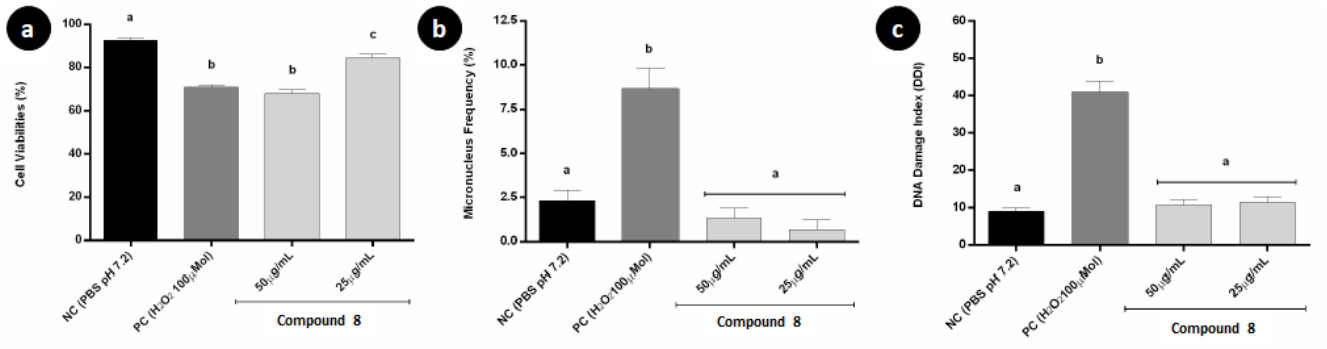



**FIG 3** Toxicological analysis; (a) cell viability, (b) micronucleus frequency, and (c) DNA damage effects of compound **8**. Bars with the same letters indicate statistical similarity (ANOVA–Tukey's test).

### 3.4.2. Haemolysis

The mean percentage of rabbit erythrocytes haemolysis (± standard deviation (SD)) of 1,4-benzenediol derivatives **2**, **5**, **8**, and **12**, at 100 μg/ml (concentration was two times higher than MIC) were 4.22 ± 0.0045, 4.25 ± 0.0052, 4.37 ± 0.0021 and 3.88 ± 0.0007, respectively.

### 3.4.3. HET-CAM

The HET-CAM test resulted in an IS for **2** (64 μg/ml), **5** (64 μg/ml), **8** (50 μg/ml), and **12** (64 μg/ml) of 2.66, 2.48, 2.94, and 2.62, respectively (see supp. info. – Figures S32 and S33), which classify these compounds as nonirritants, according to the methodology.

### 3.5. IET-CAM

The results of the IET-CAM showed eggs with an initial colony count of $10^2$-$10^3$ CFU/ml for *S. aureus* (ATCC 25923). After the incubation period, the absence of bacterial growth in the plates after treatment with 64 μg/ml of the furan-benzenediol **5** (FIG 4B) was observed. In contrast, $10^4$ to $10^5$ CFU/ml for untreated eggs (FIG 4A) was noted.

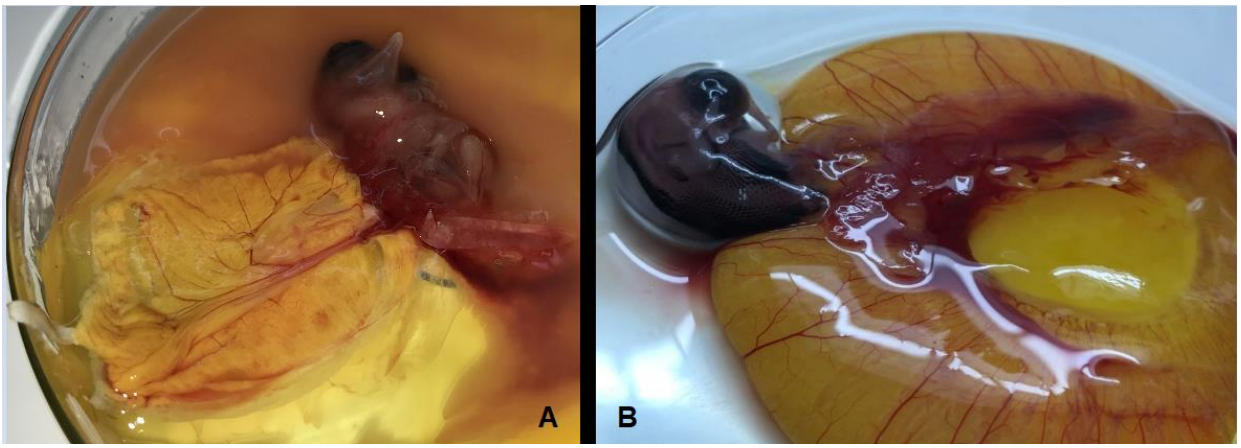



**FIG 4** Macroscopic changes in infected embryonated eggs. Eggs were infected on the 4th developmental day. (A) Control, no treatment (unviable embryo; *S. aureus* (ATCC 25923)). (B) After treatment with the furan derivative, compound **5** (64 µg/ml) - viable embryo.

## 4. Discussion

A library of 1,4-benzenediol derivatives were screened against eight fungi strains (four filamentous and four yeasts), as well as ten Gram-positive and Gram-negative bacteria. This library was prepared by the reaction of an aldehyde with 1,4-cyclohexanedione, using green chemistry conditions: $K_2CO_3$ as a base, in water:ethanol (9:1) mixture as solvent. Twelve new compounds were prepared by this methodology (see supp. info.; Scheme S1).

The fluoroquinoline-1,4-benzenediol **8** was identified as the best line of attack to the fungi strains, with the fungicidal action at 50 µg/ml for the whole panel (Table 1). This compound was further evaluated to determine its mechanism of action using the sorbitol and ergosterol assays.

Sorbitol exerts osmotic protection to the fungi cell wall (Frost et al., 1995; Batista et al., 2017). Consequently, the addition of sorbitol to the medium will decrease antifungal activity, if the drug acts on this target. In the presence of sorbitol, MICs for **8** increased abruptly, indicating that this molecule acted on the fungal cell wall of the yeasts and filamentous fungi. This effect seems to be weaker on filamentous fungi since MICs were raised just two times their initial values.

Ergosterol is an essential chemical found on the fungal membrane. Drugs, such as AMB, have an affinity to sterols. Accordingly, it is possible to determine if a new antifungal candidate is able to act on the fungal cell membrane by supplying ergosterol to the culture medium (Pippi et al., 2015; Batista et al., 2017). Mammals count on

cholesterol, instead of ergosterol, as an essential structural component of cells. Thus, targeting ergosterol is very attractive for developing selective antifungal agents (Pippi et al., 2015). The fluoroquinoline derivative **8** was shown to act on cell membranes of dermatophyte and *Candida* spp., since MICs were increased in the presence of exogenous ergosterol.

Analysis of SEM images verified specific structural changes for *C. albicans* and *M. canis* after *in vitro* treatment with compound **8** (25 μg/ml). For the dermatophyte, morphological modifications of tubular structures were noted with **8**, when compared to the untreated control (FIG 1F). For *C. albicans*, the cellular damage was observed on the blasticonidium, which was fully lysed by **8** (FIG 1C). The rupture of the cell could be related to the effect of the molecule on the cell wall and consequent death of the microorganism (fungicidal effect). The same morphological damage in fungi cells was recently observed in related studies using SEM, after treating the strains with a variety of others organic compounds (Dalla Lana et al., 2015; Batista et al., 2017).

Epifluorescence microscopy was used as an auxiliary technique to observe the possible internalization of molecules into the fungal cell. Compound **8** presents two independent aromatic rings separated by two methylene carbons, which do not allow for electrons to flow over the whole aromatic system. Meanwhile, the presence of a double bond connecting the aromatic rings of compound **24** creates a strong UV-VIS absorption band not observed with **8**. In addition, compound **24** presented neither a fungicidal nor fungistatic effect over *Candida* spp. or filamentous fungi employed in this study (MICs > 50 μg/ml, Table 1). Therefore, **24** was chosen as a fluorescent probe to test the hypothesis that 1,4-benzenediols are able to cross the cell wall and membrane of fungi cells. *C. albicans* and *M. canis* were treated with the fluorescent probe (compound **24**) and observed by epifluorescence microscopy. A great number of cells of both fungi were noted with white light, as expected, since **24** is inactive (FIG 2A and 2F). Then, after turning on the UV-light (365 nm), we noticed that cells lit up due to the presence of probe molecules inside them (FIG 2B and 2G). The UV-light faded until pictures became fully dark (FIG 2E and 2J). The UV-light degraded **24** much faster inside *C. albicans*, which indicates that *M. canis* cells demonstrated some level of UV protection.

Toxicological assays were performed using human leukocytes to identify levels of toxicity of compound **8**. The fluoroquinoline-1,4-benzenediol **8** exhibited cell viability of 70% at 50 μg/ml, which is statistically similar to 100 μM (3.4 μg/ml) of hydrogen peroxide used as a positive control (FIG 3(a-c)). However, the fluoroquinoline derivative

**8** did not induce DNA damage, in the comet assay (FIG 3(c)) which relates the genotoxicity potential of substances to affect the cell integrity and its genetic material (Batista et al., 2017). The micronucleus analysis also indicated that **8** is not capable of producing genetic mutations at 50 µg/ml (FIG 3(b)). Additionally, the fluoroquinoline-1,4-benzenediol **8** did not cause haemolysis, even at 100 µg/ml (section 2.4.2).

Toxicological studies using alternative models, such as HET-CAM it is also very advantageous to predict the toxicity of the compound to membranes (Batista et al., 2017). The HET-CAM is an *ex vivo* method alternative to the Draize rabbit eye test, which measures vascular changes in the CAM. The membrane is an analogue of the ocular conjunctive and responds to injuries caused by processes such as inflammation similarly to what is observed in the conjunctival tissue of rabbits eyes. Since substances are directly applied over the CAM, the results obtained are generally more practical than animal models (ICCVMA, 2010). Results from HET-CAM using embryonated eggs demonstrated that compounds **2**, **5**, **8**, and **12** are nonirritants of the CAM (ICCVMA, 2010).

Electron donating groups present in the aryl rings of the benzenediols were shown to be deleterious for bacterial activities. On the other hand, the best results against *S. aureus* and *E. faecalis* (MICs ≤ 32 µg/ml for at least two *S. aureus* and *E. faecalis* strains) were obtained for derivatives containing electron withdrawing groups such as halogens (compounds **18**, Table 2, and **25-27**, **31** and **36**, supp. info.; FIG S31 *vs* compounds **2**, **13** and **17**). A second aromatic ring, such as the one present on quinolines (compounds **6**, **8** and **21**, Table 2, and **24**, **33**, **34** and **42**, supp. info.; FIG S31) also resulted in benzenediol derivatives whose MICs ≥ 32 µg/ml for Gram-positive strains (Table 2). In fact, the presence of nitrogen practically did not increase Gram-positive activities (compounds **19**, **20**, **6**, **8** and **21**, Table 2 and **24**, **29**, **31**, **34**, **37**, **38**, **40**, **41** and **42,** see supp. info.; FIG S31). The only exception was noted for the 4-aminoacetylphenyl-1,4-benzenodiol **15** which demonstrated MICs 8–32 µg/ml for three Gram-positive strains (Table 2). Alkyl benzenediol derivatives were also active against Gram-positive bacteria (compounds **22**, **12** and **23**). Of these, the most promising was the hexyl-1,4-benzenediol **12** (MICs 8–64 µg/ml to all *S. aureus* and *E. faecalis* tested, Table 2). These results are similar to the ones obtained for the furan-1,4-benzenediol **5** (MICs 8–64 µg/ml, Table 2).

Although *E. coli* (ATCC 25922) is not included in the ESKAPE panel, due to its fast growth, this strain was chosen as a model for fast identification of molecules with

Gram-negative potential. 1,4-Benzenediol derivatives **2**, **5**, **19**, and **20**, presented MIC ≤ 64 μg/ml against *E. coli* (Table 2). These compounds were further screened against the entire Gram-negative ESKAPE panel. The best activity was observed for the furan-1,4-benzenediol compound **5** against *K. pneumoniae* (ATCC 700603) (MIC 8 μg/ml, Table 3) which is known to be clinically resistant to most antibiotics. The thiophene-1,4-benzenediol **32** (**5** isostere, supp. info.; FIG S21) and the benzofuran-1,4-benzenediol **35** (**5** with an extra ring, supp. info.; FIG S31) were also prepared. However, both 1,4-benzenediols were inactive against all bacteria tested.

Since the furan-1,4-benzenediol **5** was identified as the best hit to Gram-positive strains and also active against *K. pneumoniae* (ATCC 700603), this molecule was chosen to have its potency tested in an *in vivo* experiment using the CAM. Thus, the CAM was infected with *S. aureus* (ATCC 25923) after four days of embryo development. Then, after another four days, a solution of compound **5** (64 μg/ml) was added over the CAM. On the $12^{th}$ day of the experiment, eggs were opened to check the number of CFUs still present. The furan derivative **5** was able to kill the bacteria without affecting embryo development (FIG 4B). Infected eggs not treated with the furan derivative **5** did not have normal embryo maturity (FIG 4A).

*In vitro* (Schlecht et al., 2015) and *in vivo/ex vivo* (Pammi et al., 2013) studies have demonstrated a synergistic effect as a consequence of co-infection of *Candida* spp. with some Gram-positive bacteria, such as *Staphylococci* or *Enterococci*. It is believed that Gram-positive bacteria adhere or bind to the hyphae of *Candida* spp. It was shown that *C. albicans* can transport *S. aureus* into tissues, disseminating the infection in an oral co-colonization model. Therefore, co-infection of *Candida* spp. and *S. aureus* results in a more serious infection than each microorganism alone (Pammi et al., 2013). Besides the Gram-positive activities of the furan benzenediol **5**, this molecule was also active against dermatophytes screened (MICs ≤ 50 μg/ml, Table 1) and *C. albicans* (MICs 50 μg/ml, Table 1). Therefore, as a study perspective, the furan derivative **5** could be used to prepare formulations to combat infections both associated with bacteria and fungi (Silveira et al., 2017). Besides, *S. aureus* is one of the most virulent pathogens of skin and soft tissue infections. These infections may be local and progress to become invasive (Tong et al., 2015). Formulations containing the furan-1,4-benzenodiol **5** could be applied for the treatment of chronic wound infections, where higher concentrations of compounds, such as **5**, could be used to tackle *Staphylococcus* infections without cytotoxicity concerns. Finding new ways of treating wound infections is of major importance, since 1 to 2% of

populations in developing countries will develop chronic skin wounds which cost $ 25 billion/year just in the United States (Soo et al., 2017).

## 5. Conclusions

The present study determined the antifungal (filamentous fungi and yeasts of the genus *Candida*) and antibacterial activity (ESKAPE panel) of a novel library of 2-substituted-1,4-benzenediols easily synthesized in just one chemical step using inexpensive commercially available reagents and mild green chemistry conditions ($K_2CO_3$, ethanol and water). 1,4-Benzenediols demonstrated a broad spectrum of action and reduced toxicity to human leukocytes. The mechanism of antifungal action was identified targeting the cell wall and membrane of yeasts and dermatophytes. Benzenediol compounds **5** (for bacteria) and **8** (for fungi) were classified as the most promising for further *in vivo* studies. Although benzenediols presented moderate activities, 1,4-benzenediols **5**, **12**, and **22** showed an antimicrobial profile. In addition, the furan-1,4-benzenediol **5** was demonstrated to be active in an *ex vivo* model, as well as nonirritant to the mucous membranes. Therefore, the development of antimicrobials using **5** (or even **8** for fungicides) as the active component of antimicrobial formulations seems quite promising.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article is available.

## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

## ACKNOWLEDGMENTS

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## AUTHORS' CONTRIBUTIONS

Daiane F. Dalla Lana, Bruna Batista, Gabriella M. Machado, Mário L. Teixeira, Luis F.S. de Oliveira, Michel M. Machado, William Lopes, Marilene H. Vainstein Edilma E. Silva conceived, planned, and carried out the experiments. Saulo F. de Andrade, Alejandro Peixoto de Abreu Lima, Enrique Pandolfi, Gustavo P. Silveira contributed to sample preparation, and synthesis. Alexandre M. Fuentefria and Gustavo P. Silveira contributed to the interpretation of the results and supervision of all the work. Daiane F. Dalla Lana took the lead in writing the manuscript. All authors provided critical feedbacks during the manuscript preparation.

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**Supplemental Material**

# Design, synthesis, and evaluation of novel 2-substituted 1,4-benzenediol library as antimicrobial agents against clinically relevant pathogens

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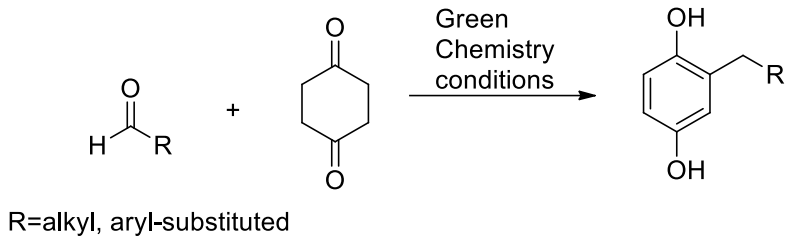
## 1. Methodologies and complementary results

### 1.1. Synthetic procedures and structural characterization

All commercially available reagents were purchased and used without further purification (Aldrich, Fluka). All solvents including, EtOH, AcOEt and *n*-hexanes were purified by distillation. All reactions were monitored by thin layer chromatography (TLC) performed on 0.25 mm silica gel glass-plates (60 $F_{254}$) using UV light, Iodine and Brady solution as visualizing agents. Flash column chromatography was carried out with silica gel (spherical, neutral, 63-210 mm grade). Yields refer to chromatographically and spectroscopically homogeneous materials. Melting points were measured on a melting point apparatus (Gallenkamp) and were uncorrected. Mass spectra (EI MS) was obtained on a Shimadzu GC-EI MS QP 1100 EX spectrometer. $^{1}$H-NMR spectra (400 MHz) and $^{13}$C-NMR (100 MHz) were recorded in the indicated solvent on a Bruker Advance DPX 400 MHz spectrometers. Chemical shifts (d) are reported in delta (d) units, parts per

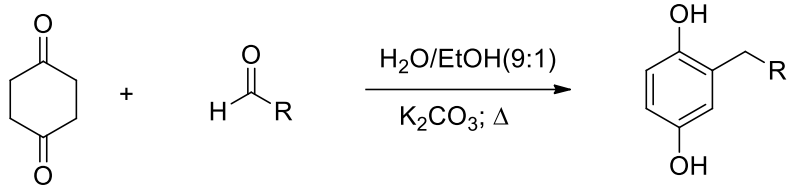
million (ppm). Chemical shifts for $^{1}$H-NMR spectra are given relative to signals for internal tetramethylsilane (0 ppm) or residual nondeuterated solvents, i.e., methanol (3.30 ppm). Chemical shifts for $^{13}$C-NMR spectra are given relative to the signal for chloroform-*d* (77.0 ppm) or dimethyl sulphoxide-$d_6$ (39.5 ppm). Multiplicities are reported by the following abbreviations: s (singlet), d (doublet), t (triplet), p (pentet), m (multiplet), dd (double doublet), dt (double triplet), br-s (broad singlet). Coupling constants (*J*) are represented in hertz (Hz). Compounds **1-3**, **9-13**, **15-18**, **21**, **22**, **25-31**,**36**, and **39** (Peixoto et al., 2016) and compounds **5**, **19**, **32**, **33**, and **38** (Rolón et al., 2018) were previously synthesized and characterized (Peixoto et al., 2016; Rolón et al., 2018). The synthesis and characterization of new compounds **6-8**, **20**, **24**, **34**, **35**, **37**, **40**, **41**, and **42** are presented (Scheme S1).




**Scheme S1. S**ynthesis of 2-substituted 1,4-benzenediols.

### 1.2. General procedure for the synthesis of 2-alkylated 1,4-benzenediol derivatives

A solution of $K_2CO_3$ (1 mmol) in a water ethanol 9:1 mixture (20 mL) is heated with stirring. Once the solution is under reflux, 1,4-cyclohexanedione (1 mmol) is gently added. Then, the aldehyde (2 mmol) is added slowly in portions. Once the reaction is complete by TLC, right after removing the heating,HCl 10%is dropwiseuntil neutral (especially with amphotheric compounds) or slightly acidic pH is reached. The aqueous solution is extracted with ethyl acetate (3X 10 mL) and the organic phase washed with brine (10 mL), dried with $Na_2SO_4$, filtered, and rotaevaporated under reduced pressure. Purification is performed by column chromatography.

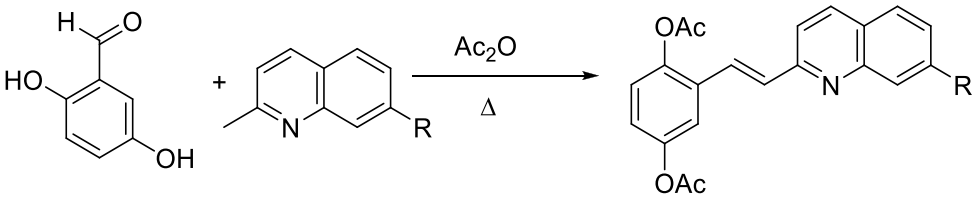



**Scheme S2**. Synthesis of 2-alkylated 1,4-benzenediol derivatives.

### 1.3. General procedure for the synthesis of (*E*)-2-(2,5-diacetoxystyryl) quinolone (Polanski et al., 2002)

2,5-dihydroxybenzaldehyde (1.0 mmol) is added to a solution of the substituted quinaldine (1.0 mmol) in acetic anhydride (3 mL/mmol). The resulting mixture is heated under reflux until the reaction is completeby TLC and then concentrated *in vacuo*. The crude is dissolved in dichloromethane (20 mL) and washed with sodium bicarbonate saturated (3x 10 mL). The aqueous solution is checked to basic pH and then the organic layer is washed with brine (10 mL), dried with $Na_2SO_4$, filtered, and rotaevaporated under reduced pressure. Purification is performed by column chromatography ($SiO_2$/ AcOEt:

Hex (3/7)).

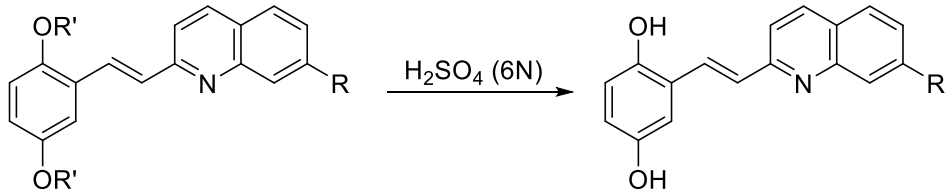



R: H,Cl,F

**Scheme S3**. Synthesis of (*E*)-2-(2,5-diacetoxystyryl) quinolines.

**1.4. General procedure for the synthesis of (*E*)-2-(2,5-dihydroxystyryl) quinoline derivatives** (Polanski et al., 2002)

(*E*)-2-(2,5-diacetoxystyryl) quinoline derivative (1 mmol) is dissolved in 6 N sulfuric acid (4 mL/mmol) and the resulting solution is heated under reflux until the reaction is complete. After cooling, the mixture is neutralized with sodium carbonate. The aqueous mixture is extracted with ethyl acetate (3x 10 mL) and the combined organic layers are washed with brine (10 mL), dried with $Na_2SO_4$, filtered, and concentrated *in vacuo*. Purification is performed by column chromatography ($SiO_2$/AcOEt: Hex (1/1)).

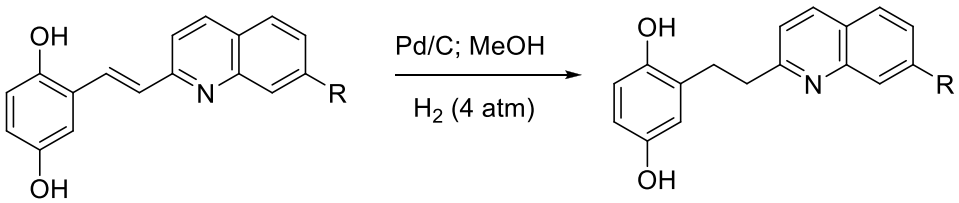



R: H,Cl,F

**Scheme S4**. Reaction of synthesis of (*E*)-2-(2,5-dihydroxystyryl) quinoline derivatives.

**1.5. General procedure for the synthesis of 2-(2,5-dihydroxyphenethyl) quinoline derivatives 3)**

A mixture of (*E*)-2-(2,5-dihydroxystyryl) quinoline derivative (0.2mmol) and 20% of Pd/C (10 wt. %) in 10 mL MeOH is shacked under 4 atm of $H_2$ during 24 hours (Scheme S5). The catalyst is filtered and the solution is concentrated *in vacuo*. Purification is performed by column chromatography ($SiO_2$/AcOEt: Hex (1/1)).




R: H,Cl,F

**Scheme S5**. Reaction of synthesis of 2-(2,5-dihydroxyphenethyl) quinoline derivatives

## 1.6. Characterization data

**1.6.1.** 2-(quinolin-2-ylmethyl)benzene-1,4-diol **6**

$^{1}$H-NMR (400MHz, $CDCl_3$; Fig. S1): δ(ppm) = 4.20 (s, 2H), 6.61 (dd, $J_1$=2.9 Hz $J_2$=8.5 Hz, 1H), 6.73 (d, $J$=2.9 Hz, 1H), 6.85 (d, $J$=8.5 Hz, 1H), 7.42 (d, $J$=8.4 Hz, 1H), 7.52 (ddd; $J_1$=8.4 Hz $J_2$=6.9 Hz $J_3$=1.1 Hz, 1H), 7.71 (ddd; $J_1$=8.4 Hz $J_2$=6.9 Hz $J_3$=1.1 Hz, 1H), 7.79 (d, $J$=8.4 Hz, 1H), 8.02 (d, $J$=8.4 Hz, 1H), 8.16 (d, $J$=8.4 Hz, 1H);$^{13}$C-NMR (100 MHz, $(CD_3)_2SO$; Fig. S2): δ(ppm) = 42.0, 115.0, 116.9, 119.0, 121.2, 126.5, 126.7, 126.9, 127.6, 127.8, 130.3, 138.3, 146.3, 149.3, 149.8, 161.4; EI MS *m/z*: 251 ($M^+$, 84), 234 (100); Mp=166-169°C; Yield: 70%.

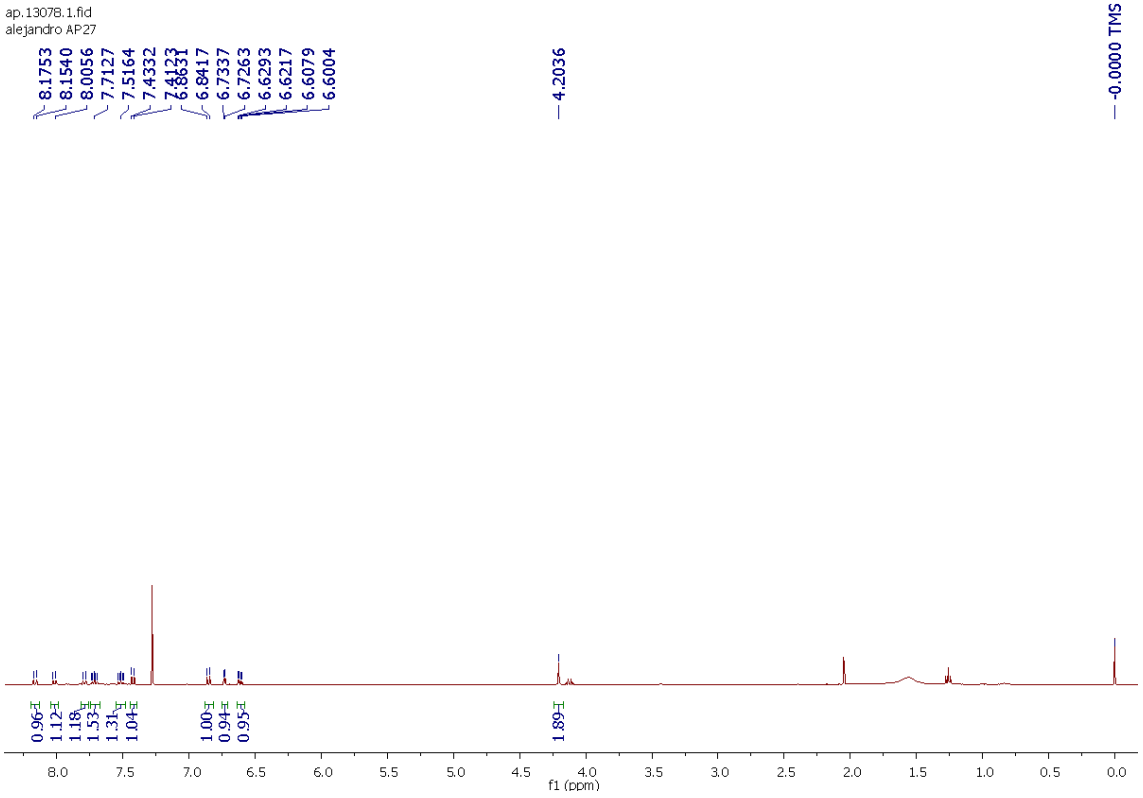



**Figure S1**.$^{1}$H-NMR spectrum of compound **6** (400MHz, $CDCl_3$).

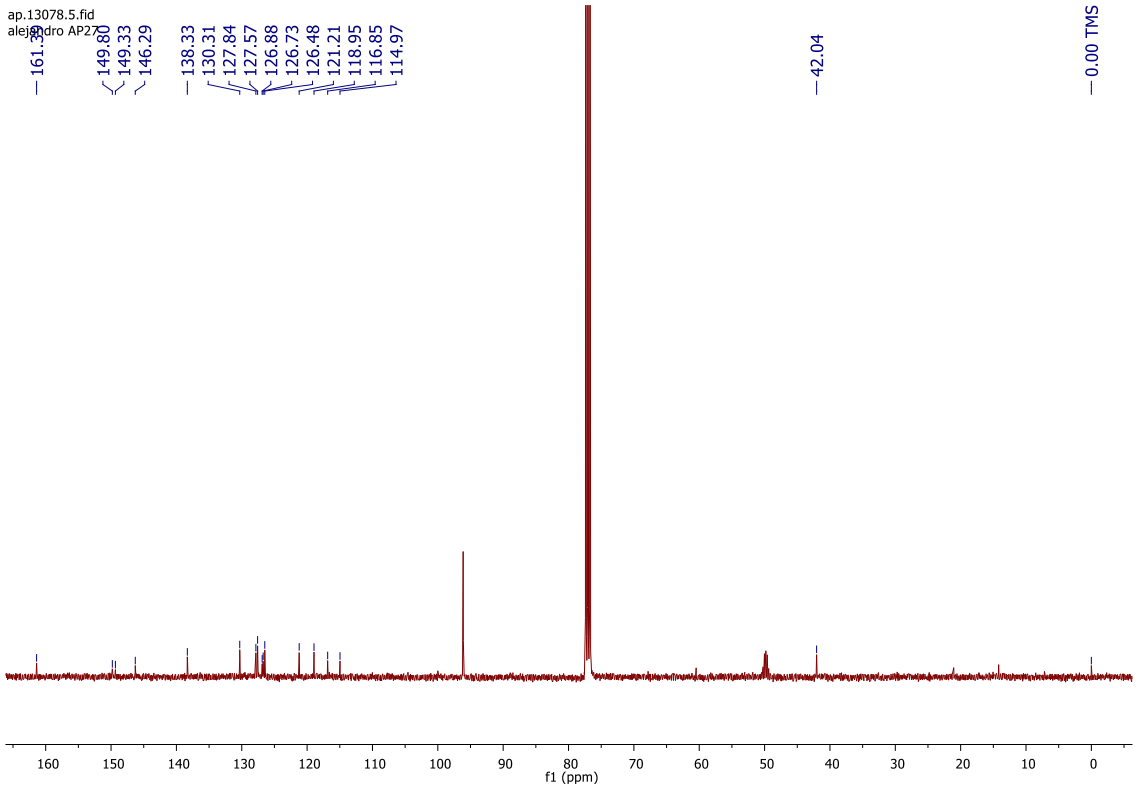



**Figure S2**. $^{13}$C-NMR spectrum of compound **6** (100 MHz, $(CD_3)_2SO$).

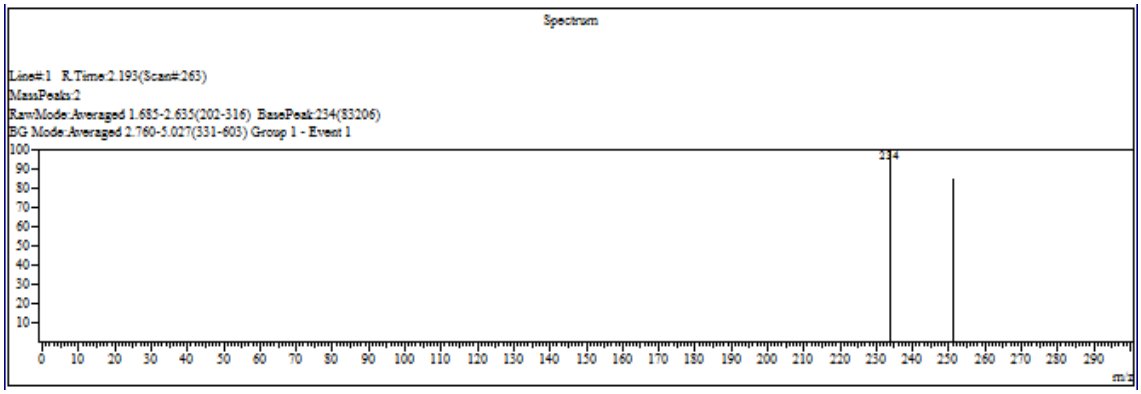



**Figure S3**. Mass spectrum (EI MS) of compound **6**.

**1.6.2.** (*E*)-2-(2-(7-chloro-2-quinolinyl)styryl)-1,4-benzenediol **7**

$^1$H-NMR (400MHz, $(CD_3)_2SO$; Fig. S17): δ (ppm) = 6.68 (d, $J_1$=2,9 Hz $J_2$=8,7 Hz, 1H), 6.77 (d, *J*=8.7 Hz, 1H), 7.04 (d, *J*=2.9 Hz, 1H), 7.41 (d, *J*=15.9 Hz, 1H), 7,64 (d, *J*=9.1 Hz, 1H), 7.98 (d, *J*=6.8 Hz, 1H), 8.05-8.09 (m, 3H), 8.51 (d, *J*=8.2 Hz, 1H); $^{13}$C-NMR (100 MHz, $(CD_3)_2SO$; Fig. S18): δ(ppm) = 113.1, 117.0, 118.3, 120.4, 122.7, 125.5, 127.1, 130.0, 133.2, 135.2, 135.3, 138.2, 138.3, 149.4, 150.0, 156.6, 156.7; EI MS *m/z*: 299 ($M^{2+}$ [$^{37}$Cl], 17), 298 (17), 297 ($M^+$ [$^{35}$Cl], 50), 296 (24), 282 (33), 281 (19), 280 (100), 163 (23); Mp=212°C dec.; Yield: 80%.

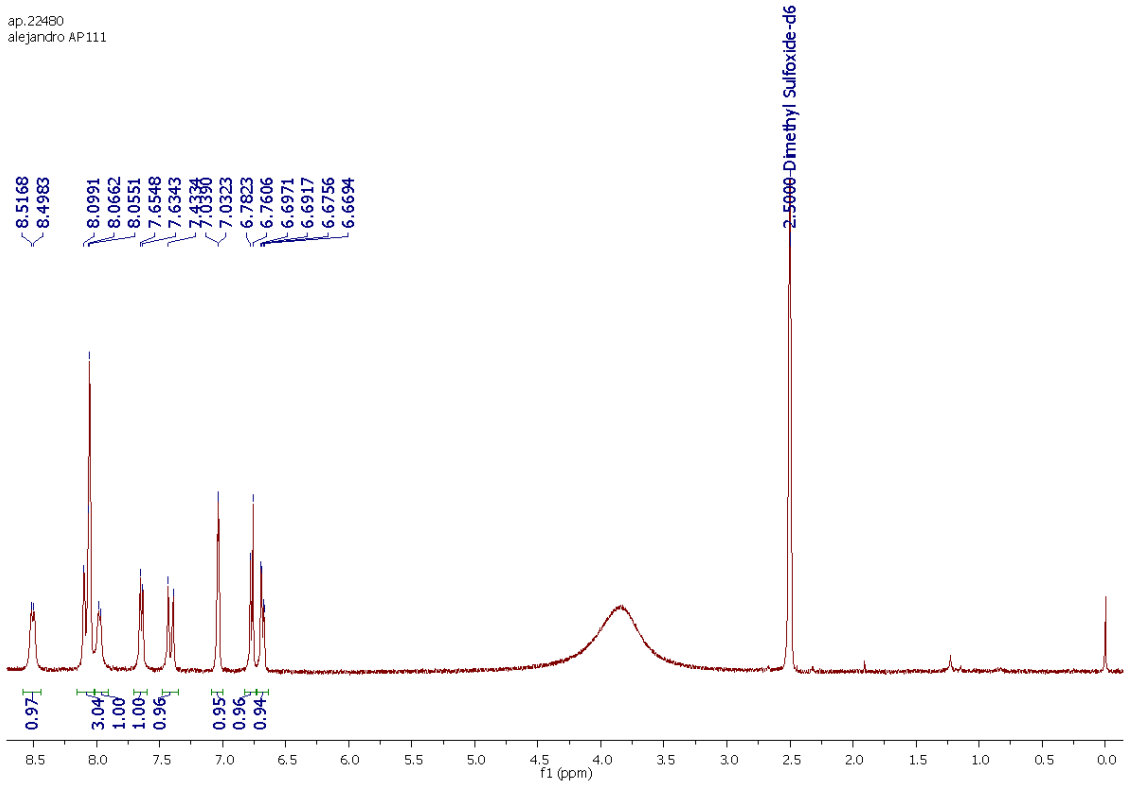



**Figure S4**.[1]H-NMR spectrum of compound **7** (400MHz, $(CD_3)_2SO$).

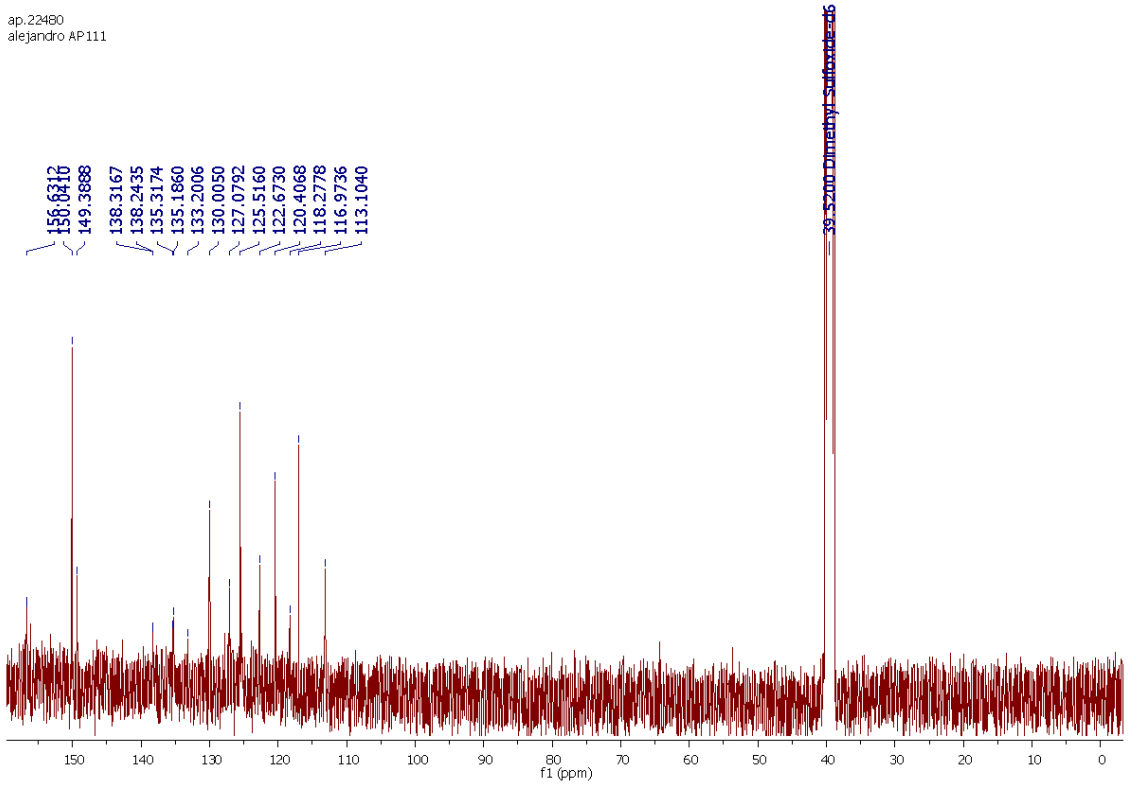



**Figure S5**. [13]C-NMR spectrum of compound **7** (100 MHz, $(CD_3)_2SO$).

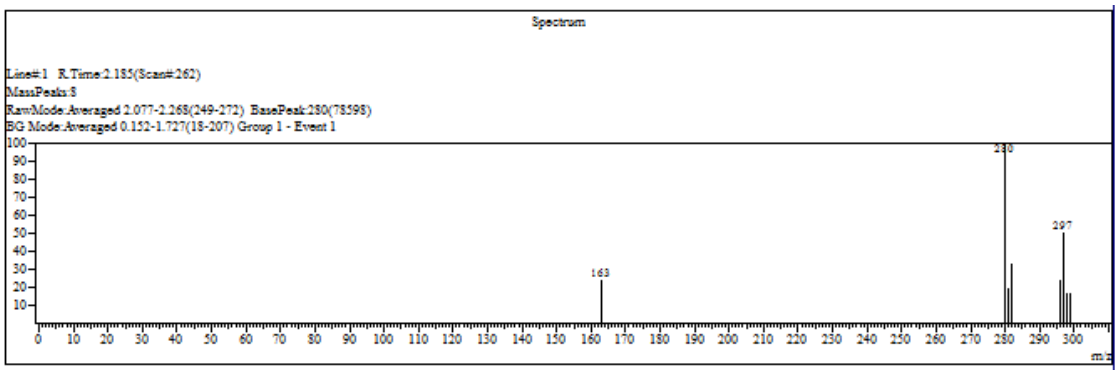



**Figure S6**. Mass spectrum (EI MS) of compound **7**.

**1.6.3.** 2-(2-(7-fluoro-2-quinolinyl)ethyl)-1,4-benzenediol **8**

$^{1}$H-NMR (400MHz, $CDCl_3$;Fig. S19): δ (ppm) = 3.10 (t, *J*=6.7 Hz, 2H), 3.36 (t, *J*=6.7 Hz, 2H), 6.58 (dd,$J_1$=3.0 Hz $J_2$=8.6 Hz, 1H), 6.68 (d, *J*=3.0 Hz, 1H), 6.75 (d, *J*=8.6 Hz, 1H), 7.27 (d, *J*=8.5 Hz, 1H), 7.31 (dt, $J_1$=2.5 Hz $J_2$=8.9 Hz, 1H), 7.74 (dd, $J_1$=2.5 Hz $J_2$=10.3 Hz, 1H), 7.78 (dd, $J_1$=6.1 Hz $J_2$=8.9 Hz, 1H), 8.07 (d, *J*=8.5 Hz, 1H); $^{13}$C-NMR (100 MHz, $CDCl_3$;Fig. S20): δ(ppm) = 27.6, 39.3, 111.4 (d, *J*=21,0 Hz), 114.3, 116.6 (d, *J*=25.2 Hz), 116.7, 117.8, 121.4 (d, *J*=2.5 Hz), 123.9, 129.4, 129.7 (d, *J*=10.0 Hz), 137.0, 147.5 (d, *J*=12.6 Hz), 147.8, 150.0, 162.5, 163.1 (d, *J*=250.5 Hz); EI MS *m/z*: 283 ($M^+$, 30), 279 (30), 161 (100); Mp=149-151°C; Yield: 38%.

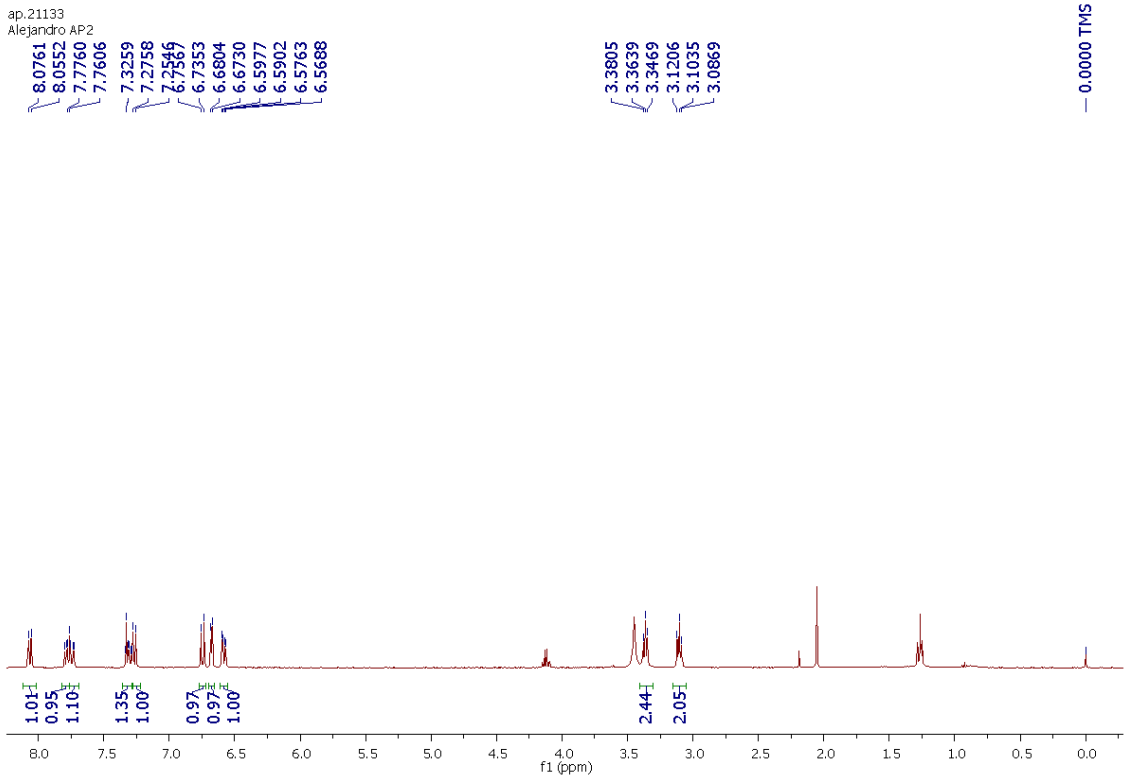



**Figure S7**.$^{1}$H-NMR spectrum of compound **8** (400MHz, $CDCl_3$).

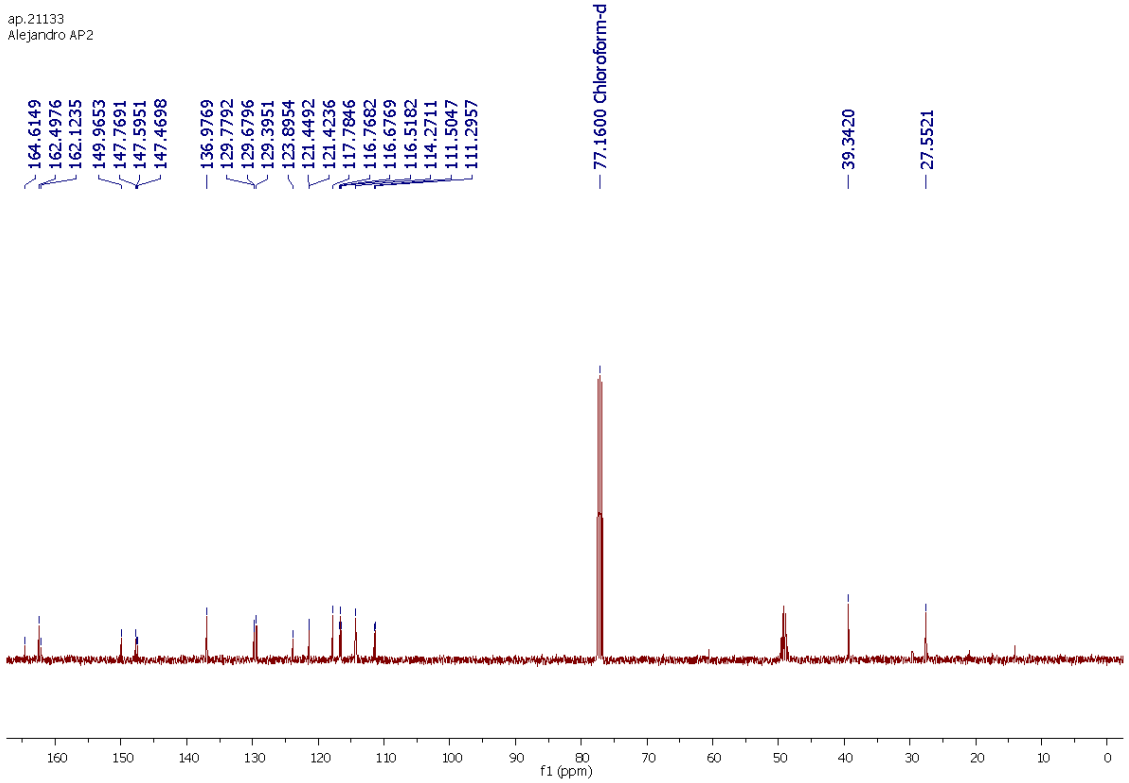



**Figure S8**. $^{13}$C-NMR spectrum of compound **8** (100 MHz, $CDCl_3$).

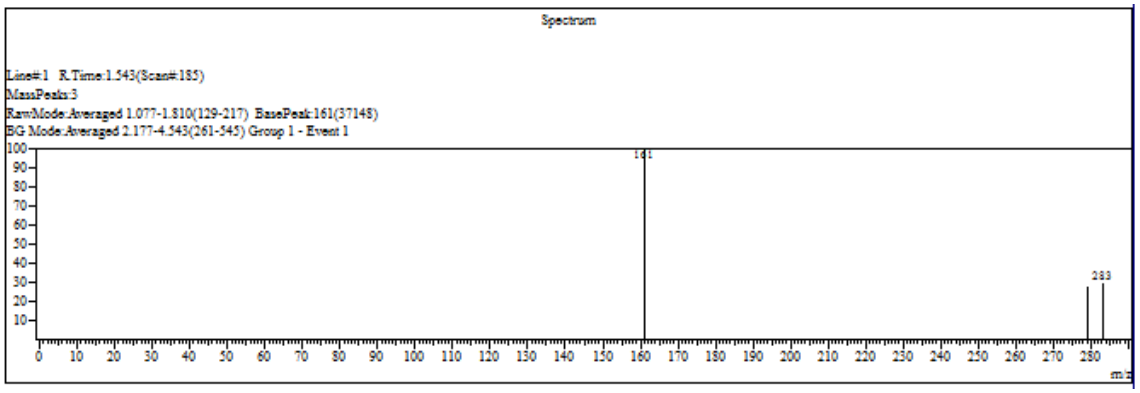



**Figure S9**. Mass spectrum (EI MS) of compound **8**.

**1.6.4.** 2-(4-pyridinemethyl)-1,4-benzenediol **20**

$^1$H-NMR (400MHz, $(CD_3)_2SO$): δ (ppm) = 3.81 (s, 2H), 6.47-6.49 (m, 2H), 6.65 (d, *J*=9.1 Hz, 1H), 7.20 (d, *J*=6,0 Hz, 2H), 8.43 (d, *J*=6,0 Hz, 2H), 8.69 (s, 1H), 8.82 (s, 1H); Mp=119-121°C; Yield: 61% (Ozaki et al., 1997a; Ozaki et al., 1997b).

**1.6.5.** 2-(2-(2-quinolinyl)ethyl)-1,4-benzenediol **21**

$^1$H-NMR (400MHz, $CDCl_3$;Fig. S11): δ (ppm) = 3.14 (t, *J*=6.6 Hz, 2H), 3.39 (t, *J*=6.6 Hz, 2H), 6.58 (dd, $J_1$=3.0 Hz $J_2$=8.6 Hz, 1H), 6.69 (d, *J*=3.0 Hz, 1H), 6.75 (d, *J*=8.6 Hz, 1H), 7.29 (d, *J*=8.5 Hz, 1H), 7.53 (ddd, $J_1$=1.1 Hz $J_2$=7.0 Hz $J_3$=8.1 Hz, 1H), 7.74 (ddd, $J_1$=1.5 Hz $J_2$=7.0 Hz $J_3$=8.4 Hz, 1H), 7.79 (dd, $J_1$=1.1 Hz $J_2$=8.1 Hz, 1H), 8.08 (d, *J*=8.4

Hz, 1H), 8.13 (d, *J*=8.5 Hz, 1H); $^{13}$C-NMR (100 MHz, $CDCl_3$; ;Fig. S12): δ(ppm) = 27.2, 39.5, 114.3, 116.8, 118.1, 122.1, 126.4, 126.9, 127.4, 127.6, 129.8, 130.1, 137.2, 146.5, 147.9, 149.9, 161.1; EI MS *m/z*: 265 ($M^+$, 26), 143 (100); Mp=142-145°C; Yield: 38%.

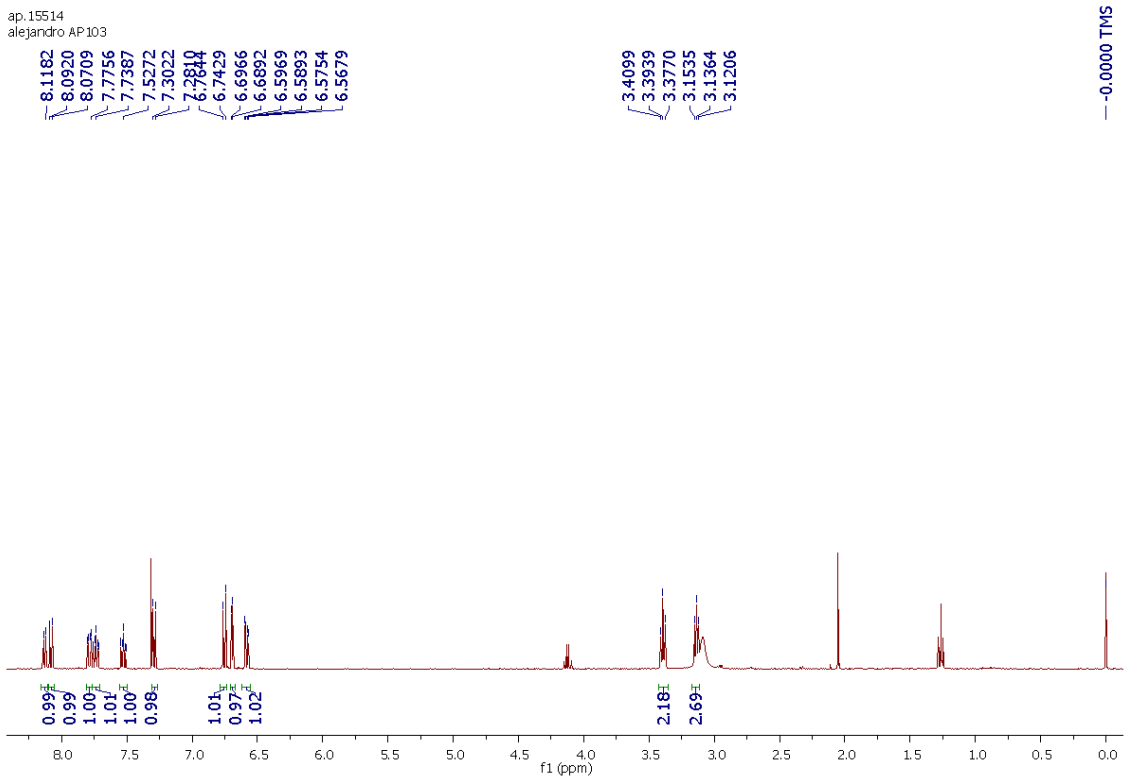



**Figure S10**.$^1$H-NMR spectrum of compound **21** (400MHz, $CDCl_3$).

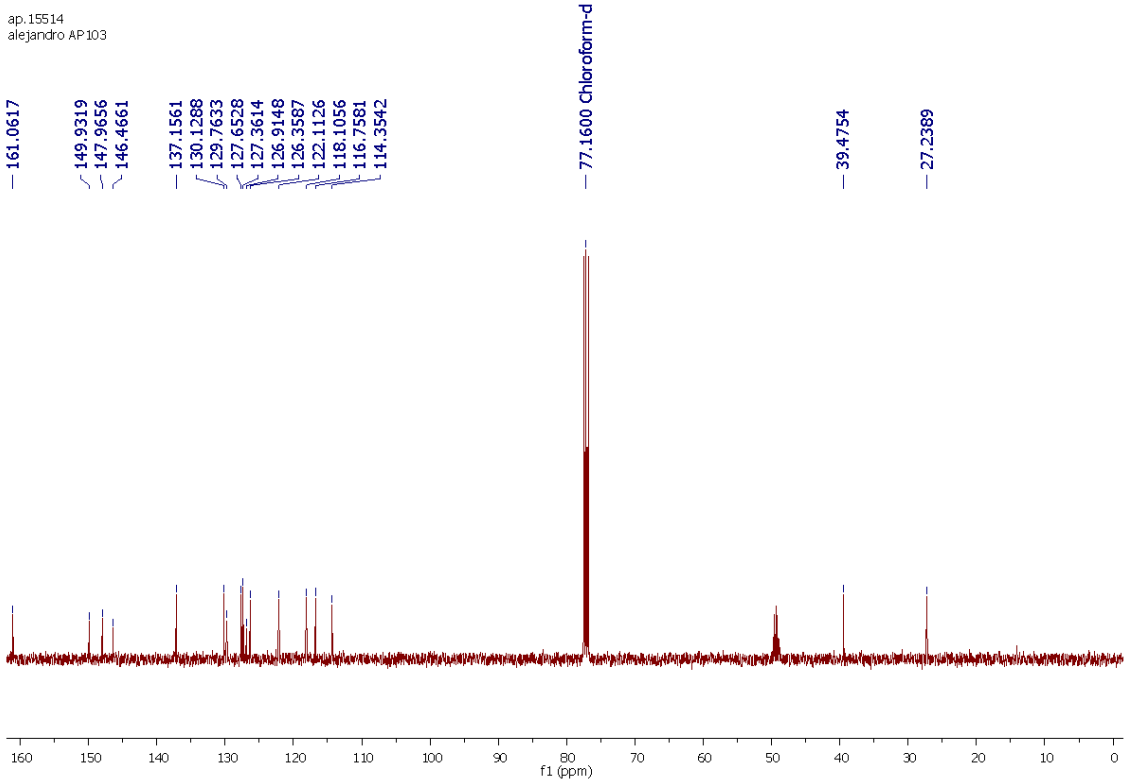



**Figure S11**. $^{13}$C-NMR spectrum of compound **21** (100 MHz, $CDCl_3$).

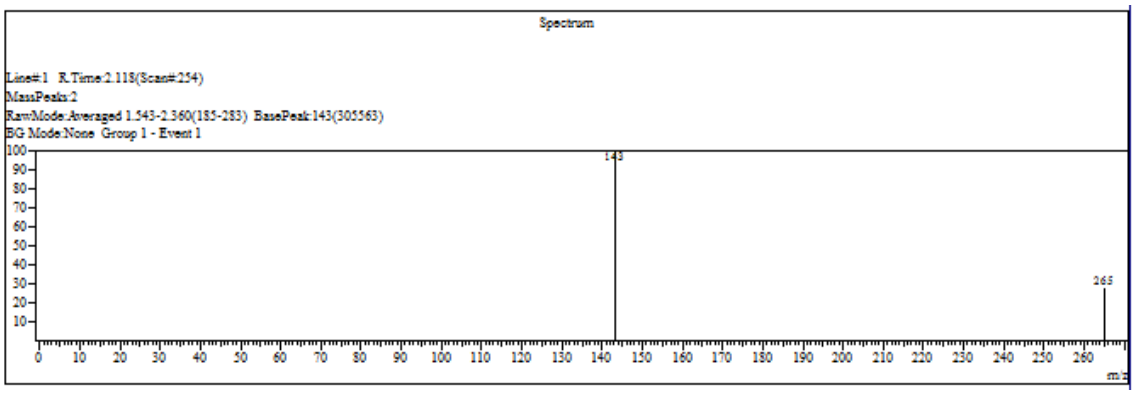



**Figure S12**. Mass spectrum (EI MS) of compound **21**.

**1.6.6.** (*E*)-2-(2-(7-fluoro-2-quinolinyl)styryl)-1,4-benzenediol **24**

$^1$H-NMR (400MHz, $CD_3)_2SO$; Fig. S15): δ (ppm) = 6.64 (dd, $J_1$=2.9 Hz $J_2$=8.6 Hz, 1H), 6.74 (d, $J$=8.6 Hz, 1H), 7.04 (d, $J$=2.9 Hz, 1H), 7.32 (d, $J$=16.4 Hz, 1H), 7.47 (td, $J_1$=2.6 Hz $J_2$=8.8 Hz, 1H), 7.71 (dd, $J_1$=2.6 Hz $J_2$=10.6 Hz, 1H), 7.76 (d, $J$=8.6 Hz, 1H), 7.99-8.04 (m, 2H), 8.36 (d, $J$=8.6 Hz, 1H), 8.86 (s, 1H), 9.34 (s, 1H); $^{13}$C-NMR (100 MHz, $(CD_3)_2SO$; Fig. S16): δ(ppm) = 111.9 (d $J$=19,8 Hz), 112.5, 116.0 (d, $J$=25.2 Hz), 116.9, 117.5, 119.8, 123.1, 124.2, 127.2, 130.4, 130.4 (d, $J$=9.9 Hz), 136.6, 148.7 (d, $J$=12.8 Hz), 148.9, 150.0, 157.2, 162.7 (d, $J$=247.0 Hz); EI MS *m/z*: 281 ($M^+$, 53), 264 (100), 147 (33), Mp=218°C dec.; Yield: 85% (2 steps).

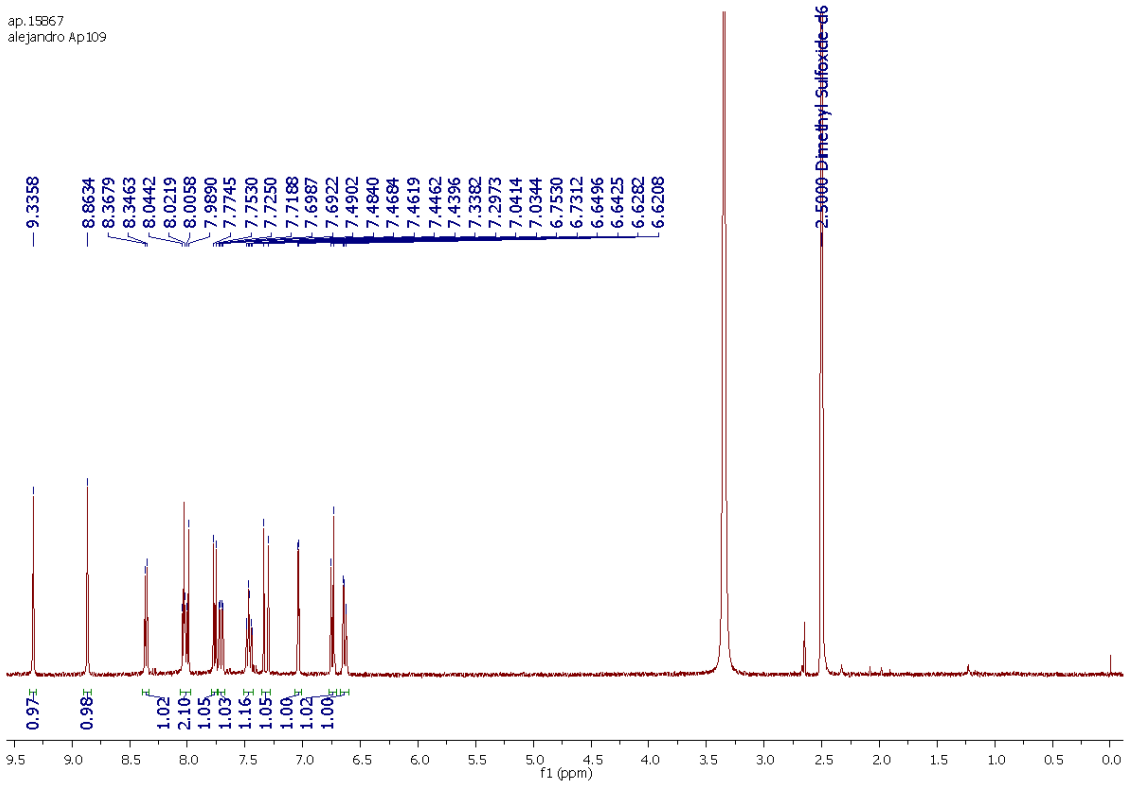



**Figure S13**.[1]H-NMR spectrum of compound **24** (400MHz, $CD_3)_2SO$).

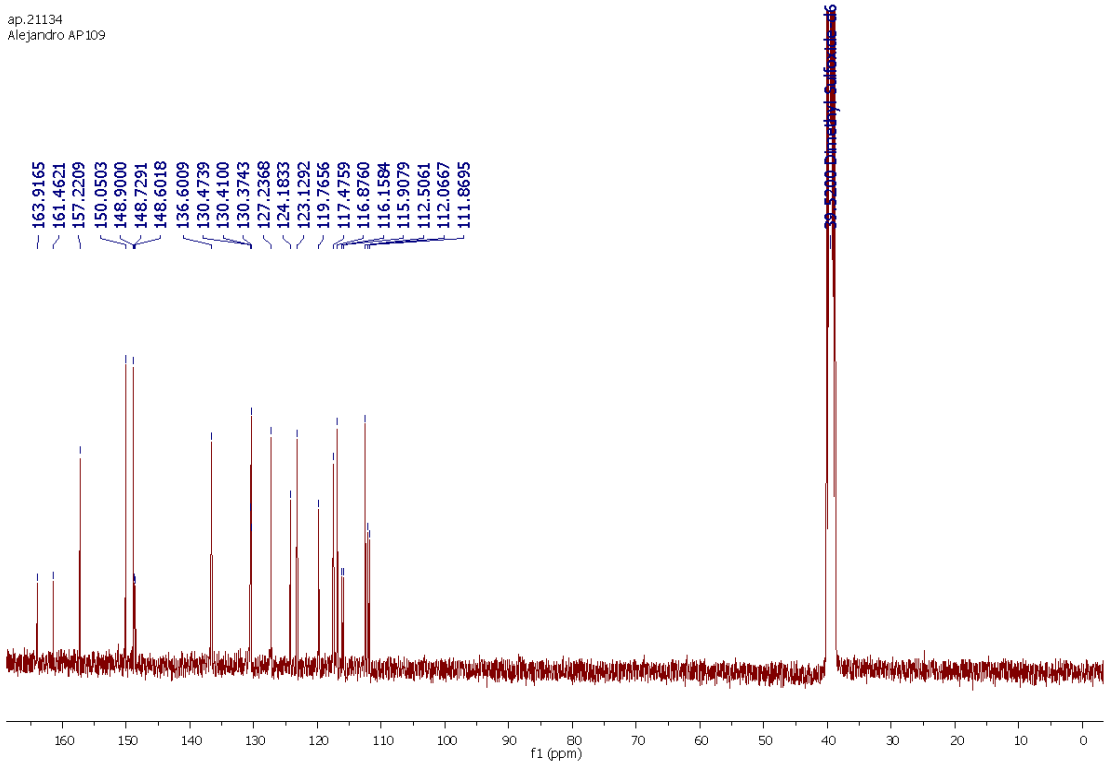



**Figure S14**. [13]C-NMR spectrum of compound **24** (100 MHz, $(CD_3)_2SO$).

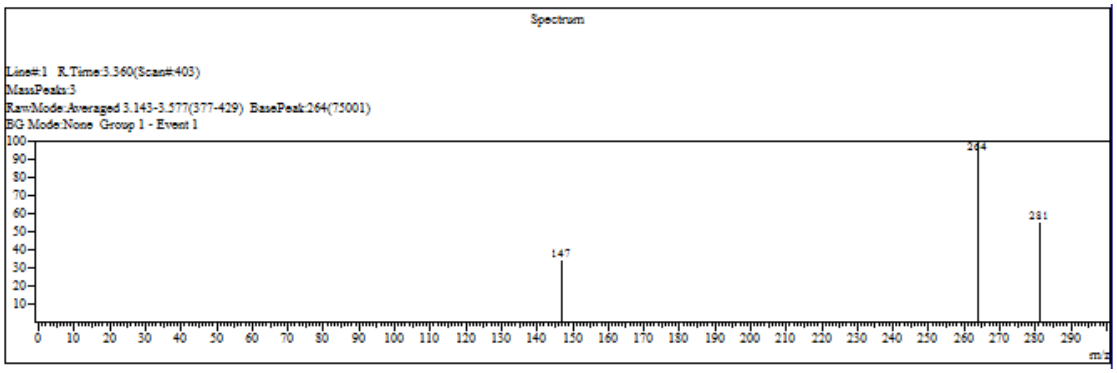



**Figure S15**. Mass spectrum (EI MS) of compound **24**.

**1.6.7.** 2-(3-(2-chloro-6-methoxy)quinolinyl)methyl-1,4-benzenediol **34**

$^{1}$H-NMR (400MHz, $(CD_3)_2SO$; Fig. S3): δ (ppm) = 3.85 (s, 3H), 4.00 (s, 2H), 6.38 (d, *J*=2.9 Hz, 1H), 6.50 (dd, $J_1$=2.9 Hz $J_2$=8.6 Hz, 1H), 6.68 (d, *J*=8.6 Hz, 1H), 7.33 (d, *J*=2.8 Hz, 1H), 7.38 (dd, $J_1$=2.8 Hz $J_2$=9.1 Hz, 1H), 7.84 (d, *J*=9.1 Hz, 1H), 7.89 (s, 1H), 8.62 (s, 1H), 8.81 (s, 1H); $^{13}$C-NMR (100 MHz, $(CD_3)_2SO$; Fig. S4): δ(ppm) = 33.0, 55.7, 105.5, 114.1, 115.8, 116.7, 122.5, 124.9, 128.5, 128.9, 132.8, 137.1, 141.7, 147.7, 148.2, 149.9, 157.8; EI MS *m/z*: 317 ($M^{2+}$ [$^{37}$Cl], 21), 315 ($M^{+}$[$^{35}$Cl], 61), 280 (100), 279 (80), 278 (56), 264 (35), 236 (30), 235 (31), 194 (30), 193 (33); Mp=198-202°C; Yield: 25%.

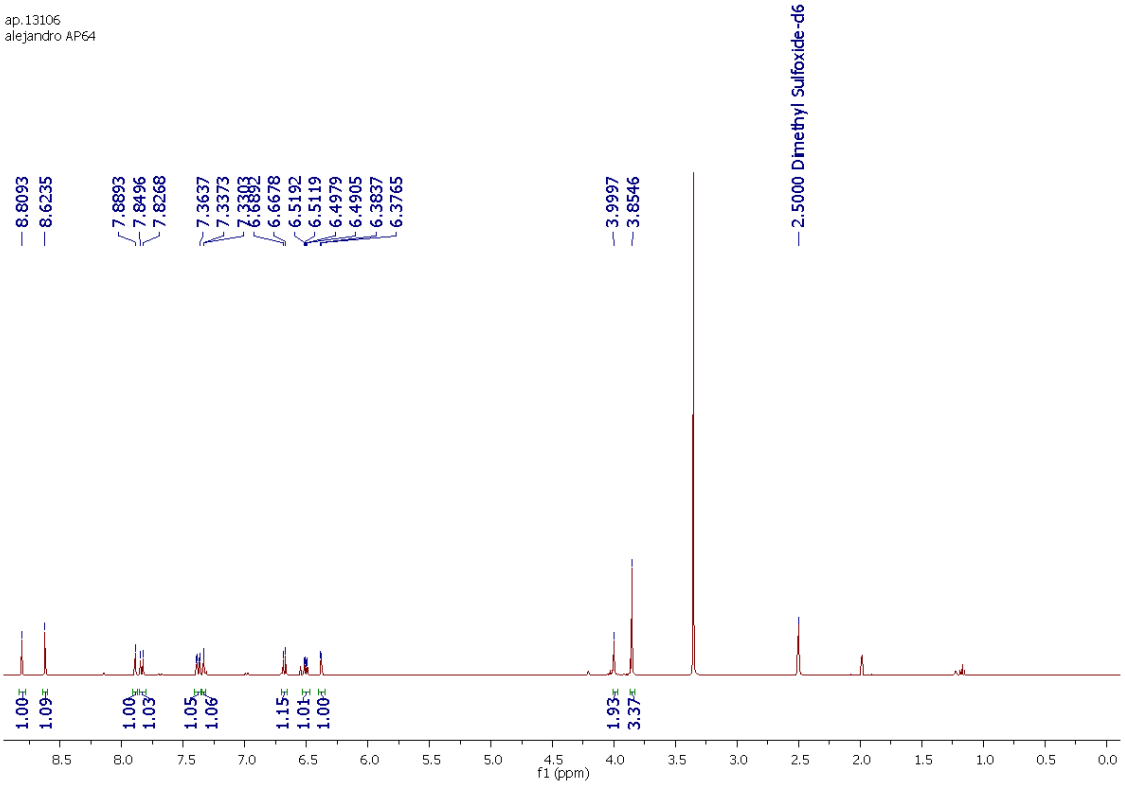



**Figure S16**.$^{1}$H-NMR spectrum of compound **34** (400MHz, $(CD_3)_2SO$).

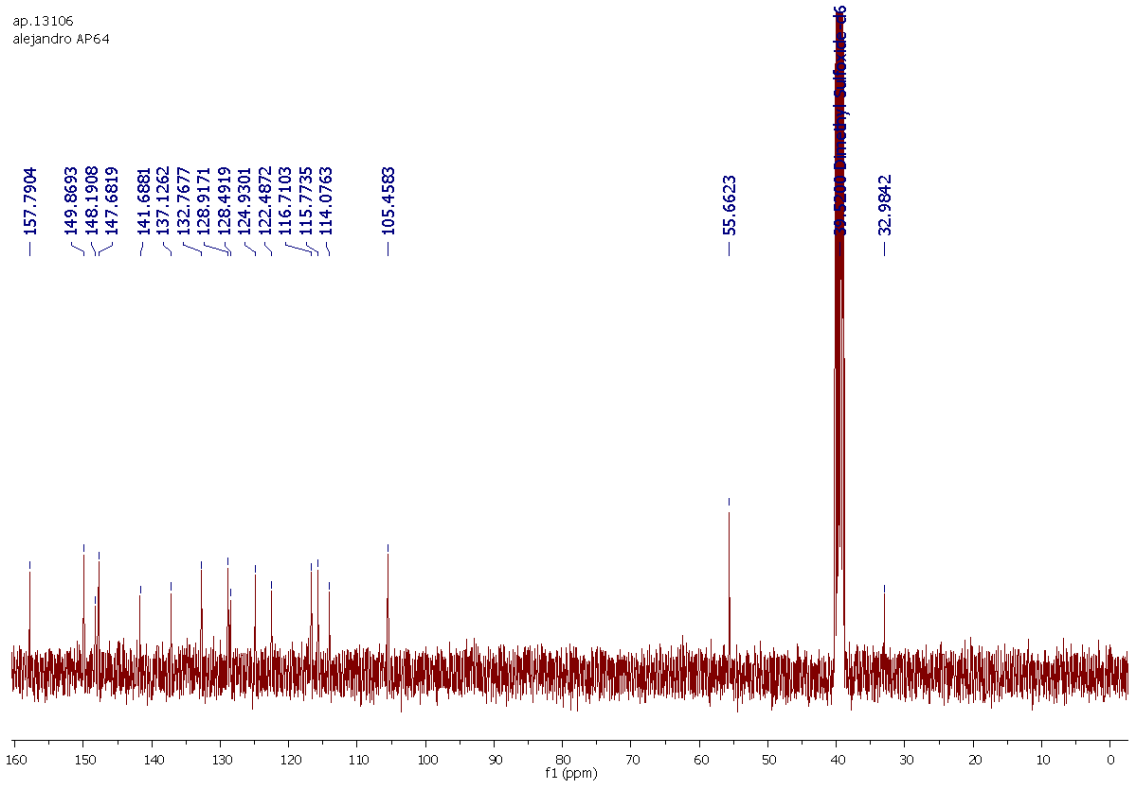



**Figure S17**. $^{13}$C-NMR spectrum of compound **34** (100 MHz, $(CD_3)_2SO$).

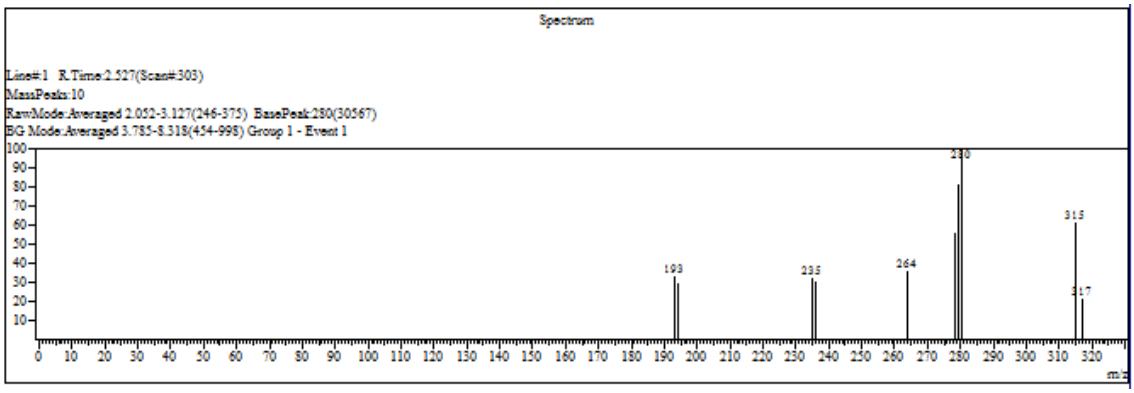



**Figure S18**. Mass spectrum (EI MS) of compound **34**.

**1.6.8.** 2-(2-benzofuranemethyl)-1,4-benzenediol **35**

$^1$H-NMR (400MHz, $CDCl_3$; Fig. S5): δ (ppm) = 4.06 (s, 2H), 6.39 (d, *J*=0.9 Hz, 1H), 6.60 (dd, $J_1$=2.9 Hz $J_2$=8.5 Hz, 1H), 6.65 (d, *J*=2.9 Hz, 1H), 6.70 (d, *J*=8.5 Hz, 1H), 7.13-7.21 (m, 2H), 7.38-7.40 (m, 1H), 7.43-7.46 (m, 1H); $^{13}$C-NMR (100 MHz, $CDCl_3$; Fig. S6) δ(ppm) = 29.0, 103.1, 110.8, 114.3, 116.1, 117.0, 120.3, 122.4, 123.1, 124.8, 129.0, 147.7, 149.7, 154.9, 157.7; EI MS *m/z*: 240 ($M^+$, 100), 122 (48), 118 (72); Mp=104-105°C; Yield: 87%.

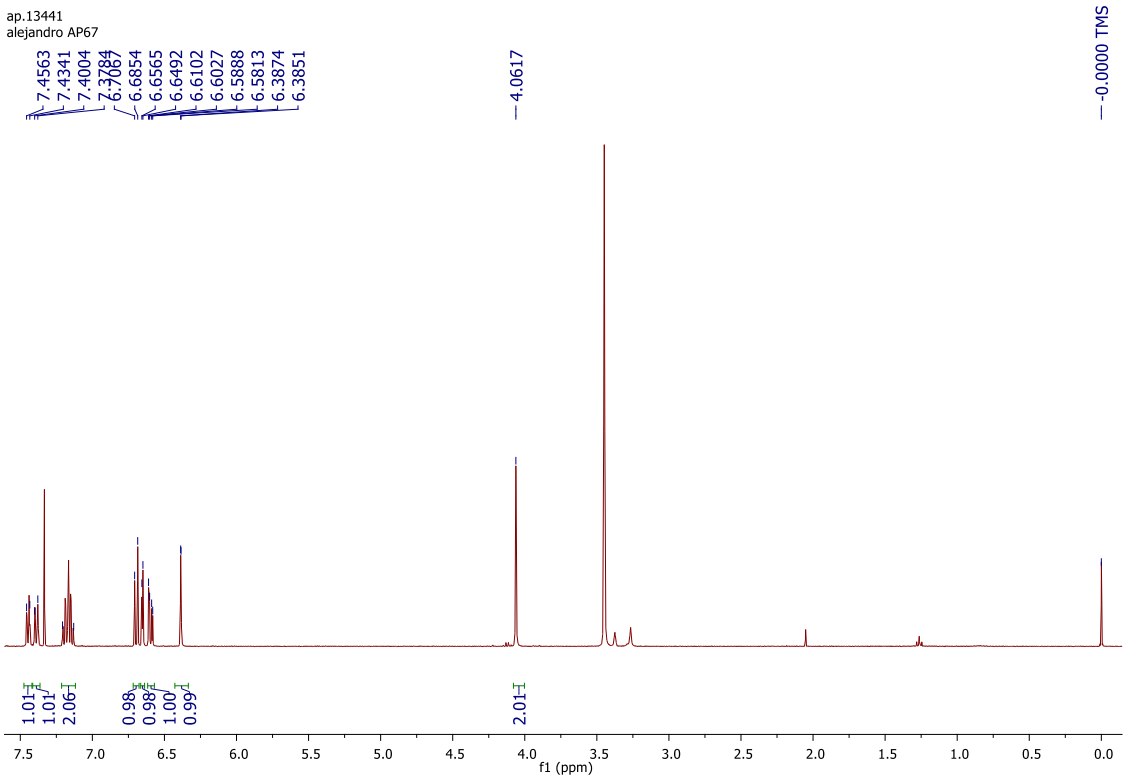



**Figure S19**.[1]H-NMR spectrum of compound **35** (400MHz, $CDCl_3$).

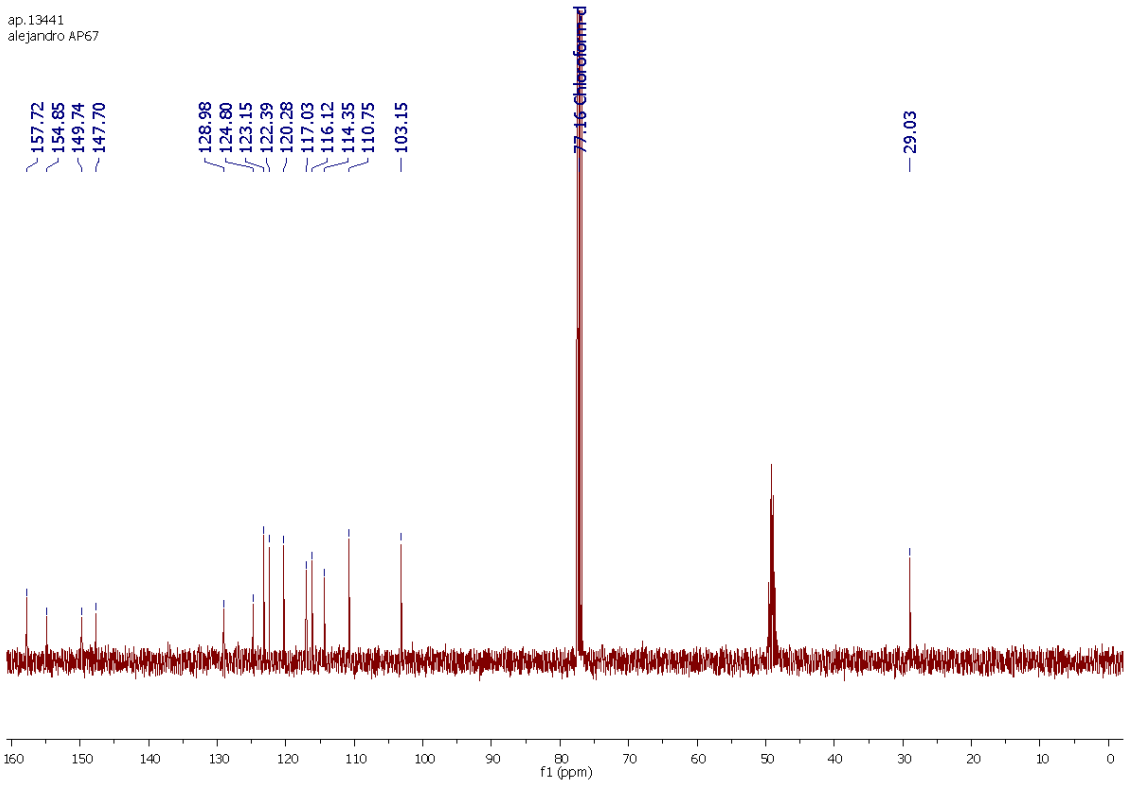



**Figure S20**. [13]C-NMR spectrum of compound **35** (100 MHz, $CDCl_3$).

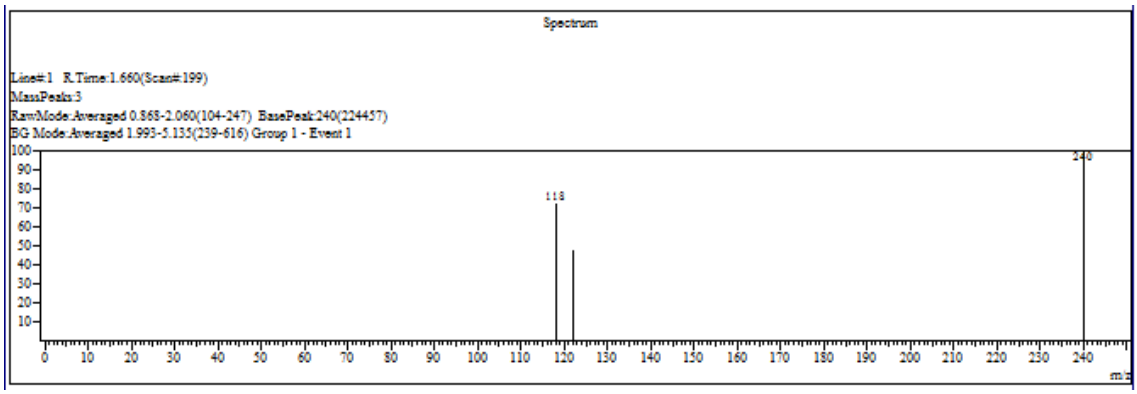



**Figure S21**. Mass spectrum (EI MS) of compound **35**.

**1.6.9.** 2-(2-chloro-3-pyridinemethyl)-1,4-benzenediol **37**

$^{1}$H-NMR (400MHz, $(CD_3)_2SO$; Fig. S7): δ (ppm) = 3.87 (s, 2H), 6.34 (d, *J*=2,9 Hz, 1H), 6.47 (dd, $J_1$=2,9 Hz $J_2$=8,6 Hz, 1H), 6.64 (d, *J*=8,6 Hz, 1H), 7.35 (dd, $J_1$=4,7 Hz $J_2$=7,6 Hz, 1H), 7.55 (dd, $J_1$=1,9 Hz $J_2$=7,6 Hz, 1H), 8.27 (dd, $J_1$=1,9 Hz $J_2$=4,7 Hz, 1H), 8.62 (s, 1H), 8.80 (s, 1H); $^{13}$C-NMR (100 MHz, $(CD_3)_2SO$; Fig. S8): δ(ppm) = 32.7, 114.0, 115.7, 116.6, 123.3, 124.9, 134.9, 139.7, 147.4, 147.6, 149.8, 150.4; EI MS *m/z*: 237 ($M^{2+}$ [$^{37}$Cl], 31), 235 ($M^+$ [$^{35}$Cl], 93), 200 (100), 198 (65); Mp=201-203°C; Yield: 33%.

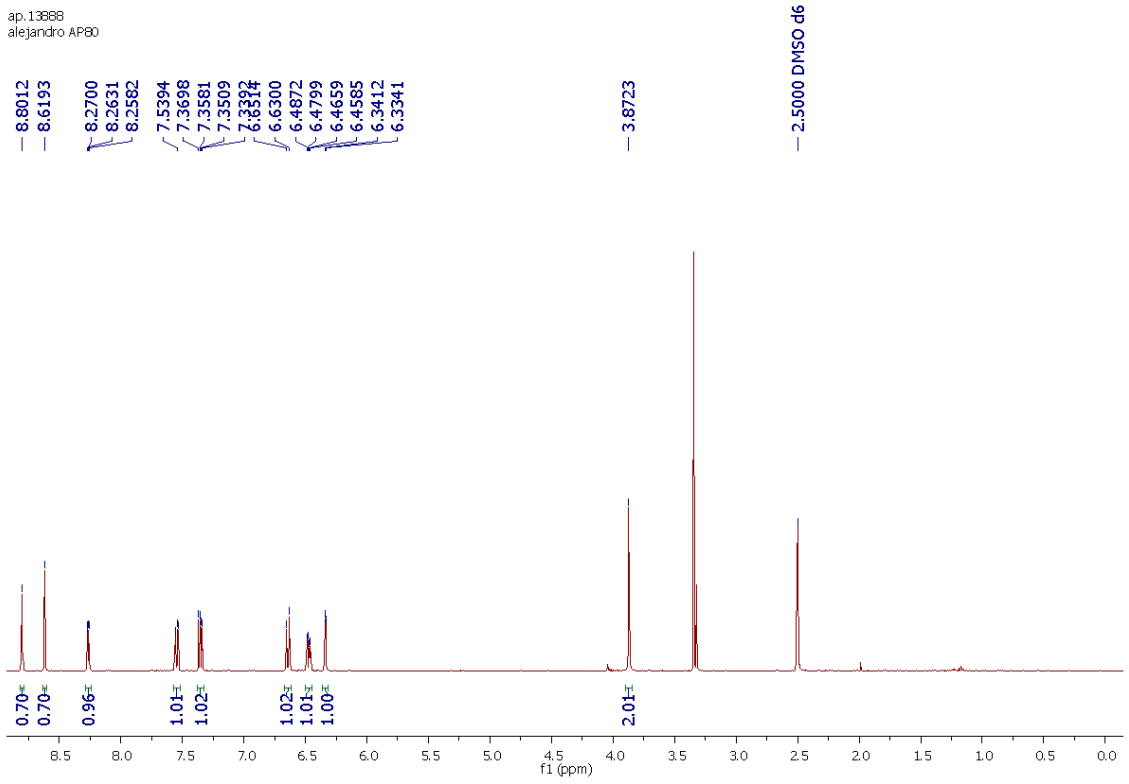



**Figure S22**.$^{1}$H-NMR spectrum of compound **37** (400MHz, $(CD_3)_2SO$).

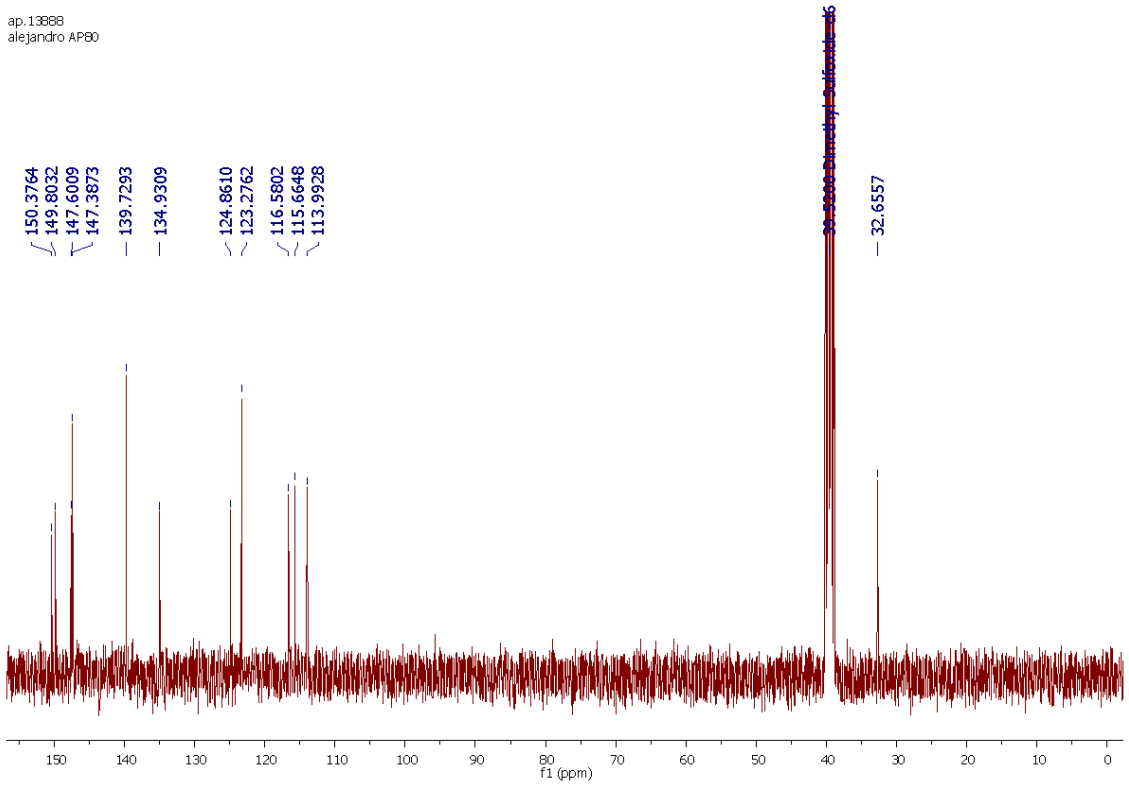



**Figure S23**. $^{13}$C-NMR spectrum of compound **37** (100 MHz, $(CD_3)_2SO$).

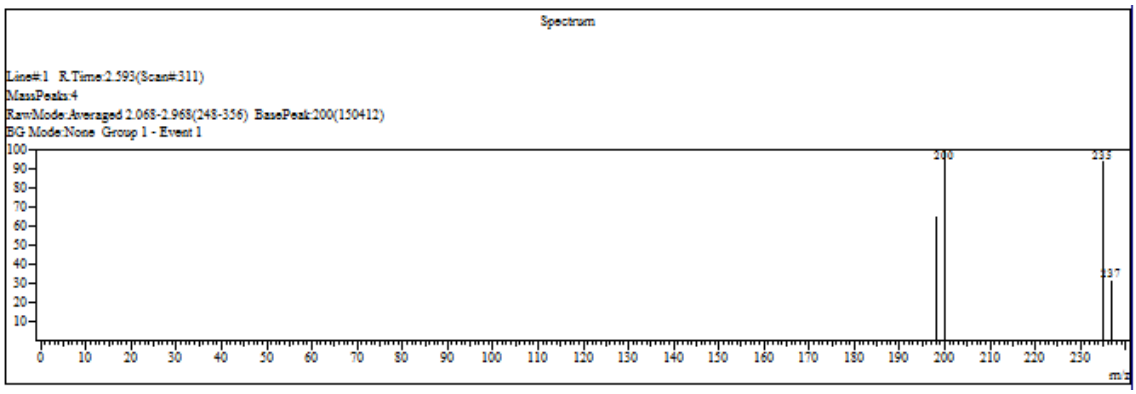



**Figure S24**. Mass spectrum (EI MS) of compound **37**.

**1.6.10.** 2-(3-pyridinylmethyl)-1,4-benzenediol **40**

$^1$H-NMR (400MHz, $(CD_3)_2SO$): δ (ppm) = 3.78 (s, 2H), 6.43 (dd, $J_1$=2.9 Hz $J_2$=8.3 Hz, 1H), 6.45 (d, $J$=2.9 Hz, 1H), 6.60 (d, $J$=8.3 Hz, 1H), 7.28 (dd, $J_1$=4.8 Hz $J_2$=7.8 Hz 1H), 7.57 (dt, $J_1$=1.9 Hz $J_2$=7.8 Hz, 1H), 8.37 (dd, $J_1$=1.5 Hz $J_2$=4.8 Hz, 1H), 8.44 (d, $J$=1.9 Hz, 1H), 8.62 (s, 1H), 8.77 (s, 1H); $^{13}$C-NMR (100 MHz, $(CD_3)_2SO$ δ(ppm): 32.7, 113.8, 115.2, 115.8, 116.8, 123.5, 127.2, 136.2, 136.9, 147.0, 147.4, 149.8, 149.9; EI MS *m/z*: 201 ($M^+$, 100), 200 (48); Mp=145-148°C; Yield: 43% (Ozaki et al., 1997a; Ozaki et al., 1997b).

**1.6.11.** (*E*)-2-(2-(2-quinolinyl)styryl)-1,4-benzenediol **41**

$^{1}$H-NMR (400MHz, $(CD_3)_2SO$; Fig. S9): δ(ppm) = 6.63 (dd, $J_1$=2.9 Hz $J_2$=8.6 Hz, 1H) 6.74 (d, $J$=8.6 Hz, 1H) 7.04 (d, $J$=2.9 Hz, 1H) 7.32 (d, $J$=16.4 Hz, 1H) 7.54 (ddd, $J_1$=12 Hz $J_2$=6.9 Hz $J_3$=8.1 Hz, 1H) 7.73 (ddd, $J_1$=1.5 Hz $J_2$=6.9 Hz $J_3$=8.4 Hz, 1H), 7.79 (d, $J$=8.6 Hz, 1H), 7.93 (dd, $J_1$=1.2 Hz $J_2$=8.4 Hz, 1H), 7.96-8.00 (m, 2H), 8,32 (d, $J$=8.6 Hz, 1H), 8.87 (s, 1H), 9.32 (s, 1H); $^{13}$C-NMR (100 MHz, $(CD_3)_2SO$; Fig. S10): δ(ppm) = 112.4, 116.8, 117.2, 120.1, 123.2, 125.9, 126.9, 127.6, 127.8, 128.6, 129.7, 129.8, 136.4, 147.7, 148.7, 150.0, 156.1; EI MS *m/z*: 263 ($M^+$, 59), 246 (100), 129 (45); Mp=215°C dec. ; Yield: 89% (2 steps).

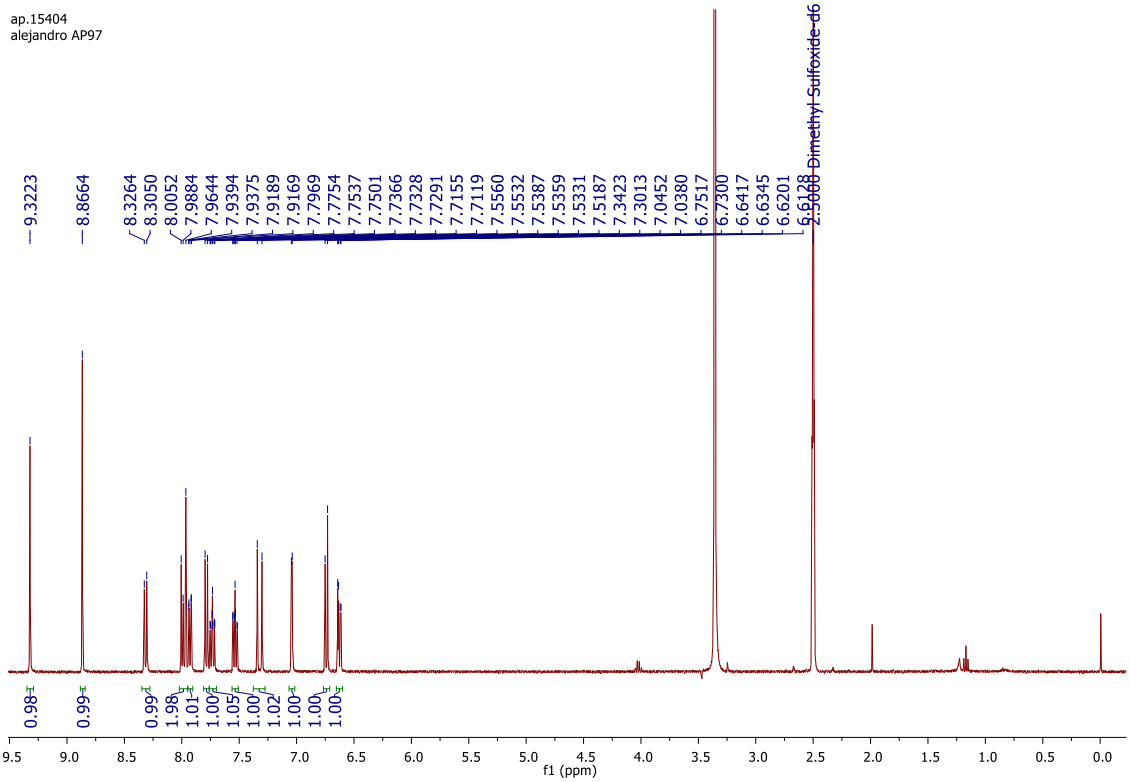



**Figure S25**.$^{1}$H-NMR spectrum of compound **41** (400MHz, $(CD_3)_2SO$).

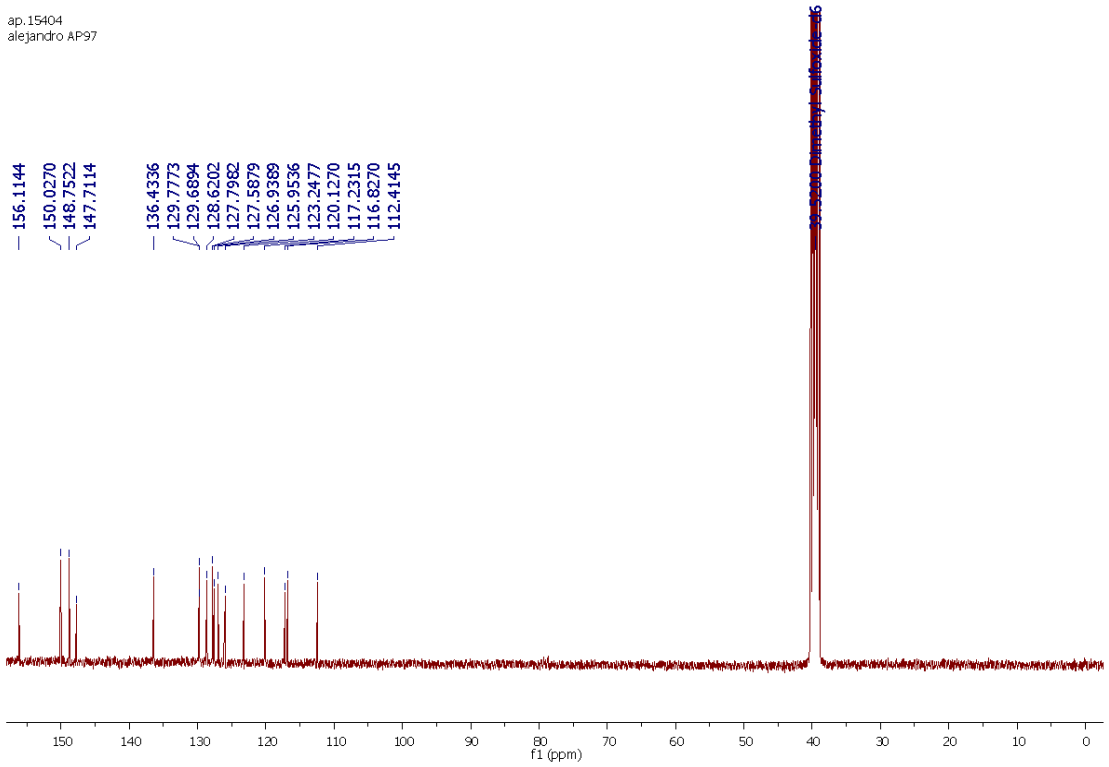



**Figure S26**. $^{13}$C-NMR spectrum of compound **41** (100 MHz, $(CD_3)_2SO$).

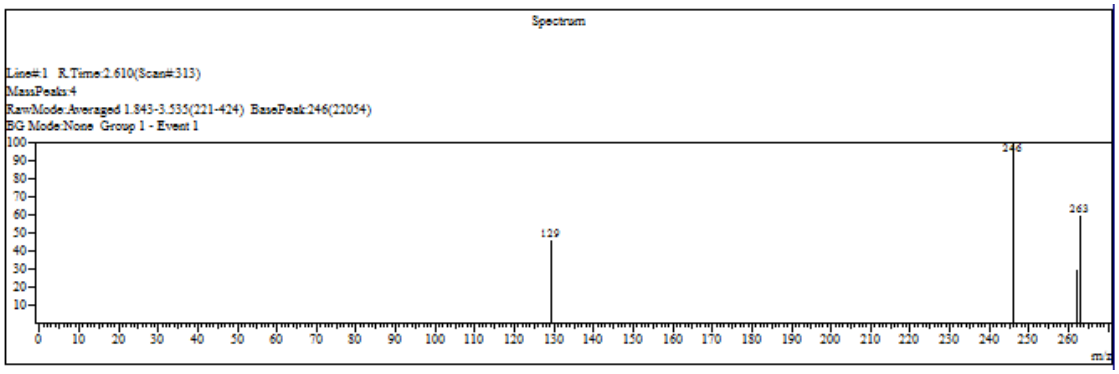



**Figure S27**. Mass spectrum (EI MS) of compound **41**.

**1.6.12.** (*E*)-2-(2-(7-fluoro-2-quinolinyl)styryl)-1,4-diacetoxybenzene **42**

$^1$H-NMR (400MHz, $CDCl_3$; Fig. S13): δ (ppm) = 2.33 (s, 3H), 2.43 (s, 3H), 7.08 (dd, $J_1$=2,6 Hz $J_2$=8.8 Hz, 1H), 7.15 (d, *J*=8.8 Hz, 1H), 7.27-7.33 (m, 2H), 7.51-7.54 (m, 2H), 7.67-7.78 (m, 3H), 8.11 (d, *J*=8.5 Hz, 1H); $^{13}$C-NMR (100 MHz, $CDCl_3$; Fig. S14): δ(ppm) = 21.1, 21.2, 113.0 (d, *J*=20.2 Hz), 116.9 (d, *J*=25.5 Hz), 119.2 (d, *J*=2.4 Hz), 119.7, 122.7, 123.9, 124.6, 127.3, 129.6 (d, *J*=10,0 Hz), 130.3, 131.5, 136.5, 146.2, 148.5, 149.3 (d, *J*=12.8 Hz), 156.2, 163.4 (d, *J*=249.8 Hz), 169.3, 169.4; EI MS *m/z*: 364 (21), 363 (88), 346 (31), 322 (21), 321 (100), 304 (51), 279 (65), 278 (85), 262 (69), 250 (26), 145 (31), 43 (43); Mp=171-173°C; Yield: 93%.

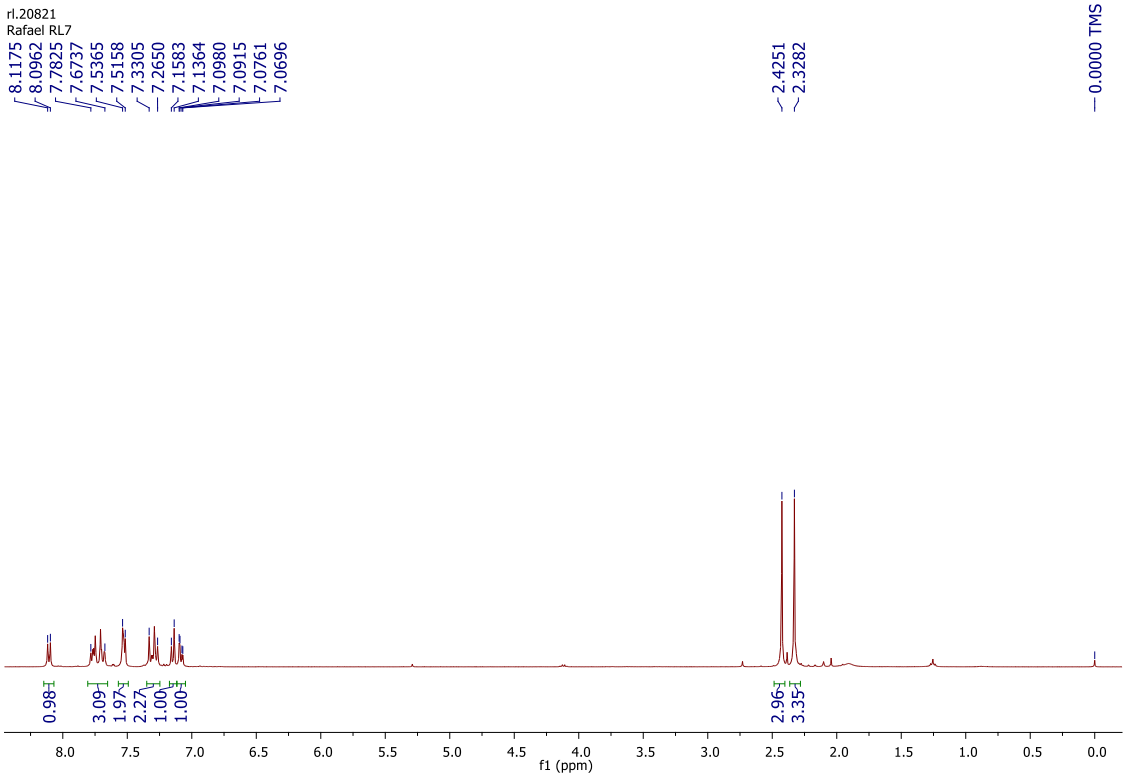



**Figure S28**.[1]H-NMR spectrum of compound **42** (400MHz, $CDCl_3$).

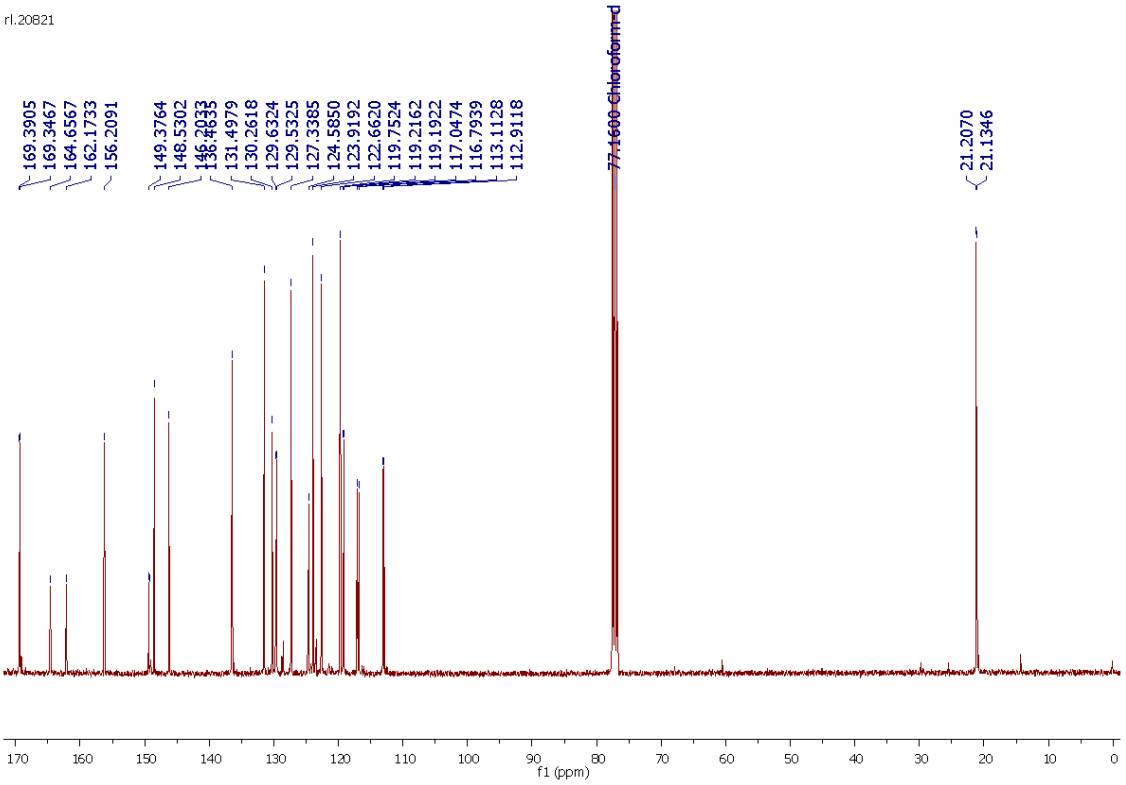



**Figure S29**. [13]C-NMR spectrum of compound **42** (100 MHz, $CDCl_3$).

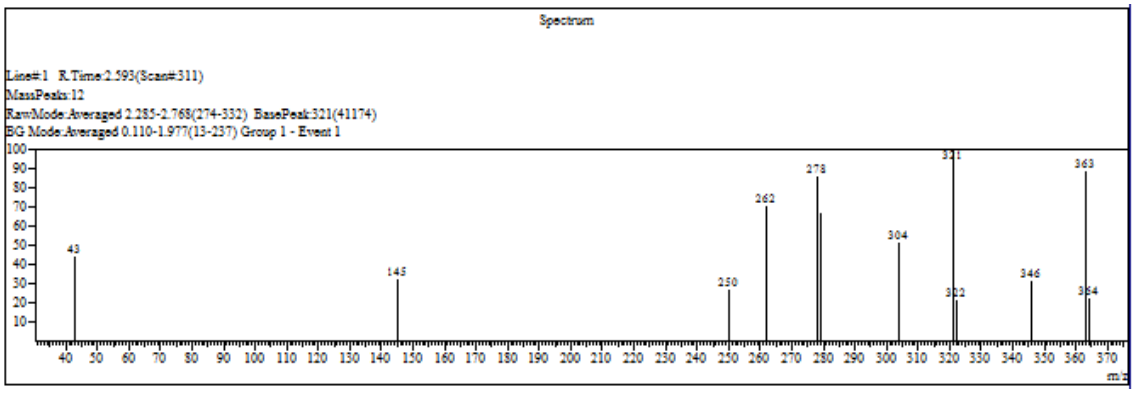



**Figure S30**. Mass spectrum (EI MS) of compound **42**.

### 1.7. 1,4-benzenediol derivatives biologically tested

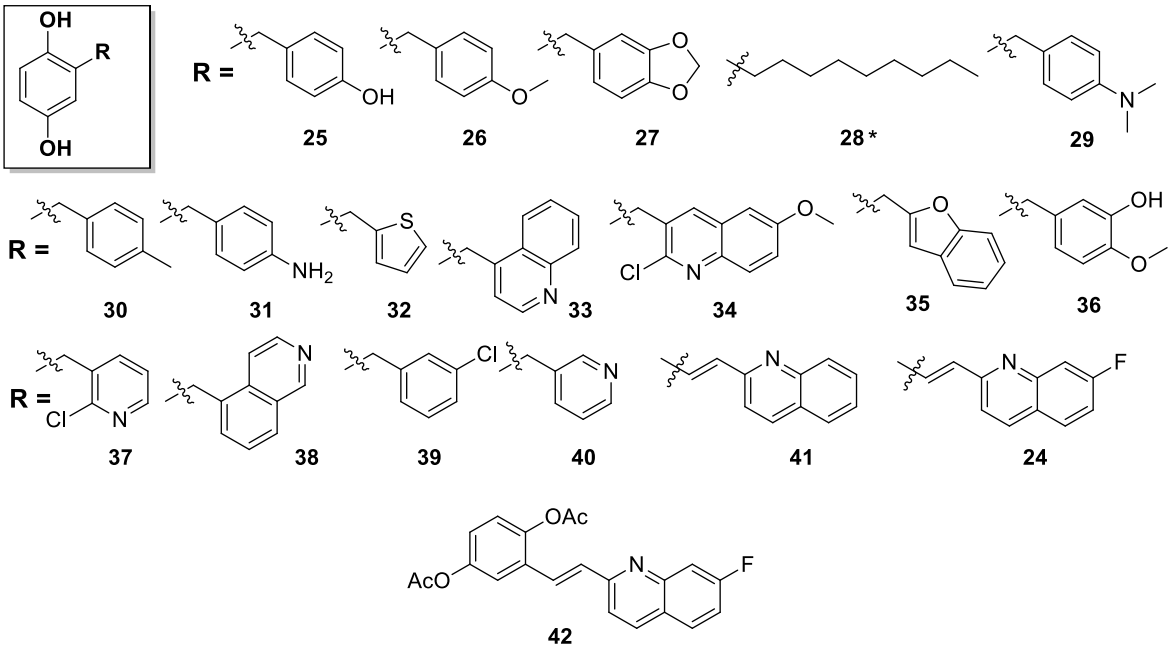



**Figure S31**. 1,4-Benzenediol derivatives (**24-42)** which neither presented *in vitro* antifungal nor antibacterial activities. ***28** was not screened against bacteria.

### 1.8. Investigation of the mechanism of antifungal action

**Table S1**. MICs (µg/mL) for the 1,4-benzenediol **8** and MECs (µg/mL) for the AFG, before and after adding sorbitol.

| **Time** | **48 h** | | | | **96 h** | | | | **168 h** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Dermatophytes** | **8** | | **AFG** | | **8** | | **AFG** | | **8** | | **AFG** | |
| **And *Candida* spp.** | **S(-)** | **S(+)** | **S(-)** | **S(+)** | **S(-)** | **S(+)** | **S(-)** | **S(+)** | **S(-)** | **S(+)** | **S(-)** | **S(+)** |
| **MCA 01** | - | - | - | - | 50 | 50 | 32 | 128 | 50 | 100 | 64 | >256 |
| **MGY 42** | - | - | - | - | 50 | 50 | 32 | 128 | 50 | 100 | 64 | >256 |
| **TME 16*** | - | - | - | - | 50 | 50 | 32 | 128 | 50 | 100 | 64 | >256 |
| **TRU 45** | - | - | - | - | 50 | 50 | 32 | 128 | 50 | 100 | 64 | >256 |
| **CA ATCC 18804** | 50 | 50 | 0.12 | 32 | 50 | 100 | 0.12 | >64 | 50 | 800 | 0.12 | >64 |
| **CK 02*** | 50 | 50 | 0.12 | 32 | 50 | 100 | 0.12 | >64 | 50 | 800 | 0.12 | >64 |
| **CG 09*** | 50 | 50 | 0.12 | 32 | 50 | 100 | 0.12 | >64 | 50 | 800 | 0.12 | >64 |
| **CT 72A*** | 50 | 50 | 0.12 | 32 | 50 | 100 | 0.12 | >64 | 50 | 800 | 0.12 | >64 |

*Multidrug-resistant and resistant fungal isolates. Abbreviations: MIC, minimal inhibitory concentration; MEC, minimal effective concentration; AFG, anidulafungin; S(-), absence of sorbitol in the test; S(+), presence of sorbitol in the test; MCA, *Microsporumcanis*; MGY, *Microsporumgypseum*; TME, *Trichophyton mentagrophytes*; TRU, *Trichophyton rubrum*; CA, *Candida albicans*; CK, *Candida krusei*; CG, *Candida glabrata*; CT, *Candida tropicalis*.

**Table S2**. MICs against eight fungi isolates for the 1,4-benzenediol **8** and AMB, in the presence and absence of ergosterol.

| **Fungi strains** | **Reading 1** | | | | | **Reading 2** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **8** | **MIC[1]** | **MIC[2]** | **MIC[3]** | **MIC[4]** | **MIC[5]** | **MIC[1]** | **MIC[2]** | **MIC[3]** | **MIC[4]** | **MIC[5]** |
| MCA 01 | 50 | 50 | 50 | 50 | 100 | 50 | 50 | 100 | 100 | 200 |
| MGY 42 | 50 | 100 | 100 | 100 | 100 | 50 | 200 | 200 | 200 | 200 |
| TME 16* | 50 | 50 | 50 | 100 | 100 | 50 | 50 | 50 | 100 | 200 |
| TRU 45 | 50 | 50 | 50 | 200 | 200 | 50 | 50 | 50 | 400 | 400 |
| CA ATCC 18804 | 50 | 100 | 200 | 400 | 400 | 50 | 100 | 400 | 400 | 400 |
| CK 02* | 50 | 50 | 200 | 400 | 400 | 50 | 50 | 400 | 400 | 400 |
| CG 09* | 50 | 50 | 200 | 400 | 400 | 50 | 100 | 400 | 400 | 400 |
| CT 72A* | 50 | 100 | 200 | 400 | 400 | 50 | 100 | 400 | 400 | 400 |
| **AMB** | **MIC[1]** | **MIC[2]** | **MIC[3]** | **MIC[4]** | **MIC[5]** | **MIC[1]** | **MIC[2]** | **MIC[3]** | **MIC[4]** | **MIC[5]** |
| MCA 01 | 0.5 | 2 | 4 | 4 | 8 | 0.5 | 2 | 4 | 4 | 8 |
| MGY 42 | 4 | 4 | 4 | 8 | 16 | 4 | 4 | 4 | 8 | 16 |
| TME 16* | 2 | 8 | 8 | 8 | 16 | 2 | 8 | 16 | 16 | 32 |
| TRU 45 | 2 | 2 | 4 | 4 | 8 | 2 | 8 | 16 | 16 | 32 |
| CA ATCC 18804 | 1 | 2 | 4 | 16 | 16 | 1 | 2 | 16 | 128 | 128 |
| CK 02* | 1 | 2 | 4 | 16 | 16 | 1 | 2 | 128 | 128 | 128 |
| CG 09* | 2 | 2 | 4 | 8 | 16 | 2 | 2 | 128 | 128 | 128 |
| CT 72A* | 0.5 | 2 | 4 | 32 | 128 | 0.5 | 2 | >128 | > 128 | > 128 |

*Multidrug-resistant and resistant fungal isolates. AMB = Amphotericin B; CA = *C. albicans* (CA ATCC 18804); CT = *C. tropicalis* (CT 72A*); CK= *C. krusei* (CK 02); CG= *C. glabrata* (CG 09); MCA= *M. canis* (MCA 01); MGY= *M. gypseum* (MGY 42); TME = *T. mentagrophytes* (TME 16*); TRU= *T. rubrum* (TRU 50). $MIC^1$ corresponds to MIC without addition of commercial ergosterol; $MIC^2$, $MIC^3$, $MIC^4$, and $MIC^5$, correspond to MIC with addition of ergosterol at the concentration of 50 μg/mL, 100 μg/mL, 150 μg/mL, and 200 μg/mL, respectively. For *Candidas*, readings 1 and 2 were performed after 2 and 5 days of incubation; for dermatophytes, readings 1 and 2 were performed after 4 and 7 days of incubation.

**1.9.Toxicicity**

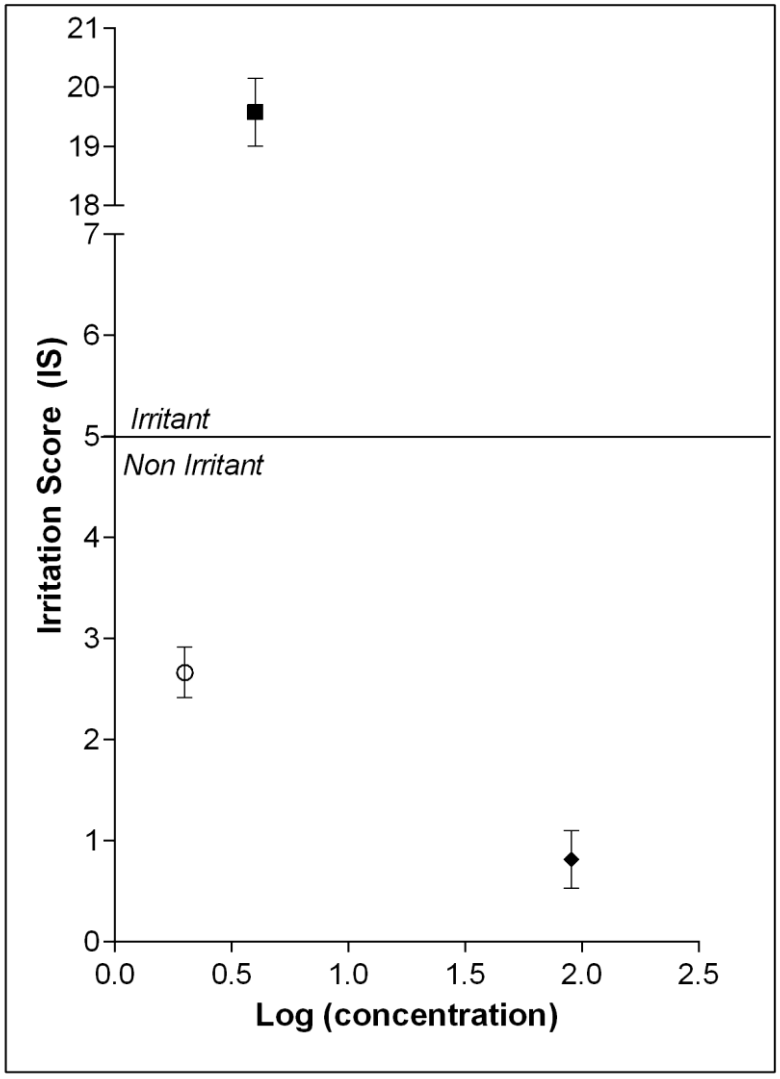



**Figure S32**. Dose-response relationship of **8** (o), negative control (0.9% NaCl) (♦) and positive control (0.1M NaOH) (□). Each point represents one experiment (n = three eggs).

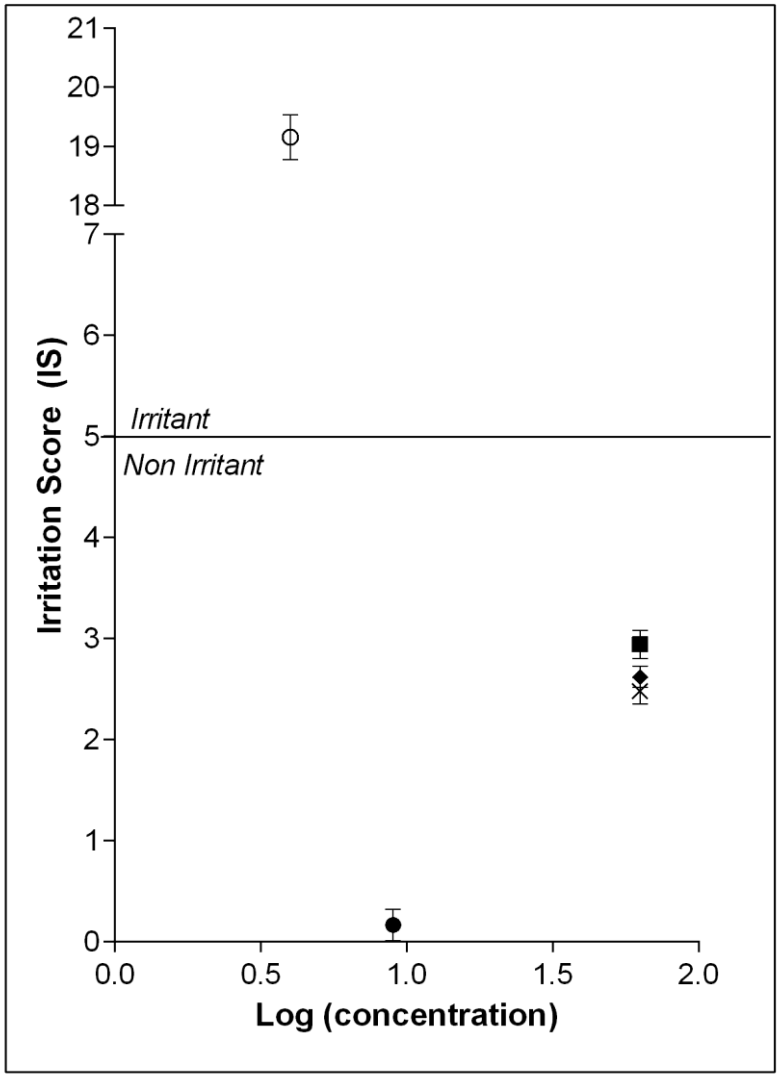



**Figure S33**. Dose-response relationship for 1,4-benzenediol derivatives. **2** (×), **5** (■), and **12** (♦), negative control (0.9% NaCl) (o) and positive control (0.1M NaOH) (□). Each point represents one experiment (n = three eggs).

**Supplemental References**

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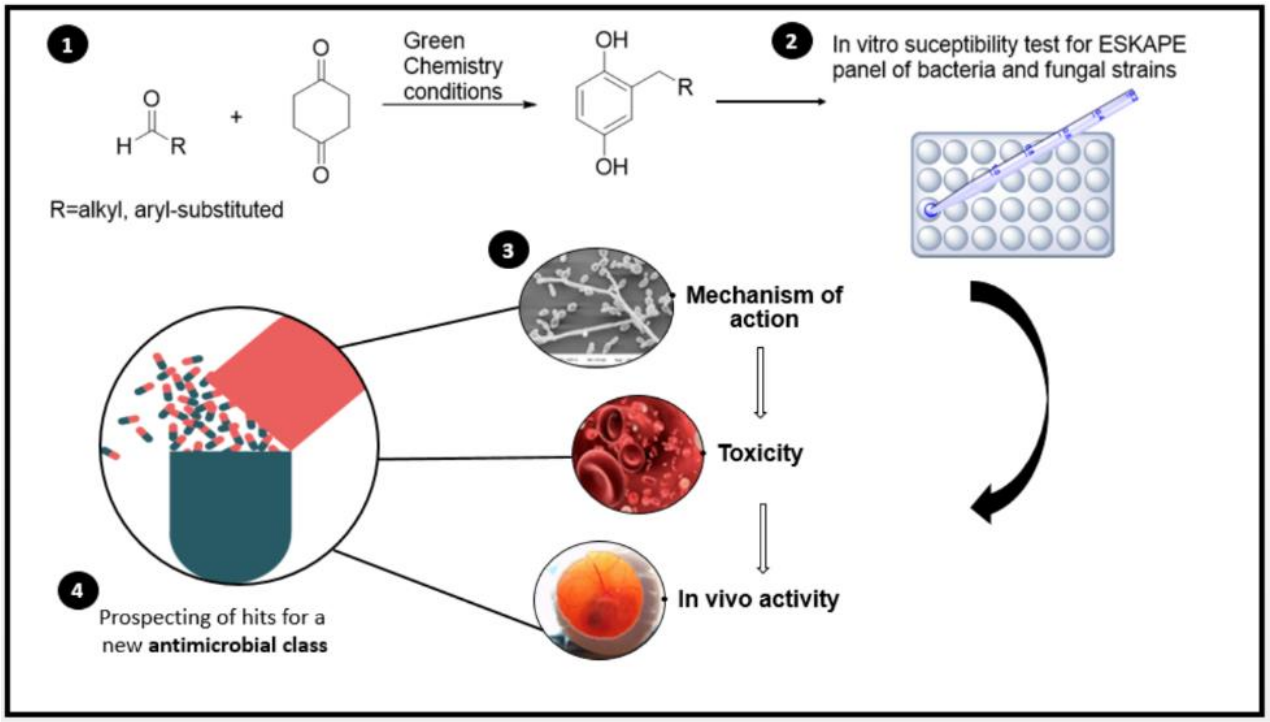
Rolón, M., Lima, A.P.A., Coronel, C., Veja, C., Pandolfi, E., Arias, A.R. The efficacy of new 2,5-dihydroxybenzyl derivatives against *Trypanosoma cruzi*, *Leishmania infantum* and *Leishmania braziliensi*, Submitted for publication in J Infect Dev Ctries. 2018.

## ANEXOS

### Graphical abstract



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## CAPÍTULO V – Invasion of human nails by *Microsporum canis* with biofilm formation

Nota: Manuscrito (*Picture of a microorganism*) submetido ao periódico Clinical Microbiology and Infection.

# Invasion of human nails by *Microsporum canis* with biofilm formation

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Keywords: Biofilm; Dermatophytes; *Microsporum canis*; Scanning electron microscoy; Onychomycosis; Human nails.

Dermatophytes are the main etiological agents of onychomycosis, known clinically for the difficult treatment. Our research group first reported biofilm formation by *Microsporum canis* [1]. Considering this finding, the purpose of this study was to evaluate for the first time the invasion process of the human nail plate by this dermatophytic species through scanning electron microscopy (SEM). The infected human nail plate (donated nails of healthy individuals, without fungal disease) was removed from the Potato Dextrose Agar (Gibco®) plate after incubation at 35 ºC with the inoculum ($10^5$ UFC/mL) for 7 days, and fixed. Nails without inoculum were also incubated under the same conditions as control. Then, the samples was washed, postfixed, dehydrated, displaced, dried, sprayed, and chemically dried. Carl Zeiss EVO® SEM (MA10, Oberkochen, Germany), operating at 10kV, was used to observe and photograph the samples.

SEM images demonstrate the human nail plate without infection, in its normal aspect (Fig 1 - A, B, and C) and the nail invasion of the hyphae of *M. canis*, it being possible to observe ultra-structurally how the pathogens colonize and explore the nail surface (Fig 1 - D, E, and F). The strategy of biofilm formation by *M. canis* in the human nail is also evident (extracellular matrix; Fig. 1 – E, and F - white arrows indicating), which favors virulence and resistance. Therefore, these results corroborate for the better knowledge of the invasion of human nails by dermatophytes, especially for the recently discovered biofilm forming species – *M. canis*, and consolidates the use of the SEM technique to analyze and confirm infectious fungal processes

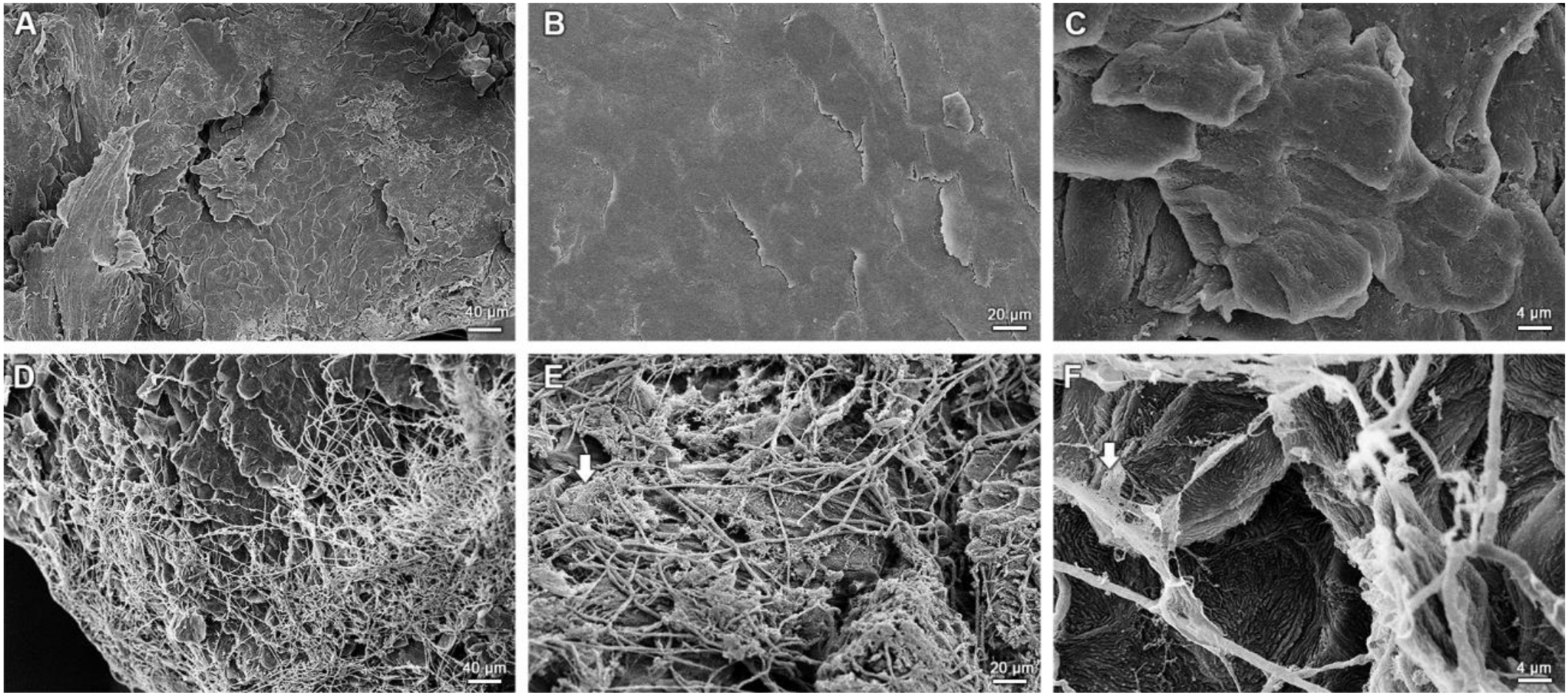


**Fig. 1**. Scanning electron micrographs of human nails invasion by *Microsporum canis* illustrating biofilm characteristics and details of the nail attack. (A, B, and C) General view of normal human nail, without fungal invasion, in different magnifications. (D, E, and F) Invasion of the hyphae of *Microsporum canis* on the nail plate, with the observation of the presence of biofilm produced by the species (indicated by white arrows), in different magnifications.

## Transparency declaration

The authors report no conflicts of interest.

## Financial support

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## Author's contribution

DFD, PR, and WL contributed to drafting the manuscript, literature review and laboratory work. MHV, and AMF contributed to drafting the manuscript and literature review.

## References

**[**1] Danielli LJ, Lopes W, Vainstein MH, Fuentefria AM, Apel MA. Biofilm formation by *Microsporum canis*. Clin Microbiol Infect 2017;23:941–2.

## ANEXO

Regras da revista (https://www.clinicalmicrobiologyandinfection.com/content/authorinfo):

*Picture of a microorganism*

High-quality images of pathogens should be of special interest, have an informative title and be accompanied by no more than 250 words and 1 reference.

**CAPÍTULO VI – Structural-functional elucidation of a novel selective fungicide applied in a topical formulation for dermatomycoses treatment**

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# Structural-functional elucidation of a novel selective fungicide applied in a topical formulation for dermatomycoses treatment

Daiane Flores Dalla Lana[a], Stefânia Neiva Lavorato[b], Laura Minussi Giuliani[c], Letícia Cruz[c], William Lopes[d], Marilene Henning Vainstein[d], Thaís Carine Ruaro[a], Igor Camargo Fontana[a], Aline Rigon Zimmer[a], Murillo de Araújo Freitas[b], Saulo Fernandes Andrade[a], Ricardo José Alves[e], Alexandre Meneghello Fuentefria[a*]

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## ABSTRACT

In this study, the antifungal activity of one hundred and twenty-one molecules, intermediates or derivatives of the 1,3-bis(aryloxy) class was investigated. The compound 1,3-bis(3,4-dichlorophenoxy)propan-2-aminium chloride (**2j**) was the most active fungicide against dermatophytes and *Candida* spp., at very low concentrations (0.39 – 3.12 µg/ml), including action on resistant and multidrug-resistant clinical strains. Compound **2j** has presented a promising toxicity profile, showing selectivity index > 10, relative to human lymphocytes. The compound was classified as non-irritant by the HET-CAM test and did not cause histopathological alterations in pig ear skin, thus presenting an excellent perspective for topical application. **2j** targets the fungal cell wall, which was confirmed by scanning electron microscopy images, which also indicated the additional ability of **2j** to inhibit the *Candida albicans* pseudohyphae formation and biofilm of *Microsporum canis*. Compound **2j** was incorporated in a hydrogel with bioadhesive potential. The results of the human skin permeation showed that **2j** remained significantly in the epidermis, ideally for the dermatomycosis treatment. Therefore, the compound **2j** demonstrated the potential for antifungal drug development, with a mechanism of action elucidated and already applied in a semisolid formulation aimed a new therapeutic option for fungal skin infections not responsive to conventional treatment.

**Keywords:** 1,3-bis(aryloxy) class, fungicide, dermatomycoses treatment, dermatophytes, *Candida* spp., hydrogel

## 1. Introduction

Dermatomycosis are fungal infections that involve the most superficial layers of the skin - stratum corneum and epidermis, and the appendages, such as hair, nails, and the mucomembranous surface (Stojanov et al., 2011; Hube et al., 2015). Positioned as 4$^{th}$ most prevalent dermatological pathology in the last years and affecting 20–25% world's population, they cause high morbidity rates (Peres et al., 2010; Sharma et al., 2015; Kim et al., 2015). They have as etiological agents mainly dermatophytic filamentous fungi and yeasts as *Candida* spp. (Vineetha et al., 2018; Metin et al., 2018).

Dermatophytes are keratinophilic fungi, comprising species of three anamorphic genera - *Microsporum*, *Trichophyton*, and *Epidermophyton* (Dalla Lana et al., 2016; Vineetha et al., 2018). *Trichophyton rubrum*, *Trichophyton mentagrophytes, and more* recently *Microsporum canis* has been identified as a biofilm-forming species, which worsens the therapeutic response (Costa-Orlandi et al., 2014; Danielli et al., 2017). In immunocompromised patients, dermatophytes are capable of causing deeper and more serious mycoses (Nir-Paz et al., 2003; Rouzaud et al., 2018), and also represent a veterinary problem (Moriello et al., 2017). *Candida* spp. are considered opportunistic fungi, which take advantage of an imbalance of the host to become pathogenic (Khosravi et al., 2018; Desai et al., 2018). *Candida albicans* and non-*albicans Candida* infections are very prevalent and reported in the literature, representing a considerable clinical problem too due to the capacity of biofilm formation (Khosravi et al., 2018; Desai et al., 2018).

The development of antimicrobial drug resistance is a relentless fact that is universal in the microbial world. The uncontrolled use of antimycotics in immunocompromised patients marked the onset of antifungal drug resistance (Pai et al., 2018). The first reported occurrences of dermatophytic resistance was with griseofulvin (Michaelides *et al*., 1961). Approximately 20% of azole resistance in dermatophytes has been observed in some countries such as the United States and India (Pai et al., 2018). Primary resistance of *Trichophyton rubrum* to terbinafine in cases of onychomycosis was described in 2003 (Mukherjee *et al*., 2003), being that still in 2018 the resistance of dermatophytes continues being reported (Martinez-Rossi et al., 2018). For *Candida* spp., in patients of chronic mucocutaneous candidiasis, resistance to ketoconazole was first verified in 1980, being that the problem of yeast resistance to azoles continues today, almost 40 years after this first report (Pai et al., 2018). Also considering the few options of antifungal drugs available, one of the main strategies of improving the mycoses therapy is overcoming antifungal resistance and all limitations involved in the in the process (Fuentefria et al., 2017; Nicola et al., 2018). Therefore, the prospection of new drugs and formulations to treat dermatomycosis is needed, to bring advances and insights that facilitate the development of novel strategies for antifungal therapy.

In this context, we propose the extensive investigation of the *in vitro* antifungal activity of one hundred twenty-one synthetic compounds of the following chemical classes: 1,3-bis(aryloxy)-2-propanol derivatives, 1,3-bis(aryloxy)-2-propanamines derivatives, 2-(2-oxo-2(arylamino)ethyl)isothiouronium chloride derivatives, 1,3-bis(aryloxy)-2-methanesulfonyloxypropane derivatives, and 1,3-bis(aryloxy)-2-azidopropane derivatives. Recently, some molecules of these classes have already

presented promising antiprotozoal activity against *Leishmania amazonensis* promastigote forms (Lavorato et al., 2017a; Lavorato et al., 2017b), so our interest in testing them in relation to fungi. We further researched the mechanism of action, toxicity and application of the most promising compound (antifungal efficacy/toxicity profile) in a hydrogel for topical treatment of dermatomycosis associated with susceptible and multidrug-resistant pathogenic fungal species.

## 2. Material and methods

### 2.1. Chemicals - General procedure for synthesis of compounds

In total, one hundred twenty-one compounds were evaluated for antifungal activity: group (1) – 1,3-bis(aryloxy)-2-propanol derivatives (**1a**–**1gg**; thirty three compounds), group (2) – 1,3-bis(aryloxy)-2-propanamines derivatives (**2a**–**2bb**; twenty eight compounds), group (3) – 2-(2-oxo-2(arylamino)ethyl)isothiouronium chloride derivatives (**3a**–**3j**; ten compounds), group (4) – 1,3-bis(aryloxy)-2-methanesulfonyloxypropane derivatives (**4a**–**4y**; twenty five compounds), and group (5) – 1,3-bis(aryloxy)-2- azidopropane derivatives (**5a**–**5y**; twenty five compounds). The compounds were synthesized according to Lavorato et al. (2017a,b). All reagents were obtained from commercial suppliers and used without further purification. Melting points were determined on Microquímica MQAPF 301 apparatus. The 1H and 13C NMR spectra were obtained on a Bruker Avance DPX-200 spectrometer. The proton and carbon chemical shifts (δ) are given with respect to TMS. IR spectra were recorded on a Spectrum One, Perkin-Elmer ATR system. Column chromatography was performed on silica gel 0.063–0.200 mm/70-230 mesh Merck. Pyridine was dried over KOH pellets. Anhydrous DMF was obtained from commercial supplier (Sigma-Aldrich). Anhydrous sodium sulfate was used as drying agent after work up when extraction was applied. The spectral characterization data of the unpublished compounds which showed antifungal activity are described below and the data of unpublished compounds without activity are shown in the supplemental information. These compounds were dissolved in DMSO (Sigma-Aldrich) and diluted in a medium assay to obtain a maximum concentration of 1% DMSO in the experiments.

**1a:** IR (ATR) $\nu_{max}$ 3555, 3071, 2944, 2881, 1614, 1587, 1504, 1454, 1257, 1107, 1041 $cm^{-1}$; $^{1}H$ NMR ($CDCl_3$, 200 MHz): $\delta$ = 7.12-6.86 (8H, m, H-3', H-4', H-5', H-6'), 4.43 (1H, qn, *J* = 5.0 Hz, H-2), 4.25 (2H, dd, *J* = 9.8 Hz, 5.0 Hz, H-1a), 4.20 (2H, dd, *J* = 9.8 Hz, 5.0 Hz, H-1b), 2.63 (1H, s, OH); $^{13}C$ NMR ($CDCl_3$, 50 MHz): $\delta$ = 153.0 (C, C-2′, *J* = 244.1 Hz), 146.7 (C, C-1', *J* = 10.7 Hz), 124.6 (CH, C-5', *J* = 3.5 Hz), 122.1 (CH, C-4', *J* = 6.8 Hz), 116.5 (CH, C-3′, *J* = 18 Hz), 115.6 (CH, C-6′, *J* = 1.5 Hz), 70.2 ($CH_2$, C-1), 68.8 (CH, C-2).

**1b**: IR (ATR) $\nu_{max}$ 3526, 3053, 2941, 1605, 1510, 1455, 1267, 1161, 1016 $cm^{-1}$; $^{1}H$ NMR ($CDCl_3$, 400 MHz): $\delta$ = 7.11-7.04 (2H, m, H-5'), 6.79-6.73 (2H, m, H-2'), 6.65-6.61 (2H, m, H-6'), 4.36 (1H, asx, *J* = 5.2 Hz, H-2), 4.11 (2H, dd, *J* = 9.6 Hz, *J* = 4.8 Hz, H-1a), 4.07 (2H, dd, *J* = 9.6 Hz, *J* = 5.6 Hz, H-1b); $^{13}C$ NMR ($CDCl_3$, 100 MHz): $\delta$ = 154.8 (C,

C-1′, *J* = 8.6 Hz, 2.1 Hz), 150.7 (C, C-3′, *J* = 246.7 Hz, 13.8 Hz), 145.6 (C, C-4’, *J* = 239.6 Hz, 12.7 Hz), 117.6 (CH, C-5′, *J* = 18.4 Hz, 1.2 Hz), 110.1 (CH, C-6′, *J* = 5.7 Hz, 3.5 Hz), 104.6 (CH, C-2′, *J* = 20.2 Hz), 69.6 ($CH_2$, C-1), 68.7 (CH, C-2).

**1c:** IR (ATR) $\nu_{max}$ 3400, 2942, 1592, 1492, 1449, 1233, 1162, 1064 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 400 MHz): $\delta$ = 7.41 (2H, t, *J* = 8.0 Hz, H-5’), 7.25 (2H, d, *J* = 8.0 Hz, H-6’), 7.18 (2H, s, H-2’), 7.11 (2H, dd, *J* = 8.0 Hz, 2.4 Hz, H-4’), 4.43 (1H, asx, *J* = 5.2 Hz, H-2), 4.27 (2H, dd, *J* = 9.6 Hz, 4.8 Hz, H-1a), 4.19 (2H, dd, *J* = 9,6 Hz, 6.0 Hz, H-1b), 2.60 (1H, d, *J* = 5.2 Hz, OH); $^{13}C$ NMR ($CDCl_3$, 100 MHz): $\delta$ = 158.7 (C, C-1′), 132.2 (C, C-3’, *J* = 32.4 Hz), 130.4 (CH, C-5’), 124.1 (C, $CF_3$, *J* = 270.6 Hz), 118.3 (CH, C-4′, *J* = 3.9 Hz), 118.2 (CH, C-6′), 111.7 (CH, C-2′, *J* = 3.8 Hz), 69.1 ($CH_2$, C-1), 68.8 (CH, C-2).

**2d:** IR (ATR) $\nu_{max}$ 2855, 1594, 1521, 1488, 1451, 1266, 1132, 1050 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 200 MHz): $\delta$ = 8.82 (3H, s, $NH_3$), 7.35 (2H, td, *J* = 8.0 Hz, 8.0 Hz, H-5’), 6.92-6.78 (6H, m, H-2’, H-4’, H-6’), 4.41-4.24 (4H, m, H-1), 3.90 (1H, qn, *J* = 4.8 Hz, H-2); $^{13}C$ NMR (DMSO-$d_6$, 50 MHz): $\delta$ = 162.8 (C, C-3′, *J* = 241.0 Hz), 159.2 (C, C-1’, *J* = 11.0 Hz), 130.8 (CH, C-5’, *J* = 9.5 Hz), 111.0 (CH, C-6’), 108.0 (CH, C-4′, *J* = 21.2 Hz), 102.4 (CH, C-2′, *J* = 24.6 Hz), 65.4 ($CH_2$, C-1), 49.0 (CH, C-2).

**2e**: IR (ATR) $\nu_{max}$ 2877, 1608, 1511, 1470, 1263, 1159, 1042 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 200 MHz): $\delta$ = 8.83 (3H, s, $NH_3^+$), 7.46-7.31 (2H, m, H-5’), 7.20-7.12 (2H, m, H-2’), 6.89-6.84 (2H, m, H-6’), 4.35 (2H, dd, *J* = 10.8 Hz, *J* = 5.0 Hz, H-1a), 4.28 (2H, dd, *J* = 10.8 Hz, *J* = 4.6 Hz, H-1b), 3.92-3.85 (1H, m, H-2); $^{13}C$ NMR (DMSO-$d_6$, 50 MHz): $\delta$ = 154.4 (C, C-1′, *J* = 7.5 Hz), 147.0 (C, C-3′), 142.1 (C, C-4’, *J* = 12.9 Hz), 117.7 (CH, C-5′, *J* = 18.1 Hz), 111.3 (CH, C-6′), 104.7 (CH, C-2′, *J* = 19.9 Hz), 66.1 ($CH_2$, C-1), 49.1 (CH, C-2).

**2i:** IR (ATR) $\nu_{max}$ $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 400 MHz): $\delta$ = 8.80 (3H, s, $NH_3$), 7.35 (2H, d, *J* = 2.4 Hz, H-3’), 7.42 (2H, dd, *J* = 8.8 H, 2.4 Hz, H-5’), 7.28 (2H, d, *J* = 8.8 Hz, H-6’), 4.47 (2H, dd, *J* = 10.4 Hz, 5.2 Hz, H-1a), 4.42 (2H, dd, *J* = 10.4 Hz, 5.6 Hz, H-1b), 3.99 (1H, aqn, *J* = 5.6 Hz, H-2); $^{13}C$ NMR (DMSO-$d_6$, 100 MHz): $\delta$ = 152.3 (C, C-1′), 129.4 (CH, C-3’), 128.3 (CH, C-5’), 125.6 (C, C-4’), 123.0 (C, C-2′), 116.0 (CH, C-6′), 66.6 ($CH_2$, C-1), 48.7 (CH, C-2).

**2j:** IR (ATR) $\nu_{max}$ 2882, 1589, 1569, 1511, 1478, 1460, 1231, 1049 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 200 MHz): $\delta$ = 8.79 (3H, s, $N\underline{H}_3^+Cl^-$), 7.56 (2H, d, *J* = 8.8 Hz, H-5’), 7.32 (2H, d, *J* = 2.8 Hz, H-2’), 7.05 (2H, dd, *J* = 8.8 Hz, 2.8 Hz, H-6’), 3.90 (1H, dd, *J* = 5.2 Hz, *J* = 4.6 Hz, H-2), 4.39 (2H, dd, *J* = 10.5 Hz, *J* = 4.6 Hz, H-1a), 4.31 (2H, dd, *J* = 10.5 Hz, *J* = 5.2 Hz, H-1b); $^{13}C$ NMR (DMSO-$d_6$, 50 MHz): $\delta$ = 157.5 (C, C-1′), 131.6 (C, C-3′), 131.1 (CH, C-5’), 123.3 (C, C-4′), 116.9 (CH, C-2′), 115.8 (CH, C-6′), 65.8 ($CH_2$, C-1), 49.0 (CH, C-2).

**2n**: IR (ATR) $\nu_{max}$ 2916, 2875, 1604, 1504, 1464, 1252, 1134, 1057 $cm^{-1}$; $^{1}H$ NMR (DMSO-$d_6$, 400 MHz): $\delta$ = 8.78 (3H, s, $NH_3$), 6.96 (2H, s, H-3'), 6.96 (2H, d, $J$ = 8.8 Hz, H-5'), 6.85 (2H, d, $J$ = 8.8 Hz, H-6'), 4.31 (2H, dd, $J$ = 10.4 Hz, 4.8 Hz, H-1a), 4.27 (2H, dd, $J$ = 10.4 Hz, 5.6 Hz, H-1b), 3.89 (1H, aqn, $J$ = 5.2 Hz, H-2); $^{13}C$ NMR (DMSO-$d_6$, 100 MHz): $\delta$ = 153.7 (C, C-1′), 131.3 (CH, C-3'), 129.7 (C, C-4'), 127.0 (CH, C-5'), 126.1 (C, C-2′), 111.5 (CH, C-6′), 65.2 ($CH_2$, C-1), 49.4 (CH, C-2), 20.1 ($CH_3$, $ArCH_3$), 15.9 ($CH_3$, $ArCH_3$).

### 2.2. Antifungal drugs

Terbinafine, amphotericin B, and anidulafungin, purity ≥ 97%, were supplied by Cristalia (Sao Paulo, Brazil), griseofulvin, purity ≥ 97%, was acquired from Wallace Pharmaceuticals (Mumbai, India), ketoconazole, purity ≥ 96%, was obtained from All Chemistry (Sao Paulo, Brazil), fluconazole purity ≥ 98% was obtained from Sigma-Aldrich (Sao Paulo, Brazil), itraconazole purity ≥ 97%, was supplied by Cassará laboratory (Dist. PHARMOS; Buenos Aires, Argentina), miconazole purity ≥ 97%, was supplied by Valdequimica Chemical Products (Sao Paulo, Brazil), and voriconazole; purity ≥ 98%, was supplied by Pfizer (Sao Paulo, Brazil). The preparation of stock and work solutions followed the recommendations of the CLSI (M38-A2, 2008). These antifungaldrugs were used as references for the assays conducted in this study.

### 2.3. Fungal strains

Yeast species of the genus *Candida* (*C. albicans* - CA ATCC 18804, *C. krusei* - CK 02*, *C. glabrata* - CG 09*, and *C. tropicalis* - CT 72A*) and dermatophytic filamentous fungi (*M. canis* - MCA 01, *Microsporum gypseum* - MGY 42, *T. mentagrophytes* - TME 16*, and *T. rubrum* - TRU 45) were selected for the broad antifungal activity screening with all molecules. Subsequently, for the most promising compound observed at screening, more clinical strains (17) were included in this study: *C. albicans* (CA 01), *C. krusei* (CK 03), *C. glabrata* (CG 05), *C. tropicalis* (CT ATCC 750), *C. parapsilosis* (CP 06, CP 07), *M. canis* (MCA 33, MCA 38), *M. gypseum* (MGY5 HCPA, MGY 50, MGY 58), *T. mentagrophytes* (TME 32, TME 40), *T. rubrum* (TRU 2 HCPA, TRU 3 HCPA, TRU 48), and *Trichophyton schoenleinii* (TSHO 3 HCPA). All species are deposited into the Mycology Collection of Universidade Federal do Rio Grande do Sul (Porto Alegre, Brazil). Standard strains of *C. albicans* (ATCC 18804) and *C. tropicalis* (ATCC 750) were obtained from American Type Culture Collection, Manassas (ATCC, VA, USA) and were included as controls.

### 2.4. *In vitro* susceptibility test

Susceptibility tests were performed for determining the Minimum inhibitory concentrations (MIC) and the minimum fungicidal concentration (MFC). The MIC was defined by the broth microdilution method, according to the protocol established for yeasts - M27-A3 (CLSI, 2008) and filamentous fungi - M38-A2 (CLSI, 2008). The inoculum of yeasts ($0.5 \times 10^3$ to $2.5 \times 10^3$ CFU/ml) and filamentous fungi ($1.0 \times 10^3$ to $3.0 \times 10^3$ CFU/ml) were prepared from cultures grown on *sabouraud dextrose agar (SDA; Kasvi, Curitiba, Brazil)* and potato dextrose agar (PDA; Neogen, Lansing, USA),

respectively. The assays were conducted in duplicate, in three independent essays, with RPMI medium, containing L-glutamine (without sodium bicarbonate; Gibco, USA), buffered to pH 7.0 with 0.165 M of 3-(N-morpholino)propanesulfonic acid (MOPS; Neon, São Paulo, Brazil), with subsequent incubation at 35 ºC. The analysed concentrations of the synthetic compounds ranged from 0.03–50 µg/ml. The reading of the results for *Candida* spp. was performed in 24–48 h, and for dermatophytes after 96 h of incubation. MIC was defined as the lowest concentration of the substance capable of inhibiting the visible fungal growth. Sterility control (drug-free medium only) and positive control (inoculum and culture medium only, for evaluation of the fungal cell viability) were used.

To determine the MFC, aliquots of each serial microdilution (corresponding to MIC, 2xMIC and 4xMIC) were spread on SDA (*Candida*) and PDA (dermatophytes), which were incubated at 35 °C and analysed. The MFC was defined as the lowest drug concentration that yielded up to three colonies (*i.e.*, ≥ 99% of the inoculum was killed) (Espinel-Ingroff et al., 2002). The assay was performed in triplicate.

### 2.5. Toxicity Profile

#### 2.5.1. Lymphocytes culture and determination of cell viability

Human lymphocytes have been isolated from peripheric blood of healthy donators (5 ml; all the consents, procedures and documents were approved by Universidade Federal do Rio Grande do Sul - Ethical Committee under authorization number 666.655/2014). These cells were maintained in RPMI-1640 medium (Sigma, St Louis, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco, Grand Island, NY, USA), 0.5% de penicillin/streptomycin e 1% de amphotericin B, at 37°C in a humidified atmosphere (5% $CO_2$). Cells were seeded in 96-well plates (5 x $10^4$ cells per well) and after 24 h of incubation, they were treated with some of the most promising compounds for 48 h (Waechter et al., 2017). The compounds were initially dissolved in DMSO and then with the medium to be tested at a maximum concentration of 50 µg/ml. The DMSO concentration was kept below 1% which was non-toxic to cells used. The viable cells counting was performed by FACSVerse™ Flow cytometer, equipped with 488 nm blue laser and BD Biosciences flow sensor (San Jose, CA, EUA) (BD FacSuite™ Software). Results were expressed as % relative to the control and $IC_{50}$ values (concentration causing 50% reduction in lymphocytes viability) and were calculated using nonlinear regression analysis with GraphPad Prism 6.0 software. The selectivity index (SI) of the compounds was calculated based on the antifungal subinhibitory concentrations (MIC/2) and $IC_{50}$ results for each compound, with the following equation 1.

**Equation 1.** SI = IC50/average of subinhibitory concentrations (MIC/2) for dermatophytes and *Candida* spp.

#### 2.5.2. Hen's Egg Test-Chorioallantoic Membrane (HET-CAM)

Fresh and fertile white eggs (Lohmann selected Leghorn, LSL) were kept under optimized incubation conditions (38–39 °C, 55–60% humidity, 10 days). On the 10$^{th}$ day,

the eggshell, around the airspace, was removed with a rotary tool (Dremel, WI). Subsequently, 0.3 ml of the most promising synthetic compound (50 µg/ml, 0.5% DMSO solution) and controls (negative control: 0.9% NaCl; positive control: 0.1 mol/l NaOH) were added to the CAM of the eggs. The irritant effect was observed at three times: 30 sec, 2 min and 5 min after application of compound and controls. The result of the irritation score (IS) was calculated according to the equation 2 and presents a maximum value of 21 (ICCVMA, 2010). The eggs were analyzed in relation to the appearance of hemorrhaging, lysis, and coagulation. Classification criterion used: 0 to 4.9 nonirritant (or practically no irritation); 5.0 to 21 irritant (moderate to severe or extreme irritation). The assay was performed in triplicate.

**Equation 2**. $$IS = \left(\left(\frac{(301-\text{hemorrhage time})}{300}\right)x5\right) + \left(\left(\frac{(301-\text{lysis time})}{300}\right)x7\right) + \left(\left(\frac{(301-\text{coagulation time})}{300}\right)x9\right)$$

### 2.5.3. Histopathological evaluation

Tissue samples from adult male pigs, freshly slaughtered at the Instituto Federal Catarinense, were used to evaluate the formation of tissue damage due to the action of the most promising synthetic compound. Pigs were slaughtered according to the rules of the Brazilian Ministry of Agriculture, respecting animal welfare (Brazil, 2013). Ear skin of pigs was used in this study. The tissues were removed within a period of 5 min after the slaughter; the hairs were carefully removed by an electric trimmer and transported to the laboratory in an ice-cold Krebs-Hepes buffer (INLAB, São Paulo, Brazil). Skin samples were mounted in Franz diffusion cells (Logan Instrument Corp., Somerset, NJ, USA) with the diffusion area of approximately 1.75 $cm^2$. The epidermal side of the skin was exposed to a phosphate-buffered saline (PBS) pH 7.0 (negative control), to 0.1 M NaOH solution (positive control), and to synthetic compound, for a period of 6 h (time that the permeation profile is more intense). The compound were dissolved in a PBS pH 7.0 at a concentrations of 20, 40, and 80 µg/ml (concentrations up to 50 times higher than MIC). The epidermal side of the skin was subjected to extract contact with this solution for a period of 6 h. Fragments of these tissues were harvested, fixed in 10% neutral-buffered formalin, processed routinely, stained with hematoxylin and eosin, and examined under light microscopy. The procedure was performed in triplicate.

## 2.6. Mechanism of action

### 2.6.1. Sorbitol protection assay

The effect of the most promising synthetic compound on the integrity of the fungal cell wall was evaluated by sorbitol protection assay. MICs were determined by the standard broth microdilution (document M38-A2 for dermatophytes, and document M27-A3 for *Candida* spp.) (CLSI, 2008), in the absence and presence of 0.8 M sorbitol (Sigma-Aldrich) added to the RPMI medium as an osmoprotectant. Microplates were incubated at 35 ºC. Experiments were carried out in duplicate. Anidulafungin was used

as positive control. MICs were measured after 4 and 7 days for dermatophytes, and after 2 and 5 days for *Candida* spp. (Frost et al., 1995; Escalante et al., 2008).

### 2.6.2. Ergosterol binding assay

The capacity of the most promising synthetic compound to complex with ergosterol in the fungal membrane was investigated by ergosterol binding assay. The MICs were determined by the standard broth microdilution, documents M27-A3 and M38-A2 (CLSI, 2008), in the absence and presence of different concentrations (50–250 µg/ml) of exogenous ergosterol (Sigma-Aldrich, St. Louis, MO, USA) first dissolved in dimethylformamide (Sigma-Aldrich, St. Louis, MO, USA) and subsequently added to RPMI medium. The plates were incubated at 35 ºC. Experiments were carried out in duplicate. Amphotericin B was used as positive control. MICs were measured after 4 and 7 days for dermatophytes, and after 2 and 5 days for *Candida* spp. (Escalante et al., 2008; Carrasco et al., 2012).

### 2.6.3. Scanning electron microscopy (SEM)

Morphological changes in *C. albicans* (ATCC 18804) and *M. canis* (MCA 01) grown in the presence of the most promising synthetic compound were determined using the macro broth dilution method, according to the documents M27-A3 and M38-A2 (CLSI, 2008). Treated and untreated (control) cells were analyzed by SEM. After the incubation period (*C. albicans*: 48 h at 35 ºC and *M. canis*: 96 h at 35 ºC), fungal cells treated with the most promising synthetic compound in subinhibitory concentrations (MIC/2) and untreated cells were washed three times with PBS (3000 rpm for 5 min. Biosystems MCD2000, Curitiba, Brazil). Anidulafungin (Pfizer, New York, USA) was used as a positive control of fungal cell damage. After washing, cells were fixed in 1 ml of modified Karnovsky's fixative adapted from Joubert et al. (2015). Then, the wells were washed three times (3000 rpm for 5 min) in 0.1 M sodium cacodylate buffered at pH 7.2 containing 0.2 M sucrose, and 2 mM $MgCl_2$ with the aid of two pipettes, which were used for addition and concurrent removal to avoid air exposure. Cells were adhered in coverslips previously functionalized with poly- L-lysine for 1 h. Adhered cells were dehydrated in a series of freshly made solutions of graded acetone 30, 50, 70, 95 (5 min) and 100% (10 min) (Dalla Lana et al., 2018; Pippi et al., 2018). Samples were then subjected to critical point drying (EM CPD 300, Leica), mounted on metallic stubs, sputtercoated with a 15–20 nm gold-palladium layer and visualized in a scanning electron microscope (Carl Zeiss EVO-MA10 Carl, Oberkochen, Germany) operating at 10 kV.

## 2.7. Hydrogel (HG) preparation and characterization

To obtain the hydrogel, Pemulen® TR2 (0.7%) was initially milled in a mortar and then dispersed in water. The dispersion was neutralized with triethanolamine (De Lima et al., 2017). The HG containing the most promising compound - **2j** (0.5 mg/g HG) was prepared by solubilizing the compound in DMSO, and then incorporated into the hydrogel base. All hydrogels were prepared in triplicate, packaged in plastic container and stored at room temperature (25 ± 2 °C).

### 2.7.1. pH determination

The pH values were evaluated in HG aqueous dispersion (10%, w/v), in potentiometer (Model pH 21, Hanna Instruments, Brazil) previously calibrated, at room temperature.

### 2.7.2. Quantification of 2j in HG

The total **2j** content in the hydrogel was assayed by diluting a sample aliquot in methanol: water (90:10) and subjecting it to shake for 15 min and sonication for 15 min. Samples were filtered through a 0.45 μm membrane and injected into the *High performance liquid chromatography* (HPLC) system. The quantitative analyses were performed on a LC-10A HPLC system (Shimadzu, Japan) equipped with a SIL-20A HT valve sample automatic injector, an UV-VIS SPD-M20A detector, a LC-20AT pump, CBM-20A system controller, a guard column, and a Inertsil® ODS-3 $C_{18}$ column (150 mm × 4.60 mm, 5 μm; 110 Å), which was kept at room temperature. The mobile phase consisted of methanol and water pH 9.0 (90:10, v/v) at isocratic flow rate (1.2 ml/min). The wavelength used for **2j** detection was 283 nm and a sample volume of 20 μl was injected into the equipment. This HPLC method was previously validated and proved to be linear ($r > 0.9998$) in the range from 2 to 16 μg/ml, specific and precise.

### 2.7.3. Spreadability determination

The spreadability was evaluated according to the methodology described by Rigo et al. (2012). The sample was placed in a central hole (1 cm diameter) of a mold glass plate that was positioned on a scanner surface (HP Officejet, model 4500 Desktop). The mold plate was removed and the sample was subsequently pressed with glass plates of known weights, in intervals of 1 min between each plate. The spreading area images were captured at each 1 min interval employing the desktop scanner. The software Image J (Version 1.49q, National Institutes of Health, USA) was used to calculate the captured images areas. Results were expressed in terms of the spreading area as a function of the cumulative weight of the plates. The spreadability factor (Sf) was also calculated and it represents the spreading capacity on a smooth horizontal surface when one gram of weight is added to it. The equation 3 was employed to calculate the Sf:

**Equation 3.** $Sf = A / W$

In which, Sf is the spreadability factor ($mm^2/g$), A is the maximum spread area ($mm^2$) after addition of the total number of plates, and W is the total weight added (g).

### 2.7.4. Evaluation of the rheological behavior

Rheology analyzes of the hydrogels were carried out at 25 ± 1 ºC using a rotational viscometer (RVDV I – PRIME model, Brookfield, USA) and a spindle RV-06. For this, about 30 g of the formulations were used. A shear stress (τ) ramp was run up and down from 6.0 to 100.0 rpm, registering 7 points. Rheograms were obtained by plotting the shear stress (τ) as a function of the shear rate (γ). The rheograms were analyzed using different flow models: Bingham (ideal plastic, $\tau = \tau o + \eta\gamma$), Casson (plastic, $\tau^{0.5} = \tau_o^{0.5} +$

$\eta^{0.5}\gamma^{0.5}$), and Ostwald (pseudoplastic, $\tau = K\gamma^{n}$), where $\tau_o$ is the yield stress, $\eta$ is the viscosity, n is the index of flow, and K is the index of consistency (Kim et al., 2003).

### 2.7.5. *In Vitro* release assay

The **2j** *in vitro* release from HG was studied through vertical Franz diffusion cells, at 32 ± 0.5 °C (n = 3) (Marchiori et al., 2017). The area for diffusion was 3.14 $cm^2$. Mixed Cellulose Esters (MCE) membrane (Unifil®; 0.45 μm pore size) was fit between donor and receptor compartments. The receptor media employed in the assay was ethanol and water (70:30) (Alvorado et al., 2015), contemplating the sink condition or a more physiological condition, respectively. A formulation amount correspondent to a 0.5 g (infinite dose) of HG containing **2j** was spread on the membrane surface. Regarding the intervals of 1, 2, 3, 4, 5, 6, 7 and 8 h, 0.5 ml receptor medium was collected and replaced by an equal volume of fresh medium to maintain sink conditions. Aliquots were analyzed by HPLC method previously developed, validated and described in the item 2.7.2.

### 2.7.6. Human skin penetration/permeation studies

Cutaneous permeation of compound **2j** was investigated using abdominal skin of female human with no damages, stretch marks or scars (obtained from plastic surgeries with permission and informed consent - Research Ethics Committee of the UFSM, CAAE: 55220016.3.0000.5346; Project approval number: 1.632.595). Immediately after surgical removal, the subcutaneous layer of the skin was removed, the skin was washed with PBS and kept in a freezer (-20 ºC) until the day of the experiment. During the experiment, with the defrosted skin (< 6 months), the **HG-2j** (0.50 g) were placed in the donor compartment of Franz diffusion cells, maintaining complete and intimate contact with the skin membrane surface (Balzus et al., 2017). The receptor compartment contained a mixture of ethanol:water (70:30 v/v) to ensure the sink condition during all the experiment. This compartment was constantly stirred and kept at 32 ºC for 8 h. Then the excess material loosely attached to the skin surface was carefully removed by the tape stripping technique - 18 pieces of adhesive tape (Scotch 3M, 35×45 mm), were used. The remained piece of skin was then separated into the epidermis and dermis by heat-separation, immersing it in a water bath at 60 ºC for 45 s (De Andrade et al, 2015). Then the dermis and epidermis were mechanically separated with the aid of a spatula and was cut in small pieces. The amount of **2j** was extracted from the stratum corneum, epidermis and dermis with methanol, using a vortex stirrer (2 min) and ultrasonic bath (15 min). Samples were filtered through a 0.45 μm membrane before HPLC analysis by a previously validated method for quantification of **2j** as described previously in section 2.7.2. All analyses were carried out right after the end of the penetration experiment, conducted with five replicates (n=5).

## 2.8. Statistical Analysis

Analysis of the data was performed with GraphPad Prism® software, version 5.0 (San Diego, CA, USA). All the results are expressed as the mean value ± the standard deviation of the mean and statistically analyzed using variance analysis (ANOVA).

Results presenting $p < 0.05$ were considered statistically different. The comparisons among the averages were performed using Tukey's test.

## 3. Results and discussion

### 3.1. *In vitro* susceptibility and structure–activity relationship

An initial screening of antifungal activity was performed with the compounds considering the concentration of 50 µg/ml. From the total of one hundred twenty-one molecules analyzed (supplementary Information, Scheme S1), eighteen (approximately 15%) of these showed anti-*Candida* spp. or anti-dermatophytes activity in concentrations ≤ 50 µg/ml (Scheme 1). Generally, they present simple structures, constituted by two aromatic moieties linked together by a spacer. The main structural differences among these substances are the nature and substitution pattern of aromatic moiety and the nature and size of the spacer. The aromatic moieties of these compounds are commonly substituted. To the best of our knowledge, there are no studies on the potential of these classes as antifungal agents, which can be prepared in four simple steps from the reaction of inexpensive epichorohydrin with appropriately substituted aryloxy molecules.

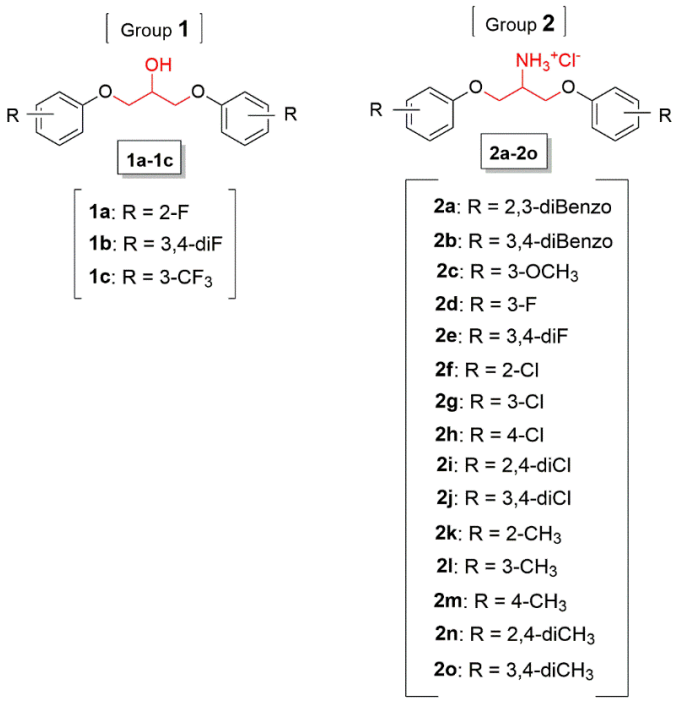
For these active molecules, the MICs (Table 1) and MFCs were determined. The active compounds were those belonging to groups 1 (1,3-bis(aryloxy)-2-propanol derivatives) and 2 (1,3-bis(aryloxy)-2-propanamines derivatives), with emphasis on group 2 in relation to the low MIC values, which can be observed in Table 1. Group 1 presented compounds with activity only for dermatophytes, while group 2 exhibited a broader action against filamentous and yeast. For group 1 the halogen substituent fluorine plays a crucial role in the antidermatophytic activity. The molecule disubstituted by fluorine (**1b**) has lower MICs when compared to monosubstituted (**1a**). The activity is even more pronounced with the substituent, also fluorinated, $CF_3$ (**1c**). All compounds of group 1 were fungistatic (MFC > 4xMIC).

For the group 2, the most promising of the entire chemical series tested, an important substituent regarding antifungal efficacy was chloride, as can be verified for the molecules **2f**, **2g**, **2h**, **2i**, and **2j**, with compound **2j** (1,3-bis(3,4-dichlorophenoxy)propan-2-aminium chloride/ $C_{15}H_{14}Cl_5NO_2$) being identified as the most potent. As **2j** was the compound that most stood out for the wide activity against filaments and yeasts with low MICS, we determined their MICs for a greater number of clinical and ATCCs strains (n = 17; Table S1). The MIC range for dermatophytes was 0.39 – 3.12 µg/ml, and for yeast was 0.78 – 1.56 µg/ml, reinforcing the data from the screening that this compound is actually active at very low concentrations. In other studies, the presence of the chloride element in chemical structures investigated as to the antifungal application has already been verified as determinant for effective activity (Dalla Lana et al., 2015; Bergamo et al., 2016; Ngo et al., 2016; Dalla Lana et al., 2018). Structurally, the position of the chlorine is important for improving the antifungal action, since the 3(*meta*),4(*para*)-diCl position of the disubstituted molecule **2j** was more advantageous (smaller MICs) than the 2(*orto*),4(*para*)-diCl position of **2i**. These results corroborate with the finding by Lavorato et al. (2017a), with some molecules of the same class investigated in this study: for meta-substituted compounds, was observed that the

electronic effect of ring substituent has a greater influence on antileishmanial activity (Lavorato et al., 2017a).

The compounds **2a**, **2b**, **2f**, **2g**, **2h**, **2j, 2k**, and **2m** were fungicides, presenting MFC = MIC values (Table 1), and so they were selected to integrate the toxicity analysis on human lymphocytes. The other compounds of groups 3, 4, and 5 had no antifungal activity up to 50 µg/ml (supplementary Information, Scheme S1).

Noteworthy, the compounds of groups 1 and 2, with emphasis on compound **2j**, showed activity against resistant yeast species and a multidrug-resistant dermatophyte (*T. mentagrophytes*; Table 1), closely related to difficult-to-treat dermatomycoses (Nigam, 2015; Drakensjö et al., 2017; Fuentefria et al., 2017). Considering the fungal species presented in Table 1, for *Candida* spp. the resistance (*) was defined based on Kuriyama et al. (2005) and CLSI breakpoints (M27-A3, 2008, and M27-S4, 2008): *C. krusei* (CK 02*) resistant to itraconazole (MIC = 1 µg/ml) and fluconazole (MIC ≥ 64 µg/ml), *C. glabrata* (CG09*) resistant to itraconazole (MIC > 4 µg/ml) and miconazole (MIC =8 µg/ml), and *C. tropicalis* (CT 72A*) resistant to itraconazole (MIC = 1 µg/ml), miconazole (MIC > 8 µg/ml), and voriconazole (MIC = 2 µg/ml). As for dermatophytes, the resistance (in the sense of reduced susceptibility) was established according to the increase in minimal inhibitory concentration (MIC) values for some clinical strains in relation to the majority, considering the following resistance threshold concentrations: terbinafine - MIC ≥ 1 µgml, griseofulvin - MIC ≥ 4 µg/ml, and ketoconazole - MIC ≥ 8 µg/ml. Consequently, the clinical isolate TME 16* was considered multidrug-resistant by the considerable elevation of MICs of three antifungal agents of different classes (MIC terbinafine = 4 µg/ml, MIC griseofulvin > 32 µg/ml and MIC ketoconazole = 16 µg/ml).




**Scheme 1.** Chemical structure of the compounds with *in vitro* antifungal activity.

**Table 1**. Minimal inhibitory concentrations of compounds that exhibited *in vitro* antifungal activity in relation to species of dermatophytes and yeasts of the genus *Candida*.

| Compounds and antifungal drugs | Dermatophytes | | | | Yeasts | | | |
|---|---|---|---|---|---|---|---|---|
| | **MCA 01** | **MGY 42** | **TME 16*** | **TRU 45** | **CA ATCC 18804** | **CK 02*** | **CG 09*** | **CT 72A*** |
| **Group 1** | | | | | | | | |
| **1a** | 50 | 25 | 25 | 50 | > 50 | > 50 | > 50 | > 50 |
| **1b** | 25 | 3.12 | 25 | 25 | > 50 | > 50 | > 50 | > 50 |
| **1c** | 3.12 | 3.12 | 3.12 | 3.12 | > 50 | > 50 | > 50 | > 50 |
| **MIC range** | 3.12 – 50 | | | | > 50 | | | |
| **Group 2** | | | | | | | | |
| **2a** | 3.12 | 0.78 | 3.12 | 6.25 | 3.12 | 3.12 | 6.25 | 3.12 |
| **2b** | 1.56 | 0.78 | 50 | 50 | 50 | 50 | 50 | 50 |
| **2c** | 50 | 50 | 50 | 50 | > 50 | > 50 | > 50 | > 50 |
| **2d** | 50 | 12.5 | 50 | 50 | > 50 | > 50 | > 50 | > 50 |
| **2e** | 25 | 3.12 | 50 | 25 | 50 | 25 | 50 | 25 |
| **2f** | 25 | 12.5 | 12.5 | 12.5 | 50 | 50 | 50 | 50 |
| **2g** | 6.25 | 1.56 | 12.5 | 6.25 | 12.5 | 12.5 | 12.5 | 12.5 |
| **2h** | 3.12 | 3.12 | 6.25 | 6.25 | 6.25 | 6.25 | 12.5 | 6.25 |
| **2i** | 50 | 50 | > 50 | > 50 | 6.25 | 25 | 12.5 | 50 |
| **2j** | 1.56 | 1.56 | 3.12 | 3.12 | 1.56 | 1.56 | 1.56 | 1.56 |
| **2k** | 25 | 6.25 | 12.5 | 25 | > 50 | > 50 | > 50 | > 50 |
| **2l** | 50 | 50 | > 50 | 50 | 50 | 50 | 50 | 50 |
| **2m** | 25 | 12.5 | 25 | 25 | 25 | 25 | 50 | 25 |
| **2n** | 0.78 | 0.78 | 0.78 | 0.78 | 12.5 | 12.5 | 12.5 | 25 |
| **2o** | 3.12 | 3.12 | 3.12 | 3.12 | 12.5 | 25 | 6.25 | 25 |
| **MIC range** | 0.78 – > 50 | | | | 1.56 – > 50 | | | |
| **Terbinafine** | 0.03 (S) | 0.03 (S) | 4 (R*) | 0.06 (S) | | | | |
| **Griseofulvin** | 1 (S) | 1 (S) | >32 (R*) | 1 (S) | | | | |
| **Ketoconazole** | 0.5 (S) | 1 (S) | 16 (R*) | 1 (S) | 0.25 (S) | 1 (S) | 0.5 (S) | 1 (S) |
| **Fluconazole** | | | | | 1 (S) | ≥ 64 (R) | 0.25 (DDS) | 2 (S) |
| **Itraconazole** | | | | | | 1 (R) | > 4 (R) | 1 (R) |
| **Miconazole** | | | | | | 0.5 (S) | > 8 (R) | > 8 (R) |
| **Voriconazole** | | | | | | | | 2 (R) |

*Multidrug-resistant and resistant fungal isolates; MIC, minimal inhibitory concentration; MCA, *Microsporum canis*; MGY, *Microsporum gypseum*; TME, *Trichophyton mentagrophytes*; TRU, *Trichophyton rubrum*; CA, *Candida albicans*; CK, *Candida krusei*; CG, *Candida glabrata*; CT, *Candida tropicalis*. R, resistance; R*, resistance in the sense of reduced susceptibility compared to other strains; S, susceptible; DDS, dose-dependent susceptibility.

## 3.2. Toxicity Profile

### 3.2.1. Lymphocytes culture

The toxicity profile was established for the fungicidal compounds of the group 2. The $IC_{50}$ values of active compounds ranged from 7.10 to 29.67 μg/ml. The results are shown in Table 2. The SI for the active compounds presented in Table 2 correspond to the ratio between the $IC_{50}$ (concentration required to inhibit 50% of the human lymphocytes viability) and the average of subinhibitory concentrations (MIC/2; MICs are presented in table 1) for dermatophytes and *Candida* spp.. Usually, a SI value higher than 10 is recommended to ensure the safety of an antimicrobial substance (Pires et al., 2013;

Vincent et al., 2016; Lavorato et al., 2017). How much higher the SI value of a substance, the better is its selective performance against the pathogen and the lower the toxicity against the studied host cell (lymphocytes; Pires et al., 2013; Vincent et al., 2016; Lavorato et al., 2017). In this study, the only compound with IS > 10 was the **2j**, presented the safest biological profile with selectivity against dermatomycosis fungi. So this compound was chose for other toxicity studies, the mechanism of action investigation and development of a semisolid formulation.

**Table 2**. *In vitro* toxicity profile ($IC_{50}$ and SI) of compounds **2a**, **2b**, **2f**, **2g**, **2h**, **2j**, **2k**, and **2m**, for dermatophytes / yeasts, respectively.

| Compounds | SC* (μg/ml) ± SD | SC* (μM) ± SD | $IC_{50}$ (μg/ml) ± SD | $IC_{50}$ (μM) ± SD | SI |
|---|---|---|---|---|---|
| **2a** | 1.66±1.12 / 1.95±0.78 | 4.36±2.95 / 5.13±2.05 | 7.10±0.52 | 18.69±0.52 | 4.28 / 3.64 |
| **2b** | 12.79±14.10 / 25±0 | 33.68±37.11 / 65.81±0 | 12.02±1.47 | 31.64±1.47 | 0.93 / 0.48 |
| **2f** | 7.81±3.13 / 25±0 | 22.41±8.96 / 71.71±0 | 22.55±1.95 | 64.68±1.95 | 2.88 / 0.90 |
| **2g** | 3.32±2.24 / 6.25±0 | 9.52±6.44 / 17.93±0 | 29.67±1.16 | 85.10±1.16 | 8.93 / 4.74 |
| **2h** | 2.34±0.90 / 3.90±1.57 | 6.71±2.58 / 11.19±4.49 | 20.22±1.76 | 58±1.76 | 8.64 / 5.18 |
| **2j** | 1.17±0.45 / 0.78±0 | 2.80±1.08 / 1.87±0 | 21.94±1.04 | 52.55±1.04 | 18.75 / 28.12 |
| **2k** | 8.59±4.69 / Inactive | 27.91±15.23 / Inactive | 8.93±0.72 | 29.01±0.72 | 1.03 |
| **2m** | 10.94±3.13 / 15.63±6.25 | 35.53±10.15 / 50.76±20.30 | 8.31±0.47 | 27±0.47 | 0.75 / 0.53 |

SC*, average of subinhibitory concentrations of fungi (MIC/2; MICs in table 1); $IC_{50}$, the concentration of each compound required to inhibit 50% of the human lymphocytes viability; SI, selective index ($IC_{50}$/SC*).

### 3.2.2. HET-CAM

The HET-CAM test resulted in an IS (equation 2) for compound **2j** (when evaluated at concentrations up to 50xMIC) of 3.14 ±0.08, which classify these compounds as nonirritant (or practically no irritation; IS ≤ 4.9) according to the methodology and showing statistical similarity with the negative control 0.9% NaCl (IS = 1.02±0.10) and significant difference when compared with the positive control (IS = 19.93±0.03). It is very relevant to carry out toxicological studies using models such as HET-CAM, which allow to discriminate various levels of toxicity by means of the IS calculation. The HET-CAM is a sensitive assay to determine the irritation effect associated with topical use. Thus, this technique becomes an alternative to other *in vivo* trials (ICCVMA, 2010; Dalla Lana et al.; Pippi et al., 2018). So, the compound **2j** showed an appropriate score confirming their potential.

### 3.2.3. Histopathological evaluation

The result of the histopathological evaluation of ear skin incubated with different concentrations (higher values of MIC) of compound **2j** showed no microscopic lesions (Figure 1), demonstrating a low topical toxicity of the compound, with images (Fig. 1 – 1A, 2A, 1B, 2B, 1C, and 2C) similar to the negative control (PBS; Fig. 1 - 1D, and 2D). Toxicological studies using models such as the *ex vivo* porcine skin model for determining the histopathological damage is very useful for predicting the potential for topical application of an antimicrobial compound (Pippi et al., 2018). Combined with the HET-

CAM result, **2j** was nonirritant and did not possess dermatologic toxicity, presenting a good perspective of application in a topical formulation for the treatment of dermatomycosis.

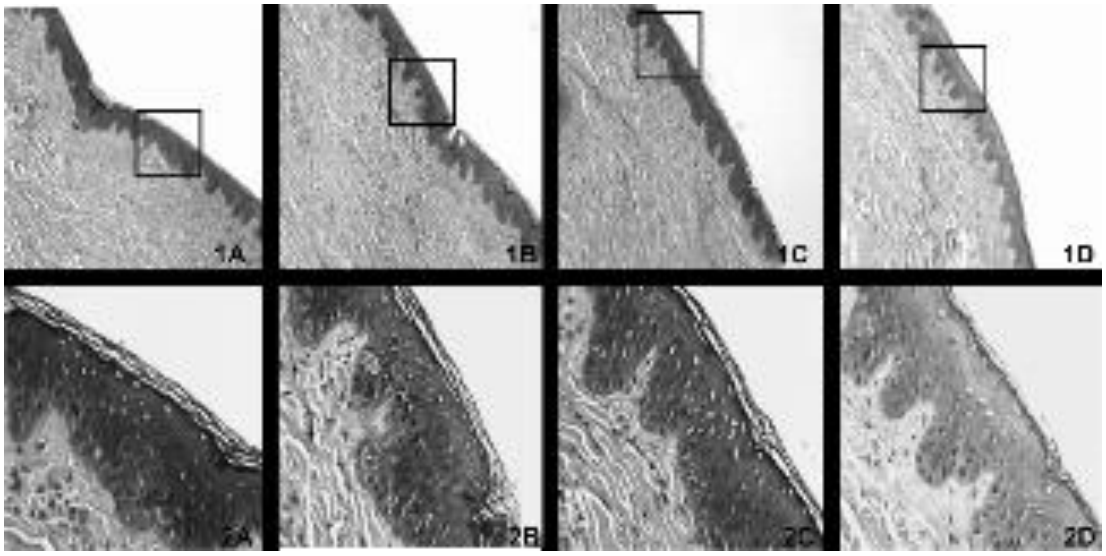



**Figure 1.** Histopathological evaluation of porcine cells treated with **2j** and negative control at 100 and 400 times magnification. 1A. Swine epidermal cells treated with **2j** (concentration 20 µg/ml) at 100 times magnification. 1B. Swine epidermal cells treated with **2j** (concentration 40 µg/ml) at 100 times magnification. 1C. Swine epidermal cells treated with **2j** (concentration 80 µg/ml) at 100 times magnification. 1D. Swine epidermal cells treated with PBS pH 7.0 at 100 times magnification. 2A. Swine epidermal cells treated with **2j** (concentration 20 µg/ml) at 400 times magnification. 2B. Swine epidermal cells treated with **2j** (concentration 40 µg/ml) at 400 times magnification. 2C. Swine epidermal cells treated with **2j** (concentration 80 µg/ml) at 400 times magnification. 2D. Swine epidermal cells treated with PBS pH 7.0 at 400 times magnification.

### 3.3. Mechanism of action

#### 3.3.1. Sorbitol protection assay

When dermatophytes and *Candida* spp. were treated with compound **2j** in a medium supplemented with sorbitol, MICs increased after seven days of incubation compared to MIC in medium without sorbitol (Table 3 and 4). MICs of **2j** increased for all dermatophytes (*M. canis*, *M. gypseum*, *T. mentagrophytes*, *T. rubrum*, and *T. schoenleinii; Table 3) and Candida* spp. *(C. albicans*, *C. krusei*, *C. glabrata*, *C. tropicalis*, and *C. parapsilosis;* Table 4) tested. After addition of sorbitol into the medium, the MIC values of anidulafungin (positive control) increased 4–32x for dermatophytes and 16–128x for yeasts, in seven days. For the anidulafungin, according to M38-A2 (CLSI, 2008), the minimum effective concentration (MEC) was determined, which is the lowest concentration of antifungal agent that leads to growth of small, rounded and compact hyphal forms compared to untreated controls. This terminology is used for echinocandins. For compound **2j**, a MIC increase profile was also observed with the presence of sorbitol

in the test: 8–32x for dermatophytes and 8–16x for yeasts, in seven days. Then, to gain insight into the capacity of active compound **2j** to interfere on fungal cell wall, this assay was conducted in the presence of the osmotic cell wall protector sorbitol. If the compound has reduced activity (increased MIC) in the presence of sorbitol, as found in this study, it previously indicates an action mechanism targeting the fungal cell wall. Since fungal but not mammalian cells possess a cell wall, these structures appeared as promissory leads for the development of selective novel antifungal compounds (Vargas et al., 2003).

**Table 3.** Effect of sorbitol on the minimum effective concentration (MEC; µg/ml) of anidulafungin and minimum inhibitory concentration (MIC) of compound **2j** against dermatophytes.

| **Dermatophytes** | **Anidulafungin** | | | | **Compound 2j** | | | |
|---|---|---|---|---|---|---|---|---|
| | Day 4 | | Day 7 | | Day 4 | | Day 7 | |
| | S (-) | S (+) | S (-) | S (+) | S (-) | S (+) | S (-) | S (+) |
| MCA 01 | 32.00 | 32.00 | 32.00 | 256.00 | 1.56 | 1.56 | 1.56 | 50.00 |
| MCA 33 | 16.00 | 16.00 | 16.00 | 128.00 | 1.56 | 6.25 | 1.56 | 25.00 |
| MCA 38 | 8.00 | 8.00 | 8.00 | 32.00 | 0.78 | 0.78 | 0.78 | 25.00 |
| MGY5 HCPA | 8.00 | 16.00 | 8.00 | 64.00 | 1.56 | 3.12 | 1.56 | 12.50 |
| MGY 42 | 32.00 | 32.00 | 32.00 | 256.00 | 1.56 | 3.12 | 1.56 | 12.50 |
| MGY 50 | 16.00 | 16.00 | 16.00 | 256.00 | 0.78 | 0.78 | 0.78 | 25.00 |
| MGY 58 | 4.00 | 4.00 | 4.00 | 64.00 | 1.56 | 1.56 | 1.56 | 25.00 |
| TME 16 | 32.00 | 32.00 | 32.00 | 128.00 | 3.12 | 3.12 | 3.12 | 25.00 |
| TME 32 | 4.00 | 8.00 | 4.00 | 64.00 | 0.78 | 0.78 | 0.78 | 12.50 |
| TME 40 | 8.00 | 8.00 | 8.00 | 64.00 | 0.39 | 0.39 | 0.39 | 6.25 |
| TRU 45 | 32.00 | 32.00 | 32.00 | 256.00 | 3.12 | 3.12 | 3.12 | 12.50 |
| TRU 2 HCPA | 16.00 | 16.00 | 16.00 | 128.00 | 1.56 | 1.56 | 1.56 | 25.00 |
| TRU 3 HCPA | 8.00 | 8.00 | 8.00 | 64.00 | 3.12 | 6.25 | 3.12 | 50.00 |
| TRU 48 | 4.00 | 8.00 | 4.00 | 64.00 | 1.56 | 1.56 | 1.56 | 50.00 |
| TSHO 3 HCPA | 4.00 | 8.00 | 4.00 | 128.00 | 0.78 | 0.78 | 0.78 | 25.00 |

MCA: *Microsporum canis*, MGY: *Microsporum gypseum*, TME: *Trichophyton mentagrophytes*, TRU: *Trichophyton rubrum*, TSCHO: *Trichophyton schoenleinii*, S (-): growth medium without sorbitol, and S (+): growth medium with sorbitol.

**Table 4.** Effect of sorbitol on the minimum inhibitory concentration (MIC; µg/ml) of anidulafungin and compound **2j** in relation to *Candida* spp.

| Yeasts | Anidulafungin | | | | Compound 2j | | | |
|---|---|---|---|---|---|---|---|---|
| | Day 2 | | Day 5 | | Day 2 | | Day 5 | |
| | S (-) | S (+) | S (-) | S (+) | S (-) | S (+) | S (-) | S (+) |
| CA ATCC 18804 | 0.12 | 0.12 | 0.12 | 4.00 | 1.56 | 3.12 | 1.56 | 25.00 |
| CA 01 | 0.12 | 0.25 | 0.12 | 4.00 | 0.78 | 0.78 | 0.78 | 12.50 |
| CK 02 | 0.12 | 0.12 | 0.12 | 8.00 | 1.56 | 6.25 | 1.56 | 12.50 |
| CK 03 | 0.06 | 0.06 | 0.06 | 1.00 | 1.56 | 3.12 | 1.56 | 12.50 |
| CG 05 | 0.06 | 0.06 | 0.06 | 1.00 | 1.56 | 1.56 | 1.56 | 25.00 |
| CG 09 | 0.12 | 0.12 | 0.12 | 8.00 | 1.56 | 1.56 | 1.56 | 12.50 |
| CT 72A | 0.12 | 0.50 | 0.12 | 16.00 | 1.56 | 3.12 | 1.56 | 25.00 |
| CT ATCC 750 | 0.06 | 0.06 | 0.06 | 4.00 | 0.78 | 1.56 | 0.78 | 12.50 |
| CP 06 | 0.06 | 0.06 | 0.06 | 4.00 | 1.56 | 1.56 | 1.56 | 12.50 |
| CP 07 | 0.12 | 0.12 | 0.12 | 16.00 | 1.56 | 3.12 | 1.56 | 25.00 |

CA: *Candida albicans*, CK: *Candida krusei*, CG: *Candida glabrata*, CT: *Candida tropicalis*, CP: *Candida parapsilosis*, S (-): growth medium without sorbitol, and S (+): growth medium with sorbitol.

### 3.3.2. Ergosterol binding assay

MICs of compound **2j** against dermatophytes (Table S2; supplementary information) and *Candida* spp. (Table S3; supplementary information) did not increase after adding different concentrations of ergosterol (50-250 µg/ml). Therefore, compound **2j** does not appear to target the fungal cell membrane, targeting only the fungal cell wall, as found in the sorbitol assay. As expected, in the presence of ergosterol, an increase until 256xMIC was observed for amphotericin B (positive control). All isolates tested behaved similarly. Amphotericin B is a well-known an antifungal that targeting the fungal membrane. This antifungal agent complex with membrane-ergosterol forming pores into the membrane, allowing permeability of ions and other elements (Scorzoni et al., 2017), so it has been selected as drug control this test.

### 3.3.3. SEM images

Further additional level of evidence on the mode of action was assessed by SEM images, treating fungal cells of dermatophytes and yeasts with compound **2j** and antifungal drugs controls and observing the possible morphological damage of these fungal cells (Fig. 2). Fungal cells were subjected to subinhibitory concentration (MIC/2) of compound **2j** and anidulafungin, the drug that also acts at the fungal cell wall.

For *C. albicans* (ATCC18804; Fig. 1– A and B; "no drug'' control), was observed that compound **2j** inhibits pseudohyphae formation (Fig. 1 – I and J), as well as anidulafungin (Fig. 1 – E and F), this being an important finding. One of the main virulence properties of *C. albicans* has been attributed to its capacity to undergo reversible morphological conversion between blastoconidia and pseudohyphae (a variety of shapes

from attached strings of yeast-like cells to long filaments with constriction at the septa) (Hwang et al., 2003). These flexible morphological spectra are thought to be required for adaptation in hosts, rapid colonization of tissues, adhesion to epithelial and endothelial cells, escape from phagocytes and immune evasion, and facilitated spread of infection. The ability of filamentous forms formation is an important factor for pathogenicity at dermatomycosis and invasive fungal infections (Lu et al., 2014). In view of this, many studies have investigated the potential of yeasts pseudohyphae inhibition as new therapies for treatment of mycoses in general (Lu et al., 2014; Pippi et al., 2018; Lee et al., 2018).

In addition to the inhibition of pseudohyphae formation in *C. albicans*, was also demonstrated for this yeast that the cell surface appeared to be altered and irregular by the compound **2j** (Fig. 1 – J) and anidulafungin (Fig. 1 – F), as evidenced by dehydrated and wilted blastoconidia with abnormal size. This finding corroborates with the previous results (sorbitol protection test) of the effect of compound **2j** especially targeting the fungal cell wall. Similar superficial changes in *C. albicans* cells have also been reported by Pippi et al. (2018), evidencing the action of the 8-hydroxyquinoline derivatives on the cell wall in that study. Cicatricial and multiple sprouts were also perceived on cells treated with **2j** (Fig. 1 – J) suggest that the normal blastoconidium division process may have been impaired resulting in single cells with multiple attempts cycle defects, which has already been verified for *Candida* cells exposed to fluconazole (Harrison et al., 2014).

Structural changes in fungi after *in vitro* treatment with compound **2j** were also evaluated by SEM. In the same way as for yeast, we identified irregular and abnormal hyphal dermatophytes **2j**-exposed (Fig. 2 – K and L). These modifications may also be a consequence of deregulation in the permeability of tubular cells, which is also observed for anidulafungin (Fig. 2 – G and H). With SEM images of *M. canis* without treatment (Fig. 2 – C and D) is possible to observe the presence of exopolimeric matrix - characterizing the biofilm, recently reported for this species by our research group (Danielli et al., 2017). The treatment with **2j** (0.78 µg/ml) was able to completely eliminate the biofilm, as well as anidulafungin (16 µg/ml), which is very promising. Fungal biofilm has demonstrated increasing drug resistance. Studies have reported an increase in resistance to antifungal agents commonly used to treat cutaneous mycoses, including dermatophytosis. Some features, including the ability to form biofilms, may be associated with dermatophyte unresponsiveness to antifungals (Peres et al., 2010; Martinez-Rossi et al., 2018). Therefore, the prospect of a drug such as compound **2j** that is able to inhibit biofilm formation of filamentous species, at very low concentrations, is selective against the pathogen as already demonstrated and still inhibits yeast virulence factors is very good to indeed have a novel treatment for nonresponsive mycoses.

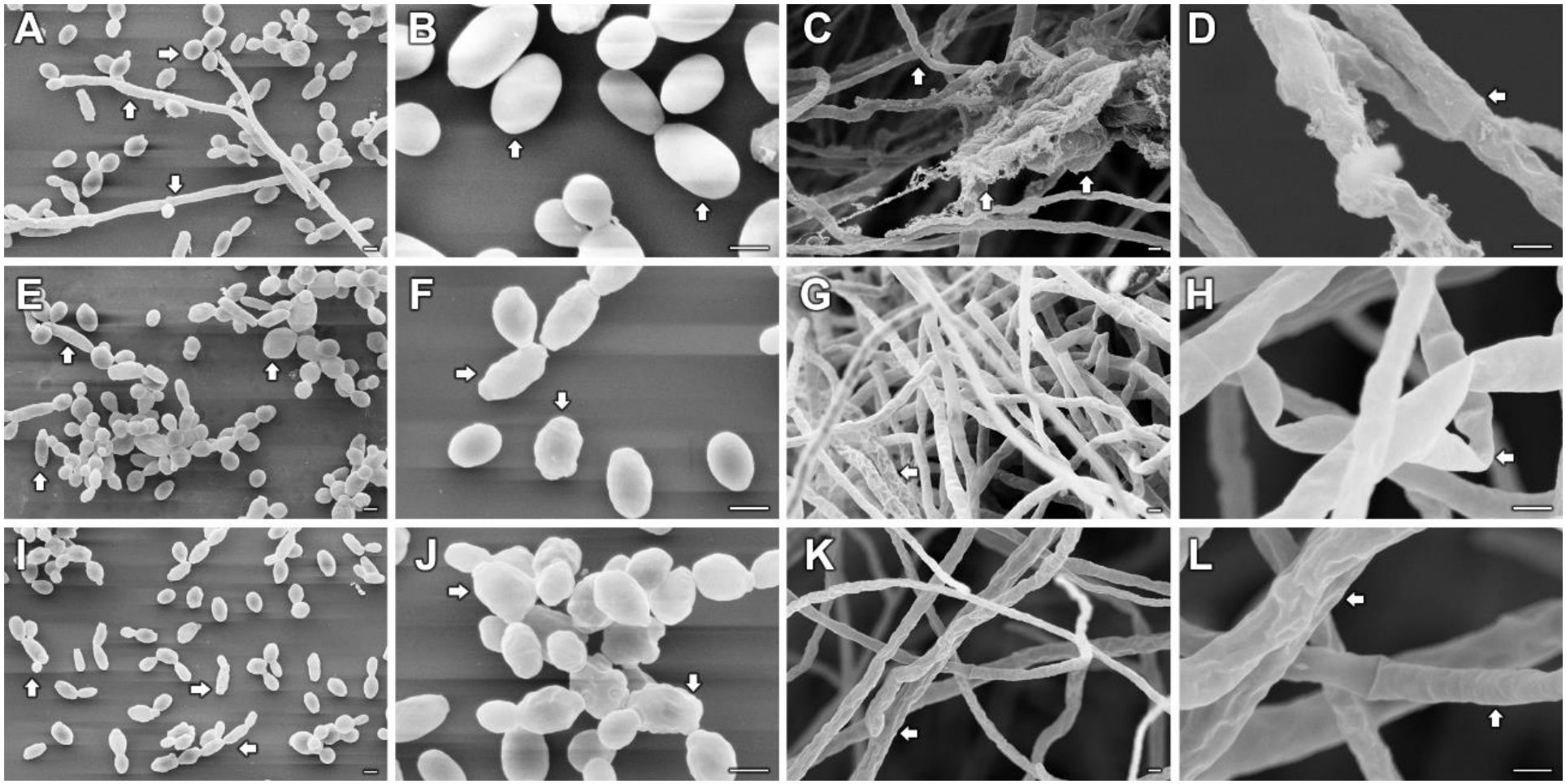



**Figure 2**. Fungal images of scanning electron microscopy (SEM): (A, B) *C. albicans* ATCC 18804 without treatment (control); (C, D) *M. canis* (MCA 01) without treatment (control); (E, F) *C. albicans* ATCC 18804 treated with anidulafungin (0.06 µg/ml); (G, H) *M. canis* (MCA 01) treated with anidulafungin (16 µg/ml); (I, J) *C. albicans* ATCC 18804 treated with compound **2j** (0.78 µg/ml); (K, L) *M. canis* (MCA 01) treated with compound **2j** (0.78 µg/ml). Bars representing 2 µm.

### 3.4. HG containing compound 2j

The hydrogel containing de compound **2j** presented a bright and homogeneous white appearance. The HPLC assay revealed **2j** contents close to the theoretical value (0.50 ± 0.06 mg/g of HG), showing that there were no losses of the active in the preparation process. The hydrogel presented pH value close to 7.0 (7.22 ± 0.11), which is adequate for cutaneous administration (Marchiori et al., 2017).

In relation to the spreadability determination, this evaluation indicates how the formulation spreads on the application site, which influences the correct transference of the dose. The value of Sf was 4.66 ± 0.60 $mm^2/g$. The complete spreadability profile is available in the supplementary information (Fig. S1), in which it is possible to observe that the applied weight is directly the spread area of HG. The rheological data obtained for **HG-2j** are presented in Fig. S2. The rheograms were compiled by plotting shear stress (mPa.s) against shear rate ($s^{-1}$) showed non-Newtonian flow with pseudoplastic behavior. The regression coefficients ($r^2$) for the different flow models of each rheogram were 0.6955 ± 0.143 (Bingham) 0.8630 ± 0.006 (Casson) and 0.9983 ± 0.001 (Ostwald). Being thus, the Ostwald flow model fitted best to the data and was chosen to describe the rheological behavior ($r^2 > 0.99$). Flow index (n = 0.628 ± 0.01) and consistency index (K = 48268 ± 2289) were also established. Accordingly, the **HG-2j** formulation is considered a no Newtonian and pseudoplastic fluid, does not need initial tension to start flowing, which is ideal for effective topical application (Marchiori et al., 2017).

The *in vitro* drug release profile of **2j** from HG using vertical Franz diffusion cells is depicted in Fig. S3. The amount of **2j** released after 8 h was 22.89 ± 3.39 $\mu g/cm^2$ demonstrating its ability to leave the hydrogel. With this profile, the compound **2j** can be easily released from the hydrogel to exert its antifungal effect in the treatment of dermatomycosis.

Lastly, in order to determine the drug location and to quantify the amount of **2j** delivered to each skin layer after the **HG-2j** application, human skin retention and permeation studies were performed using verrtical Franz diffusion cells. **2j** was analyzed in the stratum corneum and in the subjacent layers, viable epidermis and dermis, according to the tape stripping and skin extraction techniques, respectively. Fig. 3 shows the amount of **2j** accumulated in the skin after topical application. After this experiment, it was possible to observe that **2j** is more significantly retained in the outermost layers of the skin (stratum corneum and especially in the epidermis, approximately 58%) (Fig. 1), when compared to the dermis (less than 2% of **2j** amount). Noteworthy, the drug was not detected in the receptor compartment solution. Compound retained in these outermost layers of the skin is extremely advantageous for the treatment of dermatomycoses, such as dermatophytosis and superficial/cutaneous candidiasis, which possess the epidermis as the main site of infection and which are recognized as being difficult to treat (Bondaryk et al., 2013; Dalla Lana et al., 2016). This result being in the human skin brings a more real approximation of its potential therapeutic use.

Pemulen® TR2 used in the **HG-2j** formulation is a polymer of anionic character composed of acrylic block copolymers with high molecular weight and its bioadhesivity is due to its interpenetration in skin chains. Bioadhesion involves an interaction with the biological surface, which provides an intimate contact between the formulation and the

application area improving the drug bioavailability (Suresh et al., 2013; De Lima et al., 2017). Hydrogel with Pemulen®, containing clotrimazole-loaded cationic nanocapsules, has already been developed for vulvovaginal candidiasis treatment (De Lima et al., 2017). Formulations based on Pemulen® polymers deposit an occlusive layer on the skin, delivering the topical medication in the form of low-irritancy lotions and creams with elegant skin feel. Pemulen® TR2 polymer can also be used for high-clarity topical gels with ionic components (Suresh et al., 2013; Simovic et al., 1999).

Finally, hydrogel is certainly a very applied system for the incorporation of active antifungal agents, being an appropriate formulation for the purpose of treating a tissue area injured by mycosis (Winnicka et al., 2012; Sahoo et al., 2014; Shu et al., 2018). They are simple formulations of easy spreadability, which promotes patient adherence to therapy (Winnicka et al., 2012; Sahoo et al., 2014; Shu et al., 2018).

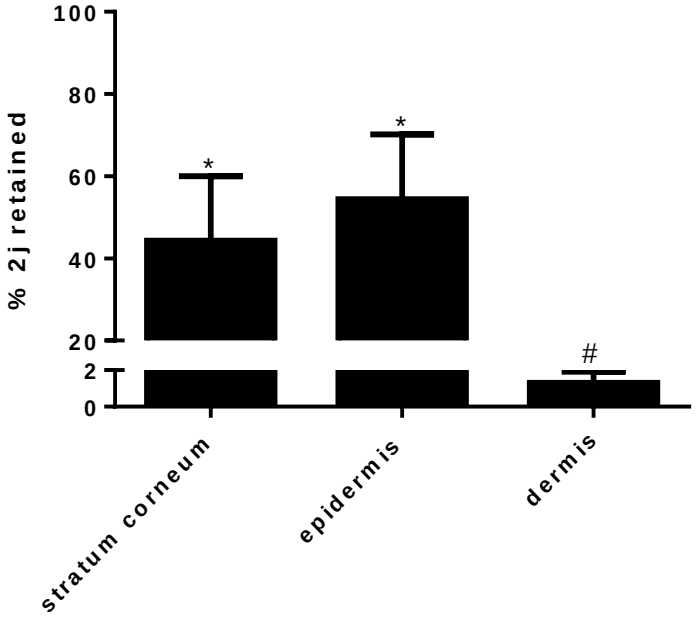



**Figure 3.** Amount of **2j** retained in the stratum corneum, epidermis, and dermis after 8 h. The values are expressed as mean ± standard deviation (n = 5) (ANOVA, Tukey's test, *$p \leq 0.05$).

## 4. Conclusions

Finally, this study provides an explanation on research and development of new chemical entities and formulation for the treatment of dermatomycoses. After observing the *in vitro* antifungal activity of one hundred twenty-one compounds and the toxicity profile, we verified that the molecule **2j** is the most promising, with selectivity against the pathogens and low MICs. **2j** appears to target the fungal cell wall and to act to inhibit virulence factors of *Candida* spp. and dermatophytes. **2j** was not irritant and therefore aimed its application in a topical formulation - a hydrogel with bioadhesive potential. The human skin permeation profile of the compound in the hydrogel was excellent, remaining mostly in the more superficial layers of the skin, which is very advantageous for topical

treatments. **2j** thus proved to have a promising antifungal activity and a desirable safety profile for use in formulations as a new alternative for fungal infections related to *Candida* spp. and dermatophytes that do not respond to currently available drugs.

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**Conflict of interest**

The authors declared that they have no competing interests.

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**Supplementary Information**

# Structural-functional elucidation of a novel selective fungicide applied in a topical formulation for dermatomycoses treatment

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## 1. Material and methods

### Chemicals - General procedure for synthesis of compounds

Spectral characterization data of the unpublished compounds that did not present antifungal activity:

Group (**1**)

**1g**: IV ($\bar{\nu}_{max}$, $cm^{-1}$): 3226 (O-H; secondary alcohol); 3051 (C-H; aromatic); 2930, 2875 ($Csp^3$-H); 1592, 1571, 1500, 1463 (C=C; aromatic); 1260, 1077 (C-O; phenylalkyl ether); 1105 (C-O; secondary alcohol); RMN de $^1H$ ($\delta$, $CDCl_3$, 200 MHz): 8,89 (dd; $^3J_{8',7'}$ = 4,2 Hz; $^4J_{8',6'}$ = 1,2 Hz; 2H; H-8'); 8,17 (dd; $^3J_{6',7'}$ = 8,4 Hz; $^4J_{6',8'}$ = 1,2 Hz; 2H; H-6'); 7,49-7,38 (m; 6H; H-3', H-4', H-7'); 7,22 (dd; $^3J_{2',3'}$ = 6,4 Hz; $^4J_{2',4'}$ = 2,4 Hz; 2H; H-2'); 4,94 (s; 3H; OH, $H_2O$); 4,78 (qa; 1H; H-2); 4,57 (dd; $^2J_{1a,1b}$ = 10,0 Hz; $^3J_{1a,2}$ = 5,4 Hz; 2H; H-1a); 4,52 (dd; $^2J_{1b,1a}$ = 10,0 Hz; $^3J_{1b,2}$ = 5,6 Hz; 2H; H-1b). 7,00 (d;$^3J$ = 9,0 Hz; 4H; H-

2’ e H-6‘); 5,05 (s; 4H; H-1); RMN de $^{13}C$ ($\delta$, $CDCl_3$, 50 MHz): 201,62 (C-2); 157,98 (C-1‘); 130,23 (C-3‘ e C-5‘); 126,66 (C-4‘); 117,23 (C-2‘ e C-6‘); 72,05 (C-1).

**1n***:* IR (ATR) $\nu_{max}$ 3383, 3013, 2940, 1684, 1663, 1586, 1509, 1463, 1265, 1131, 1024 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 200 MHz): $\delta$ = 9.86 (2H, s, CHO), 7.44 (4H, d, *J* = 8.8 Hz, H-5’), 7.42 (2H, s, H-3’), 7.05 (2H, d, *J* = 8.8 Hz, H-6’), 4.54 (1H, aqn, *J* = 5.0 Hz, H-2), 4.31 (2H, s, H-1a), 4.30 (2H, s, H-1b), 3.91 (6H, s, $OCH_3$), 2.78 (1H, s, OH); $^{13}C$ NMR ($CDCl_3$, 50 MHz): $\delta$ = 191.1 (CH, CHO), 153.5 (C, C-1′), 150.1 (C, C-2′), 130.9 (C, C-4’), 126.8 (CH, C-5′), 112.6 (CH, C-6′), 109.6 (CH, C-3′), 70.1 ($CH_2$, C-1), 68.5 (CH, C-2), 56.1 ($CH_3$, $OCH_3$).

**1o***:* IR (ATR) $\nu_{max}$ 3485, 2844, 1674, 1595, 1579, 1508, 1449, 1251, 1163, 1004 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 200 MHz): $\delta$ = 9.90 (2H, s, CHO), 7.88 (4H, d, *J* = 8.6 Hz, H-3’, H-5’), 7.17 (4H, d, *J* = 8.6 Hz, H-2’, H-6’), 4.82 (1H, s, OH), 4.44-4.25 (5H, m, H-1, H-2); $^{13}C$ NMR ($CDCl_3$, 50 MHz): $\delta$ = 191.3 (CH, CHO), 164.8 (C, C-1′), 132.6 (CH, C-3′, C-5’), 131.4 (C, C-4’), 115.9 (CH, C-2′, C-6’), 70.6 ($CH_2$, C-1), 69.0 (CH, C-2).

**1p***:* IR (ATR) $\nu_{max}$ 3397, 3284, 3149, 2972, 1600, 1543, 1507, 1454, 1263, 1170, 1137, 1008 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 200 MHz): $\delta$ = 11.31 [2H, s, HC=NNHC(=S)$NH_2$], 8.14 [2H, s, HC=NNHC(=S)$NH_2$], 8.01 [2H, s, HC=NNHC(=S)$NH_2$], 7.97 (2H, s, HC=NNHC(=S)$NH_2$], 7.51 (2H, s, H-3’), 7.14 (2H, d, *J* = 8.2 Hz, H-5’), 7.01 (2H, d, *J* = 8.2 Hz, H-6’), 5.43 (1H, d, *J* = 4.2 Hz, OH), 4.17-4.07 (5H, m, H-1, H-2), 3.81 (6H, s, $OCH_3$); $^{13}C$ NMR (DMSO-$d_6$, 50 MHz): $\delta$ = 177.6 [C, HC=NNHC(=S)$NH_2$], 149.9 (C, C-1′), 149.4 (C, C-2′), 142.5 [CH, HC=NNHC(=S)$NH_2$], 127.3 (C, C-4’), 122.1 (CH, C-5′), 112.7 (CH, C-6′), 108.9 (CH, C-3′), 70.0 ($CH_2$, C-1), 67.4 (CH, C-2), 55.8 ($CH_3$, $OCH_3$).

**1q**: IR (ATR) $\nu_{max}$ 3350, 3206, 3160, 1600, 1588, 1508, 1451, 1237, 1170, 1138, 1008 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 200 MHz): $\delta$ = 11.31 [2H, s, HC=NNHC(=S)$NH_2$], 8.11 [2H, s, HC=NNHC(=S)$NH_2$], 7.99 (2H, s, HC=NNHC(=S)$NH_2$], 7.92 [2H, s, HC=NNHC(=S)$NH_2$], 7.73 (4H, d, *J* = 8.4 Hz, H-3’, H-5’), 6.99 (4H, d, *J* = 8.4 Hz, H-2’, H-6’), 5.45 (1H, d, *J* = 4.4 Hz, OH), 4.16-4.10 (5H, m, H-1, H-2); $^{13}C$ NMR (DMSO-$d_6$, 50 MHz): $\delta$ = 177.6 [C, HC=NNHC(=S)$NH_2$], 160.0 (C, C-1′), 142.2 [CH,

HC=NNHC(=S)$NH_2$], 128.9 (CH, C-3', C-5'), 126.9 (C, C-4′), 114.7 (CH, C-2', C-6′), 69.3 ($CH_2$, C-1), 67.4 (CH, C-2).

**1t**: IR (ATR) $\nu_{max}$ 3336, 3074, 2951, 2881, 1608, 1593, 1490, 1276, 1134, 1073 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 200 MHz): $\delta$ = 7.29-7.17 (2H, m, H-4'), 6.74-6.61 (6H, m, H-2', H-5', H-6'), 4.39 (1H, aqn, $J$ = 5.6 Hz, H-2), 4.15 (2H, dd, $J$ = 9.8 Hz, $J$ = 4.8 Hz, H-1a), 4.10 (2H, dd, $J$ = 9.8 Hz, $J$ = 6.0 Hz, H-1b), 2.42 (1H, s, OH); $^{13}C$ NMR ($CDCl_3$, 50 MHz): $\delta$ = 163.8 (C, C-2′, $J$ = 244.2 Hz), 159.9 (C, C-1′, $J$ = 11.0 Hz), 130.6 (CH, C-5', $J$ = 10.0 Hz), 110.4 (CH, C-6', $J$ = 2.3 Hz), 108.4 (CH, C-4', $J$ = 21.2 Hz), 102.6 (CH, C-2', $J$ = 24.7 Hz), 69.1 ($CH_2$, C-1), 68.7 (CH, C-2).

**1u:** IR (ATR) $\nu_{max}$ 3511, 3073, 2921, 2873, 1500, 1452, 1276, 1198, 1125, 1012 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 200 MHz): $\delta$ = 7.02-6.83 (8H, m, H-2', H-3', H-5', H-6'), 4.36 (1H, aqn, $J$ = 5.6 Hz, H-2), 4.13 (2H, dd, $J$ = 9.8 Hz, $J$ = 4.8 Hz, H-1a), 4.07 (2H, dd, $J$ = 9.8 Hz, $J$ = 5.8 Hz, H-1b), 2.66 (1H, s, OH); $^{13}C$ NMR ($CDCl_3$, 50 MHz): $\delta$ = 157.7 (C, C-4′, $J$ = 237.5 Hz), 154.7 (C, C-1′, $J$ = 2.0 Hz), 116.1 (CH, C-3', C-5', $J$ = 23.9 Hz), 115.8 (CH, C-2', C-6', $J$ = 8.8 Hz), 69.6 ($CH_2$, C-1), 69.0 (CH, C-2).

**1v:** IR (ATR) $\nu_{max}$ 3304, 3091, 2945, 2884, 1604, 1508, 1459, 1260, 1207, 1142, 1031 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 400 MHz): $\delta$ = 6.97 (2H, ddd, $J$ = 9.2 Hz, 9.2 Hz, 4.8 Hz, H-6'), 6.86 (2H, ddd, $J$ = 11.2 Hz, 8.4 Hz, 4.8 Hz, H-3'), 6.79 (2H, dddd, $J$ = 9.2 Hz, 8.0 Hz, 2.8 Hz, 1.8 Hz, H-5'), 4.38 (1H, asx, $J$ = 5.6 Hz, H-2), 4.21 (2H, dd, $J$ = 9.6 Hz, $J$ = 4.8 Hz, H-1a), 4.18 (2H, dd, $J$ = 9.6 Hz, $J$ = 5.6 Hz, H-1b), 2.72 (1H, d, $J$ = 5.6 Hz, OH); $^{13}C$ NMR ($CDCl_3$, 100 MHz): $\delta$ = 157.2 (C, C-2′, $J$ = 241.3 Hz, 10.3 Hz), 153.0 (C, C-4′, $J$ = 247.9 Hz, 12.0 Hz), 143.3 (C, C-1′, $J$ = 10.8 Hz, 3.5 Hz), 116.7 (CH, C-6', $J$ = 9.4 Hz, 2.4 Hz), 110.8 (CH, C-5', $J$ = 22.5 Hz, 4.0 Hz), 105.2 (CH, C-3', $J$ = 26.6 Hz, 21.8 Hz), 71.3 ($CH_2$, C-1), 68.9 (CH, C-2).

**1z**: IR (ATR) $\nu_{max}$ 3527, 2948, 1589, 1566, 1478, 1465, 1455, 1227, 1064, 1121 $cm^{-1}$; $^1H$ NMR ($CDCl_3$, 200 MHz): $\delta$ = 7.33 (2H, d, $J$ = 9.0 Hz, H-5'), 7.03 (2H, d, $J$ = 2.6 Hz, H-2'), 6.78 (2H, dd, $J$ = 9.0 Hz, 2.6 Hz, H-6'), 4.37 (1H, s, H-2), 4.11 (4H, d, $J$ = 4.8 Hz, H-1), 2.59 (1H, s, OH); $^{13}C$ NMR ($CDCl_3$, 50 MHz): $\delta$ = 157.5 (C, C-1′), 133.2 (C, C-3′), 131.0 (CH, C-5'), 125.0 (C, C-4′), 116.7 (CH, C-2′), 114.7 (CH, C-6′), 69.3 ($CH_2$, C-1), 68.6 (CH, C-2).

**1gg:** IR (ATR) $\nu_{max}$ 3528, 3024, 2920, 2872, 1605, 1587, 1504, 1453, 1251, 1162, 1027 $cm^{-1}$; $^{1}H$ NMR (DMSO-$d_6$, 400 MHz): $\delta$ = 7.02 (2H, d, $J$ = 8.4 Hz, H-5'), 6.74 (2H, s, H-2'), 6.67 (2H, d, $J$ = 8.4 Hz, H-6'), 4.34 (1H, asx, $J$ = 5.2 Hz, H-2), 4.12 (2H, dd, $J$ = 9.6 Hz, 4.8 Hz, H-1a), 4.08 (2H, dd, $J$ = 9.6 Hz, 5.6 Hz, H-1b), 2.64 (1H, d, $J$ = 4.8 Hz, H-2), 2.22 (3H, s, $ArCH_3$), 2.19 (3H, s, $ArCH_3$); $^{13}C$ NMR (DMSO-$d_6$, 100 MHz): $\delta$ = 156.8 (C, C-1′), 138.0 (C, C-3'), 130.5 (CH, C-5'), 129.4 (C, C-4'), 116.4 (CH, C-2′), 111.7 (CH, C-6′), 69.1 ($CH_2$, C-1), 69.1 (CH, C-2), 20.2 ($CH_3$, $ArCH_3$), 19.0 ($CH_3$, $ArCH_3$).

Group (**2**)

**2o**: IR (ATR) $\nu_{max}$ 2922, 2867, 1608, 1584, 1501, 1455, 1250, 1044 $cm^{-1}$; $^{1}H$ NMR (DMSO-$d_6$, 400 MHz): $\delta$ = 8.70 (3H, s, $NH_3$), 7.05 (2H, d, $J$ = 8.4 Hz, H-5'), 6.81 (2H, d, $J$ = 2.4 Hz, H-2'), 6.72 (2H, dd, $J$ = 8.4 Hz, 2.4 Hz, H-6'), 4.26 (2H, dd, $J$ = 10.4 Hz, 5.2 Hz, H-1a), 4.21 (2H, dd, $J$ = 10.4 Hz, 6.0 Hz, H-1b), 3.86-3.80 (1H, m, H-2); $^{13}C$ NMR (DMSO-$d_6$, 100 MHz): $\delta$ = 155.9 (C, C-1′), 137.4 (C, C-3'), 130.2 (CH, C-5'), 128.9 (C, C-4'), 116.0 (CH, C-2′), 111.8 (CH, C-6′), 65.0 ($CH_2$, C-1), 49.4 (CH, C-2), 19.6 ($CH_3$, $ArCH_3$), 18.4 ($CH_3$, $ArCH_3$).

**2y**: IR (ATR) $\nu_{max}$ 3049, 2801, 2762, 1591, 1505, 1471, 1457, 1255, 1111, 1054 $cm^{-1}$; $^{1}H$ NMR (DMSO-$d_6$, 200 MHz): $\delta$ = 8.90 (3H, s, $NH_3$), 7.26-6.99 (8H, m, H-3', H-4', H-5', H-6'), 4.43 (4H, d, $J$ = 4.6 Hz, H-1), 3.94 (1H, qn, $J$ = 4.6 Hz, H-2); $^{13}C$ NMR (DMSO-$d_6$, 50 MHz): $\delta$ = 151.9 (C, C-2′, $J$ = 241.9 Hz), 145.6 (C, C-1', $J$ = 10.3 Hz), 124.9 (CH, C-4', $J$ = 3.8 Hz), 122.3 (CH, C-3', $J$ = 6.5 Hz), 116.4 (CH, C-5′), 116.0 (CH, C-6′, $J$ = 2.4 Hz), 66.4 ($CH_2$, C-1), 49.1 (CH, C-2).

**2z**: IR (ATR) $\nu_{max}$ 2876, 1599, 1504, 1463, 1251, 1201, 1095, 1047 $cm^{-1}$; $^{1}H$ NMR (DMSO-$d_6$, 200 MHz): $\delta$ = 8.95 (3H, s, $NH_3$), 6.87 (4H, s, H-3', H-5'), 6.84 (4H, s, H-2', H-6'), 4.21 (4H, s, H-1), 3.77 (1H, s, H-2); $^{13}C$ NMR (DMSO-$d_6$, 50 MHz): $\delta$ = 158.0 (C, C-2′, $J$ = 238.0 Hz), 153.8 (C, C-1'), 116.3 (CH, C-3', C-5', $J$ = 10.9 Hz), 116.0 (CH, C-2', C-6', $J$ = 4.3 Hz), 65.1 ($CH_2$, C-1), 50.8 (CH, C-2).

Group (**3**)

**3a**: IV ($\nu_{max}$, $cm^{-1}$): 3337, 3246, 3178, 3039 (N-H secondary amide and amidinium); 3064 (C-H aromatic); 2985, 2966, 2940, 2907 ($Csp^3$-H); 1698 (C=O aromatic ester); 1681 (C=O secondary amide); 1655 (N-C=N amidinium); 1595 (C=C aromatic); 1543 (C-N amide and def. angular N-H); 1508, 1474 (C=C aromatic); 1287, 1110 (C-O aromatic ester); 863 (def. angular C-H aromatic system 1,4- disubstituted); RMN de $^1$H (δ; DMSO-$d_6$; 200 MHz): 11,26 (s; 1H; ArNHCO); 9,32 (s; 4H; NH amidínio); 7,92 (d; *J*=8,4 Hz; 2H; H-2 e H-6); 7,77 (d; *J*=8,4 Hz; 2H; H-3 e H-5); 4,37-4,22 (m; 4H; H-8 e H-11); 1,33-1,26 (m; 3H; H-9). RMN de $^{13}$C (δ; DMSO-$d_6$; 50 MHz): 169,77 (C-12); 165,76; 165,28 (C-7, C-10); 142,90 (C-4); 130,30 (C-2 e C-6); 124,82 (C-1); 118,70 (C-3 e C-5); 60,53 (C-8); 34,86 (C-11); 14,23 (C-9).

**3b**: IV ($\nu_{max}$, $cm^{-1}$): 3196, 3089 (N-H secondary amide and amidinium); 1712 (C=O aromatic ester); 1689 (C=O secondary amide); 1646 (N-C=N amidinium); 1560 (C-N amide and def. angular N-H e C=C aromatic); 1287, 1110 (C-O aromatic ester); 753, 680 (C-H aromatic system 1,3- disubstituted); RMN de $^1$H (δ; DMSO-$d_6$; 200 MHz): 11,14 (s; 1H; ArNHCO); 9,34 (s; 1H; NH amidinium); 8,30 (s; 1H; H-2); 7,86 (d; *J*=7,8 Hz; 1H; H-4); 7,68 (d; *J*=7,8 Hz; 1H; H-6); 7,48 (t; *J*=7,8 Hz; 1H; H-5); 4,34-4,28 (m; 4H; H-8 e H-11); 1,31 (t; *J*=7,0 Hz; 3H; H-9). RMN de $^{13}$C (δ; DMSO-$d_6$; 50 MHz): 169,79 (C-12); 165,56; 165,49 (C-7, C-10); 138,93 (C-4); 130,52 (C-1); 129,35 (C-5); 124,46 (C-4); 123,71 (C-6); 119,64 (C-2); 60,89 (C-8); 34,70 (C-11); 14,19 (C-9).

**3c**: IV ($\nu_{max}$, $cm^{-1}$): 3187, 3036 (N-H secondary amide and amidinium); 1650 (C=O secondary amide and N-C=N amidinium); 1598 (C=C aromatic); 1548 (C-N amide, def. angular N-H, C=C aromatic); 1496 (C=C aromatic); 752, 688 (def. angular C-H monosubstituted aromatic system); RMN de $^1$H (δ; DMSO-$d_6$; 200 MHz): 10,85 (s; 1H; ArNHCO); 9,39-9,27 (m; 2H; NH amidínio); 7,60 (d; *J*=6,0 Hz; 2H; H-2 e H-6); 7,31 (s; 2H; H-3 e H-5); 7,09 (d; 1H; H-4); 4,28 (s; 2H; H-8). RMN de $^{13}$C (δ; DMSO-$d_6$; 50 MHz): 170,08 (C-9); 165,59 (C-7); 138,57 (C-1); 129,08 (C-3 e C-5); 124,25 (C-4); 119,58 (C-2 e C-6); 34,94 (C-8).

**3d**: IV ($\nu_{max}$, $cm^{-1}$): 2982, 2556 (N-H secondary amide and amidinium, O-H carboxylic acid); 1661 (C=O aromatic carboxylic acid and C=O secondary amide); 1638 (N-C=N amidinium); 1597 (C=C aromatic); 1535 (C-N amide, def. angular N-H e C=C aromatic); 1297 (C-O carboxylic acid); 951 (def. angular O-H carboxylic acid); 865 (def. angular

C-H 1,4-disubstituted aromatic system); RMN de $^{1}H$ (δ; DMSO-$d_6$; 200 MHz): 11,19 (s; 1H; ArNHCO); 9,30 (s; 4H; NH amidinium); 7,91 (d; *J*=8,4 Hz; 2H; H-2 e H-6); 7,74 (d; *J*=8,4 Hz; 2H; H-3 e H-5); 4,35 (s; 2H; H-9). RMN de $^{13}C$ (δ; DMSO-$d_6$; 50 MHz): 169,74 (C-10); 166,84; 165,68 (C-7, C-8); 142,56 (C-4); 130,47 (C-2 e C-6); 125,76 (C-1); 118,61 (C-3 e C-5); 34,86 (C-9).

**3e**: IV ($\nu_{max}$, $cm^{-1}$): 3387, 3364, 3187, 3080 (N-H secondary amide, primary sulfonamide and amidinium); 1692 (C=O secondary amide); 1657 (N-C=N amidinium); 1594 (C=C aromatic); 1520 (C-N amide, def. angular N-H amide e sulfonamide, C=C aromatic); 1320, 1161 (S=O sulfonamide); 831 (def. angular C-H 1,4-disubstituted aromatic system); RMN de $^{1}H$ (δ; DMSO-$d_6$; 200 MHz): 11,24 (s; 1H; ArNHCO); 9,30 (s; 4H; NH amidinium); 7,78 (s; 4H; $ArSO_2NH_2$, H-3 e H-5); 7,30 (s; 2H; H-2 e H-6); 4,35 (s; 2H; H-8). RMN de $^{13}C$ (δ; DMSO-$d_6$; 50 MHz): 169,71 (C-9); 165,70 (C-7); 141,44 (C-1); 138,92 (C-4); 126,78 (C-3 e C-5); 118,85 (C-2 e C-6); 34,81 (C-8).

**3f**: IV ($\nu_{max}$, $cm^{-1}$): 3085 (N-H secondary amide and amidinium); 1693 (C=O aromatic ketone); 1652 (C=O secondary amide e N-C=N amidinium); 1597 (C=C aromatic); 1538 (C-N amide, def. angular N-H e C=C aromatic); 835 (def. angular C-H 1,4-disubstituted aromatic system); RMN de $^{1}H$ (δ; DMSO-$d_6$; 200 MHz): 11,27 (s; 1H; ArNHCO); 9,35 (s; 4H; NH amidinium); 7,94-7,77 (m; 4H; H-2, H-3, H-5 e H-6); 4,37 (s; 2H; H-8); 2,50 (s; 3H; H-11). RMN de $^{13}C$ (δ; DMSO-$d_6$; 50 MHz): 196,59 (C-10); 169,73 (C-9); 165,74 (C-7); 142,85 (C-1); 132,20 (C-4); 129,53 (C-3 e C-5); 118,58 (C-2 e C-6); 34,87 (C-8); 26,46 (C-11).

**3g**: IV ($\nu_{max}$, $cm^{-1}$): 3054 (N-H secondary amide and amidinium); 1651 (C=O secondary amide, N-C=N amidinium); 1602 (C=C aromatic); 1531 (C-N amide, def. angular N-H e C=C aromatic); 1493 (C=C aromatic); 822 (def. angular C-H 1,4-disubstituted aromatic system); RMN de $^{1}H$ (δ; DMSO-$d_6$; 200 MHz): 11,10 (s; 1H; ArNHCO); 9,33 (s; 4H; NH amidinium); 7,67 (d; *J*=8,4 Hz; 2H; H-2 e H-6); 7,38 (d; *J*=8,6 Hz; 2H; H-3 e H-5); 4,32 (s; 2H; H-8). RMN de $^{13}C$ (δ; DMSO-$d_6$; 50 MHz): 169,77 (C-9); 165,37 (C-7); 137,52 (C-1); 128,76 (C-3 e C-5); 127,43 (C-4); 120,80 (C-2 e C-6); 34,67 (C-8).

**3h**: IV ($\nu_{max}$, $cm^{-1}$): 3184, 3056 (N-H secondary amide and amidinium); 1672 (C=O secondary amide); 1648 (N-C=N amidinium); 1601 (C=C aromatic); 1532 (C-N amide,

def. angular N-H e C=C aromatic); 1489 (C=C aromatic); 820 (def. angular C-H 1,4-disubstituted aromatic system); RMN de $^{1}$H (δ; DMSO-*d*$_6$; 200 MHz): 11,09 (s; 1H; ArNHCO); 9,33 (s; 4H; NH amidínio); 7,61 (d; *J*=8,2 Hz; 2H; H-2 e H-6); 7,50 (d; *J*=8,2 Hz; 2H; H-3 e H-5); 4,31 (s; 2H; H-8). RMN de $^{13}$C (δ; DMSO-*d*$_6$; 50 MHz): 169,74 (C-9); 165,37 (C-7); 137,93 (C-1); 131,66 (C-3 e C-5); 121,17 (C-2 e C-6); 115,50 (C-4); 34,70 (C-8).

**3i**: IV ($\nu_{max}$, cm$^{-1}$): 3381, 3128, 3022 (N-H secondary amide and amidinium); 1691 (C=O secondary amide); 1652 (N-C=N amidinium); 1595 (C=C aromatic); 1557 (C-N amide, def. angular N-H e C=C aromatic); 1492 (C=C aromatic, N=O); 1339 (N=O); 858 (def. angular C-H 1,4-disubstituted aromatic system); RMN de $^{1}$H (δ; DMSO-*d*$_6$; 200 MHz): 11,57 (s; 1H; ArNHCO); 9,31 (s; 4H; NH amidinium); 8,23 (d; *J*=8,4 Hz; 2H; H-3 e H-5); 7,89 (d; *J*=8,4 Hz; 2H; H-2 e H-6); 4,41 (s; 2H; H-8). RMN de $^{13}$C (δ; DMSO-*d*$_6$; 50 MHz): 169,63 (C-9); 166,03 (C-7); 144,73 (C-1); 142,57 (C-4); 125,03 (C-3 e C-5); 119,01 (C-2 e C-6); 34,97 (C-8).

**3j**: IV ($\nu_{max}$, cm$^{-1}$): 3075 (N-H secondary amide and amidinium); 1651 (C=O secondary amide e N-C=N amidinium); 1603 (C=C aromatic); 1534 (C-N amide, def. angular N-H e C=C aromatic); 1511 (C=C aromatic); 813 (def. angular C-H1,4-disubstituted aromatic system); RMN de $^{1}$H (δ; DMSO-*d*$_6$; 200 MHz): 10,86 (s; 1H; ArNHCO); 9,37 (s; 4H; NH amidinium); 7,51 (d; *J*=8,4 Hz; 2H; H-2 e H-6); 7,11 (d; *J*=8,4 Hz; 2H; H-3 e H-5); 4,29 (s; 2H; H-8); 2,24 (s; 2H; H-10). RMN de $^{13}$C (δ; DMSO-*d*$_6$; 50 MHz): 169,90 (C-9); 165,15 (C-7); 136,01 (C-4); 132,90 (C-1); 129,19 (C-3 e C-5); 119,27 (C-2 e C-6); 34,60 (C-8); 20,47 (C-10).

Group (**4**)

**4k**: IR (ATR) $\nu_{max}$ 3047, 2941, 1614, 1591, 1507, 1459, 1342, 1257, 1173, 1110, 1054 cm$^{-1}$; $^{1}$H NMR ($CDCl_3$, 200 MHz): $\delta$ = 7.13-6.90 (8H, m, H-3', H-4', H-5', H-6'), 5.26 (1H, qn, *J* = 5.4 Hz, H-2), 4.40 (2H, d, *J* = 5.4 Hz, H-1), 3.21 (3H, s, $OSO_2CH_3$); $^{13}$C NMR ($CDCl_3$, 50 MHz): $\delta$ = 152.9 (C, C-2′, *J* = 244.0 Hz), 146.1 (C, C-1', *J* = 10.7 Hz), 124.7 (CH, C-5', *J* = 3.4 Hz), 122.6 (CH, C-4', *J* = 6.6 Hz), 116.7 (CH, C-3′, *J* = 18 Hz), 115.6 (CH, C-6′), 78.3 (CH, C-2), 68.6 ($CH_2$, C-1), 38.5 ($CH_3$, $OSO_2CH_3$).

**4l**: IR (ATR) $\nu_{max}$ 3040, 2940, 1613, 1588, 1488, 1447, 1344, 1277, 1176, 1164, 1130, 1074 $cm^{-1}$; $^{1}H$ NMR ($CDCl_3$, 200 MHz): $\delta$ = 7.31-7.20 (2H, m, H-4'), 6.75-6.62 (6H, m, H-2', H-5', H-6'), 5.24 (1H, qn, *J* = 5.2 Hz, H-2), 4.31 (2H, d, *J* = 5.2 Hz, H-1), 3.15 (3H, s, $OSO_2CH_3$); $^{13}C$ NMR ($CDCl_3$, 50 MHz): $\delta$ = 163.8 (C, C-2′, *J* = 244.5 Hz), 159.2 (C, C-1′, *J* = 10.9 Hz), 130.8 (CH, C-5', *J* = 10.0 Hz), 110.3 (CH, C-6'), 108.9 (CH, C-4', *J* = 21.3 Hz), 102.7 (CH, C-2', *J* = 24.9 Hz), 77.7 (CH, C-2), 67.2 ($CH_2$, C-1), 38.7 ($CH_3$, $OSO_2CH_3$).

**4m**: IR (ATR) $\nu_{max}$ 3080, 2934, 2875, 1504, 1464, 1454, 1346, 1240, 1172, 1027 $cm^{-1}$; $^{1}H$ NMR ($CDCl_3$, 200 MHz): $\delta$ = 7.04-6.83 (8H, m, H-2', H-3', H-5', H-6'), 5.21 (1H, qn, *J* = 5.2 Hz, H-2), 4.28 (2H, d, *J* = 5.2 Hz, H-1), 3.15 (3H, s, $OSO_2CH_3$); $^{13}C$ NMR ($CDCl_3$, 50 MHz): $\delta$ = 158.0 (C, C-4′, *J* = 238.3 Hz), 154.2 (C, C-1′), 116.3 (CH, C-3', C-5', *J* = 23.3 Hz), 115.9 (CH, C-2', C-6', *J* = 7.9 Hz), 78.2 (CH, C-2), 67.7 ($CH_2$, C-1), 38.7 ($CH_3$, $OSO_2CH_3$).

**4n**: IR (ATR) $\nu_{max}$ 3093, 3069, 2946, 2891, 1608, 1560, 1513, 1458, 1344, 1262, 1158, 1024 $cm^{-1}$; $^{1}H$ NMR ($CDCl_3$, 400 MHz): $\delta$ = 7.13-7.06 (2H, m, H-5'), 6.78-6.73 (2H, m, H-2'), 6.64-6.62 (2H, m, H-6'), 5.21 (1H, qn, *J* = 4.8 Hz, H-2), 4.27 (4H, d, *J* = 4.8 Hz, H-1), 3.14 (3H, s, $OSO_2CH_3$); $^{13}C$ NMR ($CDCl_3$, 100 MHz): $\delta$ = 154.2 (C, C-1′, *J* = 8.6 Hz, 2.3 Hz), 150.8 (C, C-3′, *J* = 247.4 Hz, 13.9 Hz), 145.9 (C, C-4', *J* = 240.5 Hz, 12.5 Hz), 117.7 (CH, C-5′, *J* = 18.9 Hz), 110.1 (CH, C-6′, *J* = 5.9 Hz, 3.6 Hz), 104.8 (CH, C-2′, *J* = 20.4 Hz), 77.3 (CH, C-2), 67.7 ($CH_2$, C-1), 38.7 ($CH_3$, $OSO_2CH_3$).

**4r**: IR (ATR) $\nu_{max}$ 3042, 2940, 2883, 1593, 1566, 1478, 1452, 1345, 1231, 1176, 1057 $cm^{-1}$; $^{1}H$ NMR ($CDCl_3$, 200 MHz): $\delta$ = 7.35 (2H, d, *J* = 8.8 Hz, H-5'), 7.03 (2H, d, *J* = 3.0 Hz, H-2'), 6.78 (2H, dd, *J* = 8.8 Hz, 3.0 Hz, H-6'), 5.21 (1H, qn, *J* = 5.2 Hz, H-2), 4.29 (4H, d, *J* = 5.2 Hz, H-1), 3.15 (3H, s, $OSO_2CH_3$); $^{13}C$ NMR ($CDCl_3$, 50 MHz): $\delta$ = 156.9 (C, C-1′), 133.4 (C, C-3′), 131.2 (CH, C-5'), 125.5 (C, C-4′), 116.8 (CH, C-2′), 114.6 (CH, C-6′), 77.1 (CH, C-2), 67.4 ($CH_2$, C-1).

Group (**5**)

**5l**: IR (ATR) $\nu_{max}$ 3072, 2940, 2880, 2099, 1613, 1591, 1504, 1456, 1256, 1109, 1035 $cm^{-1}$; $^{1}H$ NMR ($CDCl_3$, 200 MHz): $\delta$ = 7.10-6.89 (8H, m, H-3', H-4', H-5', H-6'), 4.37-

4.12 (5H, m, H-1, H-2), 4.40 (2H, d, *J* = 5.4 Hz, H-1); $^{13}$C NMR ($CDCl_3$, 50 MHz): $\delta$ = 153.2 (C, C-2′, *J* = 244.3 Hz), 146.4 (C, C-1’, *J* = 10.8 Hz), 124.6 (CH, C-5’, *J* = 3.6 Hz), 122.6 (CH, C-4’, *J* = 6.7 Hz), 116.7 (CH, C-3′, *J* = 18.1 Hz), 116.1 (CH, C-6′), 69.2 ($CH_2$, C-1), 59.7 (CH, C-2).

**5m**: IR (ATR) $\nu_{max}$ 3077, 2934, 2881, 2097, 1610, 1592, 1489, 1449, 1277, 1135 cm$^{-1}$; $^1$H NMR ($CDCl_3$, 200 MHz): $\delta$ = 7.31-7.19 (2H, m, H-4’), 6.74-6.63 (6H, m, H-2’, H-5’, H-6’), 4.23-4.14 (5H, m, H-1, H-2); $^{13}$C NMR ($CDCl_3$, 50 MHz): $\delta$ = 163.8 (C, C-2′, *J* = 244.6 Hz), 159.5 (C, C-1′, *J* = 10.8 Hz), 130.6 (CH, C-5’, *J* = 9.9 Hz), 110.4 (CH, C-6’, *J* = 2.4 Hz), 108.7 (CH, C-4’, *J* = 21.2 Hz), 102.7 (CH, C-2’, *J* = 24.8 Hz), 67.8 ($CH_2$, C-1), 59.4 (CH, C-2).

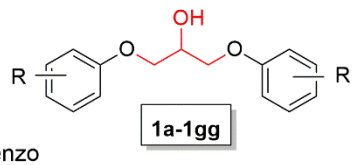
**5n**: IR (ATR) $\nu_{max}$ 3055, 2934, 2877, 2098, 1602, 1502, 1460, 1244, 1198 cm$^{-1}$; $^1$H NMR ($CDCl_3$, 200 MHz): $\delta$ = 7.04-6.84 (8H, m, H-2’, H-3’, H-5’, H-6’), 4.21-4.05 (5H, m, H-1, H-2); $^{13}$C NMR ($CDCl_3$, 50 MHz): $\delta$ = 157.9 (C, C-4′, *J* = 237.6 Hz), 154.4 (C, C-1′), 116.2 (CH, C-3’, C-5’, *J* = 23.4 Hz), 115.9 (CH, C-2’, C-6’, *J* = 8.3 Hz), 68.3 ($CH_2$, C-1), 59.6 (CH, C-2).

**5o**: IR (ATR) $\nu_{max}$ 3090, 3069, 2941, 2885, 2143, 2100, 1608, 1511, 1477, 1465, 1252, 1155, 1027 cm$^{-1}$; $^1$H NMR ($CDCl_3$, 400 MHz): $\delta$ = 7.12-7.05 (2H, m, H-5’), 6.79-6.74 (2H, m, H-2’), 6.65-6.62 (2H, m, H-6’), 4.17-4.11 (5H, m, H-1, H-2); $^{13}$C NMR ($CDCl_3$, 100 MHz): $\delta$ = 154.5 (C, C-1′, *J* = 8.5 Hz, 2.2 Hz), 150.7 (C, C-3′, *J* = 246.9 Hz, 13.8 Hz), 145.8 (C, C-4’, *J* = 240.0 Hz, 12.8 Hz), 117.7 (CH, C-5′, *J* = 18.4 Hz), 110.1 (CH, C-6′, *J* = 5.8 Hz, 3.5 Hz), 104.7 (CH, C-2′, *J* = 20.4 Hz), 68.3 ($CH_2$, C-1), 59.3 (CH, C-2).

**5s**: IR (ATR) $\nu_{max}$ 2940, 2882, 2144, 2094, 1590, 1567, 1478, 1454, 1231, 1023 cm$^{-1}$; $^1$H NMR ($CDCl_3$, 200 MHz): $\delta$ = 7.34 (2H, d, *J* = 8.8 Hz, H-5’), 7.03 (2H, d, *J* = 2.8 Hz, H-2’), 6.79 (2H, ddd, *J* = 8.8 Hz, 2.8 Hz, 0.4 Hz, H-6’), 4.20-4.11 (5H, m, H-1, H-2); $^{13}$C NMR ($CDCl_3$, 50 MHz): $\delta$ = 157.2 (C, C-1′), 133.3 (C, C-3′), 131.1 (CH, C-5’), 125.3 (C, C-4′), 116.7 (CH, C-2′), 114.7 (CH, C-6′), 68.0 ($CH_2$, C-1), 59.3 (CH, C-2).

## 2. Results

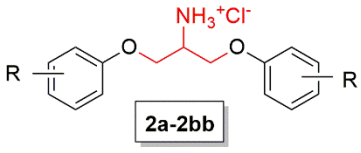
Group **1**




**1a-1gg**

**1a**: R = 2-F
**1b**: R = 3,4-diF
**1c**: R = 3-$CF_3$
**1d**: R = H
**1e**: R = 2,3-diBenzo
**1f**: R = 3,4-diBenzo
**1g**: R = [2,3-b]Pyrrole
**1h**: R = 2-CN
**1i**: R = 3-CN
**1j**: R = 4-CN
**1k**: R = 2-$OCH_3$
**1l**: R = 3-$OCH_3$
**1m**: R = 4-$OCH_3$
**1n**: R = 2-$OCH_3$,4-COH
**1o**: R = 4-COH
**1p**: R = 2-$OCH_3$, 4-CH=NNHC(=S)$NH_2$
**1q**: R = 4-CH=NNHC(=S)$NH_2$
**1r**: R = 2-$COOCH_3$
**1s**: R = 4-$COOCH_3$
**1t**: R = 3-F
**1u**: R = 4-F
**1v**: R = 2,4-diF
**1w**: R = 2-Cl
**1x**: R = 3-Cl
**1y**: R = 4-Cl
**1z**: R = 3,4-diCl
**1aa**: R = 2-$NO_2$
**1bb**: R = 3-$NO_2$
**1cc**: R = 4-$NO_2$
**1dd**: R = 2-$CH_3$
**1ee**: R = 3-$CH_3$
**1ff**: R = 4-$CH_3$
**1gg**: R = 3,4-di$CH_3$

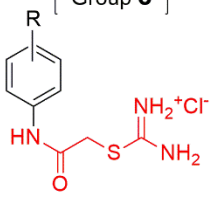
Group **2**




**2a-2bb**

**2a**: R = 2,3-diBenzo
**2b**: R = 3,4-diBenzo
**2c**: R = 3-$OCH_3$
**2d**: R = 3-F
**2e**: R = 3,4-diF
**2f**: R = 2-Cl
**2g**: R = 3-Cl
**2h**: R = 4-Cl
**2i**: R = 2,4-diCl
**2j**: R = 3,4-Cl
**2k**: R = 2-$CH_3$
**2l**: R = 3-$CH_3$
**2m**: R = 4-$CH_3$
**2n**: R = 2,4-di$CH_3$
**2o**: R = 3,4-di$CH_3$
**2p**: R = H
**2q**: R = 2-CN
**2r**: R = 3-CN
**2s**: R = 4-CN
**2t**: R = 2-$OCH_3$
**2u**: R = 4-$OCH_3$
**2v**: R = 2-$COOCH_3$
**2w**: R = 3-$COOCH_3$
**2x**: R = 4-$COOCH_3$
**2y**: R = 2-F
**2z**: R = 4-F
**2aa**: R = 3-$NO_2$
**2bb**: R = 4-$NO_2$

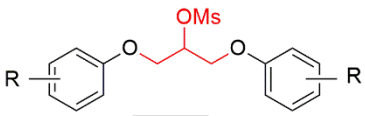
Group **3**




**3a-3j**

**3a**: R = 4-$SO_2NH_2$
**3b**: R = 4-$OCH_3$
**3c**: R = 3-$COOC_2H_5$
**3d**: R = 4-$COOC_2H_5$
**3e**: R = 4-COOH
**3f**: R = 4-$COCH_3$
**3g**: R = 4-Cl
**3h**: R = 4-Br
**3i**: R = 4-$NO_2$
**3j**: R = 4-$CH_3$

Group **4**




**4a-4y**

**4a**: R = H
**4b**: R = 2,3-diBenzo
**4c**: R = 3,4-diBenzo
**4d**: R = 2-CN
**4e**: R = 3-CN
**4f**: R = 4-CN
**4g**: R = 2-$OCH_3$
**4h**: R = 3-$OCH_3$
**4i**: R = 4-$OCH_3$
**4j**: R = 2-$COOCH_3$
**4k**: R = 4-$COOCH_3$
**4l**: R = 2-F
**4m**: R = 3-F
**4n**: R = 4-F
**4o**: R = 3,4-diF
**4p**: R = 2-Cl
**4q**: R = 3-Cl
**4r**: R = 4-Cl
**4s**: R = 3,4-diCl
**4t**: R = 2-$NO_2$
**4u**: R = 3-$NO_2$
**4v**: R = 4-$NO_2$
**4w**: R = 2-$CH_3$
**4x**: R = 3-CH3
**4y**: R = 4-$CH_3$

Group **5**


**5a-5y**

**5a**: R = H
**5b**: R = 2,3-diBenzo
**5c**: R = 3,4-diBenzo
**5d**: R = 2-CN
**5e**: R = 3-CN
**5f**: R = 4-CN
**5g**: R = 2-$OCH_3$
**5h**: R = 4-$OCH_3$
**5i**: R = 2-$OCH_3$, 4-COH
**5j**: R = 2-$COOCH_3$
**5k**: R = 4-$COOCH_3$
**5l**: R = 2-F
**5m**: R = 3-F
**5n**: R = 4-F
**5o**: R = 3,4-diF
**5p**: R = 2-Cl
**5q**: R = 3-Cl
**5r**: R = 4-Cl
**5s**: R = 3,4-diCl
**5t**: R = 2-$NO_2$
**5u**: R = 3-$NO_2$
**5v**: R = 4-$NO_2$
**5w**: R = 2-$CH_3$
**5x**: R = 3-$CH_3$
**5y**: R = 4-$CH_3$

**Scheme S1.** Chemical structure of all compounds evaluated in relation to *in vitro* antifungal activity.

**Table S1**. Minimal inhibitory concentrations of compound **2j** in relation of dermatophytes and yeasts of the genus *Candida*.

| **Dermatophytes (n=11)** | **MIC 2j** |
|---|---|
| *Microsporum canis* (MCA 33) | 1.56 |
| *Microsporum canis* (MCA 38) | 0.78 |
| *Microsporum gypseum* (MGY5 HCPA) | 1.56 |
| *Microsporum gypseum* (MGY 50) | 0.78 |
| *Microsporum gypseum* (MGY 58) | 1.56 |
| *Trichophyton mentagrophytes* (TME 32) | 0.78 |
| *Trichophyton mentagrophytes* (TME 40) | 0.39 |
| *Trichophyton rubrum* (TRU 2 HCPA) | 1.56 |
| *Trichophyton rubrum* (TRU 3 HCPA) | 3.12 |
| *Trichophyton rubrum* (TRU 48) | 1.56 |
| *Trichophyton schoenleinii* (TSHO 3 HCPA) | 0.78 |
| **$MIC_{50}$** | 1.56 |
| **MIC range** | 0.39 – 3.12 |
| ***Candida* spp. (n=6)** | **MIC 2j** |
| *Candida albicans* (CA 01) | 0.78 |
| *Candida krusei* (CK 03) | 1.56 |
| *Candida glabrata* (CG 05) | 1.56 |
| *Candida tropicalis* (CT ATCC 750) | 0.78 |
| *Candida parapsilosis* (CP 06) | 1.56 |
| *Candida parapsilosis* (CP 07) | 1.56 |
| **$MIC_{50}$** | 1.56 |
| **MIC range** | 0.78 – 1.56 |

$MIC_{50}$, minimal concentration that inhibits approximately 50% of the strains.

**Table S2.** Effect of different concentrations of external ergosterol (50-250 µg/ml) on the minimum inhibitory concentration (MIC; µg/ml) of Amphotericin B and compound **2j** against dermatophytes.

| Dermatophytes | 4 days | | | | | | | | | | | | 7 days | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Amphotericin B | | | | | | Compound 2j | | | | | | Amphotericin B | | | | | | Compound 2j | | | | | |
| | Ergosterol concentration (µg/ml) | | | | | | Ergosterol concentration (µg/ml) | | | | | | Ergosterol concentration (µg/ml) | | | | | | Ergosterol concentration (µg/ml) | | | | | |
| | 0 | 50 | 100 | 150 | 200 | 250 | 0 | 50 | 100 | 150 | 200 | 250 | 0 | 50 | 100 | 150 | 200 | 250 | 0 | 50 | 100 | 150 | 200 | 250 |
| MCA 01 | 0.50 | 1.00 | 2.00 | 4.00 | 16.00 | 64.0 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.00 | 32.00 | 64.00 | 64.00 | 256.00 | 256.00 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 |
| MCA 33 | 2.00 | 4.00 | 4.00 | 8.00 | 16.00 | 32.0 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 2.00 | 32.00 | 64.00 | 64.00 | 256.00 | 256.00 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 |
| MCA 38 | 1.00 | 1.00 | 2.00 | 4.00 | 16.00 | 32.0 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 2.00 | 32.00 | 64.00 | 64.00 | 128.00 | 256.00 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 |
| MGY5 HCPA | 1.00 | 1.00 | 2.00 | 4.00 | 16.00 | 32.0 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.00 | 16.00 | 32.00 | 32.00 | 64.00 | 64.00 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 |
| MGY 42 | 4.00 | 16.00 | 32.00 | 32.00 | 32.00 | 64.0 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 4.00 | 64.00 | 128.00 | 128.00 | 128.00 | 128.00 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 |
| MGY 50 | 0.25 | 0.25 | 1.00 | 1.00 | 4.00 | 16.0 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.50 | 16.00 | 32.00 | 32.00 | 128.00 | 128.00 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 |
| MGY 58 | 1.00 | 1.00 | 1.00 | 2.00 | 4.00 | 16.0 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.00 | 16.00 | 64.00 | 64.00 | 128.00 | 128.00 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 |
| TME 16 | 2.00 | 2.00 | 4.00 | 4.00 | 16.00 | 32.0 | 3.12 | 3.12 | 3.12 | 3.12 | 3.12 | 3.12 | 2.00 | 8.00 | 32.00 | 32.00 | 64.00 | 128.00 | 3.12 | 3.12 | 3.12 | 3.12 | 3.12 | 3.12 |
| TME 32 | 0.50 | 1.00 | 2.00 | 4.00 | 16.00 | 32.0 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.50 | 16.00 | 32.00 | 32.00 | 128.00 | 256.00 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 |
| TME 40 | 1.00 | 1.00 | 2.00 | 4.00 | 16.00 | 32.0 | 0.39 | 0.39 | 0.39 | 0.39 | 0.39 | 0.39 | 2.00 | 32.00 | 64.00 | 64.00 | 128.00 | 128.00 | 0.39 | 0.39 | 0.39 | 0.39 | 0.39 | 0.39 |
| TRU 45 | 2.00 | 4.00 | 4.00 | 4.00 | 16.00 | 32.0 | 3.12 | 3.12 | 3.12 | 3.12 | 3.12 | 3.12 | 2.00 | 32.00 | 64.00 | 64.00 | 128.00 | 128.00 | 3.12 | 3.12 | 3.12 | 3.12 | 3.12 | 3.12 |
| TRU 2 HCPA | 0.25 | 0.25 | 1.00 | 2.00 | 16.00 | 32.0 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 0.25 | 4.00 | 16.00 | 16.00 | 64.00 | 128.00 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 |
| TRU 3 HCPA | 0.25 | 0.25 | 1.00 | 2.00 | 4.00 | 16.0 | 3.12 | 3.12 | 3.12 | 3.12 | 3.12 | 3.12 | 0.25 | 8.00 | 32.00 | 32.00 | 128.00 | 128.00 | 3.12 | 3.12 | 3.12 | 3.12 | 3.12 | 3.12 |
| TRU 48 | 0.25 | 0.25 | 0.50 | 2.00 | 16.00 | 32.0 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 0.25 | 8.00 | 32.00 | 32.00 | 128.00 | 128.00 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 |
| TSHO 3 HCPA | 0.25 | 0.25 | 1.00 | 1.00 | 4.00 | 16.0 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.50 | 8.00 | 32.00 | 32.00 | 64.00 | 128.00 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 |

MCA: *Microsporum canis*, MGY: *Microsporum gypseum*, TME: *Trichophyton mentagrophytes*, TRU: *Trichophyton rubrum*, TSCHO: *Trichophyton schoenleinii.*

**Table S3.** Effect of different concentrations of external ergosterol (50-250 µg/ml) on the minimum inhibitory concentration (MIC; µg/ml) of amphotericin B and compound **2j** against *Candida* spp.

| *Candida* spp. | **2 days** | | | | | | | | | | | | **5 days** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Amphotericin B** | | | | | | **Compound 2j** | | | | | | **Amphotericin B** | | | | | | **Compound 2j** | | | | | |
| | Ergosterol concentration (µg/ml) | | | | | | Ergosterol concentration (µg/ml) | | | | | | Ergosterol concentration (µg/ml) | | | | | | Ergosterol concentration (µg/ml) | | | | | |
| | 0 | 50 | 100 | 150 | 200 | 250 | 0 | 50 | 100 | 150 | 200 | 250 | 0 | 50 | 100 | 150 | 200 | 250 | 0 | 50 | 100 | 150 | 200 | 250 |
| CA ATCC 18804 | 1.00 | 2.00 | 4.00 | 8.00 | 16.00 | 32.00 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.00 | 64.00 | 128.00 | 256.00 | 256.00 | 256.00 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 |
| CA 01 | 0.50 | 1.00 | 1.00 | 2.00 | 8.00 | 16.00 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.50 | 16.00 | 64.00 | 128.00 | 128.00 | 128.00 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 |
| CK 02 | 1.00 | 4.00 | 4.00 | 8.00 | 16.00 | 32.00 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 2.00 | 64.00 | 128.00 | 256.00 | 256.00 | 256.00 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 |
| CK 03 | 0.50 | 1.00 | 2.00 | 4.00 | 16.00 | 32.00 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.00 | 32.00 | 64.00 | 128.00 | 128.00 | 128.00 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 |
| CG 05 | 0.25 | 0.50 | 0.50 | 1.00 | 4.00 | 16.00 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 0.25 | 16.00 | 64.00 | 128.00 | 128.00 | 128.00 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 |
| CG 09 | 2.00 | 4.00 | 8.00 | 8.00 | 16.00 | 64.00 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 2.00 | 128.00 | 128.00 | 128.00 | 128.00 | 128.00 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 |
| CT 72A | 0.50 | 1.00 | 1.00 | 4.00 | 16.00 | 32.00 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 0.50 | 32.00 | 64.00 | 128.00 | 128.00 | 128.00 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 |
| CT ATCC 750 | 0.25 | 0.50 | 0.50 | 1.00 | 4.00 | 16.00 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.25 | 16.00 | 32.00 | 64.00 | 64.00 | 64.00 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 |
| CP 06 | 0.25 | 1.00 | 2.00 | 4.00 | 16.00 | 32.00 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 0.50 | 32.00 | 64.00 | 128.00 | 128.00 | 128.00 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 |
| CP 07 | 0.50 | 1.00 | 1.00 | 2.00 | 4.00 | 16.00 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 0.50 | 32.00 | 64.00 | 128.00 | 128.00 | 128.00 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 | 1.56 |

CA: *Candida albicans*, CK: *Candida krusei*, CG: *Candida glabrata*, CT: *Candida tropicalis*, CP: *Candida parapsilosis*.

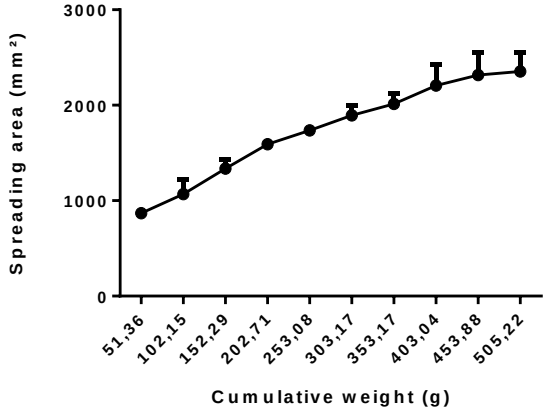



**Fig. S1** Spreadability ($mm^2$) of hydrogel formulation with compound **2j**.

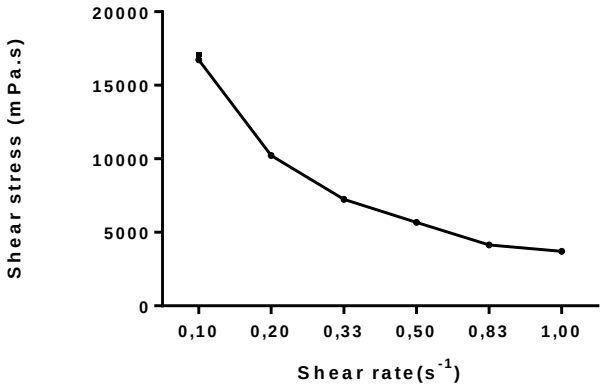



**Fig. S2** Rheological behavior of hydrogel formulation with compound **2j**.

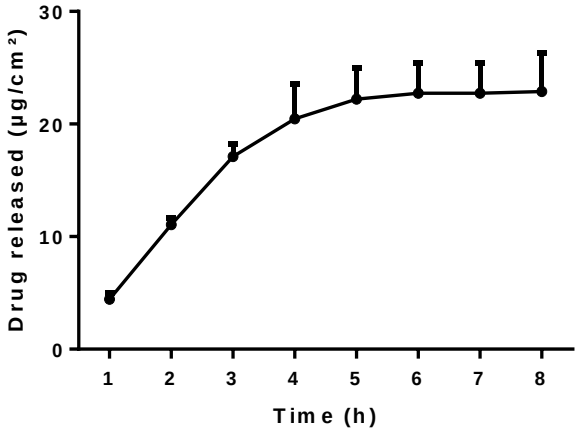



**Fig. S3** *In vitro* **2j** release profile from the semisolid formulation **HG-2j** using vertical Franz diffusion cells (n = 3).

**CAPÍTULO VII – Bisaryloxypropanamines derivative exhibiting protective effect against fungal infection in *Drosophila melanogaster* minihost model**

Nota: Manuscrito em fase de redação. Posteriomente será submetido ao periódico Mycoses.

**Bisaryloxypropanamines derivative exhibiting protective effect against fungal infection in *Drosophila melanogaster* minihost model**

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## ABSTRACT

**Background:** The number of deaths due to systemic fungal infections is increasing alarmingly, which is aggravated by the limitations of traditional treatments and the multidrug-resistance. Therefore, the research and development of new therapeutic options against pathogenic fungi is an urgent need. **Objectives:** To evaluate the fungicidal activity of a synthetic compound derived from 1,3-bis(aryloxy)-2-propanamines (**2j**), through time-kill studies and pharmacokinetic/pharmacodynamic (PK/PD) modeling. The protective effect of the compound was also evaluated, using the *Drosophila melanogaster* minihost model of candidiasis. **Methods:** Mathematical modeling of time-kill data of compound **2j** was performed to obtain pharmacodynamic characteristics. Additionally, Toll-deficient *D. melanogaster* flies were infected with a *Candida albicans* strains and treated with **2j**. **Results:** We observed that compound **2j** demonstrated a time- and dose-dependent fungicidal effect against *Candida* spp. and dermatophytes, even at low concentrations, and rapidly achieved kill rates reaching the maximum effect in less than one hour. The efficacy of the compound against systemic candidiasis in *D. melanogaster* flies was comparable to that achieved by fluconazole. **Conclusions:** These results support the potential of compound **2j** as a systemic antifungal agent candidate and serve as a starting point for further studies involving mammalian animal models.


**Keywords:** 1,3-bis(aryloxy)-2-propanamines, antifungal, dermatophytes, *Candida* spp., time kill, PK/PD modeling, fruit fly.

## 1. Introduction

Global mortality rates due to systemic infections by human fungal pathogens are increasing alarmingly.[1,2] In the last decade, the yeast *Candida albicans* has become the fourth leading cause of hospital-acquired bloodstream diseases.[1,2,3] Although *C. albicans* is still the most isolated species in intensive care units, there is currently a growing trend of non-*albicans* candidemia (NAC) infection. NAC account for an average of approximately 40% of nosocomial cases.[4] The major risk factors for candidemia include invasive procedures, multisite yeast colonization, broad-spectrum antibiotics, and immunosuppressive pathologies. Increased hospital costs and prolonged length of stay associated with invasive candidiasis contribute to a major financial burden, which is believed to exceed billions annually.[4,5] Dermatophytes are keratinophilic fungi that cause

benign and common forms of ringworm worldwide. However, they can lead to serious and rare diseases in immunocompromised patients.[6] Severe forms include invasive and/or extensive dermatophytosis.[7,8,9,10]

Current antifungal drugs suffer from a number of drawbacks, including toxicity to the host, high costs, and a limited spectrum of action.[11] These restrictions are further aggravated by antifungal resistance, an increasingly reported and worrying fact.[12,13,14] The emergence of strains resistant both to newer members of the echinocandin class and to azole drugs is considered a major threat by the Centers for Disease Control and Prevention (CDC).[15] Therefore, better antifungal therapies are needed to address this escalating problem and are key factors in optimizing patient outcomes.[11,16]

In this context, we propose the applied mathematical modeling on time-kill data of *Candida* spp. and dermatophytes to obtain pharmacodynamic characteristics of a novel synthetic molecule (compound **2j**; 1,3-bis(3,4-dichlorophenoxy)propan-2-aminium chloride) belonging to class of 1,3-bis(aryloxy)-2-propanamine derivatives. Recently, some compounds of this class have already presented promising antiprotozoal activity against *Leishmania amazonensis* promastigote forms.[17] We further researched the potential protective effect of compound **2j** using the *Drosophila melanogaster* minihost model of systemic candidiasis.

## 2. Materials and methods

### 2.1. Synthesis of compound 2j

The compound (Fig. 1) was synthesized according to Lavorato et al. (2017)[17] and was dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich) and diluted in sterile distilled water to obtain a maximum concentration of 1% DMSO for the experiments.

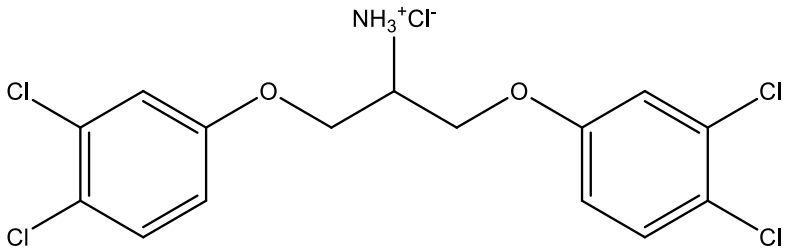


**Fig. 1**. Chemical structure of compound **2j**.

**2.2. Time-kill curves**

A time-kill assay was carried out with yeast species of the genus *Candida* (*C. albicans* - CA ATCC 18804, and *C. tropicalis* - CT 72A*) and dermatophytic filamentous fungi (*Microsporum canis* - MCA 01, and *Trichophyton mentagrophytes* - TME 16*). All fungal species are deposited in the Mycology Collection of the research group in Applied Mycology (Universidade Federal do Rio Grande do Sul, Brazil). For *Candida* spp. the resistance was defined based on Kuriyama et al. (2005)[18] and CLSI breakpoints,[19,20] considering the following minimum inhibitory concentrations (MICs): CT 72A resistant to itraconazole (MIC = 1 µg/mL; Cassará laboratory, Argentina), miconazole (MIC > 8 µg/mL, Valdequimica, Brazil), and voriconazole (MIC = 2 µg/mL; Pfizer, Brazil). For dermatophytes, the resistance (or decreased susceptibility) was established according to the increase in minimal inhibitory concentration (MIC), considering the following resistance threshold concentrations: terbinafine (Cristalia, Brazil) - MIC ≥ 1 µg/mL, griseofulvin (Cristalia, Brazil) - MIC ≥ 4 µg $mL^{-1}$, and ketoconazole (Cristalia, Brazil) - MIC ≥ 8 µg/mL. Consequently, the clinical isolate TME 16 was considered multidrug-resistant (MIC terbinafine = 4 µg/mL, MIC griseofulvin > 32 µg/mL and MIC ketoconazole = 16 µg/mL). The procedures were conducted with RPMI 1640 medium (Gibco®) buffered with MOPS. Compound **2j** was tested over a range of concentrations: 0 (control - drug free), 1, 2, 4 and 8 times the MIC for each clinical strain.[21,22] The MIC was defined according to the microdilution technique of the document M27-A3 (CLSI, 2008)[19] for yeasts and M38-A2 (CLSI, 2008)[23] for dermathophytes.

For yeasts, prior to time-kill evaluation, isolates were subcultured twice on Sabouraud dextrose agar (SDA) with chloramphenicol for 24 to 48 h at 35 °C. The inoculum was adjusted spectrophotometrically at 530 nm to the density of a 0.5 McFarland turbidity standard. The adjusted inoculum suspension was diluted 1:10 in RPMI 1640 medium containing the appropriate concentrations of **2j**, and tubes with the test solution were incubated at 35 °C without agitation; the final volume was 2 mL. This procedure yielded an initial inoculum ranging from 1–5 x $10^5$ colony-forming units [CFU]/mL and **2j** concentrations of 1.56 (MIC), 3.12 (2xMIC), 6.24 (4xMIC), and 12.48 (8xMIC) µg/mL, for both species – *C. albicans* (CA ATCC 18804) and *C. tropicalis* (CT 72A). At predetermined time points (0, 2, 4, 8, 12, 24, 36, and 48 h), an aliquot was aseptically removed from the control tube (drug free) and each tube with a test solution and serially diluted 10-fold ($10^{-1}$ to $10^{-6}$) with sterile water. Then, 20 µL was plated on

SDA plates for determination of CFU counts (drop method). Colony counts were determined following incubation at 35 °C for 24 to 48 h.

For filamentous fungi, prior to time-kill evaluation, isolates were subcultured twice on potato dextrose agar (PDA) plates for 96 h at 35 °C. The inoculum was adjusted to 1–3 × $10^3$ CFU/mL in RPMI 1640 medium with or without (control) the desired amount of compound **2j**, and tubes with the test solution were incubated at 35 °C; the final volume was 2 mL. Concentrations of **2j** were 1.56 (MIC), 3.12 (2xMIC), 6.24 (4xMIC), and 12.48 (8xMIC) μg/mL for *M. canis* (MCA 01) and 3.12 (MIC), 6.24 (2xMIC), 12.48 (4xMIC), and 24.96 (8xMIC) μg/mL for *T. mentagrophytes* (TME 16). At predetermined time points (0, 3, 6, 12, 24, 48, and 96 h), an aliquot was aseptically removed from both the control tube (drug free) and each tube with a test solution and serially diluted 10-fold ($10^{-1}$ to $10^{-3}$) with sterile water. Then, 20 μL directly from the test solution and each dilution solution were plated onto a PDA plate, using a Drigalski spreader. Plates were incubated at 35 °C and colonies were counted at 96 h.

The CFU for each incubation time-point per mL was plotted as a function of time, resulting in time–kill curves, including the standard deviations. A fungicidal effect was considered when there was a decrease of ≥ 99.9% in the $\log_{10}$ of the number of CFU/mL compared with starting inoculum. The results were reported as the mean colony counts from triplicate experiments.

### 2.3. Pharmacokinetic/pharmacodynamic (PK/PD) modeling

Time-kill curve data of compound **2j** for *C. albicans* (CA ATCC 18804), *C. tropicalis* (CT 72A), *M. canis* (MCA 01), and *T. mentagrophytes* (TME 16) were applied for the mathematical modeling. An *in vitro* kinetic model was used to investigate the antifungal efficacy of constant drug concentrations during 48 h for yeasts (inoculum of 1 – 5 x $10^5$ CFU/mL), and 96 h for dermatophytes (inoculum of 1–3 × $10^3$ CFU/mL). The selection of **2j** concentrations tested was based on their MIC values (MIC, 2xMIC, 4xMIC, and 8xMIC). A control experiment with untreated fungus was run simultaneously.

Adapted $E_{max}$ models described by Treyaprasert et al. (2007),[24] Li et al. (2009),[25] and Joaquim et al. (2018)[26] were used in the modeling of *C. albicans* (with k and and $N_{max}$ fixed), *C. tropicalis, M. canis* (k fixed), and *T. mentagrophytes* (k fixed), according to the equation 1:

**Eq. (1)**

$$\frac{\mathrm{d}N}{\mathrm{d}t} = \left[k_0 \left(1 - \frac{N}{N_{max}}\right) - \left(\frac{K_{max}C}{EC_{50}^{h} + C}\right)\right] N$$

Where d$N$/d$t$ is the variation in number of fungi as a function of time, $k_0$ ($h^{-1}$) is the fungal growth rate constant in the absence of compound **2j**, $k_{max}$ ($h^{-1}$) the maximum killing rate constant (maximum effect), $EC_{50}$ (μg/mL) the concentration of **2j** necessary to produce 50% of maximum effect, $C$ (μg/mL) the concentration of **2j** at any time ($t$), $N$ ($\log_{10}$ CFU/mL) the number of viable fungal cells, and $N_{max}$ is the maximum number of fungal cells. If necessary, the model is adjusted in adaptation rate terms ($x$, $y$), and incorporate a Hill factor ($h$) that modifies the steepness of the slopes and smoothes the curves.[24,25,26]

The mathematical modeling of the kill curve data was performed with the non-linear regression software program Scientist® 3.0 (Micromath, Salt Lake, UT, USA). Graphs were visually inspected for quality of fit and model selection criterion (MSC), coefficient of determination ($R^2$) and correlation between measured and calculated data points.

### 2.4. Fungal infection in *Drosophila melanogaster* minihost model

#### 2.4.1. *Drosophila melanogaster* stocks

Toll-deficient transheterozygote mutants were generated by crossing flies carrying a thermosensitive allele of Tl (*Tl*$^{r632}$) with flies carrying a null allele of Tl (*Tl*$^{l\text{-}RXA}$), as described by Chamilos et al. (2008).[27] Both fly strains were a gift from Dimitrios P. Kontoyiannis (University of Texas MD Anderson Cancer Center). Standard procedures were followed for storing and manipulating flies,[27,28] with the exception of the diet used.[29]

#### 2.4.2. Fungal strain and antifungal drug control

The *C. albicans* ATCC18804 strain was used for infecting the flies. Fluconazole (purity ≥ 98%), used as a positive control, was obtained from Sigma-Aldrich (Sao Paulo, Brazil) and solved in sterile distilled water.

### 2.4.3. Drug protection experiments

To determine whether the compound **2j** protect flies against systemic candidiasis, groups of Toll-deficient female flies (age: 2 to 4 days) were put into vials without food for 6–8 h to starve them and then transferred to vials with food containing 100 µL of **2j** (1 mg/mL), fluconazole (1 mg/mL), or sterile PBS (control). After 24 h, a needle that had been dipped into a 0.85% NaCl solution containing $1x10^8$ *C. albicans* cells/mL was used to puncture the dorsal side of the thorax of the flies. Flies were then transferred into vials containing fly food mixed with the compound of interest, fluconazole, or sterile PBS and maintained at 29 °C. Survival of the flies was assessed daily for seven days after infection in all experiments. Flies that died within 3 h of injection were considered to have died as a result of the procedure and were excluded from the survival analysis.[27,28] Positive control was provided by infecting flies that were not exposed to antifungal compounds. Furthermore, prior to the protection experiments, the uninfected flies were exposed to food containing 1 mg/mL of compound **2j** and fluconazole to verify the toxicity of the drugs. Each experiment was performed at least in quadruplicate, in four independent trials, using 35–45 flies.

### 2.4.4. Assessment of tissue fungal burden

Quantification of *C. albicans* cells in the tissues of flies was performed after seven days of infection. Ten flies from each group were collected and separated in live and dead flies and ground in 1 mL of 0.85% NaCl solution. Serial dilutions were done in triplicate and plated onto SDA with chloramphenicol. The number of CFU was counted after 24 h of incubation at 35 °C. Results shown are the mean of 4 experiments.

### 2.4.5. Statistical analysis

We pooled the survival data from our four independent biological repeat sets of injection, as no significant differences were observed between repeats ($p > 0.1$, log-rank test), therefore providing a more robust analysis of the lifetime data. Survival curves were plotted using Kaplan-Meier analysis and significant differences between survival data were identified using the log-rank test (GraphPad Prism software; version 6.01). CFU values were $\log_{10}$ transformed before statistical analysis. We compared treatment groups using the nonparametric Kruskal-Wallis test. We considered $p < 0.05$ to be statistically significant.

## 3. Results

### 3.1. Killing curves and PK/PD modeling

A time-kill plot (Fig. 2–5) of the interaction of compound **2j** with *Candida* and dermatophyte species tested revealed that **2j** exhibited concentration and time dependent fungicidal activity ( ≥ 99.9% decrease in the $\log_{10}$ of the number of CFU/mL compared with starting inoculum; support information – Fig. S1), including the killing action against resistant (*C. tropicalis* - CT 72A) and multidrug-resistant (*T. mentagrophytes* - TME 16) clinical strains.

The compound achieves the maximum fungicidal effect very fast: 0.28 h, 0.37 h, 0.24 h, and 0.29 h for *C. albicans* (ATCC 18804), *C. tropicalis* (72 A), *M. canis* (MCA 01), and *T. mentagrophytes* (TME 16), respectively.

The determined pharmacodynamic parameters for each individual fungal strain are presented in Table 1.

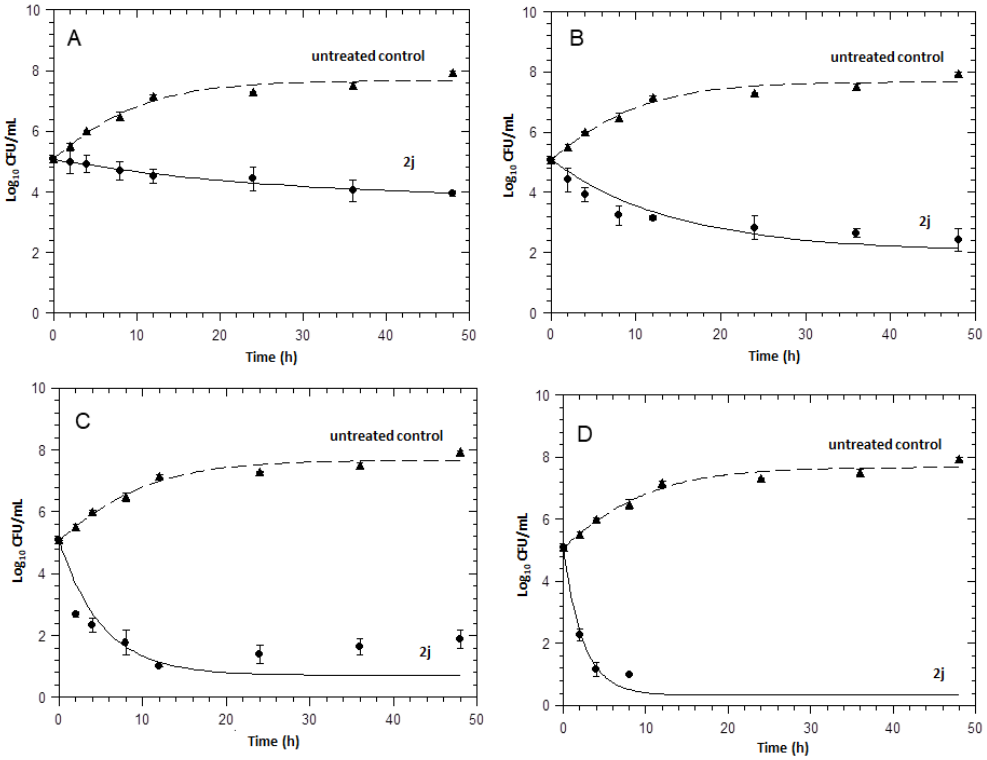


**Fig. 2.** Fitted curves resulting from modeling fungal growth (dashed line) and time-kill curve plots of synthetic compound **2j** (full line) against *Candida albicans* ATCC 18804 (A-D) (A: MIC; B: 2xMIC; C: 4xMIC; D: 8xMIC). MIC: minimum inhibitory concentration; CFU: colony-forming units. MIC = 1.56 μg/mL.

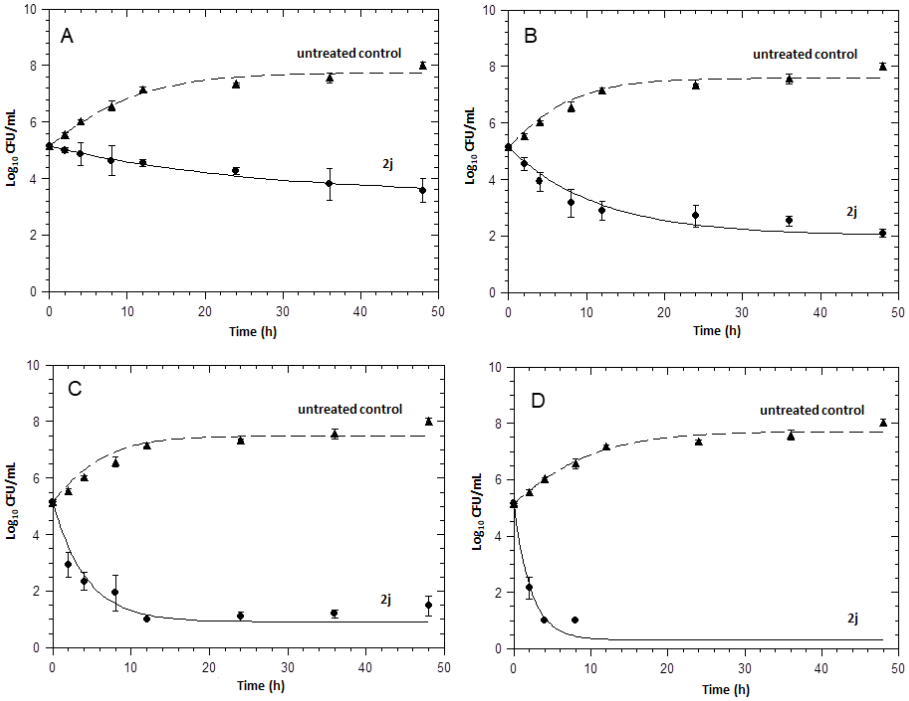


**Fig. 3.** Fitted curves resulting from modeling fungal growth (dashed line) and time-kill curve plots of synthetic compound **2j** (full line) against the multidrug-resistant clinical strain *Candida tropicalis* 72A (A-D) (A: MIC; B: 2xMIC; C: 4xMIC; D: 8xMIC). MIC: minimum inhibitory concentration; CFU: colony-forming units. MIC = 1.56 μg/mL. .

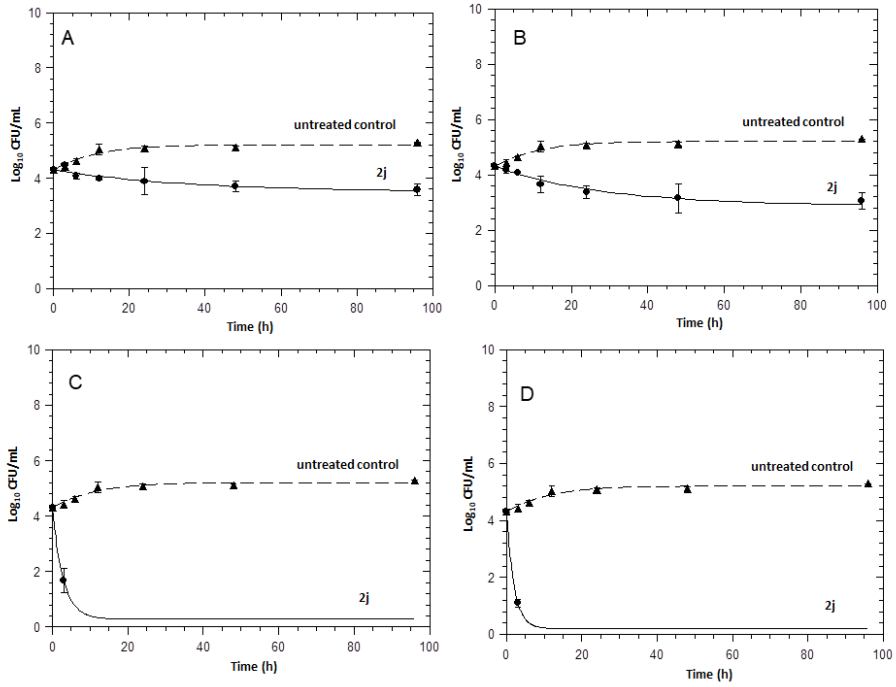


**Fig. 4.** Fitted curves resulting from modeling fungal growth (dashed line) and time-kill curve plots of synthetic compound **2j** (full line) against *Microsporum canis* 01 (A-D) (A: MIC; B: 2xMIC; C: 4xMIC; D: 8xMIC). MIC: minimum inhibitory concentration; CFU: colony-forming units. MIC = 1.56 μg/mL.

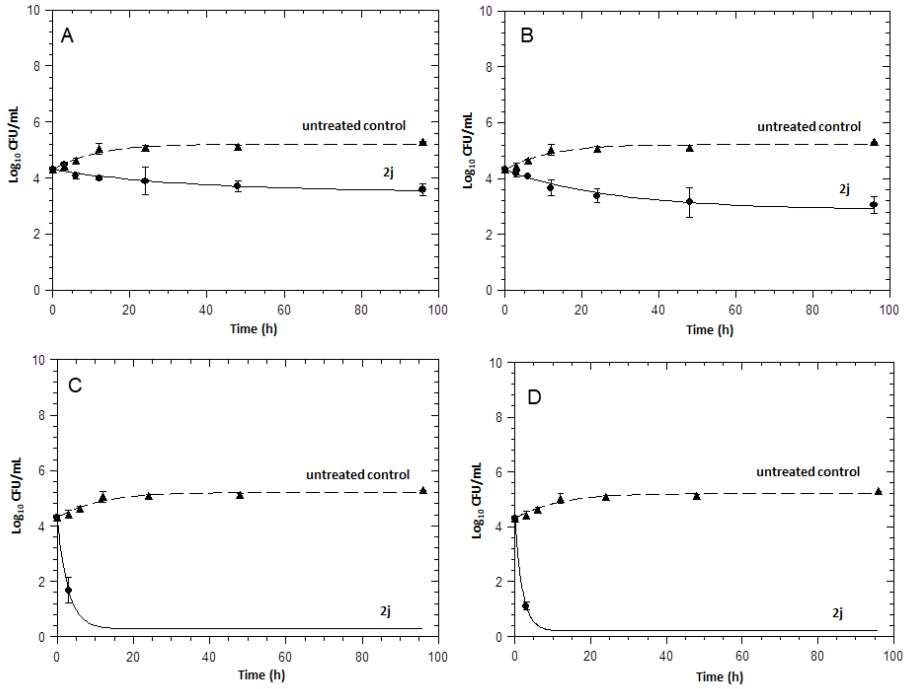


**Fig. 5.** Fitted curves resulting from modeling fungal growth (dashed line) and time-kill curve plots of synthetic compound **2j** (full line) against the multidrug-resistant clinical strain *Trichophyton mentagrophytes* 16 (A-D) (A: MIC; B: 2xMIC; C: 4xMIC; D: 8xMIC). MIC: minimum inhibitory concentration; CFU: colony-forming units. MIC = 3.12 µg/mL.

**Table 1.** Pharmacodynamic parameters determined for compound **2j** against yeasts and dermatophytes, and goodness of fit criteria.

| **Parameters** | ***C. albicans* ATCC 18804** | ***C. tropicalis* 72 A*** | ***M. canis* 01** | ***T. mentagrophytes* 16*** |
|---|---|---|---|---|
| MIC[#] (µg/mL) | 1.56 ± 0.00 | 1.56 ± 0.00 | 1.56 ± 0.00 | 3.12 ± 0.00 |
| $K_0$ ($h^{-1}$) | 0.14 ± 0.00 | 0.16 ± 0.04 | 0.10 ± 0.00 | 0.15 ± 0.00 |
| $K_{max}$ ($h^{-1}$) | 2.48 ± 0.51 | 1.89 ± 1.83 | 2.84 ± 2.61 | 2.37 ± 2.16 |
| $EC_{50}$ (µg/mL) | 4.71 ± 0.38 | 3.13 ± 0.75 | 3.59 ± 0.26 | 4.69 ± 0.24 |
| $N_{max}$ ($log_{10}$CFU/mL) | 7.67 ± 0.00 | 7.65 ± 0.13 | 5.21 ± 0.00 | 5.11 ± 0.00 |
| $x$ ($h^{-1}$) | - | - | - | - |
| $y$ ($h^{-1}$) | - | - | - | - |
| $h$ | 3.28± 0.62 | - | - | - |
| Model selection criterion/$R^2$ | 3.44/1.00 | 3.46/1.00 | 3.44/1.00 | 3.33/1.00 |

[#]Minimum inhibitory concentration was previously determined by broth microdilution. *Multidrug-resistant clinical strains; $k_0$: fungal growth rate constant in the absence of compound **2j**; $k_{max}$: maximum killing rate constant (maximum effect); $EC_{50}$: **2j** concentration necessary to produce 50% of maximum effect; $N_{max}$: maximum number of fungal cells; $x$, $y$: adaptation rate terms; $h$: Hill factor; $R^2$: coefficient of determination.

### 3.3. Protective effect against fungal infection in *Drosophila melanogaster* minihost model

Experiments showed that the compound **2j** and fluconazole were found to be nontoxic to Toll-deficient *D. melanogaster* flies at 1 mg/mL - concentration used (data not shown). The efficacy of **2j** treatment on Toll-deficient flies infected with *C. albicans* is showed in Fig. 6A. Infected flies fed with food containing compound **2j** had a significantly higher survival rate than untreated control flies ($p < 0.001$). Indeed, response to treatment with the compound **2j** and fluconazole was identical ($p > 0.05$). Nonetheless, CFU count did not differ among the groups (Fig. 6B).

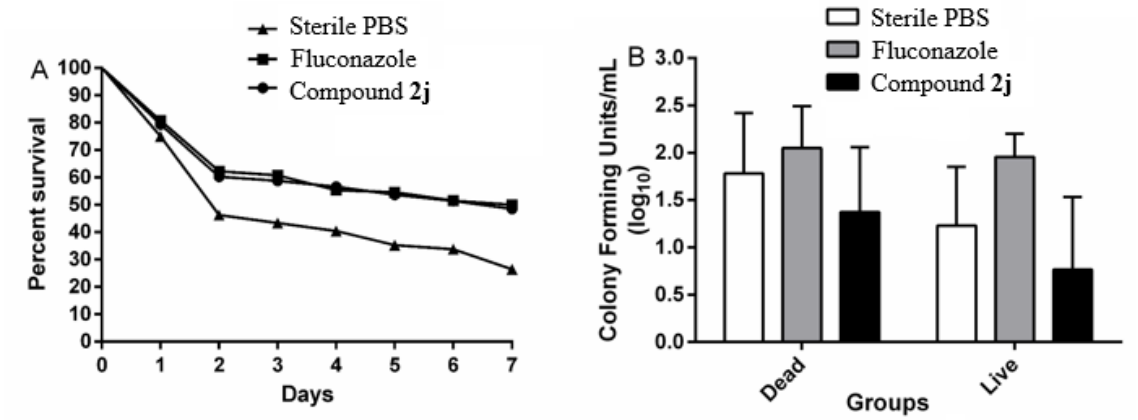


**Fig. 6**. A) Survival of *Tl*-deficient flies infected with *Candida albicans* and treated with compound **2j**, fluconazole, or sterile PBS (control). Data shown are the means of four independent experiments. Compound **2j** and fluconazole groups were statistically different from the Sterile PBS group ($p < 0.001$; log-rank test). B) Fungal burden (expressed as mean $\log_{10}$ and standard error of the mean (SEM) of colony-forming units per mL) of dead and live flies at day 7 of experiment. No statistical difference was observed among the groups ($p > 0.05$; Kruskal Wallis test).

## 4. Discussion

From the dynamic time kill curves of yeasts after exposure to compound **2j** (Fig. 2 – 3), was observed that the concentration of 12.48 µg/mL (8xMIC) was associated with achieving the cidal endpoint (≥ 99.9% reduction in CFU) at 0.28 h (*C. albicans*) and at 0.37 h (*C. tropicalis*) of exposure to **2j**. For filamentous fungi, the fungicidal concentration was 6.24 µg/mL (4xMIC) for *M. canis* and 12.48 µg/mL (4xMIC) for *T. mentagrophytes*, after 0.24 h and 0.29 h of *in vitro* contact with the compound. Rapid fungicidal action may be a valuable advantage for treatment of systemic infections that require the implementation of a therapy that has immediate action due to the patient's severity.[3] Joaquim et al. (2018)[26] modeling of 8-hydroxyquinolines derivatives time kill data, observed that one of the most promising compounds (molecule **5b**) in the study

reached the maximum fungicidal effect for *C. albicans*, *M. canis*, and *T. mentagrophytes* after 7, 17, and 0.25 h, respectively.

In none of the **2j** tested concentrations there was an increase in the number of CFU/mL. For *C. albicans* ATCC 18804 (Fig. 2) and *C. tropicalis* 72A (Fig. 3), similar kinetic profiles were observed. At concentrations ranging from MIC to 4xMIC, the growth was slightly less than observed with the untreated control (Fig. 2 – 3, A – C). For these concentrations of **2j**, the number of CFU/mL remained constant or with a small decrease over time, with the greatest reduction in CFU/mL more perceptible at 8xMIC (Fig. 2 – 3, D; Fig. S1).

For *M. canis* (Fig. 4B) and *T. mentagrophytes* (Fig. 5B), until 2xMIC concentration, **2j** was fungistatic for 96 h, while from 4xMIC onwards (Fig. 4 – 5, C) the fungicidal effect initiated (Fig. 4 – 5, D; Fig. S1). Thus, time-kill data revealed that **2j** begins to exert the fungicidal action against dermatophytes in lower concentrations when compared to yeasts. Although the fungicidal activity varied among test fungal isolates, it was observed that fungicidal capacity increased for all strains with increasing concentrations and exposure time to this compound.

The PK/PD modeling results indicate that the mathematical model chosen are appropriate for fitting the data. Data showed that **2j** was highly effective as $EC_{50}$ was 4.71 µg/mL (*C. albicans*), 3.13µg/mL (*C. tropicalis*), 3.59 µg/mL (*M. gypseum*), and 4.69 µg/mL (*T. mentagrophytes*) - concentrations very close to the MIC values (Table 1). Similar doses were also reported by Joaquim et al.[26] in relation to dynamic interaction of new 8-hydroxyquinolines against *C. albicans*, *M. canis*, and *T. mentagrophytes*, which corroborates the use of this type of model to predict the dose-response efficacy. This approach of combining *in vitro* time kill with PK/PD modeling might serve to delineate future studies in animals in order to define the dosage of the compound to be applied. $E_{max}$ models are useful to describe the relationship between concentration and effect[25,26] and have been shown to be successfully applied in our study with **2j**.

Summarize, for all four pathogens tested, modified $E_{max}$ model was found to be adequate to describe the observed kill curve data. Simulations based on previous preclinical trials can predict clinical outcome and help to come up with dose recommendations. [24,25,26] Complement to this, PK/PD modeling has become a prediction tool to evaluate the maximum antimicrobial effect, considering important variables in this process.[24,25,26] Thus, the combination of the time kill data with this PK/PD strategy, together with the pre-established MICs, may improve the understanding of the dynamic

interaction of a new antifungal candidate with the pathogens. [21,22,24,25,26] However, further work needs to be completed in order to fully understand the PK/PD of **2j**.

According to the findings of **2j** protection experiments in the *Drosophila melanogaster* minihost model, we observed a protective effect of compound **2j** – approximately 50% of the Toll-deficient flies treated with this compound or fluconazole survived at the end of the experiment, whereas less than 30% of flies survived with sterile PBS (control) (Fig. 6). Survival curves of both **2j** (MIC for *C. albicans* ATCC 18804 = 1.56 μg/mL) and fluconazole (MIC for *C. albicans* ATCC 18804 = 1.0 μg/mL) differed statistically from the PBS group, demonstrating the efficacy of compound **2j** as a promising agent in the therapy of systemic and disseminated mycoses, mainly in immunocompromised patients. This survival profile of flies was similar to that found by Chamilos et al. (2006)[30] using fluconazole. They found that fluconazole had a significant protective effect in flies infected with a susceptible *C. albicans* strain, but this antifungal agent did not fully protect the infected flies since survival did not exceed 70% at day 7 of the experiment.[30] Although survival rates with **2j** or fluconazole treatments were promising, the number of CFU/mL ($\log_{10}$) did not differ among the groups, including PBS. The determination of CFU/mL ($\log_{10}$) is a primordial factor for confirmation of the systemic infectious process, in this case caused by yeast *C. albicans*.

In conclusion, using dynamic time–kill experiments, we found for the synthetic compound **2j** a dose- and time-dependent fungicidal action for all species of dermatophytes and *Candida* evaluated, including resistant (*C. tropicalis*) and multidrug-resistant (*T. mentagrophytes*) clinical strains. The maximum fungicidal effect is achieved in less than one hour exposure of the microorganism to compound **2j**, having an extremely rapid effect. Simulated curves still predicted that **2j** would exert prolonged fungicide activity against all four fungal species (*C. albicans*, *C. tropicalis*, *M. canis*, and *T. mentagrophytes*). The $EC_{50}$ values obtained by combination of time-kill studies with mathematical model were similar to MIC values, which clarifies the potential of this molecule belonging to class 1,3-bis(aryloxy)-2-propanamine derivatives. Additionally, **2j** successfully protected Toll-deficient *Drosophila* fruit flies infected with *C. albicans*, demonstrating the antifungal efficacy of the compound in systemic treatment. Thus, the compound **2j** is promising antifungal candidate for the therapeutic of fungal infections, being this an important initial study for further researches of pharmacokinetics in mammalian animal models.

**Acknowledgements**

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**Conflict of interest**

No conflict of interest declared.

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**Supplementary information**

# Bisaryloxypropanamines derivative exhibiting protective effect against fungal infection in *Drosophila melanogaster* minihost model

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**Results**

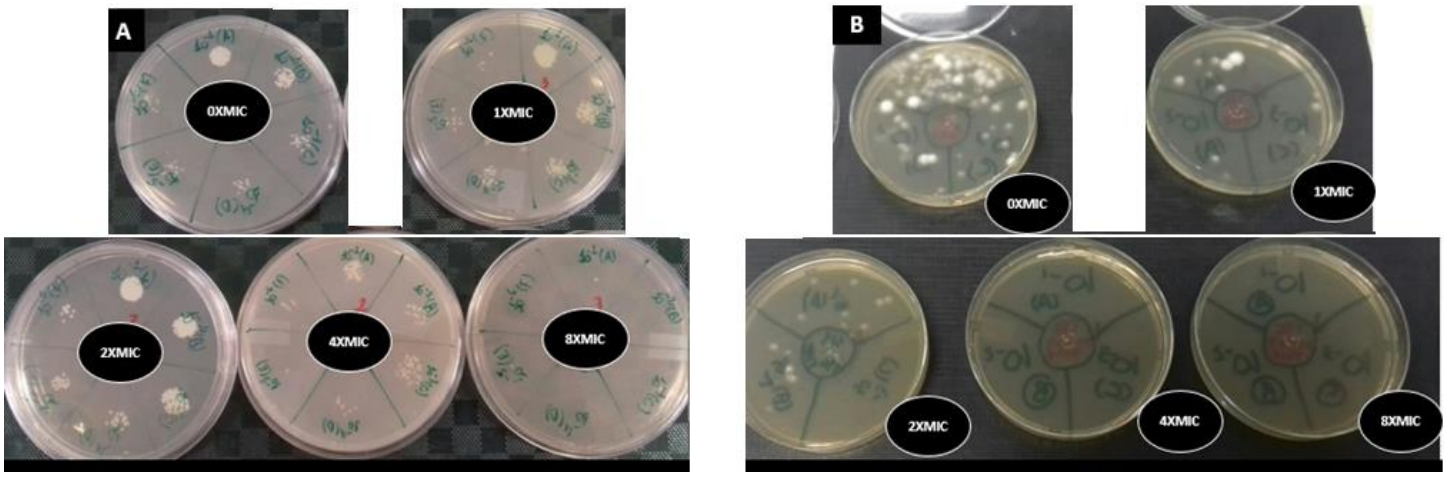


**Figure S1**. Time kill experiment images of the growth of *C. tropicalis* (CT 72A; A) and *T. mentagrophytes* (TME 16; B) treated *in vitro* with compound **2j** at different concentrations (0xMIC – drug free control, 1xMIC, 2xMIC, 4xMIC and 8xMIC; MIC = 1.56 μg/mL for CT 72A and MIC = 3.12 μg/mL for TME 16), in a maximum experiment time of 48 h for yeasts and 96 h for dermatophytes.

# DISCUSSÃO GERAL

A incidência das infecções fúngicas vêm aumentando gradativamente nos últimos 30 anos, tornando-se um relevante problema de saúde pública no mundo todo. Muitas dessas infecções são responsáveis por elevadas taxas de morbi-mortalidade, principalmente em pacientes que apresentam comprometimento imunológico (PARKER et al., 2014; GAFFI, 2018; WHO, 2018; FUENTEFRIA et al., 2018).

O aumento da resistência dos microrganismos, em geral, aos agentes antimicrobianos é uma das maiores preocupações entre cientistas e médicos, sendo que no âmbito da resistência das espécies fúngicas, o problema é ainda mais grave, pois o arsenal de fármacos antifúngicos disponíveis além de restrito, apresenta muitas limitações, sendo necessária a pesquisa e desenvolvimento de novas terapias antifúngicas que apresentem eficácia, toxicidade seletiva para o patógeno e baixo custo (BOSTROM et al. 2012; ANEJA et al. 2016). Entre as estratégias investigadas, a síntese de novos compostos vem sendo muito aplicada para obtenção de protótipos que podem vir a complementar a terapia convencional ou ser uma nova opção de antifúngico (TEGOS; MYLONAKIS, 2012; DHANASEKARAN; THAJUDDIN; PANNEERSELVAM, 2015; GARVEY et al., 2015; CAMPOY; ADRIO, 2017; FUENTEFRIA et al., 2018).

Nesse contexto, considerando-se que a síntese de compostos orgânicos constitui um importante processo na descoberta e no desenvolvimento de novos fármacos (SANGI, 2016), propôs-se a investigação detalhada da atividade antifúngica de variadas classes de compostos sintéticos (TAs, δ-Ls, 1,4-BZs e 1,3-BXPs). Inicialmente, fez-se um uma triagem com aproximadamente 200 substâncias oriundas de processos sintéticos, chegando-se a essas quatro classes que apresentaram moléculas com ação *in vitro*, em concentrações ≤ 50 μg/mL, contra dermatófitos e *Candida* spp., dois grupos de fungos de extrema importância clínica e elevada incidência (WATANABE et al., 2001; MARUYAMA et al., 2002; VANDER; HOSSAIN; GHANNOUM, 2003; MUKHERJEE et al., 2003; CORTEZ et al., 2012; SILVA et al., 2012; ATAIDES et al., 2012; SARDI et al., 2013; PFALLER; DIEKEMA, 2007; HOLLENBACH, 2008; MOHD et al., 2018). Para a prospecção de novos antifúngicos, além da atividade, a toxicidade e o mecanismo de ação são passos subsequentes e essenciais que devem ser investigados, para uma análise completa do quanto uma classe ou molécula realmente seria promissora (TEGOS; MYLONAKIS, 2012), o que foi realizado no

presente estudo. Espera-se de um novo antifúngico que ele tenha amplo espectro, que seja fungicida, atóxico e que apresente a possibilidade de múltiplas vias de administração (TEGOS; MYLONAKIS, 2012; CALDERONE et al., 2014; ROEMER; KRYSAN, 2014; DENNING; BROMLEY, 2015; DEL POETA, 2016).

Compostos em uma mesma classe química, em geral, apresentam uma estrutura básica em comum, "*scaffold*" ou farmacóforo, que confere a atividade biológica. As substâncias pertencentes as mesmas classes diferem entre si por grupos químicos periféricos substituintes que conferem melhores propriedades farmacológicas e espectro de atividade (FISCHBACH; WALSH, 2009). Estruturalmente, comparando-se as quatro classes químicas estudadas, para todas observa-se como estrutura base um anel aromático, com ligações duplas conjugadas, e para alguns compostos em particular a presença de anéis heterocíclicos– ambas as condições estão presentes em outras substâncias que reconhecidamente possuem atividades biológicas importantes (SLANINOVÁ et al., 2001; CERNUCHOVÁ et al., 2005; RONKETTI et al., 2007; XIA et al., 2010; RESCIFINA et al., 2012; ABBEHAUSEN et al., 2013).

Em relação aos testes de suscetibilidade, comparando-se todas as classes investigadas no presente estudo, do total de 189 compostos, 48 desses (≈25%) apresentaram atividade antifúngica em concentrações ≤ 50 µg/mL (concentração limite definida para a triagem inicial). 1,3-BXPs apresentaram os menores valores de CIM (0,39 a 50 µg/mL), com ação também frente a isolados clínicos resistentes e multirresistentes, sendo, portanto, o grupo de moléculas de maior destaque quanto ao potencial antifúngico (Capítulo VI). Devido a isso, o composto mais promissor do referido grupo químico foi selecionado para o desenvolvimento de uma formulação semissólida visando uma alternativa de tratamento tópico para dermatomicoses e demais estudos foram conduzidos para investigação de um possível e adicional uso sistêmico do mesmo.

Para as três demais classes investigadas (TAs, δ-Ls e 1,4-BZs), os compostos mais ativos, contra dermatófitos e *Candida* spp., demonstraram faixa de CIM de 25 a 50 µg/mL (Capítulos II, III e IV, respectivamente). Os "*hits*" de cada classe, ou seja, as moléculas que mais se destacaram para a atividade biológica em questão, em relação às demais do grupo, poderão servir de estrutura modelo para um possível melhoramento estrutural a fim de obterem-se compostos com valores menores de CIM, como já evidenciado em outros

estudos da literatura (LAFLEUR et al., 2011; SINGH et al., 2016; PIPPI et al., 2017). Neste sentido, um outro recurso interessante já visando uma futura aplicação, seria utilizar a nanotecnologia, testando os compostos em uma nanoformulação (QADIR, 2017), como foi feito para a molécula mais promissora da classe dos TAs (Capítulo II). O composto cloro-derivado teve os valores de CIM reduzidos em até 64 vezes quando em uma nanoemulsão, que foi devidamente desenvolvida e caracterizada. FERNANDEZ-CAMPOS et al. (2013) observaram efeito semelhante para uma nanoemulsão de nistatina, que apresentou melhor atividade antifúngica (CIM 2 vezes menor), em comparação com o fármaco livre (FERNANDEZ-CAMPOS et al., 2013). Adicionalmente, para outros antifúngicos comerciais, tais como clotrimazol, econazol e anfotericina B, já foi constatado que os nanossistemas são muito vantajosos por melhorar a biodisponibilidade e efeito, bem como reduzir a toxicidade (FUKUI et al., 2003; FRONZA; CAMPOS; TEIXEIRA, 2004; PANDEY et al., 2005; NAHAR et al., 2008; VAN DE VEM ET AL., 2012; SOLIMAN et al., 2017).

Nanoemulsões são misturas isotrópicas de fármacos, lipídios e surfactantes, geralmente com cossolventes hidrofílicos e com diâmetro de partícula variando de 10 a 500 nm (MUNDADA; PATEL; SAWANT, 2016; SOLIMAN et al., 2017). Essas nanoformulações possuem características interessantes para a administração de agentes antifúngicos, como tamanho pequeno de partícula, boa estabilidade cinética, alta capacidade de solubilização de fármacos e penetração aprimorada através da pele (HUSSAIN et al., 2016). Além disso, podem ser utilizadas como alternativa aos nanotransportadores lipídicos menos estáveis (por exemplo, lipossomas) (HUSSAIN et al., 2014; MAHTAB et al., 2016). A alta área superficial devido ao reduzido tamanho das partículas, além da natureza lipofílica da formulação são fatores que melhoram a permeação do fármaco através das membranas biológicas, resultando em melhor eficácia (THAKKAR et al., 2015; SOLIMAN et al., 2017). As nanoemulsões também podem permitir a liberação tópica de antifúngicos para maximizar os efeitos locais dos medicamentos e reduzir os danos tóxicos associados (HUSSAIN et al., 2016; SOLIMAN et al., 2017).

Em virtude de sua versatilidade e ampla gama de propriedades, as nanoformulações podem superar muitas das características farmacológicas desfavoráveis de fármacos, que têm limitações importantes relacionadas ao seu

espectro de atividade, propriedades físico-químicas, biofarmacêuticas e interações medicamentosas (SOLIMAN et al., 2017). Além disso, poderiam aumentar a penetração do antifúngico através da pele e, assim, ajudar a erradicar infecções fúngicas profundas (MBAH; BUILDERS; ATTAMA, 2014). Outras características atrativas incluem liberação sustentada, maior estabilidade do fármaco, direcionamento para tecido infectado, redução de efeitos adversos fora do alvo e prolongamento do tempo de permanência do ativo no sangue (CHANG et al., 2015; WICKI et al., 2015; SOLIMAN et al., 2017).

Quanto as demais propriedades biológicas das classes de moléculas avaliadas, para os TAs já foram relatadas atividade antibacteriana (SILVEIRA et al., 2012; SÁ et al., 2014) e antitumoral (FORTES et al., 2016). Para as δ-Ls, propriedades antimicrobianas e antitumorais também já foram observadas (WANI et al., 1980; UMEZAWA et al., 1984; OHKUMA et al., 1992; KISHIMOTO et al., 2005; TANAKA et al., 2007; YANG et al., 2011; JANECKA et al., 2012; MODRANKA et al., 2012). Para os 1,4-BZs, algumas das atividades já identificadas para a classe são antioxidante (DAI; MUMPER, 2010), antitumoral (DAI, MUMPER, 2010) e antiprotozoária (ROLDOS et al., 2008; DE ARIAS et al., 2012; SERNA et al., 2015). Para os 1,3-BXPs, atividade antiprotozoária, contra *Leishmania amazonenses* e *Trypanosoma cruzi* já foram reportadas (LAVORATO et al., 2015; LAVORATO, 2016; LAVORATO et al., 2017; LAVORATO; DUARTE; DE ANDRADE, 2017). Essas atividades biológicas evidenciadas na literatura nos motivaram a investigar o potencial antifúngico das referidas classes.

A melhor compreensão dos mecanismos da ação antifúngica das moléculas mais promissoras foi uma das etapas subsequentes ao teste de suscetibilidade. De modo geral, dois alvos foram avaliados: parede e membrana celular fúngica. As moléculas das classes das δ-Ls e 1,4-BZs apresentaram determinada ação na parede e/ou membrana, dependente do tipo de substituinte da estrutura; compostos da classe dos TAs demonstraram mecanismo de ação relacionado à complexação com o ergosterol fúngico e para os 1,3-BXPs foi observado como alvo a parede celular. Para dois dos compostos que demonstram ação na parede (composto **2j -** 1,3-BXPs e composto **8** - 1,4-BZs), o efeito observado para ambos foi fungicida, ressaltando-se nesse caso a importância desse alvo para a morte das espécies fúngicas analisadas. A saber,

as equinocandinas, classe fungicida, têm como alvo um complexo enzimático envolvido na síntese da parede celular fúngica. Recentes atualizações de diretrizes para tratamento de infecções fúngicas recomendam as equinocandinas como terapêutica de primeira linha para candidemia (LARKIN; DHARMAIAH; GHANNOUM, 2018). Atualmente, existem dois novos antifúngicos em desenvolvimento que visam a síntese de 1,3-β-glucana: acetato de rezafungina (anteriormente CD101) e SCY078 (LARKIN; DHARMAIAH; GHANNOUM, 2018). Como as equinocandinas só estão disponíveis para administração intravenosa, buscam-se novos membros para a classe atuante a nível de parede celular fúngica, com biodisponibilidade oral satisfatória ou que ainda possam ser utilizados topicamente (LARKIN; DHARMAIAH; GHANNOUM, 2018).

Nas espécies fúngicas, a estrutura celular é semelhante à dos demais eucariotos, constituída basicamente por uma membrana, um citoplasma com as organelas distribuídas aleatoriamente por todo interior celular e um compartimento especial, o núcleo, que armazena o material genético. As células podem ser unicelulares, como as leveduras, ou então pluricelulares formando conjuntos de hifas, septadas ou não, denominadas de micélio, como no caso dos fungos filamentosos. Tanto as células leveduriformes, quanto o micélio estão envolvidos por uma camada protetora externa denominada de parede celular, quimicamente diferente da parede encontrada em vegetais, ainda que possa exercer os mesmos tipos de funções (JUNQUEIRA; CARNEIRO, 2005; MADIGAN; MARTINKO; PARKER, 2004; FUKUDA et al., 2009). Desse modo, a parede exerce uma fundamental função de proteção a células fúngicas, além de ser exclusiva dos patógenos, por isso a sua importância como alvo terapêutico específico, na investigação da atividade antifúngica de novos compostos (FUENTEFRIA et al., 2018).

Para o TA avaliado quanto ao mecanismo de ação antifúngico (Capítulo II), foi evidenciada somente atividade por complexação com o ergosterol da membrana celular fúngica, em um processo que se equipara a anfotericina B, utilizada como antifúngico controle, no ensaio realizado. O fundamento do teste consistiu em detectar se um composto atua por ligação ao ergosterol da membrana dos fungos, fornecendo-se ergosterol exógeno às substâncias-teste, que se possuem afinidade por esteróis, formam rapidamente um complexo com

o ergosterol comercial fornecido, evitando-se assim a complexação com o ergosterol próprio da membrana celular fúngica; como consequência, um aumento da CIM é observado para esses compostos (CARRASCO et al., 2012).

Os agentes antifúngicos tradicionais, como os azólicos e os polienos, são dirigidos contra alvos moleculares envolvidos na síntese e na estabilidade da membrana dos fungos. Como a membrana de células fúngicas é predominantemente composta de esteróis, glicerofosfolipídios e esfingolipídios, o papel dos lipídios na patogênese e a identificação de alvos para terapias melhoradas foram amplamente explorados por pesquisadores durante os últimos anos (OUF et al., 2013; SANT et al., 2016; CHEN et al., 2018b). A membrana celular fúngica contém tipicamente o ergosterol como componente principal, em contraste com o colesterol que é o principal esterol presente nas células animais (SANT et al., 2016). Resultados semelhantes aos encontrados para as classes investigadas nos Capítulos II, III, IV e VI, quanto ao mecanismo de ação, foram também reportados para moléculas sintéticas de outras classes, analisadas pelo nosso grupo de pesquisa (PIPPI et al., 2017; BATISTA et al., 2017). Mais estudos são necessários no sentido de verificar detalhes da ação na membrana ou parede celular fúngica, concluindo se é a nível enzimático ou envolve outro processo que leva a alterações morfológicas na célula fúngica, como as evidenciadas nas imagens de microscopia eletrônica de varredura (MEV), após tratamento *in vitro* com os compostos mais representativos de cada classe estuda neste trabalho. Com as imagens de MEV das principais moléculas mais promissoras, o dano celular ficou muito evidente, com efeito de ruptura de blastoconídios, formação de poros em hifas, inibição de pseudo-hifas, dentre outras consequências às células de *Candida* spp. e dermatófitos analisados.

Dois compostos estruturalmente semelhantes, diferenciando-se apenas por um átomo ou posição que este ocupa na molécula, podem apresentar diferenças quanto às suas propriedades físico-químicas e, consequentemente, quanto à ação biológica, tanto do ponto de vista quantitativo como qualitativo. A ação terapêutica de medicamentos ocorre por interações destes com sistemas biológicos e é dependente de fatores relacionados com sua estrutura química e características físico-químicas. Estes fatores, sejam eles de caráter eletrônico, estérico ou hidrofóbico, influenciam a relação dos compostos com a biofase e a sua distribuição nos compartimentos que compõem o sistema biológico ou alvo.

Esse processo fundamenta o planejamento de novas substâncias que possuam perfil terapêutico mais adequado às necessidades atuais (TAVARES, 2004; FUENTEFRIA, 2018).

Em relação à toxicidade, os resultados para todas as classes foram promissores, quando as moléculas foram avaliadas nas CIMs e/ou concentrações superiores. A molécula **11** dos TAs não foi irritante (membrana cório-alantoide/HET-CAM), nem mutagênica, nem ocasionou inviabilidade celular e hemólise significativa, embora tenha apresentado dano ao DNA dose-dependente. Para as δ-Ls, a toxicidade foi dose-dependente para a viabilidade de leucócitos humanos, mas nenhum dos compostos foi mutagênico, genotóxico ou irritante (HET-CAM). Para os 1,4-BZs, os efeitos da molécula **8** em relação à viabilidade celular foi dose-dependente, no entanto, o composto não demonstrou ser genotóxico e nem mutagênico, em células leucocitárias humanas e também não ocasionou hemólise. Complementarmente, não foi irritante, quando analisada pelo método HET-CAM. O composto **2j**, pertencente aos 1,3-BXPs, foi o único da referida classe química que apresentou índice de seletividade (IS) maior que 10. Essa informação é apresentada no Capítulo VI. Um elevado valor de IS é uma característica importante para candidatos a fármacos. Um valor de IS maior que dez é recomendado para que se garanta a segurança de um novo antimicrobiano (PIRES et al., 2013; VINCENT et al., 2016; LAVORATO, 2016). Logo, **2j** atende aos requisitos de segurança estabelecidos na literatura. Ainda para **2j**, não foi observado dano histopatológico em pele de orelha de porco após exposição ao composto e nem efeito irritante (HET-CAM).

De modo geral, a toxicidade a leucócitos humanos e eritrócitos de coelho foi verificada visando a possibilidade de futura administração oral de formulações a serem desenvolvidas, contendo os compostos mais promissores, e a toxicidade à membrana cório-alantoide e histopatológico, para observação, exemplificadamente, da segurança para uma futura aplicação tópica (CHANDRIKA et al., 2018; OLIVEIRA et al., 2018).

Quanto às técnicas aplicadas, uma das metodologias mais utilizadas para avaliação de possíveis danos mutagênicos causados por fármacos ou novas biomoléculas é o teste de micronúcleo (MN). A investigação da frequência de MN é o padrão de teste de mutagenicidade OECD – *Guidelines for the testing of chemicals/ section 4: Health Effects – Mammalian Erythrocytes Micronucleos*

(Test: nº 474) e recomendado pelo *Gene-Tox Program, Environmental Protection Agency* – EPA/US, pela sua capacidade e precisão em detectar possíveis agentes clastogênicos (quebra de cromossomos) e aneugênicos (segregação cromossômica anormal) (FENECH, 2000; RIBEIRO; SALVADORI; MARQUES, 2003). Os micronúcleos são definidos como pequenas massas esféricas, extranucleares, constituídas de cromatina não incorporada ao núcleo principal da célula, durante as etapas finais da mitose, servindo como um biomarcador de dano cromossomal, instabilidade genética e, eventualmente, se houver uma exposição crônica à substância tóxica, risco de câncer (FENECH, 2000).

Igualmente importante no screening de toxicidade de novos compostos, o teste cometa é um método extremamente sensível para estudos de danos ao DNA em células individualizadas (MUKHOPADHYAY et al., 2004; COLLINS, 2009). Alterações genéticas são quase sempre prejudiciais (excetuando-se os casos de compostos que agem contra células neoplásicas), especialmente em organismos multicelulares, nos quais a modificação está mais inclinada a perturbar o desenvolvimento e a fisiologia extremamente complexa de um organismo (SPIVAK; COX; HANAWALT, 2008). Enquanto o ensaio do cometa detecta lesões reversíveis, o teste de MN detecta lesões mais persistentes no DNA ou efeitos aneugênicos que não podem ser reparados (HARTMANN et al., 2003).

Então, a partir da relevância experimental desses testes realizados, incluindo a determinação do índice de seletividade em relação a linfócitos humanos, alguns dos compostos não terem apresentado indícios de mutagenicidade e genotoxicidade, nas concentrações que foram efetivos contra as espécies fúngicas patogênicas, é um resultado certamente muito promissor para uma possível bioaplicação em formulações. Apesar disso, ainda há uma evidente necessidade de avaliações adicionais de citotoxicidade, genotoxicidade e mutagenicidade dessas substâncias, considerando outros modelos, principalmente utilizando-se parâmetros *in vivo*, e concentrações mais elevadas, para determinação de um perfil toxicológico mais completo.

Todos os demais testes adicionais realizados para os manuscritos deste estudo, além da tríade suscetibilidade-mecanismo de ação-toxicidade, incluíram: para os TAs, análise da atividade antiquimiotáxica; para os TAs e 1,4-BZs, ensaio da eficácia *in vivo* em modelo alternativo de infecção em membrana cório-

alantoide de ovo de galinha; para os 1,4-BZs, a inclusão de cepas bacterianas na avaliação da atividade – para observar se os compostos exerciam ação antimicrobiana; para as δ-Ls, análise quimiométrica, correlacionando-se as CIMs e propriedades químicas mais importantes para a atividade e por fim, para os 1,3-BXPs, incorporação do composto mais ativo em um hidrogel, modelagem farmacocinética/farmacodinâmica (PK/PD) a partir dos dados do *time kill* e avaliação do efeito protetor em modelo de infecção fúngica sistêmica em mosca-das-frutas.

Associado ao efeito antifúngico, os TAs apresentaram importante efeito antiquimiotáxico, indicando ação na fase aguda do processo inflamatório (MEDZHITOV, 2008). Essa propriedade complementar auxilia tanto no processo de cura de uma infecção fúngica, bem como no alívio dos sintomas. Ao acessar o tecido do hospedeiro, os dermatófitos induzem uma resposta imune pelos queratinócitos, responsável pelo desencadeamento do processo inflamatório do organismo em face ao agressor (PERES et al., 2010). No entanto, quando a resposta inflamatória é exacerbada e prolongada, acarreta em prejuízo ao sistema de defesa e à evolução terapêutica (ROMANI, 2011; KIM et al., 2015).

O ensaio de infecção *in vivo* em membrana cório-alantoide de ovo de galinha embrionado foi realizado pela primeira vez, e ainda está em fase de padronizações, tendo como inspiração o próprio ensaio de toxicidade HET-CAM. O HET-CAM é uma alternativa *in vitro* ao teste *in vivo* de Draize (determinação da irritação ocular em coelhos), que imita as alterações vasculares na membrana corio-alantóide. Este método qualitativo avalia o potencial de irritação de produtos químicos. A membrana cório-alantoide responde à lesão com um processo inflamatório semelhante ao do tecido conjuntival do olho de coelho. Determina-se um composto com relativa baixa toxicidade dérmica, em determinada concentração, quando ele não é considerado irritante pelo HET-CAM, pois a membrana cório-alantoide é extremamente sensível, então isso é um forte indício de que a substância também não será irritante para a membrana epidérmica ou mucosas, por exemplo (OLIVEIRA et al., 2018).

Através da aplicação de ferramentas quimiométricas, observou-se a formação de grupamentos de moléculas das δ-Ls com maior potencial de atividade e que o LogP (lipofilicidade) seria uma das propriedades mais importantes para o desempenho do efeito biológico e pela maior interação com

membranas fúngicas. As análises quimiométricas auxiliam no estabelecimento de uma relação entre um grande número de amostras, considerando sua complexidade química, a fim de estabelecer tendências, definir marcadores e quimiotipos (KONG et al., 2009; PODUNAVAC-KUZMANOVIĆ et al., 2012; SCOTTI et al., 2012).

Para os 1,3-BXPs, considerando o composto **2j**, a sua incorporação em um hidrogel (Capítulo VI) representa uma real aplicação da molécula mais promissora do estudo em uma forma farmacêutica simples e que vem sendo muito utilizada com outros agentes antifúngicos, visando tratamento tópico de infecções fúngicas (WINNICKA et al., 2012; SAHOO et al., 2014; MAHATA et al., 2014; ALDAWSARI et al., 2015; SHU et al., 2018). O posterior estudo, em relação ao potencial de **2j** para uso sistêmico (capítulo VII) contitui-se da determinação das curvas de morte (time kill), análise PK/PD, e efeito sobre a candidíase sistêmica em *Drosophila melanogaster* Toll-deficientes. **2j** demonstrou ser fungicida para todas as espécies fúngicas (dermatófitos e *Candida* spp.), atingindo o efeito máximo de morte fúngica em menos de 1h. Em relação à candidíase sistêmica em *D. melanogaster*, **2j** apresentou eficácia estatisticamente comparável ao fluconazol no controle do processo infeccioso. Esses resultados servem de base para estudos adicionais que complementem a hipótese de uso sistêmico do composto.

O ensaio *in vitro* denominado de *Time kill* é empregado para a caracterização da farmacodinâmica antifúngica de determinada substância, consistindo na análise de curvas de morte fúngica em função do tempo. Com isso, é possível determinar se um composto exerce ação fungicida ou fungistática, dependente ou não da dose e do tempo. **2j** teve o seu efeito fungicida evidenciado em tal experimento, que foi dose e tempo dependente. Fármacos antifúngicos que apresentam o efeito antifúngico dependente de tais variáveis, como **2j**, incluem poliênicos e equinocandinas (ANDES et al., 2003).

Além disso, a modelagem PK/PD é considerada uma abordagem muito útil para a determinação da posologia de antifúngicos. Essa técnica é desenvolvida e aplicada com base em simulações e previsão de efeito, assim como na otimização de posologias para diversos fármacos, através da combinação de parâmetros farmacocinéticos e propriedades farmacodinâmicas,

que permitam uma correlação matemática entre os elementos concentração-efeito-tempo (DE ARAÚJO, 2008).

*D. melanogaster* tem sido utilizada para estudos de resposta imune do hospedeiro, virulência de microrganismos e eficácia de novos agentes como um modelo de infecção (MERKEL, 2018a). Este díptero possui duas importantes vias de sinalização da resposta imune para o processo de resistência microbiana, a via Imd e a via Toll. O gene da imunodeficiência (Imd) parece ser mais importante contra bactérias, enquanto que os fungos são induzidos pelos componentes da via Toll (LEMAITRE et al., 1995; HOFFMANN; REICHHART, 2002). Mosca-das-frutas deficientes da sinalização Toll, portanto, apresentam muita utilidade em estudos de patogênese e pesquisa e desenvolvimento de fármacos frente a espécies fúngicas (CHAMILOS et al., 2008; ARVANITIS et al., 2013). A semelhança do sistema imune inato de *D. melanogaster* com o de humanos, corrobora a importância deste modelo para essas finalidades. Desse modo, *D. melanogaster* apresenta-se como um modelo promissor e já consolidado na pesquisa, envolvendo uma série de investigações de patologias de origem fúngica, tais como *Cryptococcus neoformans* (APIDIANAKIS et al., 2004), *Aspergillus* (LIONAKIS et al., 2005), *Candida* spp. (CHAMILOS et al., 2006), *Fusarium* spp. e *Scedosporium* (LAMARIS et al., 2007), *Mucorales* (CHAMILOS et al., 2008) e mais recentemente *Malassezia* spp. (MERKEL, 2018b).

Em suma, moléculas de variadas classes químicas apresentaram potencial antifúngico contra dermatófitos e *Candida* spp. Alguns compostos tiveram ainda efeito mais pronunciado pela ação antiquimiotáxica associada ou por estarem em nanoformulações. 1,3-BXPs foi o grupo de moléculas de maior destaque em relação à atividade antifúngica, com os menores valores de CIM. Em relação ao mecanismo, os compostos no geral apresentaram como alvo a parede e/ou membrana celular fúngica, dependendo do tipo de substituinte e classe de cada substância. Quanto à toxicidade a leucócitos humanos e irritação à membrana (HET-CAM), os resultados são promissores, principalmente quanto a uma futura aplicação tópica. O desenvolvimento de uma formulação semissólida com o composto mais promissor de todos (**2j**), bem como os estudos iniciais de modelagem PK/PD e efeito protetor em modelos alternativos de infecção fúngica, reafirmam a potencialidade de uma nova opção segura e eficaz

para o tratamentode de dermatomicoses de origem dermatofítica e leveduriforme.

Os estudos adicionais de revisão bibliográfica sobre dermatofitose e imagens de MEV, demostrando o processo de invasão ungueal por *Microsporum canis*, são complementares ao eixo central do estudo que consistiu de uma grande triagem de mais de uma centena de compostos, caracterização do mecanismo de ação, perfil de toxicidade; e por fim, desenvolvimento de um produto de aplicação tópica, no caso um hidrogel, com o composto mais promissor e subsequentes análises do seu potencial adicional para uso sistêmico.

# CONCLUSÕES E PERSPECTIVAS

- Pela revisão da literatura sobre dermatofitoses, observou-se que essas micoses cutâneas figuram entre as infecções fúngicas de maior ocorrência. O quadro clínico mais comum inclui despigmentação, placas anulares, prurido e perda de cabelo. O tratamento, em geral, está relacionado ao uso de antifúngicos tópicos e/ou sistêmicos, apresentando como problemática o surgimento de espécies multirresistentes (Capítulo I).

- Para a classe dos tiocianatos alílicos: das 15 moléculas avaliadas, o composto **11** (4-clorofenil substituído) exibiu amplo espectro de ação antifúngica e significativa atividade antiquimiotáxica, com 100% de redução na migração de leucócitos. A faixa de CIM dos compostos foi de 25 a 50 µg/mL, com mecanismo de ação relacionado à complexação com o ergosterol fúngico. Com a nanoemulsão contendo o composto **11**, observou-se melhora da atividade antifúngica *in vitro* em aproximadamente 64 vezes para dermatófitos e quatro vezes para *Candida* spp. A molécula **11** não foi mutagênica nem ocasionou inviabilidade celular e hemólise significativa, embora tenha apresentado dano ao DNA dose-dependente. Além disso, não foi irritante e inibiu 100% o crescimento fúngico no modelo de infecção alternativo de dermatofitose, em membrana cório-alantoide de ovo embrionado de galinha (Capítulo II).

- Para a classe das δ-lactonas: dois dos compostos da série apresentaram os menores valores de CIM (25-50 µg/mL) e mais amplo espectro de atividade contra fungos filamentosos e leveduras, incluindo espécies resistentes e multirresistentes. O mecanismo de ação foi relacionado a parede e membrana celular, com ação alvo específica dependente do tipo de halogênio presente na estrutura. Os danos às células fúngicas foram corroborados por imagens de MEV, que evidenciaram células lisadas e completamente alteradas. A toxicidade foi dose-dependente para a viabilidade de leucócitos humanos, mas nenhum dos compostos foi mutagênico, genotóxico ou irritante, quando avaliado em concentrações superiores a CIM (Capítulo III).

- Para a classe dos 1,4-benzenodiós 2-substituídos: 23 compostos apresentaram atividade *in vitro* contra fungos e/ou bactérias. As moléculas

com o mais amplo espectro de ação *in vitro* e com os menores valores de CIM, contra bactérias e fungos foram a "**5**" e a "**8**", respectivamente. O composto **5** demostrou eficácia *in vivo* na redução total da carga bacteriana, no modelo alternativo de infecção em ovos de galinha embrionados. O mecanismo antifúngico, para a molécula **8**, foi relacionado à parede e membrana celular, corroborado por imagens de MEV, que revelaram alterações consideráveis na forma celular após tratamento *in vitro* com o composto. Os efeitos do composto **8** na viabilidade celular foram dose-dependentes, no entanto, a molécula não demonstrou ser genotóxica e nem mutagênica, em células leucocitárias humanas e não ocasionou hemólise, nas concentrações avaliadas. Complementarmente, não foi irritante, quando analisada pelo método HET-CAM (Capítulo IV).

- Em relação a avaliação da capacidade de invasão ungueal (unhas humanas) por *Microsporum canis*, através de imagens de MEV, observou-se que essa espécie é capaz de colonizar a lâmina ungueal, incluindo o processo de formação de biofilme, recentemente descoberto e reportado para esta espécie dermatofítica (Capítulo V).

- Para os 1,3-BXPs, após observar a atividade antifúngica *in vitro* de cento e vinte e um compostos e o perfil de toxicidade, verificou-se que a molécula **2j** é a mais promissora de todas as moléculas analisadas neste estudo, com seletividade contra os patógenos (IS > 10) e baixíssimos valores de CIM (0,39 a 3,12 µg/mL), inclusive contra isolados clínicos resistentes e multirresistentes. **2j** tem como alvo a parede celular fúngica e adicionalmente inibe fatores de virulência de *Candida* spp. e dermatófitos. **2j** não foi irritante (HET-CAM) e, portanto, a incorporamos em um hidrogel com potencial bioadesivo, para o tratamento de dermatomicoses. O perfil de permeação em pele humana do composto no hidrogel foi excelente, permanecendo principalmente nas camadas mais superficiais da pele, o que é muito vantajoso para tratamentos tópicos (Capítulo VI). Além disso, usando experimentos dinâmicos de *time kill*, **2j** apresentou uma ação fungicida dose e tempo dependente. O efeito fungicida máximo foi alcançado em menos de uma hora de exposição do microrganismo ao

composto. Os valores de $EC_{50}$ obtidos pela modelagem PK/PD foram semelhantes aos valores de CIM. Ainda, **2j** protegeu de modo eficaz mosca-das-frutas Toll-deficientes em modelo de candidíase sistêmica (Capítulo VII). Assim, o composto **2j** é um candidato antifúngico promissor para a terapêutica de infecções fúngicas associadas a dermatófitos e *Candida* spp..

- Como perspectiva do estudo, espera-se aprofundar ainda mais as análises para uso sistêmico do composto **2j,** com farmaconinética/farmacodidâmica envolvendo modelos em mamíferos, bem como avaliar de modo mais detalhado a toxicidade e eficácia no combate de infeções fúngicas, considerando-se incluir ensaios biológicos *in vivo*. Adicionalmente, visa-se a aplicação do composto em esmalte para tramento de onicomicoses, com avaliação da eficácia em modelo de infecção fúngica em casco bovino.

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