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CAMILLA PINHEIRO DE MENEZES CALDAS

INVESTIGAÇÃO DO MECANISMO DA ATIVIDADE ANTIFÚNGICA DO MONOTERPENO CITRAL FRENTE A CEPAS DE *Cladosporium spp* *e Cladophialophora carrionii*

JOÃO PESSOA – PB

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Tese apresentada ao Programa de Pós-graduação em Produtos Naturais e Sintéticos Bioativos, Centro de Ciências da Saúde, Universidade Federal da Paraíba, em cumprimento aos requisitos necessários para a obtenção do título de Doutor em Produtos Naturais e Sintéticos Bioativos. Área de concentração: Farmacologia.

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Tese de Doutorado aprovada em: 14/02/2017.

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*“*O futuro pertence àqueles que acreditam na beleza de seus sonhos."

Eleanor Roosevelt

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RESUMO

CALDAS, C. P. M. **INVESTIGAÇÃO DO MECANISMO DA ATIVIDADE ANTIFÚNGICA DO MONOTERPENO CITRAL FRENTE A CEPAS DE *Cladosporium spp* E *Cladophialophora carrionii*.** Tese (Doutorado em Produtos Naturais e Sintéticos Bioativos – Área de Concentração: Farmacologia) - Universidade Federal da Paraíba, João Pessoa, 2017.

Fungos dematiáceos estão associados com infecções superficiais de pele e tecidos moles até sepse, com elevada mortalidade. A importância clínica, epidemiológica e terapêutica dispensada às micoses causadas por fungos dematiáceos impulsionam estudos que visam à descoberta de novos agentes antifúngicos. Entre eles os monoterpenos se destacam por possuírem amplo reconhecimento do seu efeito antimicrobiano. O citral é um monoterpeno com conhecidas propriedades farmacológicas, incluindo ação antifúngica. Neste contexto, este estudo teve como objetivo investigar a atividade antifúngica desse monoterpeno, seus possíveis mecanismos de ação, o efeito da associação com antifúngicos contra *Cladophialophora carrionii* e *Cladosporium* spp, bem como determinar, através de análise teórica, *in silico*, o seu perfil farmacocinético e outras possíveis atividades farmacológicas. Os ensaios da atividade antifúngica foram realizados por meio da triagem microbiológica de 8 fitoconstituíntes; determinação da concentração inibitória mínima (CIM) e concentração fungicida mínima (CFM) do citral pela técnica de microdiluição; medida do crescimento micelial radial em diferentes intervalos de tempo; inibição da germinação de conídios. A ação do citral sobre a parede celular fúngica (ensaio com Sorbitol) e sobre a membrana plasmática fúngica (complexação com o ergosterol) foram investigadas. Também foram realizados estudos *in silico* e avaliado o efeito da associação do citral com antifúngicos (anfotericina B e voriconazol) pelo método de *checkerboard.* Na triagem microbiológica o citral apresentou melhor atividade antifúngica contra as 10 cepas testadas, sendo selecionado para dar continuidade a investigação antifúngica. A CIM do citral variou entre 128 e 256 µg/mL para *C. carrionii* e *C. sphaerospermum,* para *C. oxysporum* a CIM foi de 128 para as três cepas testadas e a CIM de *C. cladoporioides* foi de 64 µg/mL. A CFM do citral variou entre 256 e 1024 µg/mL para *C. carrionii* e *C. sphaerospermum*, foi de 256 µg/mL para *C. oxysporum* e 128 µg/mL para *C. cladosporioides.* Os resultados também mostraram que o citral inibiu significativamente o desenvolvimento micelial e a germinação dos conídios das quatro espécies testadas. Na investigação do mecanismo de ação foi evidenciado que os valores de CIM de citral contra *Cladosporium spp.* e *C. carrionii* permaneceram inalterados na presença de sorbitol 0.8 M sugerindo que este monoterpeno não atua através da inibição da síntese da parede celular fúngica. Por outro lado, os resultados do ensaio sobre a membrana plasmática mostraram que o citral interage com o ergosterol. No estudo *in silico*, o citral demonstrou uma boa biodisponibilidade oral, bem como importantes atividades farmacológicas. A associação citral-voriconazol foi indiferente, enquanto citral-anfotericina B foi antagonista para todas as cepas testadas. Diante dos resultados, sugere-se que o citral atua sobre a membrana de *Cladosporium* spp e *C. carrionii*, por um mecanismo que envolve a complexação com o ergosterol. Dessa maneira, esse monoterpeno apresenta-se como promissor agente antifúngico, em especial em casos de micoses causadas por fungos dematiáceos.

Palavras-chave: Citral. Fungos Dematiáceos. *Cladosporium*. *Cladophialophora.* Antifúngicos. Ergosterol.

ABSTRACT

CALDAS, C. P. M. **RESEARCH INTO THE ANTIFUNGAL ACTIVITY MECHANISM OF THE MONOTERPENE CITRAL AGAINST STRAINS OF *Cladosporium* spp AND *Cladophialophora carrionii*.** Thesis (Doctorate in Natural and Synthetic Bioactive Products - Concentration Area: Pharmacology) - Federal University of Paraíba, João Pessoa, 2017.

Dematiaceous fungi are associated with superficial infections of the skin and soft tissues, and include sepsis with high mortality. The clinical, epidemiological and therapeutic importance given to the mycoses caused by dematiaceous fungi drive studies aimed at discovering new antifungal agents. Among them the monoterpenes are distinguished and enjoy broad recognition of their antimicrobial effect. Citral is a monoterpene with known pharmacological properties, including antifungal action. In this context, this study aimed to investigate the antifungal activity of this monoterpene, its possible mechanisms of action, and the effect of association with certain antifungals against *Cladophialophora carrionii* and *Cladosporium spp, a*s well as to determine, through theoretical analysis, in silico, its pharmacokinetic profile and other possible pharmacological activities. Tests of antifungal activity were performed by microbiological screening of eight (8) phytochemicals; to determine the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of citral by microdilution technique; measuring the radial mycelium growth at different time intervals; and measuring spore germination inhibition. The actions of citral on the fungal cell wall (Sorbitol assay), and the fungal cell membrane (ergosterol complexation) were also investigated. We also carried out *in silico* studies and rated the association effect of citral with antifungals (amphotericin B and voriconazole) using the checkerboard method. In microbiological screening, citral showed better antifungal activity against 10 tested strains, being selected to continue in antifungal research. The MIC of Citral ranged between 128 and 256 ug/ml for *C. carrionii,* and *C. sphaerospermum.* For *C. oxysporum* the MIC was 128 ug/ml for all three tested strains, and the MIC for *C. cladoporioides* was 64 ug/mL. The MFC of citral varied between 256 and 1024 ug/ml for *C. carrionii* and *C. sphaerospermum*, it was 256 ug/ml for *C. oxysporum*, and 128 ug/ml for *C. cladoporioides*. The results also showed that citral significantly inhibits mycelial growth and spore germination for the four species tested. In the mechanism of action investigation it was shown that the MIC values of citral against *Cladosporium spp.* and *C. carrionii* remained unchanged in the presence of 0.8 M sorbitol suggesting that this monoterpene does not act by inhibiting the synthesis of the fungal cell wall. However, the test results on the plasma membrane showed that citral interacts with ergosterol. In the *in silico* study, citral showed good oral bioavailability, as well as important pharmacological activities. The citral-voriconazole association was indifferent and the citral-amphotericin B association was antagonistic for all strains tested. From the results, it is suggested that citral acts on the *Cladosporium spp*. and *C. carrionii* membrane through a mechanism involve ergosterol complexation. The monoterpene is presented as a promising antifungal agent, in particular, for cases of mycosis caused by dematiaceous fungi.

Keywords: Citral. Dematiaceous Fungi. *Cladosporium. Cladophialophora*. Antifungals. Ergosterol.

LISTA DE FIGURAS

Evaluation of Citral's Antifungal Activity and Mode of Action against *Cladophialophora carrionii*

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Evaluation of Antifungal Activity of citral associated with antifungals Amphotericin B and Voriconazole, against *Cladophialophora carrionii* and *Cladosporium spp*

LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

AIDS	Síndrome da imunodeficiência adquirida
ASD	Ágar Sabouraud Dextrose
CCS	Centro de Ciências da Saúde
CFM	Concentração Fungicida Mínima
CIM	Concentração Inibitória Mínima
Cm	Centímetro
CMRVS	Coleção de Microrganismos de Referência em Vigilância Sanitária
CSD	Caldo Sabouraud Dextrose
DMSO	Dimetilsulfóxido
E.P.M.	Erro Padrão da Média
FDA	Food and Drug Administration
FIOCRUZ	Fundação Oswaldo Cruz
^{o}C	Grau(s) Celsius
ICIF	Índice da concentração inibitória fracionada
INCQS	Instituto Nacional de Controle de Qualidade em Saúde
LM	Laboratório de Micologia
NaCl	Cloreto de Sódio
µg	Micrograma
µL	Microlitro
µg/mL	Micrograma por mililitro
µm	Micrômetro
mm	Milímetro
M	Molar
MDCKs	Madin-Darby canine kidney
OMS	Organização mundial de saúde
%	Percentual
Ppm	Partes por milhão
PASS	Previsão do Espectro de Atividade para Substâncias
Pa	Probabilidade de ser ativo
Pi	Probabilidade de ser inativo
PM	Peso Molecular
RPM	Rotação por minuto
RPMI	Roswell Park Memorial Institute
UFC	Unidades Formadoras de Colônias
UFC/mL	Unidades Formadoras de Colônias por mililitro
UFPB	Universidade Federal da Paraíba
UFPE	Universidade Federal de Pernambuco
UV	Ultravioleta

OBS.: Os termos não listados nesta relação encontram-se descritos no texto.

SUMÁRIO

Introdução

1 INTRODUÇÃO

As infecções fúngicas oportunistas adquirem, na prática médica, importância cada vez maior, principalmente em ambientes hospitalares e em serviços especializados com procedimentos invasivos, estados de imunossupressão induzida pela quimioterapia antineoplásica, AIDS, transplantes ou hemopatias diversas. Sua incidência tem aumentado nas últimas décadas, pois esta população em geral é mais exposta a fatores que favorecem a infecção micótica (BROWN et al., 2012; KHAN et al., 2010).

Avanços nos cuidados com a saúde ao longo dos últimos anos permitem a sobrevida de pacientes imunocomprometidos, levando a um aumento no número de pessoas com risco de adquirir infecções fúngicas oportunistas, e a tendência parece ser que este número continue a crescer (SILVA, PAULA, ESPINDOLA, 2009). A magnitude desta epidemia em curso foi estimada em milhões de seres humanos que sofrem as consequências de uma doença fúngica com substancial morbidade e mortalidade (DENNING, BROMLEY, 2015; VOS et al., 2012; BROWN et al., 2012).

Os fungos dematiáceos pertencentes aos gêneros *Cladosporium* e *Cladophialophora* são encontrados como contaminantes em diversos ambientes. São importantes fitopatógenos e sua ligação com a saúde humana e animal está principalmente relacionada com quadros de patologias oportunistas quando do desenvolvimento de severa imunossupressão (TASIC, TASIC, 2007; CORREIA et al., 2010; BAKHESHWAIN et al., 2011).

As principais infecções fúngicas, causadas por esses fungos, são as feo-hifomicoses, a cromoblastomicose e quadros alérgicos, que são infecções crônicas difíceis de tratar, devido ao longo período de tratamento, às limitadas opções terapêuticas, às condições da imunidade do indivíduo e à relativa resistência do fungo aos antifúngicos habitualmente utilizados, podendo, portanto, ocorrer recidivas (ZAITZ et al., 2012; REVANKAR, SUTTON, 2010).

A terapêutica antifúngica, apesar de mais diversificada ainda apresenta algumas limitações no que diz respeito à toxicidade sobre as células humanas, pois tanto o hospedeiro mamífero quanto os fungos invasores são eucarióticos, tornando difícil desenvolver antifúngicos específicos que visem seletivamente apenas o agente patogênico (KHAN, AHMAD, CAMEOTRA, 2013) além dos casos de

resistência, tanto intrínseca como adquirida, por parte dos fungos (NISHI et al., 2009).

Nesta perspectiva, existe uma procura crescente por agentes antifúngicos novos e eficazes, justificando a intensa busca por novos medicamentos de várias fontes, incluindo produtos naturais que sejam mais eficazes e menos tóxicos do que aqueles já em uso (KHAN, AHMAD, CAMEOTRA, 2013; RAJPUT, KARUPPAYIL, 2013; PERFECT, 2016).

Os produtos naturais têm se destacado como excelentes alternativas para este propósito devido a sua diversidade química e por constituírem uma importante fonte de novos compostos biologicamente ativos (GUIMARÃES, MOMESSO, PUPO, 2010), contendo uma série de substâncias que podem ser usadas para o tratamento não só das infecções fúngicas, mas também de diferentes doenças infecciosas (SAAD, 2010).

Assim, a descoberta de produtos naturais contendo princípios ativos com ação antifúngica pode representar uma nova alternativa para a produção e o uso de fármacos no combate aos agentes infecciosos (KHAN, et al., 2012; MENDES, 2011).

Dentre os produtos naturais pode-se citar os monoterpenos, que são os principais constituintes da maioria dos óleos essenciais, compreendendo uma ampla série de substâncias que apresentam diversas atividades farmacológicas (CERQUEIRA et al., 2011; SANTOS et al., 2011).

O citral (3,7-dimetil-2,6-octadienal), monoterpeno acíclico natural, é uma mistura de dois monoterpenos aldeídos acíclicos isômeros geométricos - geranial e neral que pode ser encontrado nos óleos essenciais de diversas plantas aromáticas, empregadas na medicina popular, e que tem revelado importantes atividades farmacológicas em especial atividade antifúngica (SADDIQ, KHAYYAT, 2010; ZHOU et al*.,* 2014*;* LEITE et al., 2014; SOUSA et al., 2016).

Diante da importância clínica e alta incidência das micoses causadas por fungos dematiáceos, aumento do número de indivíduos imunocomprometidos, surgimento de cepas resistentes, toxicidade dos antifúngicos existentes, é de fundamental importância a busca por novos agentes antifúngicos mais eficazes, menos tóxicos, sendo os produtos derivados de plantas medicinais excelentes alternativas para esse propósito. Assim, o objetivo deste estudo foi investigar a atividade antifúngica e o possível mecanismo de ação do monoterpeno citral contra cepas de *Cladosporium spp.* e *Cladophialophora carrionii*.

Objetivos

2 OBJETIVOS

2.1 Objetivo geral

Investigar a atividade antifúngica, *in vitro,* do monoterpeno citral, isolado e em associação com antifúngicos padrões, frente a cepas de *Cladophialophora carrionii*, *Cladosporium oxysporum*, *Cladosporium sphaerospermum* e *Cladosporium cladosporidioides,* o seu possível mecanismo de ação e a análise teórica do seu perfil farmacológico e farmacocinético.

2.2 Objetivos específicos

- Realizar triagem antifúngica de 8 fitoconstituíntes, escolhendo dentre estes, o que apresentar melhor perfil antifúngico para aprofundar o estudo;
- Determinar, através de análise teórica, *in silico*, o perfil farmacocinético e outras possíveis atividades farmacológicas do citral;
- Determinar a Concentração Inibitória Mínima (CIM) do citral frente a cepas de *Cladosporium spp.* e *Cladophialophora carrionii*;
- Determinar a Concentração Fungicida Mínima (CFM) do citral frente a cepas de *Cladosporium spp.* e *Cladophialophora carrionii;*
- Avaliar o efeito do citral sobre a cinética de crescimento micelial radial;
- Avaliar o efeito do citral na germinação dos conídios fúngicos;
- Estudar os possíveis modos de ação do citral com ênfase nos seus efeitos sobre a parede celular e a membrana plasmática fúngica;
- Avaliar os possíveis efeitos da associação do citral com os antifúngicos voriconazol e anfotericina B (*Checkerboard*);

Referencial Teórico

3 REFERENCIAL TEÓRICO

3.1 Considerações gerais sobre os fungos

Os fungos são micro-organismos eucariontes, uni ou pluricelulares e heterotróficos pertencentes ao Reino Fungi (MEIRELES, NASCENTE, 2009). Apresentam espessa parede celular constituída por glicoproteínas e polissacarideos, principalmente glucano e quitina, além de uma membrana celular, cujo principal componente é o ergosterol (BOWMAN, FREE, 2006).

Possuem ampla distribuição na natureza, podendo ser encontrados dispersos no meio ambiente, nos vegetais, no ar atmosférico, no solo, na água, nos animais e nos alimentos. Suas espécies sofrem em sua incidência variações conforme a localidade, estação do ano, grau higroscópico do ar, entre outras (LACAZ et al., 2002; SIDRIM, ROCHA, 2012).

Estima-se que aproximadamente 800.000 espécies de fungos tenham sido descritas, dentre essas, cerca de 400 espécies são patogênicas para humanos e animais (QUINN et al., 2011).

Nesse contexto, os fungos e seus metabólitos interessam à medicina sob vários aspectos, a saber: a) como agentes de hipersensibilidade imediata ou tardia, b) como agentes bem definidos de micoses - infecções fúngicas, c) como agentes de micetismo, por intoxicações por fungos macroscópicos e d) como agentes de micotoxicoses, pela ingestão contínua ou prolongada de alimentos contaminados por fungos produtores de micotoxinas (ZAITZ et al., 2012).

Os fungos de interesse médico, agentes de micoses, são de dois tipos morfológicos: multicelular, produzindo estruturas filamentosas microscópicas sendo designados de fungos filamentosos, ou unicelulares como são conhecidas as leveduras. Existem ainda os fungos dimórficos, que se apresentam sob ambas as formas, dependendo principalmente da temperatura, mas sob a influência também do teor de CO_2 e das condições nutricionais (SULLIVAN, MORAN, COLEMAN, 2005; BRASIL, 2004).

Segundo os tecidos, órgãos e indivíduos atingidos, as infecções fúngicas são classificadas como oportunistas, superficiais ou disseminadas. As infecções oportunistas atingem exclusivamente indivíduos com sistemas imunitários debilitados. Nos casos de infecções superficiais os microrganismos estão confinados

às camadas exteriores dos epitélios. E quando penetram essa barreira, entrando na corrente sanguínea e disseminando-se por todo o corpo, são consideradas infecções disseminadas ou sistêmicas (SULLIVAN, MORAN, COLEMAN, 2005).

Apesar dos humanos possuírem um nível elevado de imunidade inata em relação aos fungos, com exceção dos causadores de infecções na pele, unhas e cabelo, a situação sofre uma considerável alteração quando os alvos são indivíduos com sistemas imunitários muito debilitados, como os encontrados em ambientes hospitalares (DEACON, 2006).

Desde o final do século passado, evidenciou-se um aumento considerável de micoses de caráter invasivo e de difícil tratamento, representando uma significativa causa de morbidade e mortalidade em pacientes criticamente doentes (ZAITZ et al., 2012).

Dentre estes grupos de micoses oportunistas, merecem destaque, atualmente, aquelas causadas por fungos escuros, denominados dematiáceos. Mais de cem espécies e sessenta gêneros destes fungos estão implicados em um amplo espectro de infecções humanas (REVANKAR, 2007).

3.2 Fungos dematiáceos

Fungos demácios, dematiáceos ou pretos são grupos heterogêneos de fungos que pertencem à família Dematiaceae e são encontrados no solo ou vegetais em decomposição. Têm coloração naturalmente acastanhada em decorrência da presença de pigmento melânico (di-hidroxinaftalenomelanina) em sua parede celular. Este pigmento, além de constituir um elemento fotoprotetor, é considerado um fator de virulência do fungo, uma vez que protege os organismos do estresse ambiental (exposição a metais pesados, dissecação, condições hiperosmóticas e temperaturas extremas, do antagonismo de outros organismos, da limitação de nutrientes, do choque de pH e contra a radiação ionizante e UV (ZAITZ et al., 2012; SIDRIM, ROCHA, 2012; HENSON et al., 1999).

Os fungos dematiáceos têm a propriedade de digerir as proteínas da epiderme, causando várias lesões de pele, desde pequenas manchas avermelhadas até severas erupções que podem abrir caminho para a disseminação desses fungos, causando a proliferação das lesões, podendo comprometer parcial ou totalmente alguns órgãos (ESPINEL-INGROFF et al., 1986).

Embora sejam causas incomuns de doenças, fungos dematiáceos foram cada vez mais reconhecidos como importantes patógenos, com a maioria dos relatos ocorridos nos últimos 20 anos (REVANKAR, SUTTON, 2010). Até 2008, mais de 130 espécies de 70 gêneros têm sido associada a infecções em humanos e animais (KUMAR, HALLIKERI, 2008). Os gêneros mais frequentemente envolvidos em infecções humanas incluem *Bipolaris, Curvularia, Exserohilum, Alternaria* e *Cladosporium* (REVANKAR, SUTTON, 2010).

O espectro de doenças com as quais estão associados também ampliou e incluem desde infecções superficiais de pele e tecidos moles até sepse disseminada, com elevada mortalidade (YEW et al., 2014). As infecções mais comuns incluem feo-hifomicoses, as cromomicoses, eumicetomas, doença alérgica, infecções superficiais e profundas locais, pneumonia, abscesso cerebral, e infecção disseminada. Para algumas infecções em indivíduos imunocompetentes, tais como sinusite fúngica alérgica e abscesso cerebral, eles estão entre os agentes etiológicos mais comuns (ZAITZ et al., 2012; REVANKAR, SUTTON, 2010; AFROZ et al., 2010).

Infecções fúngicas sistêmicas por fungos dematiáceos são raras em comparação com candidíase sistêmica e aspergilose. No entanto, os fungos dematiáceos estão sendo cada vez mais reconhecidas como patógenos humanos invasivos (DE HOOG, VICENT, GORBUSHINA, 2013) especialmente em receptores de transplante de órgão (LEVIN et al., 2004; KINDO, RAMALAKSHMI, GIRI, 2013).

As feo-hifomicoses, de acordo com McGinnis (1983) englobam importante, distinto e heterogêneo grupo de infecções micóticas causadas por fungos dematiáceos, que vão desde o comprometimento superficial até doenças em órgãos profundos, nas quais os agentes etiológicos ocorrem nos tecidos como células leveduriformes, pseudo-hifas e hifas demacioides curtas ou alongadas, regulares, distorcidas ou dilatadas ou em combinação com qualquer destas formas (LACAZ et al., 2002; SIDRIM, ROCHA, 2012).

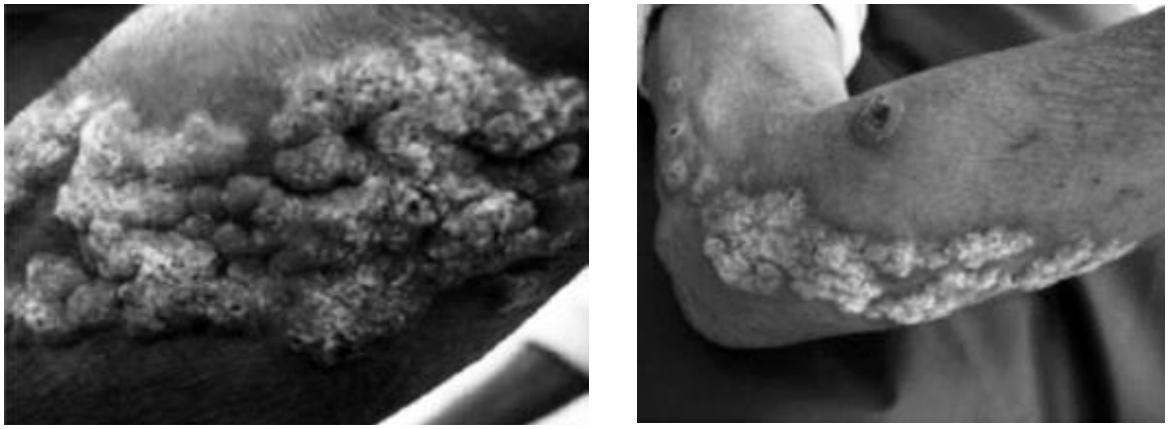
Vários casos de feo-hifomicose foram relatados no passado, e a sua incidência pode ser crescente, particularmente em pacientes imunossuprimidos (REVANKAR et al., 2002) no entanto, casos também foram relatados em hospedeiros imunocompetentes (PALAOGLU et al., 1993; WALZ et al., 1997). As taxas de mortalidade são elevadas, independentemente do estado imunológico do paciente (REVANKAR et al., 2002).

Os achados clínicos das feo-hifomicoses são bastante variáveis, dependendo do padrão imunológico do hospedeiro, sítio anatômico acometido e espécie fúngica implicada, podendo assim ser classificada como superficial, subcutânea e sistêmica ou invasivas. As formas invasivas mais comuns são pulmonares e cerebrais. As principais espécies fúngicas envolvidas nesses quadros são *Cladosporium spp.*, *Piedraia hortae*, *Curvularia spp.*, *Exophiala jeanselmei*, *Wangiella dermatitidis*, *Alternaria* spp., *Bipolaris hawaiiensis* (CASTRO, OLIVEIRA, LOPES, 2013; PATEL et al., 2006; SINGH et al., 2005).

Cromomicose ou cromoblastomicose é uma infecção micótica crônica, granulomatosa, de evolução lenta, com supurações da pele e tecido subcutâneo, causada pela implantação traumática de uma variedade de fungos demátiáceos que formam corpos esclerótícos no tecido (AMEEN, 2009).

As lesões iniciais, relacionadas ao local da inoculação, são pápulas ou nódulos que evoluem para lesões verrucosas que, ao se confluírem, formam placas verrucosas de aspecto tumoral. Na evolução, que pode ser lenta e progressiva, as lesões tendem a crescer centrifugamente, cicatrizando ou ulcerando na parte central (Figura 1) (QUEIROZ-TELLES et al., 2009). As complicações do quadro crônico inflamatório incluem a fibrose, o linfoedema e a infecção bacteriana secundária (BONIFAZ et al., 2010). Sintomas de dor e prurido podem estar presentes (QUEIROZ-TELLES et al., 1980). No exame histopatológico, há infiltrado granulomatoso, com presença de microabcessos e células gigantes que, em seu interior, observa-se grande número de corpos arredondados castanho-escuros, isolados ou em divisão (corpos fumagoides) (QUEIROZ-TELLES et al., 2009; ALMEIDA et al., 2014).

Figura 1 - Lesão verrucosa de base eritematosa no braço direito.



Fonte: ALMEIDA et al., 2014.

As interações entre o fungo e o hospedeiro, bem como a resistência imunológica, a quantidade de inóculo e a virulência do fungo, determinam alterações imunopatológicas importantes para controlar sua disseminação (MATTE et al., 1997; GIMENES et al., 2006). As lesões de cromomicose infectam-se com facilidade, podendo desencadear erisipela, linfedema, elefantíase e, ocasionalmente, carcinoma espinocelular (MINOTTO et al., 2001).

Esta micose apresenta distribuição global, com predominância em regiões tropicais e subtropicais. O maior número de casos está concentrado no continente Africano e nas Américas, em países como África do Sul, Brasil, Costa Rica e Madagascar (QUEIROZ-TELLES et al., 2013; TORRES-GUERRERO et al., 2012). No Brasil, a cromoblastomicose não é considerada doença de notificação compulsória, registrando-se um número aproximado de 520 casos na literatura (MOUCHALOUAT et al., 2011). Os estados com as maiores médias anuais de casos são: Rio Grande do Sul, Paraná, Maranhão e Pará (QUEIROZ-TELLES et al., 2011).

A cromoblastomicose é considerada uma doença ocupacional, ligada às atividades rurais e florestais, que acomete especialmente trabalhadores de baixa renda desprovidos de calçados e vestimentas adequados para a realização destas atividades (QUEIROS-TELLES et al., 2011).

A faixa etária mais acometida está entre 30 e 50 anos (QUEIROZ-TELLES et al., 2009). Os trabalhadores rurais são acometidos com maior frequência em decorrência da exposição contínua, ou seja, maior tempo de contato com o agente

etiológico presente no solo e na vegetação (MATTE et al., 1997; CORREIA et al., 2010).

O prognóstico quanto à vida é bom, porém as ulcerações, linfedema, elefantíase e a cronicidade podem ser responsáveis por incapacidade funcional do membro afetado (CORREIA et al., 2010; ZAITZ, 2012).

Os principais agentes etiológicos descritos para as cromomicoses são: *Fonsecaea pedrosoi*, *Fonsecaea compacta*, *Phialophora verrucosa*, *Cladophialophora carrionii*, *Rhinocladiella aquaspersa*, sendo *Fonsecaea pedrosoi* a espécie mais frequentemente isolada de casos em regiões tropicais, seguida por *Phialophora verrucosa* e *Cladophialophora carrionii* (AMEEN, 2010; AZAD et al., 2011; KIM et al., 2011; LU et al., 2013; ROJAS et al., 2015).

3.2.1 Gênero *Cladosporium*

O gênero *Cladosporium*, criado por Link, em 1816, é um dos maiores e mais heterogêneos gêneros de *Hyphomycetes* (DUGAN, SCHUBERT, BRAUN, 2004). Esse gênero abrange muitas espécies de fungos contaminantes e oportunistas dematiáceos, normamente identificados como contaminantes do ar e encontrados ubiquamente como saprófitas no solo e em materiais em decomposição, principalmente folhas e hastes vegetais (ZALAR et al., 2007).

Muitas espécies são conhecidas por serem patógenos de plantas, enquanto outros são regularmente encontrados como contaminantes e agentes de deterioração nos alimentos ou produtos industriais, podendo ser isolados a partir de uma vasta gama de substratos, tais como terra, pedras, tijolos, bem como os têxteis, papel e couro, além de ser frequentemente associados com queixas asmáticas (BENSCH et al., 2012; BROWN, HYDE, GUEST, 1998; EL-MORSY, 2000; SCHUBERT, 2005; GUTAROWSKA, 2014).

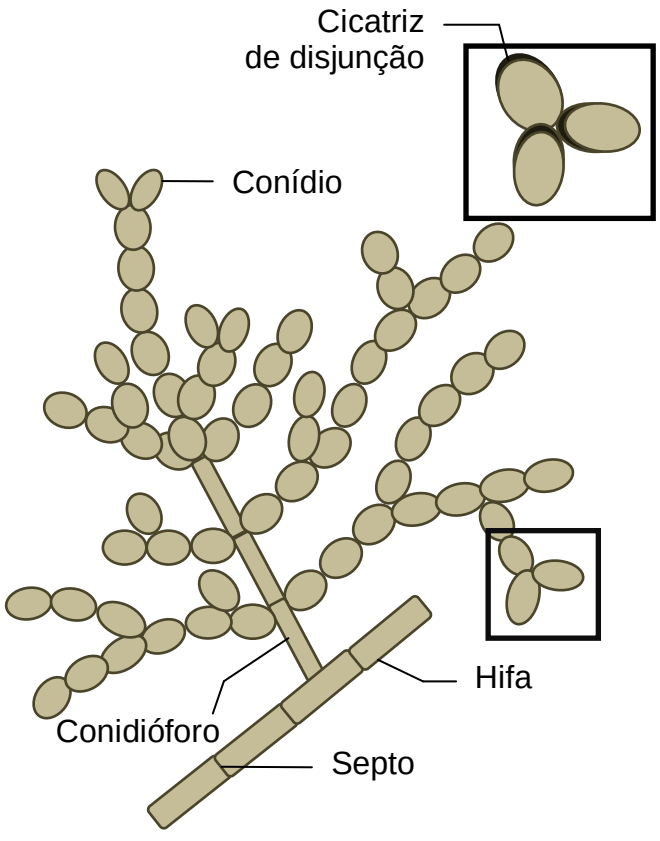
Este gênero compreende mais de 189 espécies, com uma distribuição mundial, estando entre os fungos mais comumente isolados do ambiente em quase todo lugar do mundo, podendo ser isolado a partir de praticamente qualquer fonte ambiental e localização geográfica (CROUS et al., 2014; BENSCH et al., 2012, 2015).

Conídios de espécies de *Cladosporium* representam o mais comum componente de fungos isolados do ar, uma vez que, são pequenos e bem

adaptados para se espalhar facilmente em grandes números por longas distâncias. São importantes alérgenos, e em grandes quantidades, podem afetar gravemente as pessoas asmáticas e com doenças respiratórias, já que a exposição prolongada pode enfraquecer o sistema imunológico (FARR et al., 1989; MULLINS, 2001; FLANNIGAN, 2001; FAIRS et al., 2010).

Na fase saprófita, formam hifas septadas e escuras, com conidióforos laterais e terminais de tamanhos variados. A conidiação é do tipo *Cladosporium*, isto é, apresenta conidióforos de comprimento variado, eretos e variadamente ramificados, próximo ao ápice. Os conidióforos são de vários tamanhos e podem ser septados. Eles são pretos esverdeados claros e levemente dilatados nas extremidades distais. Produzem cadeias longas e ramificadas de conídios ovais e de paredes lisas e finas, à maneira de uma árvore, ou seja, as cadeias ramificam onde quer que um conídio produza dois brotos, ao em vez de um. Os blastoconídios podem apresentar cicatrizes escuras, nas disjunções ou nos hilos de onde se desgarram do conidióforo, ou de um outro conídio (Figura 2) (FISHER, COOK, 2001).

Figura 2 – Conidiação do tipo *Cladosporium.*



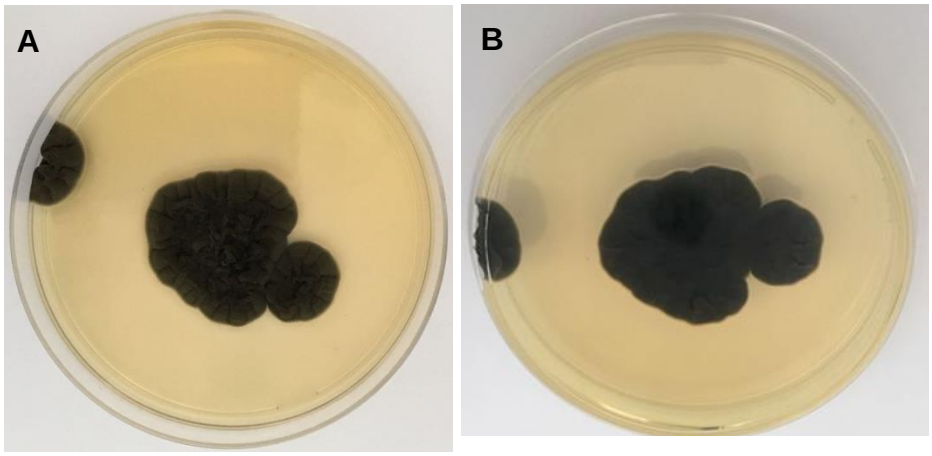
Fonte: Adaptado de FISHER, COOK, 2001.

Os conídios podem ser produzidos em cadeias, sendo catenulados, podem ser muitas vezes solitários em algumas espécies, onde os conídios são mais largos e algumas vezes são ramificados em cadeia acropleurógena, são simples, cilíndricos, ovóides, doliformes, fusiformes, elipsóides, esféricos ou sub-esféricos. Possuem coloração marrom olivácea escuro ou marrom, a superfície do conídio pode ser lisa, verrugosa ou equinulada com 0–3 septos ocasionalmente (BENSH et al., 2012; ELLIS, 1971).

Nos tecidos aparecem como células leveduriformes, pseudo-hifas, hifas verdadeiras ou qualquer combinação dessas formas, com coloração castanha (ZAITZ et al., 2012).

São fungos de crescimento lento, atingindo a maturidade dentro de 14-21 dias. Caracterizam-se pela produção de colônias efusas ou ocasionalmente puntiformes, com superfícies planas, aveludadas, circulares, de crescimeto lento e enrrugado, que vão do verde oliva ao marrom escuro e reverso preto (Figura 3) (TAMSIKAR, NAIDU, SINGH, 2006).

Figura 3 – Cultura de *Cladosporium* em ágar sabouraund dextrose.



A: Micélio aéreo. B: Reverso da colônia

Fonte: MENEZES, 2017.

Apesar de sua prevalência, apenas um número limitado de espécies têm sido documentados como agentes de infecções micóticas em humanos. As espécies bem conhecidas como patogênicas em humanos, anteriormente conhecidas como *C. bantiana, C. carrionii* e *C. devriesii,* caracterizada pela ausência de conidióforos, e cicatrizes conidiais não pigmentadas, foram reclassificadas no

gênero *Cladophialophora* (DE HOOG et al., 1995; BENSH et al., 2012). De modo que, atualmente, as espécies de *Cladosporium* de interesse médico, associadas com doença humana são *C. sphaerospermum, C.cladosporioides, C. oxysporum* e *C. herbarum* (DE HOOG et al., 2011).

Estas espécies geralmente estão associadas à rinite e asma alérgica (CHEN et al, 2014; VICENDESE et al, 2014), quadro de feo-hifomicoses superficiais, cromoblastomicoses, feo-hifomicoses profundas (SANG et al, 2012;. GUGNANI et al, 2006;. NAMRATHA, NADGI, KALE, 2010; MARTINEZ-HERRERA et al., 2015; SOSA et al., 2012), e mas raramente podem causar infecções disseminadas (CHEN et al, 2013;. LALUEZA et al., 2011, SANDOVAL-DENIS et al., 2015).

3.2.1.1 Cladosporium sphaerospermum

C. sphaerospermum é uma espécie saprofítica, cosmopolita que habita uma variedade de ambientes. É encontrada ubiquamente em ambientes naturais e provocados pelo homem, tais como ar interior e exterior, o solo, a vegetação em decomposição, pintura, silicone e têxteis, podendo também está presente nas plantas, animais e seres humanos (ZALAR et al., 2007).

C. sphaerospermum é um importante fitopatógeno, sendo muito prejudicial para as culturas vegetais (AHMED, 2015). Para os seres humanos e animais, nem todas as cepas são patogênicas, entretanto algumas cepas podem provocar ocasionalmente feo-hifomicose cutânea e cerebral independentemente do estado imunitário do hospedeiro (SOUMAGNE et al., 2015; MADURI et al., 2015; TASIC, MILADINOVIC, 2007).

Esta espécie é um dos alérgenos mais amplamente distribuídos em todo o mundo, sendo responsáveis por graves problemas em pacientes com doenças respiratórias (SEGERS et al., 2015; NG et al., 2012). Podendo causar doenças alérgicas das vias respiratórias, enfisema pulmonar e lesões intrabrônquica (YEW et al., 2016; YANO, KOYABASHI, KATO, 2003).

3.2.1.2 Cladosporium cladosporioides

C. cladosporioides é um fungo, dematiáceo, saprofítico amplamente distribuído nos mais variados ambientes que ocasionalmente pode provocar uma

variedade de infecções clínicas em seres humanos e animais imunocomprometidos e imunocompetentes. As manifestações clínicas variam dependendo da imunidade do hospedeiro e dos tecidos atingidos (SHI et al., 2016).

Dentre as infecções fúngicas causadas por essa espécie podemos destacar as feo-hifomicoses subcutâneas (SANG et al., 2012, NATH et al., 2015; ZHOU et al., 2016), ceratomicose (CHEW et al., 2009), onicomicose (SHI et al., 2016), abcessos cerebrais (KANTARCIOGLU et al., 2002; SHIMIZU et al., 1982), infecções no couro cabeludo (ZELLER et al., 2015) e infecções pulmonares (KWON-CHUNG, SCHWARTZ, RYBAK, 1975). Os esporos são alérgenos relevantes, que podem prejudicar os pacientes com asma (SHI et al., 2016).

3.2.1.3 *Cladosporium oxysporum*

C. oxysporum é um fungo saprofítico que frequentemente cresce em vários substratos (BURNETT, HUNTER, 1972). Esta espécie é muitas vezes encontrados como contaminantes da culturas vegetais (ZHENG et al., 2014) e citado em casos de doença cutânea e subcutânea (ROMANO et al., 1999; GUGNANI et al., 2006).

3.2.2 Gênero *Cladophialophora*

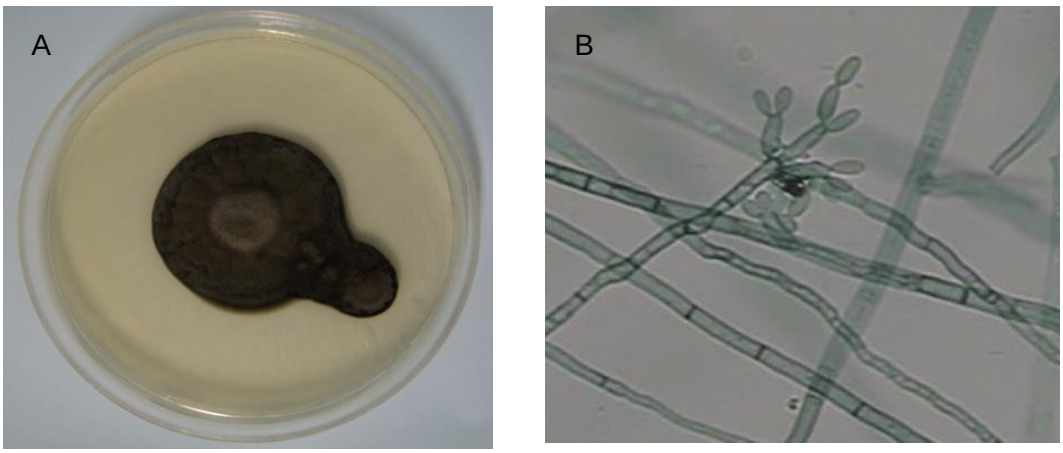
O gênero *Cladophialophora* compreende um grande numero de espécies fúngicas saprofíticas, bem como espécies de importante significado clínico. Algumas espécies são altamente relevantes porque podem causar infecção sistêmica em indivíduos saudáveis, tais como: cromoblastomicose, feo-hifomicose subcutânea, encefalite fatal e infecções disseminadas (BADALI et al., 2008; REVANKAR, SUTTON, 2010; FERNANDES et al., 2007).

Esse gênero é caracterizado por: (1) a ausência de conidióforos, "células *shield*", ou hilo proeminente (pontos de fixação); (2) a capacidade para crescer em meios contendo ciclo-heximida; e (3) tendo cadeias secas, não-frágeis de conídios (REVANKAR, SUTTON 2010).

Nos tecidos aparecem como grandes células globosas, ovaladas de parede espessa, de coloração castanha, apresentando ou não septação interna com 5-12 µm de diâmetro, denominadas de corpos fumagóides (FISHER, COOK, 2001; ZAITZ et al., 2012).

Macromorfologicamente, as colônias de *Cladophialophora* são de crescimento moderadamente lento, apresentam coloração verde-oliva, verde-acinzentadas ou lavanda, com pigmento reverso negro-azeviche. Colônias mais velhas podem tornar-se cinza-escuras ou negras tanto na superfície quanto no reverso. A superfície é recoberta com micélio aéreo curto, que lhe confere uma textura aveludada ou algodonosa. A topografia pode ser espalhada, dobrada ou elevada. E crescem a uma temperatura variável de 30-40 ^{o}C a depender da espécie fúngica (Figura 4A) (FISHER, COOK, 2001; BADALI et al., 2008).

Figura 4 – Características morfológicas de *C. carrionii.*



(A) Macromorfologia da colônia após 15 dias de crescimento em ASD a 28 ^{o}C.
(B) Micromorfologia (400x).

Fonte: MENEZES, 2012.

As hifas apresentam um pigmento marrom-esverdeado nas preparações sem corantes. Elas são septadas e têm 4µm de largura. Os conídios variam de acordo com a espécie, podendo ser ovais, elipsoide, fusiforme, levemente pontudos com paredes lisas, apresentam tamanhos variados e, muitas vezes, apresentam septos (Figura 4B). São laterais ou terminais, de cor marrom-esverdeada clara, dispostos em cadeias ramificadas que podem ser longas ou curtas. As cadeias são geralmente coerentes e as cicatrizes de conídios apresentam uma pigmentação em pálido. Além disso, a maioria dos conidióforos em espécies de *Cladophialophora* são pouco diferenciados (DE HOOG et al., 2000).

Esse gênero foi recentemente reavaliado por sequenciamento multilocus e atualmente contém sete espécies associadas com infecções humanas (BADALI et al., 2008).

Cladophialophora bantiana é o agente causador de inúmeros casos de feohifomicose cerebral muitas das quais ocorrem em indivíduos imunocompetentes e a maioria dos quais são fatais (CHAKRABARTI et al., 2016). *C. carrionii* e a recentemente descrita *C. samoensis* são agentes de cromoblastomicose. Espécies menos comuns, ocasionalmente, implicados em micoses profundas e superficiais incluem, *C. ArXII, C. boppii, C. devriesii, C. emmonsii, C. modesta* e *C.saturnica. C. yegresii* é uma espécie-irmã do meio ambiente intimamente relacionado com *C. carrionii* (REVANKAR, SUTTON, 2010; DE HOOG et al., 1995, 2015).

3.2.2.1 Cladophialophora carrionii

A espécie *C. carrionii* é encontrada no solo, na madeira e nos vegetais em decomposição. Sua distribuição é universal, mais frequente nas zonas tropicais e subtropicais, mas ocasionalmente, é encontrada nas zonas temperadas (DE HOOG et al., 2007).

Infecções com *C. carrionii* ocorrem, sobretudo, nas pessoas que trabalham ao ar livre, sem a proteção de roupas adequadas, sapatos e luvas. Os fungos penetram na pele através de algum ferimento ou pequenos traumatismos, tais como cortes, espinhos infectados, lascas de madeira. Exposições repetidas e má saúde geral podem contribuir para o desenvolvimento da infecção (CORREIA et al., 2010).

C. carrionii é um dos agentes etiológicos da cromoblastomicose, de feo-hifomicoses superficiais e quadros alérgicos. As infecções causadas por *C. carrionii* são crônicas e aparecem como micoses na pele, tecidos subcutâneos e unhas (ROY et al., 2015; DENG et al., 2014; ROSEN et al., 2016; CORREIA et al., 2010).

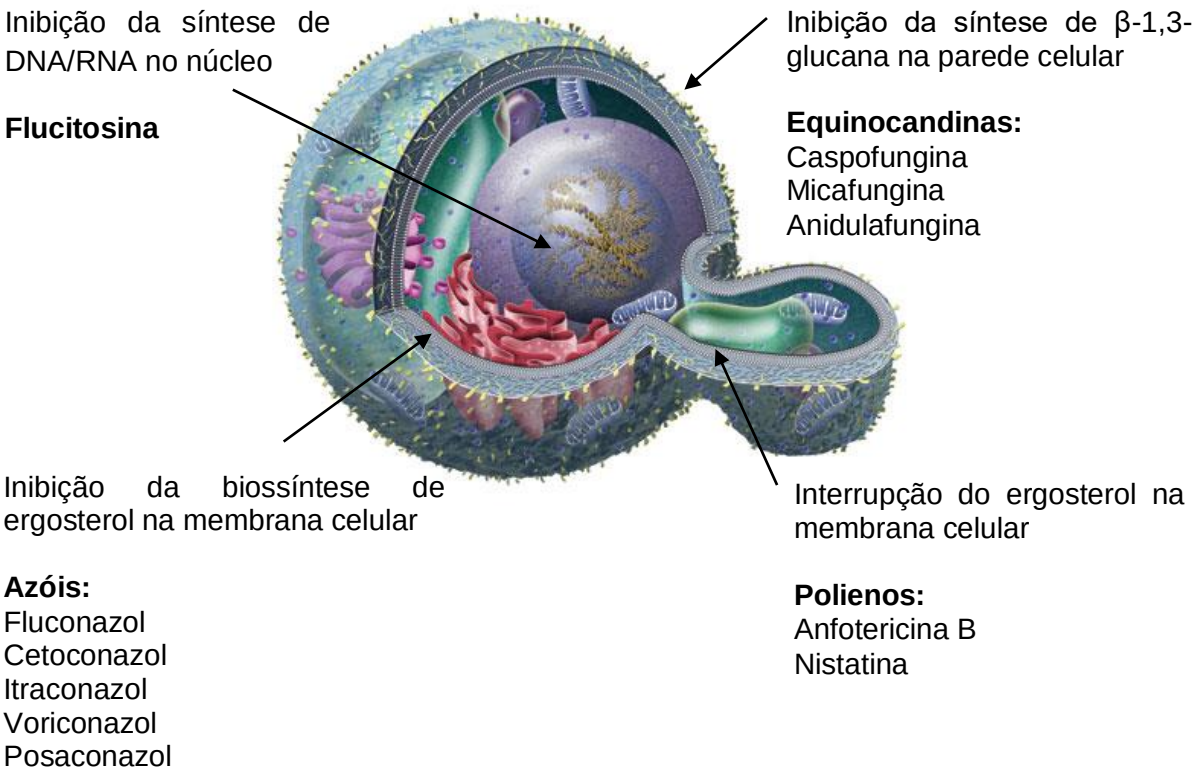
Casos de cromoblastomicose causada por *C. carrionii* são comumente encontrados na Austrália, Venezuela, Costa Rica, Colombia, Cuba, Madagascar e Brasil (DE HOOG et al., 2007; ROSEN et al., 2016). No Brasil, o Rio Grande do Sul, São Paulo, Rio de Janeiro, Minas Gerais e estados da região amazônica são áreas endêmicas (MOUCHALOUAT et al., 2008; CORREIA et al., 2010).

3.3 Terapia antifúngica

Nas últimas décadas, o aumento do número de pacientes imunocomprometidos levou ao aparecimento de muitas formas de infecções fúngicas. Sendo, atualmente, essas infecções são uma das principais causas de morbidade e mortalidade. Além disso, há uma quantidade restrita de antifúngicos disponível e um aumento no desenvolvimento da resistência a drogas antifúngicas (SCORZONI et al., 2016). Sendo assim, o início precoce da terapia antifúngica correta tem um impacto direto no resultado do paciente (PATEL et al., 2009).

Os agentes terapêuticos atuais podem ser amplamente classificados em dois grupos: o primeiro, os antibióticos antifúngicos que ocorrem naturalmente, tais como os poliênicos e as equinocandinas, e o segundo, os fármacos sintéticos, incluindo os azóis e as pirimidinas fluoradas. Os principais alvos das drogas antifúngicas são a parede celular, membrana plasmática e núcleo da célula fúngica, conforme figura 5 (MURRAY et al., 2014).

Figura 5 - Alvos de drogas antifúngicas na parede celular, membrana e núcleo.



Fonte: Adaptado de https://www.sciencenews.org/article/i-mold.

Os derivados poliênicos (nistatina e anfotericina B), introduzidos na década de 1950, representam a mais antiga família de fármacos antifúngicos. A atividade antifúngica dos poliênicos é mediada por meio da sua ligação ao ergosterol, um componente essencial da membrana citoplasmática fúngica (MATHEW, NATH, 2009). A ligação resulta na formação de canais aquosos e não aquosos que aumentam a permeabilidade da membrana, causando o vazamento de componentes celulares, incluindo proteínas e cátions monovalentes e divalentes, através desses poros, o que leva à perda de potencial de membrana e, finalmente, a morte da célula (CHANDRASEKAR, 2011; MOHR et al., 2008; MATHEW, NATH, 2009; BENNET, 2006).

Contemplando a maior classe de antifúngicos sintéticos, as drogas azólicas são constituídas por duas famílias que diferem no número de átomos de nitrogênio no seu anel azol: os imidazólicos (dois átomos de nitrogênio) como o miconazol, cetoconazol e clotrimazol e econazol; e os triazólicos (três átomos de nitrogênio), como fluconazol, voriconazol, itraconazol, posaconazol e isavuconazol (MURRAY et al., 2014; NETT, ANDES, 2016). De uma forma geral, esta classe impede a síntese do ergosterol (principal componente da membrana dos fungos que confere controle na permeabilidade celular), por inibir a enzima C-14-α-desmetilase, o que causa acúmulo de 14-α-metilesterois (produto tóxico as células fúngicas), levando a inibição do crescimento fúngico (CUENCA-ESTRELLA, 2010).

Outra classe de drogas que também atuam inibindo a síntese do ergosterol são as alilaminas (naftifina e terbinafina) que atuam ligando-se a enzima esqualeno epoxidase, promovendo a redução do ergosterol e acúmulo do esqualeno nas células fúngicas (ODDS et al., 2003). Podem apresentar ação fungistática ou fungicida. O efeito fungistático está relacionado com a síntese de esterol e o efeito fungicida é proporcionado principalmente pelo acúmulo de esqualeno, o que promove a destruição da membrana celular (BIANCALANA et al., 2011; GUERRA et al., 2012).

As equinocandinas representam a mais nova classe de antifúngicos desenvolvida, e incluem a caspofungina, micafungina e anidulafungina (KAUFFMAN, CARVER, 2008; MOHR et al., 2008; MUÑOZ et al., 2010). Diferentemente da anfotericina B e dos azólicos, as equinocandinas têm como alvo farmacológico a parede celular do fungo. Elas atuam interferindo na síntese da parede celular fúngica por meio da inibição não-competitiva da enzima ligada à síntese de β-1,3-glucano,

que é um dos principais componentes da parede celular fúngica (ROGERS, FROST, 2009). A diminuição da síntese de β-1,3-glucano resulta em desestabilização da parede celular fúngica, lise e morte celular (SABLE et al., 2008; CORTÉS; RUSSI, 2011).

A griseofulvina penetra na célula, e no núcleo interagindo com os microtúbulos desfazendo o fuso mitótico, o que inibe a multiplicação do fungo. A flucitosina gera na célula a 5-fluorouracil, que inibe a enzima timidilato-sintetase e consequentemente a síntese de DNA, levando à morte celular (CHANDRASEKAR, 2011). Geralmente esta droga é empregada em associação com a anfotericina B (ODDS et al., 2003).

Em se tratando de infecções por fungos dematiáceos a terapia é prolongada e varia de acordo com a síndrome clínica. Infecção local pode ser curada com excisão isolada, enquanto a doença sistêmica é muitas vezes refratária ao tratamento, sendo necessária, em geral a combinação de terapias (REVANKAR, SUTTON, 2010).

A diversidade de quadros de feo-hifomicose impõe condutas particulares, de modo que o tratamento das feo-hifomicoses é bastante variável no que se refere ao tipo, localização e à extensão da lesão, bem como ao fungo envolvido e ao estado imunitário do paciente, dependendo, portanto da forma clínica (REVANKAR, SUTTON, 2010).

O tratamento de primeira escolha para as feo-hifomicoses é a terapia sistêmica oral com um antifúngico da classe do azólicos, sendo o fármaco de escolha o itraconazol em esquema prolongado (GOPINATHAN et al., 2002; ZHOU et al., 2016). A terbinafina, antifúngico pertencente a classe das alilaminas também tem sido utilizada com sucesso, particularmente em pacientes que não responderam a terapia com azólicos (AGGER, ANDES, BURGERS, 2004).

Em casos de infecções mais graves pode-se associar a anfotericina B e o voriconazol (CHEN et al., 2013). Lesões localizadas devem ser tratadas cirurgicamente ou com infiltrações locais de anfotericina B e para casos disseminados emprega-se a associação de anfotericina B e 5-fluorocitosina (REVANKAR et al., 2002; DENG et al., 2016).

A cromoblastomicose é uma das infecções fúngicas mais difíceis de tratar, sendo o tratamento longo e podendo ter recidivas, sendo assim, o tratamento pode variar dependendo de cada situação e da extensão da lesão. O sucesso do tratamento para cromoblastomicose está relacionado com o agente causador, a

forma clínica e gravidade das lesões. Atualmente existem três modalidades de tratamento, isto é, tratamento físico (crioterapia e exérese cirúrgica), quimioterápico ou a possibilidade da combinação dessas terapias (BONIFAZ, PAREDES-SOLIS, SAUL, 2004; ZANINI, 2012).

A crioterapia promove destruição dos tecidos acometidos por congelamento e alterações da resposta imunológica (MORAES, VELHO, MAGALHÃES, 2008). A cirurgia por exérese deve abranger margem de segurança na superfície e na profundidade. A eletrocirurgia e o laser de CO_2 também podem ser utilizados. A excisão cirúrgica é o modo mais eficaz de terapia, independentemente de se a infecção é do tipo subcutânea ou profundamente invasiva (KINDO et al., 2013).

O tratamento oral com antifúngicos pode ser isolado ou associado com procedimentos cirúrgicos (BONIFAZ et al., 1997). No tratamento farmacológico existem alguns fármacos indicados para o combate dessa doença, sendo o grupo dos azólicos os antifúngicos de primeira escolha para a terapia, sendo o itraconazol o mais utilizado na clínica (BONIFAZ et al., 2001; KUMARASINGHE, 2000; QUEIROZ-TELLES et al., 2009). Atualmente azólicos de novas gerações, como o voriconazol, vem sendo utilizados no tratamento dessas infecções e tem mostrado bons resultados contra os fungos dematiáceos (KINDO et al., 2013).

Uma variedade de outros tratamentos também foram bem sucedidos, incluindo cetoconazol, flucitosina, e anfotericina B (ATTAPATTU, 1997; JACYK et al., 1979; MINOTTO et al., 2001). No entanto, a taxa de cura global foi de apenas 57% em uma grande série de 100 casos do Brasil, apesar do uso de múltiplas modalidades (MINOTTO et al., 2001). Recentemente, a terbinafina tem sido bastante utilizada. Em casos refratários, os melhores resultados foram obtidos com a associação dos antifúngicos itraconazol e terbinafina em doses altas, por 6 a 12 meses, de modo que alguns especialistas recomendam isso como terapia de primeira escolha para a doença moderada a grave (BONIFAZ et al., 2005; TAN et al., 2015).

O uso do itraconazol, para reduzir o tamanho das lesões, combinado com criocirurgia subsequente também representa uma alternativa de tratamento para pacientes com lesões extensas (BONIFAZ, CARRASCO-GERARD, SAUL, 2001; ALMEIDA et al., 2014). Em geral, é recomendado para pequenas lesões, especialmente aqueles longe das dobras para evitar fibrose e retráteis cicatrizes

secundárias. A criocirurgia combinado com antimicóticos é uma alternativa muito eficaz, especialmente para doentes com múltiplas lesões (ROSEN et al., 2016).

Nos países onde os antifúngicos sistêmicos não estão facilmente disponíveis ou são muito caros, o uso de somente de uma forma sistemática de crioterapia durante vários meses levou a boas taxas de cura (CASTRO, PIMENTEL, LACAZ, 2003).

De modo que o sucesso do tratamento está relacionado ao agente causal, à forma clínica e a extensão das lesões (QUEIROZ-TELLES, et al., 2009; GUPTA, TABORDA, SANZOVO, 2002; BONIFAZ, PAREDES-SOLIS, SAUL, 2004).

3.4 Resistência aos antifúngicos

O aumento do uso de antifúngicos induziu uma maior pressão seletiva sobre cepas fúngicas e a resistência antifúngica está se tornando um problema emergente. Por um lado, existe a resistência intrínseca, e por outro lado, o desenvolvimento de resistências secundárias e espécies sensíveis têm sido substituídas por outras resistentes, mudando a epidemiologia das infecções fúngicas (LASS-FLORD, 2009).

De modo que a resistência aos antifúngicos é um problema sério a nível clínico, levando à necessidade de descobrir novos alvos que possibilitem o desenvolvimento de novos antifúngicos. O diagnóstico tardio e o relativo número reduzido de classes de antifúngicos terapeuticamente disponíveis favorecem a mortalidade, que é atribuída às infecções sistêmicas. Aliado a isso, a resistência fúngica aos agentes disponíveis torna-se um problema para alguns grupos de pacientes, especialmente os imunocomprometidos (CANNON et al., 2009).

Os fungos desenvolvem diversos mecanismos para sobreviver à exposição a drogas antifúngicas. Isso é preocupante, devido o limitado número de antifúngicos clinicamente úteis, e a crescente população de indivíduos imunocomprometidos vulneráveis ao risco de infecção fúngica (LAFAYETTE et al., 2010).

Ao contrário dos mecanismos de resistência aos agentes antibacterianos, não há evidência de que os fungos são capazes de destruir ou modificar os agentes antifúngicos como meio de adquirir resistência. Da mesma forma, os genes de resistência antifúngica não são transmissíveis de célula a célula da maneira que ocorre com muitos genes de resistência bacteriana. Entretanto, é evidente que

modificações na membrana plasmática reduzindo a permeabilidade ou captação da droga, alterações estruturais no sítio alvo, aumento no efluxo das drogas ou alteração nos níveis intracelulares dos alvos são mecanismos importantes de resistência a agentes antifúngicos, da mesma forma que eles são à resistência antibacteriana (MURRAY, 2014; DEISING, REIMANN, PASCHOLATI, 2008).

Mecanismos de resistência aos azólicos têm sido descritos tanto antes da exposição a essa classe de antifúngicos quanto de forma adquirida durante a terapêutica (ALCAZAL-FUOLI, MELLADO, 2014). A taxa de resistência adquirida aos azólicos vem aumentando, particularmente para espécies de *Candida*. Durante a última década a frequência de cepas de *C. glabrata* resistentes ao fluconazol aumentou de 9% para 14% (PFALLER et al., 2003; PFALLER et al., 2009). A resistencia cruzada é bem comum, com a maioria dos isolados resistentes ao fluconazol apresentando resistência ao voriconazol (NETT, ANDES, 2016).

Nos últimos anos a frequência de *Aspergillus fumigatus* resistente aos azólicos vem aumentando significativamente, sobretudo na Europa, com as taxas de resistência variando em torno de 20 %, a depender da região geográfica (PERLIN, SHOR, ZHAO, 2015, VAN DER LINDEN et al, 2015)

O principal mecanismo de resistência aos azólicos para *Aspergillus*, *Candida*, e *Cryptococcus* spp envolve a mutação do alvo da droga azólica, a enzima C-14-α-desmetilase (PERLIN, SHOR, ZHAO, 2015). Para *Aspergillus* spp, isso geralmente leva à resistência a todos os medicamentos azólicos. Contudo, para *Candida* spp, a modificação deste alvo da droga pode levar à resistência ao fluconazol sozinho, ou a resistência a um subconjunto de azóis. Um segundo mecanismo de resistência, a supra-regulação de bombas de efluxo, também tem sido demonstrado que promove a resistência aos medicamentos através de uma diminuição dos níveis intracelulares da droga (NETT, ANDES, 2016).

A resistência aos polienos permanece pouco expressiva apesar da utilização extensiva por mais de 30 anos (NETT, ANDES, 2016). A resistência secundária à anfotericina B tem sido descrita em *C. tropicalis*, *C. parapsilosis*, *C. lusitaniae* e *C. haemulonii* (ELLIS, 2002).

Além disso, vários patógenos emergentes, como *A. terreus*, *Fusarium* spp são intrinsecamente resistentes a anfotericina B (ALASTRUEY-ISQUIERDO, 2008; CUENCA-ESTRELLA et al., 1999). O mecanismo de resistência à anfotericina B tem sido associado com uma diminuição do conteúdo de ergosterol em membranas de

fungos, principalmente devido a alterações na via da biossíntese do ergosterol. Também tem sido sugerido que a resistência a anfotericina B pode estar relacionada com a desregulação das mitocôndrias fúngicas (MESA-ARANGO, SCORZONI, ZARAGOZA, 2012).

Os mecanismos de resistência a flucitosina envolvem mudança na enzima citosina permease (codificada pelo gene FCY2), responsável pela internalização da droga na célula fúngica; ou alterações na enzima citosina desaminase (codificada pelo gene FCY1), responsável pela conversão de flucitosina a 5 - fluorouracil; ou mudanças na enzima uracila fosforibosil transferase, que é responsável pela transformação de 5-fluorouracil em monofosfato de 5-fluorouridina (codificada pelo gene FUR1), que impossibilita o comprometimento a nível de RNA e síntese proteica da célula fúngica (ESPINEL-INGROFF, 2008). O que vem sendo observado é que especificamente a maioria destes mecanismos é direcionada a *C. albicans*, porém resistência em *C. glabrata,* também vem sendo evidenciada, por mutações em outros genes (WHITE, 2007; PFALLER, 2012).

A resistência as equinocandinas ainda é pouco expressiva. Entretanto, resistência intrínseca a equinocandinas tem sido descrita em isolados de *Candida parapsilosis,* e *Cryptococcus neoformans* (ZHANEL et al., 1997). Embora seja menos comum, também tem sido relatada resistência adquirida durante o tratamento com essa classe de antifúngico em infecções por *Candida* spp. A maioria dos casos envolvem *C. glabrata* resistente a equinocandina embora outras espécies, tais como *C. albicans, C. tropicalis* e *C. krusei* , também tem se mostrado capaz de desenvolver resistência secundária (DANNAOUI et al., 2012; FORASTIERO et al., 2013). Mutações pontuais são descritas em duas regiões “hot-spot” (HS1 e HS2) do gene FKS1 (codificador do complexo β-1,3-D-glucano sintase) e em menor proporção em FKS2, genes que codificam as enzimas alvo destas drogas, estão associados ao mecanismo de resistência a esses antifúngicos (PARK et al., 2005; PERLIN, 2007; PFALLER, 2012).

Tendo em vista a crescente importância clínica e epidemiológica dispensada às infecções micóticas, o aumento nos casos de resistência fúngica e a necessidade de tratamentos mais eficazes e menos tóxicos para os indivíduos acometidos, numerosas pesquisas vêm sendo desenvolvidas na perspectiva de se obter novos produtos antifúngicos. Essa situação tem encorajado a adoção de novas

terapêuticas, dentre elas o uso mais extenso dos produtos naturais, em especial das plantas medicinais (ODDS et al., 2003; BANSOD, RAI, 2008).

3.5 Produtos naturais

Os produtos naturais são utilizados pela humanidade, desde a antiguidade, como importantes ferramentas nos procedimentos das terapias naturais, objetivando a busca por alívio e cura de doenças através do uso de ervas e consistindo, possivelmente, uma das primeiras formas de utilização dos produtos naturais (MUKHERJEE et al., 2010).

Os produtos naturais e seus derivados têm sido reconhecidos por muitos anos como fontes de agentes terapêuticos e de diversidade estrutural (LAHLOU, 2013). Durante a última década, as plantas medicinais - com as características de vários constituintes, ações e alvos - têm se expandido a nível mundial e ganhou popularidade considerável. Sendo assim, os produtos naturais à base de plantas fornecem uma fonte inigualável de diversidade química para a descoberta de moléculas biologicamente ativas importantes e interessantes (LI, 2010).

Em todo o mundo, o uso de plantas medicinais contribui significativamente com os primeiros cuidados com a saúde, embora seus constituintes químicos nem sempre sejam conhecidos (OLIVEIRA, BARROS, NETO, 2010). Estima-se que aproximadamente 80 % da população mundial empregam frequentemente a medicina tradicional em suas necessidades primárias de saúde, especialmente àquelas que se utilizam de terapias que envolvem o uso de fitoterápicos. (BAGETTA et al., 2010).

Sabe-se que a utilização de plantas medicinais tem aumentado progressivamente em todo o mundo devido a fatores como facilidade de utilização e aquisição. Segundo a Organização Mundial de Saúde (OMS), a medicina tradicional é muito popular em todas as regiões do mundo e seu uso está se expandindo rapidamente, mesmo em países desenvolvidos (EDDOUKS et al., 2012).

As plantas possuem uma vasta capacidade biossintética, o que faz delas uma valiosa fonte de compostos terapêuticos (SCHMIDT et al., 2008). Muitos dos quais constituem modelos para síntese de grande número de fármacos. Sendo assim, estes produtos, encontrados na natureza, revelam grande diversidade em termos de estrutura e de propriedades físico-químicas e biológicas, o que permite a elaboração

de novos fármacos com funções terapêuticas diversificadas (BRESOLIN, CECHINEL FILHO, 2003).

Substâncias derivadas de plantas são utilizadas no tratamento de várias doenças, contudo, o potencial de uso como fonte de novas drogas é ainda pouco explorado. Segundo Braz Filho (2010), estima-se que somente de 5-15% destas espécies foram investigadas, restando assim, uma fonte natural de inúmeros organismos vivos contendo significativo número de substâncias inéditas e forte potencial de produtos naturais bioativos, incluindo-se fontes de matérias-primas para o desenvolvimento de modernas drogas terapêuticas.

Os produtos químicos produzidos pelos vegetais podem ser divididos em dois grandes grupos. Os primeiros, denominados metabólitos primários ou macromoléculas, são essenciais a sobrevivência dos vegetais. Incluem os lipídeos, protídeos, glicídeos e nucleotídeos, que têm funções vitais bem definidas. Os produtos do metabolismo primário, através de rotas biossintéticas diversas e frequentemente desconhecidas, originam, às custas de energia, o segundo grupo de compostos químicos – os metabólitos secundários ou micromoléculas - que geralmente apresentam estrutura complexa, baixo peso molecular, marcantes atividades biológicas e, diferentemente daqueles do metabolismo primário, são encontrados em concentrações relativamente baixas e em determinados grupos de plantas (POSER, MENTZ, 2004).

Os metabólitos secundários possuem propriedades biológicas importantes e estão diretamente envolvidas nos mecanismos que permitem a adequação da planta ao seu meio. As substâncias pertencentes a essa classe de metabólitos possuem diversas funções biológicas, tais como defesa contra herbívoros e microrganismos, proteção contra raios UV, atração de polinizadores ou animais dispersores de sementes (FUMAGALI et al., 2008).

O uso de metabólitos secundários de plantas vem crescendo e conquistando o mercado e a preferência dos consumidores por apresentarem benefícios à saúde, bem como menores impactos ao meio ambiente (PEREIRA et al., 2008).

Os metabólitos secundários apresentam várias atividades biológicas. Muitos são de importância comercial tanto na área farmacêutica quanto nas áreas: alimentar, agronômica e de perfumaria. Entre os metabólitos secundários, os principais grupos encontrados com atividade biológica são os alcalóides, flavonóides, cumarinas, taninos, quinonas e óleos essenciais (PEREIRA, 2006).

Dentre os produtos naturais empregados em abordagens terapêuticas, os óleos essenciais (OE), utilizados frequentemente na aromaterapia, são descritos como produtos com grande potencial terapêutico e farmacológico. (EDRIS, 2007).

3.5.1 Óleos essenciais e terpenos

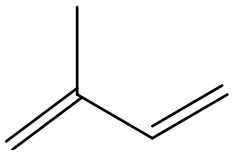
Os óleos essenciais, um complexo aromático que contém uma mistura de diversas moléculas orgânicas, oriundos do metabolismo secundário das plantas apresentam importantes propriedades biológicas exercidas sobre animais, plantas e seres humanos (BASER; BUCHBAUER, 2010); sendo este perfil, um reflexo da sua rica composição química, que varia desde hidrocarbonetos, alcoóis simples e terpênicos, aldeídos, cetonas, fenóis, ésteres até compostos com enxofre (SIMÕES, SPITZER, 2010; VILLA et al., 1998).

Os óleos essenciais podem conter cerca de 20 a 60 componentes em concentrações bastante diferentes. Destes, dois ou três podem ser considerados componentes majoritários por estarem em concentrações bastante elevadas (20 a 70%) em comparação com outros componentes presentes em quantidades mínimas. Geralmente, estes componentes majoritários determinam as propriedades biológicas do óleo essencial (BAKKALI et al., 2008).

A grande maioria dos óleos essenciais é constituída de derivados fenilpropanóides ou de terpenos, sendo estes últimos, os predominantes (SIMÕES; SPITZER, 2010).

Assim sendo, os terpenos se enquadram como o grupo mais importante na composição dos óleos essenciais. Biossinteticamente são derivados de condensação de unidade de isopreno C_5 (Figura 6) a partir da via do ácido mevalônico. Os terpenos são classificados segundo a quantidade de unidades isoprênicas existente no seu esqueleto de carbono. Os terpenos mais frequentemente encontrados são monoterpenos (C_{10}), sesquiterpenos (C_{15}) e diterpenos (C_{20}). No entanto, a partir do momento, em que esses contêm compostos de oxigênio na molécula, tais como grupos hidroxila, carbonila, cetona e aldeído, resultam em compostos denominados como terpenóides (BAKKALLI et al., 2008).

Figura 6 - Isopreno, molécula base dos terpenos.



Fonte: SIMÕES, SPITZER, 2010.

Os terpenos são empregados na indústria na produção de perfumes e cosméticos, além de apresentarem efeitos farmacológicos (EDRIS, 2007). São conhecidos desde a antiguidade por possuir propriedades antifúngicas cuja utilização pode representar um avanço contra os mecanismos de resistência que inativam antifúngicos padrões (CASTRO, 2010; SAAD et al., 2010; TEMPONE et al., 2008).

Os terpenos mais frequentes nos óleos essenciais são os monoterpenos (cerca de 90 % dos óleos voláteis), como o mentol, linalol e citral, presentes na hortelã (*Mentha x piperita,* Lamiaceae), alfazema (*Lavandula angustifolia* L., Lamiaceae) e no capim-limão (*Cymbopogon citratus*, Poaceae) e os sesquiterpenos (SINGH et al., 2015; VALERIANO et al., 2012; KUMAR et al., 2013).

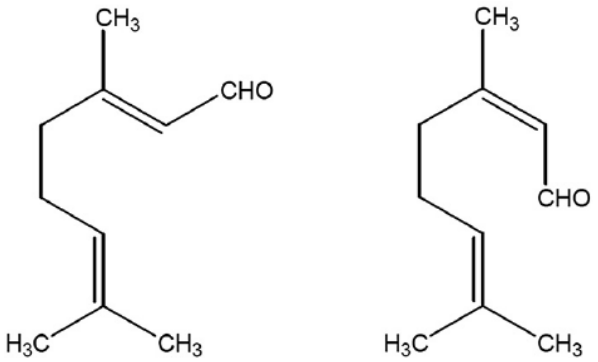
Os monoterpenos podem ser divididos em três subgrupos: acíclicos (mirceno, linalol, geraniol, citral), monocíclicos (alfa-terpineol e terpinoleno) e bicíclicos (alfa-pineno, tujona, cânfora e fenchona). São altamente voláteis, sendo arrastados pelo vapor de água livres de outros componentes, sendo utilizados por suas características organolépticas marcantes (BANDONI, CZEPAK, 2008; SIMÕES, SPITZER, 2010).

E embora sejam moléculas estruturalmente simples, são atribuídas várias atividades farmacológicas aos terpenos (CERQUEIRA et al., 2011; SANTOS et al., 2011; SIQUEIRA et al., 2006).

3.5.1.1 Citral

O Citral (3,7-dimetil-2,6-octadienal) é uma mistura de dois monoterpenos aldeídos acíclicos isômeros geométricos - geranial (trans-citral ou citral A) e neral (cis-citral ou citral B) (Figura 7) (SADDIQ, KHAYYAT, 2010).

Figura 7 – Estrutura química do *cis*- e *trans*-citral



Geranial (*trans*-citral) Neral (*cis*-citral)

Fonte: MANGPRAYOOL, SAJEERA, NUANNOI, 2013.

Como um monoterpeno acíclico natural, o citral pode ser encontrado nos óleos de diversas plantas aromáticas, empregadas na medicina popular como as espécies de *Cymbopogon citratus* (KUMAR et al., 2013), *Melissa officinalis*, (MENEZES et al., 2015), *Lippia Alba* (STASHENKO et al., 2014), *Zingiber officinale* (FASS et a., 2014).

Têm sido relatados inúmeros efeitos farmacológicos, em especial importantes atividades antimicrobianas, contra *Escherichia coli* (SOMOLINOS et al., 2009; BELDA-GALBIS et al., 2013, SHI et al., 2016), *Listeria innocua* (BELDA-GALBIS et al., 2013), *Staphylococcus aureus, Penicillium italicum* e *Rhizopus stolonifer* (SADDIQ, KHAYYAT, 2010), *Colletotrichum musae*, *Colletotrichum gloeosporioides* e *Fusarium subglutinans* (GARCIA et al., 2008), *Penicillium digitatum* (FAN et al., 2014), *Trichophyton mentagrophytes* (PARK et al., 2009), *Aspergillus flavus, Aspergillus fumigatus,* (LUO et al., 2004; MESA-ARANGO et al., 2009) *Geotrichum citri-aurantii (*ZHOU et al*.,* 2014) e *Candida* spp. (FERREIRA et al., 2009; LEITE et al., 2014; LIMA et al., 2012; SOUSA et al., 2016), atividade anti-*Trypanosoma cruzi* (CARDOSO, SOARES, 2010, ARMAS et al., 2016) e anti-*Leishmania* (MACHADO et al., 2012).

O citral é geralmente reconhecido como aditivo alimentar seguro, e foi aprovado para uso em alimentos pelo Food and Drug Administration (FDA). Além disso, o citral foi registrado por Comissão Europeia para o uso como aromatizante em alimentos porque o seu uso não apresenta risco para a saúde do consumidor

(BURT, 2004). E pode tornar-se um ingrediente antimicrobiano adequado para uso mais amplo na indústria de alimentos (SOMOLINOS et al., 2009).

Além dessas importantes atividades biológicas, estudos relatam a baixa toxicidade deste monoterpeno. Em um estudo de 24 h sobre a toxicidade aguda oral em dose única, a DL_{50} do óleo essencial de *Cymbopogon citratus*, cujo constituinte majoritário é o citral, foi cerca de 3500 mg/kg. No estudo de 21 dias de toxicidade oral de dose repetida, não foram observadas alterações significativas no peso corporal, pesos de órgãos, histologia (cérebro, coração, rins, fígado, pulmões, estômago, baço e bexiga urinária), urina ou bioquímica clínica em ratos. Da mesma forma, os dados do ensaio cometa em células de sangue periférico não mostrou qualquer efeito genotóxico (COSTA et al., 2011).

Estudos realizados por Ganev (2010) investigaram os possíveis efeitos tóxicos cumulativos do citral devido a utilização aguda, por 14 dias. Para tanto, avaliou parâmetros macroscópicos de toxicidade, como evolução do peso corporal, mortalidade, e normalidade dos órgãos (coração, pulmões, fígado, rins e fígado). Em todos os parâmetros toxicológicos analisados, o citral não demonstrou alterações quando comparado ao grupo controle.

Lima (2011) observou que o citral apresentou baixa toxicidade frente ao experimento de toxicidade aguda. Não demonstrou efeito oxidante nem antioxidante, quando testados com as hemácias humanas. Mas apresentou citoxicidade frente às células de mamíferos MDCKs (Madin-Darby canine kidney).

Portanto, o citral, considerado molécula de valor comercial, configurada ao papel de ingrediente em perfumes, produtos de higiene, cosméticos e em alimentos como flavorizantes (MARQUES et al., 2013; XIA et al., 2013); somada a sua baixa toxicidade e ao perfil ativo do qual vem sendo relatado, permite vislumbrar o seu grande potencial ao desenvolvimento de um novo medicamento com possível propriedade antifúngica e que possa ser utilizado na terapêutica de infecções fúngicas.

Apesar de muitos relatos sobre as propriedades antimicrobianas do citral, existem poucas investigações sobre a sua atividade sobre fungos dematiáceos, o modo de ação antifúngica deste monoterpeno e estudos de associação dele com antifúngicos, o que o torna um interessante alvo de estudo.

Considerando a importância das infecções fúngicas, especialmente as causadas por fungos dematiáceos, e as dificuldades no seu tratamento, bem como o

aumento da resistência aos antifúngicos, torna-se necessário o estudo de novos fármacos naturais e/ou sintéticos com propriedades antifúngicas. Neste contexto, o presente estudo se mostra de grande relevância, uma vez que pretende investigar novas alternativas terapêuticas para o tratamento de doenças fúngicas, com amplo espectro de ação, pouco tempo de uso e mínimos efeitos adversos.

Material e Métodos

4 MATERIAL E MÉTODOS

4.1 Local de Trabalho

As atividades laboratoriais propostas neste estudo foram realizadas no Laboratório de Micologia, Departamento de Ciências Farmacêuticas, Centro de Ciências da Saúde, Universidade Federal da Paraíba.

4.2 Espécies Fúngicas

Para realização dos ensaios de atividade antifúngica foram selecionadas três cepas de *Cladophialophora carrionii* (URM 2871, 0212, CQ02), três cepas de *Cladosporium oxysporum* (URM 5234, URM 6056, URM 5412), quatro cepas de *Cladosporium sphaerospermum* (URM 5962, URM 5455, URM 5350, URM 6120) e uma cepa de *Cladosporium cladosporioides* (INCQS 40188), sendo as cepas *C. carrionii* URM 2871, *C. oxysporum* URM 5234, URM 6056, URM 5412 e *C. sphaerospermum* URM 5962, URM 5455, URM 5350, URM 6120 pertencentes à coleção de culturas da Micoteca do Departamento de Micologia (URM), Centro de Ciências Biológicas, Universidade Federal de Pernambuco, as cepas *C. carrionii* 0212 e CQ02 pertencentes à coleção de culturas do Laboratório de Micologia, Departamento de Ciências Farmacêuticas, Centro de Ciências da Saúde, Universidade Federal da Paraíba. E a cepa *C. cladosporioides* INCQS 40188 pertencente à Coleção de Microrganismos de Referência em Vigilância Sanitária (CMRVS), Instituto Nacional de Controle de Qualidade em Saúde (INCQS), Fundação Oswaldo Cruz (FIOCRUZ).

Todas as cepas foram mantidas em Ágar Sabouraud Dextrose – ASD inclinado (DIFCO Laboratories Ltda, USA) a temperatura ambiente (28 ºC) e sob refrigeração (4 ºC).

4.3 Fitoconstituíntes

Os fitoconstituíntes (citral, (-) citronelal, (+) citronelal, β-cariofileno, geraniol, linalool, β-cimeno e α-pineno) utilizados nesta pesquisa foram adquiridos da Sigma-

Aldrich® Ltda (São Paulo, SP, Brasil). Os fitoconstituintes foram testados na concentração inicial de 1024 µg/mL e solubilizados em 2 % de Tween 80 (INLAB®) e dimetilsulfóxido (DMSO) (MERCK®) numa proporção de até 0,5% do volume final.

4.4 Antifúngicos sintéticos

Para o controle de avaliação da atividade antifúngica dos produtos naturais testados, foram utilizados como padrão os seguintes antifúngicos sintéticos: anfotericina B, itraconazol e voriconazol, obtidos da Sigma-Aldrich® (São Paulo-SP).

As soluções foram preparadas no momento da execução dos testes, para alcance das concentrações desejadas. Os antifúngicos foram testados na concentração inicial de 1024 µg/mL e solubilizados em dimetilsulfóxido (DMSO) (MERCK®) numa proporção de até 0,5% do volume final.

4.5 Meios de Cultura

Para manutenção das cepas selecionadas e realização dos ensaios de atividades biológicas foram usados o meio sólido Ágar Sabouraud Dextrose - ASD (DIFCO Laboratories Ltda, USA), o Caldo Sabouraud Dextrose – CSD (DIFCO Laboratories Ltda, USA), e o caldo RPMI-1640-L-glutamina, sem bicarbonato de sódio, adquirido da Sigma-Aldrich, São Paulo, SP, Brasil.

Os meios foram solubilizados em água destilada e esterilizados em autoclave, a 121 ^{o}C, 1 atm por 15 minutos, conforme orientações do fabricante.

4.6 Inóculo

Para preparação do inóculo, as cepas fúngicas selecionadas foram mantidas no meio de cultura, por 10-14 dias, a temperatura de 28 ºC para atingirem um crescimento satisfatório. As recentes colônias fúngicas foram devidamente cobertas com 5 mL de solução salina estéril (NaCl 0,85 % p/v), e as suspensões feitas por suaves agitações e raspagens com auxílio de uma alça de platina em “L”. A mistura resultante de conídios e fragmentos de hifas foi retirada e transferida para tubos de

ensaio esterilizados (FERNÁNDEZ-TORRES et al., 2002; SANTOS, HAMDAN, 2005).

Em seguida, essas suspensões foram agitadas por 2 minutos com auxílio do aparelho Vortex (FANEM). Após agitação, cada suspensão teve sua turbidez comparada e ajustada àquela apresentada pela suspensão de sulfato de bário do tubo 0,5 da escala McFarland, a qual corresponde a um inóculo de aproximadamente 10^6 unidades formadoras de colônias/mL (UFC/mL). Por fim, foi realizado contagem celular em câmara de Neubauer e as suspensões ajustadas no espectrofotômetro (Leitz-Fhotometer 340-800), para conter aproximadamente $1x10^6$ UFC/mL. (CLEELAND, SQUIRES, 1991; HADACEK, GREGER, 2000; ODDS, 1989; SAHIN et al., 2004).

4.7 Triagem microbiológica

Para avaliação da atividade antifúngica, realizou-se uma triagem dos fitoconstituintes citral, (-)-citronelal, (+)-citronelal, β-caryophyllene, geraniol, linalool, β-cymeno, α-pineno pela técnica de microdiluição em caldo, conforme metodologia descrita no item 4.9.1. O monoterpeno citral foi selecionado para dar continuidade aos estudos por apresentar melhor atividade antifúngica sobre as 11 cepas testadas.

4.8 Ensaios *in silico*

4.8.1 PASS online

Previsão do espectro de atividade para substâncias (PASS) online é um software projetado para avaliar o potencial biológico geral de uma molécula orgânica sobre o organismo humano. Ele fornece previsões simultâneas de muitos tipos de atividades biológicas com base na estrutura dos compostos orgânicos. O espectro de atividade biológica de um composto químico é o conjunto de diferentes tipos de atividade biológica, que refletem os resultados de interação do composto com várias entidades biológicas. *Pass online* dá várias facetas da ação biológica de um composto, obtendo os índices Pa (probabilidade " de ser ativo") e Pi (probabilidade "de ser inativo") estimando a categorização de um composto potencial em ser

pertencente à subclasse de compostos ativos ou inativos, respectivamente (SRINIVAS et al., 2014).

4.8.2 Análise teórica dos parâmetros farmacocinéticos

Para a análise dos parâmetros farmacocinéticos a estrutura do citral foi submetido ao estudo *in silico* usando o programa Osíris Property Explorer (http://www.organic-chemistry.org/prog/peo/drugScore.html).

Nesta análise foram determinados o potencial de *druglikeness* e o *drug-score* que são relacionados a descritores topológicos, e outras propriedades como cLogP e massa molecular (TECKTO, 2005).

Na análise dos parâmetros farmacológicos, foi avaliado a biodisponibilidade oral teórica do citral, pela “Regra dos Cinco” de Lipinski, que estabelece que pelo menos três de quatro requisitos deva ser apresentados para que o composto possua uma boa biodisponibilidade. Assim, para que compostos sejam absorvidos, devem possuir peso molecular < 500 daltons (Da), coeficiente de partição octanol/água calculado (cLogP) < 5, número de aceptores de ligação hidrogênio (nALH) ≤ 10 e número de grupos doadores de ligação hidrogênio (nDLH) ≤ 5 (LIPINSKI et al., 2001). A esta predição, foi empregada o programa Molinspiration Cheminformatics (http://www.molinspiration.com/cgi-bin/properties).

4.9 Ensaios de atividade antifúngica *in vitro* sem alvos específicos

4.9.1 Determinação da Concentração Inibitória Mínima (CIM)

A determinação da concentração inibitória mínima foi realizada utilizando-se o Citral, fitoconstituínte selecionado na triagem microbiológica, frente oito cepas de *Cladosporium* spp. e três cepas de *C.carrionii*.

Os ensaios foram realizados por meio da técnica de microdiluição em caldo, utilizando placas de 96 orifícios estéreis e com tampa (CLEELAND, SQUIRES, 1991; ELOFF, 1998; HADACEK, GREGER, 2000).

Em cada orifício da placa, foi adicionado 100 µL do meio líquido caldo RPMI-1640-L-glutamina, sem bicarbonato de sódio. Em seguida, 100 µL da emulsão do

citral na concentração inicial de 2048 µg/mL foram dispensados nas cavidades da primeira linha da placa. E por meio de uma diluição seriada em razão de dois, foram obtidas as concentrações de 1024, 512, 256, 128, 64, 32, 16, 8 e 4 µg/mL, de modo que na primeira linha da placa encontrava-se a maior concentração e na última, a menor concentração. Por fim, foi adicionado 10 µL do inóculo de aproximadamente 1-5 x 10^6 UFC/mL das espécies fúngicas nas cavidades, onde cada coluna da placa correspondeu a uma cepa fúngica, especificamente. Paralelamente, foi realizado o mesmo ensaio com os antifúngicos anfotericina B, itraconazol e voriconazol nas mesmas concentrações.

Para verificar a ausência de interferência nos resultados pelos solventes, DMSO e Tween 80, utilizados na preparação da emulsão do fitoconstituínte, foi realizado um controle no qual foram colocados nas cavidades 100 µL do caldo RPMI-1640-L-glutamina, DMSO (0,5 %), Tween 80 (2 %) e 10 µL da suspensão fúngica. Um controle de microrganismo foi realizado colocando-se nas cavidades 100 µL do caldo RPMI-1640-L-glutamina, 100 µL de água destilada estéril e 10 µL do inóculo de cada espécie. Também foi realizado um controle de esterilidade do meio, no qual foi colocado 200 µL do caldo RPMI-1640-L-glutamina em um orifício, na ausência da suspensão dos fungos.

As placas foram assepticamente fechadas e incubadas a 28 ^oC por 5 dias (e confirmada em 7 dias), sem anaerobiose, para realização da leitura. As CIMs para o citral e os antifúngicos foram definidas como a menor concentração capaz de inibir visualmente o crescimento fúngico verificado nos orifícios quando comparado com o crescimento controle. Os ensaios foram realizados em duplicata e o resultado expresso pela média aritmética das CIM's obtidas nos dois ensaios. (SANTOS, HAMDAN, 2005).

Com base nos resultados da CIM os produtos naturais podem ser classificados quanto ao seu potencial antimicrobiano, onde substâncias com CIM ≤ 500 µg/mL são consideradas com forte atividade antimicrobiana, com 500 µg/mL < CIM ≤ 1500 µg/mL possuem moderada atividade e CIM > 1500 µg/mL são consideradas com fraca atividade (SARTORATTO et al., 2004).

4.9.2 Determinação da Concentração Fungicida Mínima (CFM)

A concentração fungicida mínima do citral foi determinada para as 11 cepas em estudo, pelo método de microdiluição (DENNING et al., 1992; RASOOLI, ABYANEH, 2004). Após a leitura da CIM em 7 dias, alíquotas de 20 µL foram retiradas de cada cavidade da placa de microdiluição onde não houve crescimento fúngico e foram transferidas para placas de 96 cavidades previamente preparadas com 100 uL do caldo RPMI-1640. As placas foram seladas em condições assépticas e incubadas a 28 ºC por 5 dias. A CFM foi definida como a menor concentração do citral, em que não houve crescimento, visível quando subcultivadas em placas de 96 poços contendo caldo RPMI-1640 sem produtos antifúngicos. Paralelamente, foi realizado o mesmo ensaio com os antifúngicos anfotericina B e voriconazol. Os experimentos foram realizados em duplicata.

Por meio da razão entre CFM/CIM podemos descobir a natureza da ação de uma substância com atividade antifúngica, podendo esta ter um efeito fungistático ou fungicida. Caso a razão resulte num valor ≤ 4 o efeito é fungicida, no entanto se for > 4 a substância tem perfil fungistático (SIDDIQUI et al., 2013).

Sendo assim, após a determinação da CIM e CFM, foram selecionadas 4 cepas frente as quais o citral apresentou um efeito fungicida (*C. carrionii* URM 2871, *C. oxysporum* URM 5234, *C. sphaerospermum* URM 6120 e *C. cladosporioides* INCQS 40188) para dar continuidade ao estudo de atividade antifúngica desse monoterpeno.

4.9.3 Determinação da cinética de crescimento micelial

A inibição do crescimento micelial fúngico foi determinada utilizando-se a técnica de diluição em meio sólido. Este estudo baseou-se na medida do crescimento micelial radial em ASD adicionado do monoterpeno nas concentrações CIM, CIM x 2 e CIM x 4, em diferentes intervalos de tempo. Para a execução da técnica, inicialmente, uma porção de 2 mm de diâmetro foi tomado de uma cultura com crescimento de 7-14 dias em Ágar Sabouraud Dextrose, a 28 ^{o}C, e colocada no centro de uma placa de Petri estéril com meio ASD adicionado do citral, nas diferentes concentrações. O sistema foi incubado a temperatura ambiente. Em

diferentes intervalos de tempo (0, 2, 4, 6, 8, 10, 12 e 14 dias) após incubação, o crescimento micelial radial da colônia fúngica foi medido e o resultado expresso em milímetros (mm).

Os controles foram realizados por meio da medida do crescimento micelial em ASD na ausência do citral, ou adicionado com anfotericina B ou voriconazol (CIM, CIM x 2 e CIM x 4). Foram realizados dois experimentos independentes em diferentes ocasiões e os resultados representam a média ± erro padrão dos dois experimentos (ADAN et al., 1998; THYÁGARA, HOSONO, 1996; DAFERERA, ZIOGAS, POLISSION, 2003).

4.9.4 Interferência sobre a germinação de conídios

Para avaliar a interferência do citral, voriconazol e anfotericina B sobre a germinação de conídios, diferentes concentrações dos produtos teste correspondentes a CIM, CIM x 2 e CIM x 4 foram adicionados em ependorf estéreis contendo 500 µL de caldo RPMI-1640, em seguida foram homogeneamente misturadas com 500 µL da suspensão dos conídios fúngicos determinada em camara de Neubauer e ajustada a 10^6 conídios/mL. Foi utilizado um controle de microrganismos apenas com água destilada. As amostras foram incubadas a 28 ^oC e após 48 h o número de conídios germinados e não germinados foi determinado em camara de Neubauer e o percentual de inibição da germinação dos conídios foi calculado, comparando os resultados obtidos no experimento teste com os resultados do experimento controle. A análise foi realizada em microscópio óptico comum (Zeiss® model Primo Star). Cada análise foi realizada em duplicata e os resultados expressos como a média das duas repetições (SURENDER et al., 1987; RANA et al., 1997; SHARMA, TRIPARTHI, 2008).

4.10 Ensaios de atividade antifúngica *in vitro* com alvos específicos

4.10.1 Ação do citral na parede celular fúngica

Para investigar a ação do citral sobre a parede celular fúngica foi realizado ensaio com sorbitol, um protetor osmótico usado para estabilizar os protoplastos de fungos. Este método baseia-se na medida dos danos que produtos com atividade

antifúngica produzem aos componentes da parede celular fúngica. Caso o produto atue de alguma forma sob a parede celular do fungo, ele provocará lise de suas células quando na ausência de um estabilizador osmótico, mas permitirá seu crescimento na presença desse suporte osmótico. Dessa maneira, este ensaio compara as CIM's dos produtos antifúngicos na ausência e presença de sorbitol a 0,8 M.

A determinação da CIM do citral, na presença do sorbitol (0,8 M), foi realizada pelo método de microdiluição, utilizando placas de microtitulação contendo 96 cavidades, com fundo em forma de "U" e em duplicata, semelhante ao item 4.9.1. Em cada orifício da placa, foram adicionado 100 µL do meio líquido CSD previamente adicionado de sorbitol (PM = 182,17) (Sigma-Aldrich®), ambos duplamente concentrados. Posteriormente, 100 µL da solução dos produtos, também duplamente concentrados, foram dispensados nas cavidades da primeira linha da placa. E por meio de uma diluição seriada a uma razão de dois, foram obtidas as concentrações dos produtos e, no caso do sorbitol, uma concentração final de 0,8 M em cada cavidade. Por fim, foram adicionados 10 µL do inóculo das espécies nas cavidades, onde cada coluna da placa refere-se a uma cepa fúngica, especificamente. Um controle de microrganismo foi realizado colocando-se nas cavidades 100 µL do mesmo CSD e sorbitol (0,8 M), 100 µL de água destilada estéril e 10 µL do inóculo de cada espécie. Um controle de esterilidade também foi realizado, onde foi colocado 200 µL do CSD em um orifício sem a suspensão dos fungos. As placas foram assepticamente fechadas e incubadas a 28 °C por 5 dias para ser realizada a leitura (FROST et al., 1995; ZACCHINO, 2001).

4.10.2 Ação do citral na membrana celular – Interação com ergosterol

Muitos fármacos disponíveis para o uso clínico interagem diretamente com o ergosterol, ocasionando a ruptura da membrana celular fúngica e perda de conteúdo intracelular. Para determinar se o citral se liga a esteróis da membrana fúngica, a CIM deste produto foi determinada com e sem a adição de ergosterol exógeno. Se a atividade do produto for causada pela ligação ao ergosterol, o ergosterol exógeno impedirá a ligação com o ergosterol da membrana fúngica e como consequência, a CIM desse produto tende a aumentar na presença do ergosterol exógeno em relação ao ensaio controle. Caso a CIM do produto permaneça inalterada na

presença de diferentes concentrações exógenas de ergosterol, sugere-se que este composto não age ligando-se ao ergosterol da membrana.

A determinação da CIM do produto contra cepas de *Cladosporium* spp. e *Cladophilophora carrionii* foi realizada por microdiluição, utilizando placas de microdiluição contendo 96 cavidades, com fundo em forma de “U” e em duplicata semelhante ao protocolo exposto no item 4.9.1. O meio de cultura foi utilizado na ausência e na presença de 400 µg/mL de ergosterol (Sigma-Aldrich®). Um controle de microrganismo foi realizado colocando-se nas cavidades 100 µL do mesmo meio de cultura e ergosterol nas mesmas concentrações e 10 µL do inóculo de cada espécie. Um controle de esterilidade também foi realizado, onde foi colocado 200 µL do meio de cultura em um orifício sem a suspensão dos fungos. As placas foram seladas e incubadas a 28 °C por 5 dias para ser realizada a leitura (ESCALANTE et al., 2008).

4.11 Análise da associação do citral com antifúngicos padrões

4.11.1 Determinação do Índice de Concentração Inibitória Fracionada (Método de *Checkerboard*)

As associações do citral com os antifúngicos comerciais (anfotericina B ou voriconazol) foram testadas em triplicata contra *C. carrionii* URM 2871, *C. oxysporum* URM 5234, *C. sphaerospermum* URM 6120 e *C. cladosporioides* INCQS 40188), utilizando a técnica de microdiluição *checkerboard* (ELIOPOULOS, MOELLERING, 1991). Inicialmente, 100 µL de caldo foram adicionados nos poços da placa de microdiluição. Em seguida, foi adicionado o monoterpeno no sentido vertical e o antifúngico (anfotericina B ou voriconazol) no sentido horizontal da placa. Cada produto foi testado em diferentes concentrações (CIM x 8, CIM x 4, CIM x 2, CIM, CIM ÷ 2, CIM ÷ 4 e CIM ÷ 8). Por fim, foi adicionado 10 µL da suspensão fúngica. As placas foram incubadas a 28 ºC por 5 dias.

Para avaliar a interação de cada combinação, o ICIF (Índice da Concentração Inibitória Fracionada) foi calculado através da soma do CIF^A + CIF^B, onde A representa o citral e B a anfotericina B ou voriconazol. O CIF^A, por sua vez, é calculado através da relação CIM^A combinado/CIM^A sozinho, enquanto que o CIF^B =

CIM^B combinado/CIM^B sozinho. Este índice é interpretado da seguinte forma: sinergismo (≤0,5), aditividade (>0,5 e <1), indiferença (≥1 e <4) ou antagonismo (≥4,0) (LEWIS et al., 2002).

4. 12 Análise Estatística

Os resultados obtidos nos experimentos tiveram seus valores expressos em média ± erro padrão da média (e.p.m.) e analisados empregando-se o teste *t* de Student não pareado, para análise de duas colunas. Os resultados foram considerados significativos quando $p < 0,05$. Para a análise dos dados utilizou-se o programa estatístico GraphPad Prisma versão 5.0®.

Resultados e Discussão

5. 1 Antifungal Activity of Phytochemicals against Species of *Cladosporium* and *Cladophialophora*

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Original Research Article

Antifungal Activity of Phytochemicals against species of *Cladosporium* and *Cladophialophora*

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ABSTRACT

Cladosporium species are ubiquitous, saprobic dematiaceous fungi, associated with human and animal opportunistic infections. *Cladosporium* has been known to be one of the most airborne fungi causing respiratory allergies diseases, particulary asthma and rhinitis. Antifungal compounds of natural origin, such as terpenes, have received much attention in recente times. They are a promising therapeutic tool for treating fungal infections, and are known for their antimicrobial properties. **Aims:** In this context, the present study aims to evaluate the in vitro antifungal activity of eight phytochemicals commonly found in *Melissa officinalis* L. essential oil (citral, (-) citronelal, (+) citronelal, β-caryophyllene, geraniol, linalool, β-cymene, α-pinene) against ten species of *Cladosporium* and *Chadophialophora*. **Methodology:** Antifungal susceptibility testing was performed with the phytochemicals at a concentration of 1.024 µg/mL. Microbiological screening was performed based on the broth microdilution technique. **Results:** Through analysis of results, it is observed that citral showed the best activities of the as species of *Cladosporium* studied. **Conclusion:** citral representing a new possibility in the arsenal of products for treatment of fungal infections caused by these fungi.

Keywords: *Phytochemicals; Citral; Antifungal; Cladophialophora carrionii, Cladosporium oxysporum, Cladosporium sphaerospermum.*

1. INTRODUCTION

Fungal infections are becoming more frequent because of expansion of at-risk populations and use of treatment modalities that permit longer survival of these patients [1].

Cladosporium species are among the most common fungal inhabitants worldwide, being isolated from almost any environmental source and geographic location [2].

The most common *Cladosporium* species are primarily isolated from soil and plant material, where they are frequently encountered as saprobes or secondary invaders on follicular lesions, concomitant with other plant pathogenic fungi [2-4]. However, several species are important pathogens of plants and some are also able to affect animals including humans [5-7].

Cladosporium is usually associated with allergic rhinitis and asthma [8,9] or localized superficial or deep lesions [10-13], but rarely can cause disseminated infections [14,15]. They are difficult to treat due to long treatment periods, limited treatment options, resistance to common antifungal agents, and their greater prevalence among immunocompromised patients. All of these characteristics invite recurrences [16,17].

There exists a clear need for more, therapeutically effective antifungals. Actually, plants have been an interesting alternative to source of new biologically active compounds [18-20]. The plants produce numerous and varied organic compounds including monoterpenes and sesquiterpenes compounds present in essential oils, of which the majority does not directly participate in the plant's growth and development and are generally called secondary metabolites [21,22]

Melissa officinalis L., member of Lamiaceae family, is one of the well known aromatic medicinal plant species. The essential oil is a well-known antibacterial and antifungal agent [23-25]. There have been some previous reports on the chemical constitutions of *M. officinalis.* According to these studies, the major components of the essential oil of *M. officinalis* were citral (geranial and neral), and citronellal [25,26], (*B*)-caryophyllene [27], caryophyllene oxide [27-28], linalool [29], geraniol [30], thymol [31], α-pinene [27], β-pinene [28,32], carvacrol and *iso*-menthone [33].

Previous studies in our laboratory with *M. officinalis* L. essential oil showed strong antifungal activity of this oil against *C. carrionii* strains (Menezes *et al*., 2015). Therefore, the aim of the present work was to investigate the antifungal activity of eight phytochemicals commonly found in *M. officinalis* L. essential oil against strains *of Cladosporium.*

2. MATERIAL AND METHODS

2.1 Phytochemicals and Synthetic Antifungal

The phytochemicals (citral, (-) citronelal, (+) citronelal, β-caryophyllene, geraniol, linalool, β-cymene, α-pinene) and amphotericin B (standard drug) were acquired from Sigma-Aldrich®. All them were dissolved in 2% Tween 80 (INLAB®) and up to 0.5 % dimethyl sulfoxide – DMSO (MERCK®) in sterile distilled water to obtain 2.048 µg/mL solutions.

2.2 Cladosporium Samples

For testing of antifungal activity were selected and used ten species of *Cladosporium. Cladophialophora carrionii* strains (URM 2871, 0212, CQ 02), *Cladosporium oxysporum* strains (URM 5234, URM 6056, URM 5412) and *Cladosporium spherospermum* strains (URM 5962, URM 5455, URM 5350, URM 6120) were taken from the Microorganisms Collection of the Mycology Laboratory, at the Department of Pharmaceutical Sciences, Health Sciences Center, Federal University of Paraíba, Brazil and from the Pernambuco (Brazil) Federal University, Biological Sciences Center – Mycology Department fungal collection (URM).

The fungal cultures were maintained on Sabouraud Dextrose Agar - SDA (DIFCO®) at room temperature (28° to 30°C) and under refrigeration (4°C).

2.3 Inoculum

Stock inoculate suspensions of the *Cladosporium* strains were prepared from 10-days old sabouraud dextrose agar (Difco Lab., USA) cultures grown at 28 ºC. Fungal colonies were covered with 5 mL of sterile saline solution (0.9 %), the surface was gently scraped with a sterile loop, and the saline solution with the fungal elements was transferred to a sterile tube. These suspensions were shaken for 2 min using a vortexer, and allowed to stand for 5 min to allow hyphal fragments to fall out of the suspensions so that the supernatant containing the conidia could be collected. Tubes containing the inocula were standardized to 0.5 McFarland scale (1-5 x 10^6 CFU/mL). Mold conidia were counted using a hemocytometer. The inocula of the conidial suspensions were adjusted using sterile NaCl 0.9 % to contain approximately 10^6 CFU/mL [34,35, 36].

2.4 Antifungal susceptibility testing

Antifungal susceptibility testing was performed with the phytochemicals (citral, (-) citronelal, (+) citronelal, β-caryophyllene, geraniol, linalool, β-cymene, α-pinene) at a concentration of 1.024 µg/mL. Antifungal susceptibility testing was performed based on the broth microdilution technique [34,36]. Sterile 96-U-shaped-well microplates were used and each well of the plates contained 100 µL of Sabouraud dextrose broth - SDB (DIFCO®). Then, 100 µL of the phytochemicals and antifungal drugs (2.048 µg/mL) were individually added to each line of wells, so that each line of wells corresponded to a phytochemical tested. Finally, 10 µL of fungal inoculum of each strain of *Cladosporium* were added to wells, so that each column corresponded to a strain. The microplates were incubated at 28 °C being selected those phytochemicals who showed better inhibition profile visual growth of microorganisms after seven days incubation. The standard antifungal was amphotericin B (1.024 µg/mL). Negative control (Sabouraud dextrose broth without drugs) was performed to confirm the viability of the conidia. Sensitivity control for Tween 80 and DMSO was also performed. The assays were performed in triplicates, and the geometric mean values were calculated.

3. RESULTS AND DISCUSSION

The results of the antifungal susceptibility testing of phytochemicals against *Cladosporium* strains are summarized in Table 1.

In 1.024 µg/mL, the concentration of phytochemicals used, it was found that citral showed better antifungal activity, inhibiting the growth of 100 % of the *Cladosporium* strains tested. The phytochemicals (+) citronellal, linalool and α-pinene were able to inhibit the growth of at most 3 strains.

The (-) citronellal inhibited the growth of 2 strains (*C. carrionii* URM 2871 and *C. carrionii* CQ 02), the β-caryophyllene and geraniol, inhibited only strains *C. sphaerospermum* URM 5962 and *C. carrionii* URM 2871, respectively and β-cymene was not able to inhibit the growth of any of the strains tested, at this concentration.

The term phytochemical relates to chemical compounds, non-nutritive, which naturally occur in plants and exhibit biological activity [37]. Studies involving phytochemicals are of great importance, because they facilitate the utilization of individual components, instead of a mixture like in essential oils, giving more

predictability and probably less collateral effects. Several studies point to the various activities of phytochemicals, which are: antimicrobial, antioxidant, anti-inflammatory, analgesic, cardioprotective, anti-hemorrhagic, hepatoprotective, antitussive, antitumor, immunostimulating, anticancer, antiviral, among other [38-44].

Citral (3,7-dimethyl-2-6-octadienal) is the name given to a mixture of two geometric isomers: (2E)-3,7-dimetilocta-2,6-dienal (geranial, *trans*-citral, citral A) and (2Z)- 3,7-dimetilocta-2,6-dienal (neral, *cis*-citral, citral B), which are acyclic α, β-unsaturated monoterpene aldehydes that occur naturally in many essential citrus fruit oils and in other herbs or spices [45].

The citral aroma is stronger and sweeter than that of lemon [46]. Geranial has a strong lemon odor while neral has a sweeter, yet less intense lemon odor. Due to its characteristic lemon aroma, citral has become a flavoring substance of great importance, a heavily used rawmaterial for the pharmaceutical, food, perfume, and cosmetics industries [47,48]. Also it has emerged as the active component of citrus essential oils against pathogens [49].

Citral was reported by presenting antifungal activity [50-52], antibacterial [53,48], anti- *Leshimania* [54] anti-*Trypanosoma cruzi* [55] and insecticide [56].

In the present study, citral showed activity against *Cladosporium* strains, confirming the results obtained in previous studies. Zheng *et al*. [57] demonstrated the antimicrobial activity of citral front of fungal strains of *Penicillium digitatum*. Such phytochemical has brought an action against strains of methicillin-resistant *Staphylococcus aureus*, *Penicillium italicum* and *Rhizopus atolonifer* [58]. In recent studies, citral showed in vitro antifungal potential against strains of *Candida albicans* [59].

Knowing that there are few studies on the activity of essential oils and their phytochemicals in dematiaceous fungi, particularly on fungi of the *Cladosporium* genus, and that caused them infections are increasingly common around the world, this work will enable a contribution to scientific research, in respect to the pharmacological research of new antifungal products derived from natural products against these fungi.

Table 1. Antifungal activity of phytochemicals against species of Cladosporium - microdilution technique.

Microorganisms	**Phytochemicals (1.024 µg/mL)**										
	citral	(-) citronellal	(+) citronellal	β-caryophyllene	geraniol	linalool	β-cymene	α-pinene	**Amphotericin B**	**Culture control**	**Medium control**
C. carrionii URM 2871	-	-	+	+	-	+	+	-	-	+	-
C. carrionii 0212	-	+	+	+	+	+	+	+	+	+	-
C. carrionii CQ 02	-	-	+	+	+	+	+	+	-	+	-
C. oxysporum URM 5234	-	+	+	+	+	+	+	+	-	+	-
C. oxysporum URM 6056	-	+	+	+	+	-	+	+	+	+	-
C. oxysporum URM 5412	-	+	+	+	+	-	+	+	-	+	-
C. sphaerospermum URM 5962	-	+	+	-	+	-	+	-	-	+	-
C. sphaerospermum URM 5455	-	+	-	+	+	+	+	+	-	+	-
C. sphaerospermum URM 5350	-	+	-	+	+	+	+	-	-	+	-
C. sphaerospermum URM 6120	-	+	-	+	+	+	+	+	+	+	-

(+): Microbial growth in culture medium (-): Absence of microbial growth

4. CONCLUSION

The results obtained in this study show the pharmacological potential of plant products, particularly, the antifungal potential of citral against *Cladosporium* and *Cladophialophora*. The citral could appear as promising compound to be inserted in pharmaceutical formulations applied to control the survival and dissemination of etiological agents of superficial or systemic opportunistic mycoses. Moreover, the results of this study show the necessity of accomplishment of researches addressed to the evaluation of antimicrobial properties of this phytochemical in different pathogenic microorganisms.

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5.2 Atividade antifúngica *in silico* e *in vitro* do monoterpeno citral

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Atividade antifúngica *in silico* e *in vitro* do monoterpeno citral

Antifungal activity *in silico* and *in vitro* of citral monoterpene

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.**RESUMO**

Introdução: as pesquisas com monoterpenos têm aumentado com a necessidade de novas ferramentas terapêuticas para o combate das infecções fúngicas oportunistas, como as causadas pelos fungos dematiáceos pertencentes ao gênero *Cladosporium*. **Objetivo:** avaliar o potencial antifúngico do monoterpeno citral. **Métodos:** a determinação da CIM (Concentração inibitória mínima) das substâncias, foi realizada através da técnica da microdiluição em caldo. Utilizou-se duas cepas de *C. oxysporum* e duas cepas de *C. sphaerospermum*. Todas as cepas de micro-organismos utilizados neste estudo fazem parte da Micoteca URM do Departamento de Micologia, Centro de Ciências Biológicas, Universidade Federal de Pernambuco. Foi realizado controle de viabilidade das cepas ensaiadas, e também controle de sensibilidade destas cepas frente à ação de antimicrobianos considerados padrões na utilização clínica. **Resultados:** pode-se observar que todos os compostos avaliados apresentaram uma CIM_{50} (Concentração Inibitória Mínima para 50% das cepas testadas) de 128 µg/mL para as cepas fúngicas testadas. **Conclusão:** conclui-se que o monoterpneo citral pode se tornar uma alternativa para o tratamento de infecções fúngicas causadas por cepas de *Cladosporium*.

Palavras-chave: antifúngico; monoterpeno; citral; *Cladosporium oxysporum; Cladosporium sphaerospermum.*

ABSTRACT

Introduction: research with monoterpenes have increased the need for new therapeutic tools to combat opportunistic fungal infections such as those caused by fungi dematiaceous belonging to the *Cladosporium* genus. **Objective:** To evaluate the antifungal potential of citral monoterpene. **Methods:** The determination of the MIC (minimum inhibitory concentration) of the substance was performed using microdilution broth technique. We used two strains of *C. oxysporum* and two strains of *C. sphaerospermum.* All the strains of microorganisms used in this study are part of URM Culture Collection of the Department of Mycology, Center of Biological Sciences, Federal University of Pernambuco. Control was carried viability of the tested strains, and also control sensitivity of these strains opposite antimicrobial action patterns considered in the clinical use. **Results:** It can be seen that all the compounds evaluated showed a MIC_{50} (Minimum Inhibitory Concentration for 50% of tested strains) to 128 µg/mL for the tested fungal strains. **Conclusion:** It was concluded that citral monoterpene can become an alternative for the treatment of fungal infections caused by strains of *Cladosporium*.

Keywords: antifungal; monoterpene; citral; *Cladosporium oxysporum, Cladosporium sphaerospermum.*

INTRODUÇÃO

Os produtos químicos produzidos pelos vegetais podem ser divididos em dois grandes grupos. Os primeiros, denominados metabólitos primários ou macromoléculas, são essenciais a todos os seres vivos, e o segundo grupo de compostos químicos – os metabólitos secundários ou micromoléculas - que geralmente apresentam estrutura complexa, baixo peso molecular, marcantes atividades biológicas e, diferentemente daqueles do metabolismo primário, são encontrados em concentrações relativamente baixas e em determinados grupos de plantas (POSER, MENTZ, 2004).

Os metabólitos secundários apresentam várias atividades biológicas. Muitos são de importância comercial tanto na área farmacêutica quanto nas áreas alimentar, agronômica e de perfumaria, entre outras. Entre os metabólitos secundários, os principais grupos de compostos encontrados com atividade biológica são os alcaloides, flavonoides, cumarinas, taninos, quinonas e óleos essenciais (PEREIRA, 2006). Quimicamente, a maioria dos óleos essenciais é constituída de derivados fenilpropanóides ou de terpenóides, sendo os monoterpenos (cerca de 90% dos óleos voláteis) e os sesquiterpenos os mais frequentes (SIMÕES, SPITZER, 2010).

Dentre os monoterpenos relatados na literatura destaca-se o citral, um monoterpeno acíclico natural, citral está presente no óleo essencial de várias espécies de plantas, incluindo limão e laranja (FISHER, PHILLIPS, 2008). E é o

principal componente do óleo de *Melissa officinalis* pertencente à família *Lamiaceae* (SADDIQ, KHAYYAT, 2010). O citral é um monoterpeno com conhecidas propriedades farmacológicas, como: anti-tumoral (FARAH et al., 2010; CHAOUKI et al., 2009; XIA et al., 2013; DUDAI et al., 2005), broncodilatador (MANGPRAYOOL, KUPITTAYANANT, CHUDAPONGSE, 2013), inseticida (ABRAMSON, ALDAMA, SULBARAN, 2007) e antimicrobiana (SOMOLINOS et al., 2009; BELDA-GALBIS et al., 2013).

O gênero Cladosporium abrange muitas espécies de fungos contaminantes e oportunistas dematiáceos que são encontrados ubiquamente como saprófitas no solo e em materiais em decomposição (SCHUBERT, 2005).

As espécies *C. cladosporioides*, *C. herbarum*, *C. oxysporum*, *C. carrionii* e *C. sphaerospermum* têm sido observadas como responsáveis primárias por quadro de feo-hifomicoses superficiais (onicomicoses, ceratites, etc.), cromoblastomicoses e feo-hifomicoses profundas (meningites, quadro pulmonares, etc.) (DE HOOG et al., 2000; KWON-CHUNG, SCHWARTZ, RYBAK, 1975; NAMRATHA et al., 2010; CORREIA et al., 2010).

Nesse contexto, este estudo teve como objetivo avaliar *in silico* e *in vitro* o potencial antifúngico do monoterpeno citral contra cepas de *C. oxysporum* e *C. sphaerospermum.*

MATERIAL E MÉTODOS

LOCAL DA PESQUISA

A pesquisa foi realizada no Laboratório de Micologia do Departamento de Ciências Farmacêuticas (DCF), Centro de Ciências da Saúde (CCS), Universidade Federal da Paraíba (UFPB), no período de maio de 2015 a junho de 2016.

CEPAS FÚNGICAS

Para realização dos ensaios de atividade antifúngica *in vitro* foram selecionadas 2 cepas de *C. oxysporum* (URM 5412 e URM 6056) e 2 cepas de *C. sphaerospermum* (URM 5350 e URM 5455) pertencentes à coleção de culturas da

Micoteca URM do Departamento de Micologia, Centro de Ciências Biológicas, Universidade Federal de Pernambuco. Todas as cepas foram mantidas em Ágar Sabouraud Dextrose – ASD inclinado (DIFCO Laboratories Ltda, USA) a temperatura ambiente (28 ºC) e sob refrigeração (4 ºC).

Foi utilizado um inóculo fúngico de aproximadamente 1 - 5 x 10^6 UFC/mL padronizado de acordo com a turbidez do tubo 0,5 da escala de McFarland (CLEELAND, SQUIRES, 1991; HADACEK, GREGER, 2000; CLSI, 2002).

MEIOS DE CULTURA

Foram utilizados os meios ágar Sabouraud dextrose (ASD) e caldo Sabouraud dextrose (CSD) obtidos da Difco Laboratories (Detroit, MI, EUA) e RPMI-1640-L-glutamina (sem bicarbonato de sódio) da Sigma-Aldrich® (São Paulo, SP, Brasil), preparados conforme as instruções do fabricante.

MONOTERPENO E ANTIFÚNGICOS

O monoterpeno (citral) e os antifúngicos (anfotericina B e voriconazol) foram adquiridos da Sigma-Aldrich®. As emulsões, nas diferentes concentrações, foram preparadas no momento de execução dos ensaios, dissolvendo-os primeiramente em dimetilsulfóxido (DMSO) a 0,5% e Tween 80 (Sigma-Aldrich®) a 2%, e utilizando água destilada estéril para alcançar a concentração desejada.

PASS ON LINE

Previsão do espectro de atividade para substâncias (PASS) online (http:// www.way2drug.com/passonline) é um software destinado para avaliar o potencial biológico geral de uma molécula orgânica sobre o organismo humano. Este programa fornece previsões simultâneas de muitos tipos de atividades biológicas com base na estrutura dos compostos orgânicos. O espectro de atividade biológica de um composto químico é o conjunto de diferentes tipos de atividade biológica, que refletem os resultados de interação do composto com várias entidades biológicas. *Pass online* dá várias facetas da ação biológica de um composto, obtendo os índices Pa (probabilidade " de ser ativo") e Pi (probabilidade "de ser inativo") estimando a

categorização de um composto potencial em ser pertencente à subclasse de compostos ativos ou inativos, respectivamente (SRINIVAS et al., 2014).

PASS oferece previsões com base na probabilidade de novos efeitos e mecanismos de ação com espectro de atividade requerido entre os compostos a partir de bases de dados internas, antigas e comerciais. Pass online prevê o espectro de atividade biológica para as impressões modificadas, com base em sua fórmula estrutural, juntamente com diferentes descritores como antifúngica, antiviral, anti-helmíntico, antiprotozoários, etc., por isso, é possível estimar se os novos compostos têm um efeito particular (SRINIVAS et al., 2014).

DETERMINAÇÃO DA CONCENTRAÇÃO INIBITÓRIA MÍNIMA (CIM)

A concentração inibitória mínima do citral, bem como dos antifúngicos anfotericina B e voriconazol foi determinada pela técnica da microdiluição em caldo CLEELAND, SQUIRES, 1991; HADACEK, GREGER, 2000; CLSI, 2002; Eloff, 1998). Foram utilizadas placas de 96 orifícios estéreis e com tampa. Em cada orifício da placa, foi adicionado 100 µL de RPMI-1640 duplamente concentrado. Em seguida, 100 µL da emulsão dos produtos, também duplamente concentrado, foram dispensados nas cavidades da primeira linha da placa. E por meio de uma diluição seriada a uma razão de dois, foram obtidas concentrações de 1024 µg/mL até 0,5 µg/mL, de modo que na primeira linha da placa se encontra a maior concentração e na última, a menor concentração. Por fim, foi adicionado 10 µL do inóculo de aproximadamente 1-5 x 10^6 UFC/mL das espécies nas cavidades, onde cada coluna da placa refere-se a uma cepa fúngica, especificamente.

Paralelamente, foi realizado controle de viabilidade das cepas ensaiadas (100 µL do mesmo RPMI-1640 duplamente concentrado e 10 µL do inóculo de cada cepa). E para verificar a ausência de interferência nos resultados pelos agentes emulsificantes utilizados na solubilização do monoterpeno, foi feito um controle no qual foi colocado nas cavidades 100 µL do RPMI-1640, DMSO (0,5%), Tween 80 (2%) e 10 µL da suspensão. Um controle de esterilidade do meio também foi realizado, colocando-se 100 µL do RPMI-1640 em cavidades sem a suspensão fúngica.

As placas foram assepticamente fechadas e incubadas a 28 °C por 5-7 dias para a realização da leitura. A CIM foi definida como a menor concentração capaz de inibir o crescimento fúngico visualmente verificado nos orifícios quando comparado com o crescimento controle. Os ensaios foram realizados em triplicata e a média geométrica foi calculada.

RESULTADOS

A análise dos valores de “Pa” no estudo *in silico* do citral revela que este monoterpeno apresenta uma probabilidade de atividades farmacológicas anti-infecciosa, em especial, atividade antifúngica (Pa=0,429 e Pi= 0,044) (Tabela 1).

Tabela 1- Valores de Pa e Pi para o monoterpeno citral.

Valor de Pa	Valor de Pi	Atividades farmacológicas
0,395	0,005	Antiviral (CMV)
0,340	0,066	Antiviral (Herpes)
0,281	0,103	Antiviral (Influenza)
0,363	0,143	Antiviral (Picornavirus)
0,228	0,120	Antiviral (Poxvirus)
0,735	0,002	Antiviral (Rhinovirus)
0,233	0,081	Antiprotozoal
0,235	0,129	Antiprotozoal (Coccidial)
0,335	0,079	Antiprotozoal (Leishmania)
0,230	0,035	Antiprotozoal (Plasmodium)
0,194	0,068	Antiprotozoal (Toxoplasma)
0,353	0,060	Antiprotozoal (Trypanosoma)

0,421	0,014	Antihelmíntico
0,136	0,126	Antihelmíntico (Fasciola)
0,260	0,150	Antihelmíntico (Nematodes)
0,429	0,044	Antifúngico
0,377	0,036	Antibacteriano

Observando os valores da CIM (Concentração Inibitória Mínima) do citral para as cepas de *C. oxysporum* e *C. sphaerospermum* pode-se perceber que o composto testado apresenta uma CIM de 128 µg/mL para três cepas avaliadas (*C. oxysporum* URM 6056 e URM 5412, *C. sphaerospermum* URM 5350) e de 256 µg/mL para uma das cepas testadas (*C. sphaerospermum* URM 5455). Além disso, observa-se que os antifúngicos padrões, voriconazol e anfotericina B, foram também capazes de inibir o crescimento fúngico, exceto para a cepa *C. oxysporum* URM 6056, que foi resistente a maior concentração testada (1024 µg/mL) dos dois antifúngicos padrão (Tabela 2).

Tabela 2 – Resultados da CIM do citral, voriconazol e anfotericina B contra *C. oxysporum* e *C. sphaerospermum.*

Cepas de Cladosporium	Citral µg/mL CIM	Voriconazol µg/mL CIM	Anfotericina B µg/mL CIM	*C
C. oxysporum URM 6056	128	>1024	>1024	+
C. oxysporum URM 5412	128	8	16	+
C. sphaerospermum URM 5350	128	16	64	+
C. sphaerospermum URM 5455	256	16	16	+

*C - Controle de crescimento do micro-organismo em RPMI-1640, DMSO (0,5%) e Tween 80 (2%), sem monoterpeno ou antifúngicos.

DISCUSSÃO

Os terpenos são constituintes que fazem parte da composição dos óleos essenciais, que por sua vez são empregados na indústria na produção de perfumes e cosméticos, além de apresentarem efeitos farmacológicos (EDRIS, 2007).

Diferentes testes têm sido utilizados para avaliar as propriedades farmacológicas das substâncias. Neste contexto, destacam-se os testes que utilizam os modelos *in silico* (expressão usada com o significado de “executado em computador”), que são rápidos, reprodutíveis e normalmente baseados em biorreguladores humanos, garantindo assim a segurança para a utilização do produto natural como um futuro fármaco (ANGELO, MAX, MARKUS et al., 2006; SRINIVAS et al., 2014). Neste estudo, a análise *in silico* do monoterpeno citral demonstrou várias promissoras atividades farmacológicas desta substância contra diferentes agentes infecciosos, em especial os fungos.

Estes dados computacionais, por sua vez, foram confirmados com o estudo *in vitro* frente a diferentes cepas de *C. oxysporum* e *C. sphaerospermum*, contra as quais o monoterpeno testado apresentou valores de CIM de 128 e 256 µg/mL.

De acordo com Aligianis et al. (2001) e Sartoratto et al. (2004), produtos com CIM até 500 µg/mL são considerados com forte poder antimicrobiano; produtos com CIM entre 600 e 1500 µg/mL –moderado poder antimicrobiano, e produtos com CIM acima de 1500 µg/mL - fraco poder antimicrobiano. Desta forma, pode-se afirmar que o citral apresenta um forte efeito antifúngico contra as cepas de *C. oxysporum* e *C. sphaerospermum.*

Este potencial antifúngico *in silico* e *in vitro* do citral encontra-se de acordo com outros estudos que já demonstraram o potencial antimicrobiano deste composto contra outras espécies fúngicas, como por exemplo *Penicillium digitatum* (FISHER, PHILLIPS, 2008), *Trichophyton mentagrophytes* (PARK et al., 2009), *Aspergillus flavus, Aspergillus fumigatus* (LUO et al., 2004; MESA-ARANGO et al., 2009).

CONCLUSÕES

Portanto, ao analisar os dados obtidos nesta pesquisa *in silico* e *in vitro* pode-se perceber que o monoterpeno citral apresenta uma promissora atividade antimicrobiana, em especial antifúngica contra espécies de *C. oxysporum* e *C. sphaerospermum.*

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5. 3 Evaluation of Citral's Antifungal Activity and Mode of Action against *Cladophialophora carrionii*

O artigo será submetido na Brazilian Journal of Pharmaceutical Science.

Evaluation of Citral's Antifungal Activity and Mode of Action against *Cladophialophora carrionii*

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ABSTRACT

Cladophialophora carrionii is one of the most frequent etiologic agents of human chromoblastomycosis, a chronic cutaneous disease. Such fungal infections are difficult to treat and underline the need for new antifungal treatments. Citral is a monoterpene with known pharmacological properties, including antimicrobial activeity. This study aimed to investigate the antifungal activity of citral against *C. carrionii* URM 2871. The minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) were determined by broth microdilution techniques. Citral was tested to evaluate its effects on *C. carrionii* mycelia growth and germination of fungal conidia. We also investigated possible citral action on cell walls (0.8 M sorbitol) and cell membranes (citral to ergosterol binding).The MIC_{50} and MFC_{50} of citral were respectively 128 μg/mL and 512 μg/mL. The study shows that citral is capable of inhibiting both mycelia growth and germination of conidia for *C. carrionii*, and affects the structure of fungal cell membranes. Citral showed *in vitro* antifungal potential against strains of *C. carrionii*. Citral's mechanism of action involves ergosterol. Further study is needed to completely describe its effects before future use as a component of new antifungals.

Keywords: Monoterpene, Citral, Antifungal, *Cladophialophora carrionii*, Chromoblastomycosis.

INTRODUCTION

Cladophialophora carrionii is saprobic dematiaceous fungi, associated with opportunistic human and animal infections. This fungus is one of the relatively common causative agents of chromoblastomycosis, a chronic, progressive, polymorphic implantation mycosis of skin and subcutaneous tissue, causing hyperproliferation leading to verrucous nodular clinical features, and is histologically characterized by muriform cells. Infection occurs after the etiologic agent gains entrance through a traumatic lesion (Abliz *et al*., 2004; Queiroz-Telles *et al*., 2009; Rubin *et al*., 1991).

The disease is seen worldwide, but most reports are from tropical and subtropical areas with greater prevalences in Africa and Latin America (Esterre *et al*., 1996; Silva, Sousa, Rozental, 1999; Bonifaz, Carrasco-Gerard, Saul, 2001). The lesions associated with the disease are polymorphic or hyperkeratotic and in therapy must be differentiated from those associated with other infectious or autoimmune disorders (Deng *et al*., 2014). The infection is very difficult to treat. Various therapies are commonly applied, but there is no standard treatment (Queiroz-Telles *et al*., 2009).

The increased incidence of these fungal infections, especially dangerous hospital-acquired infections and infections in immunocompromised patients, has underlined the need for new antifungal treatments. There has been growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants (George, Selitrennikoff, 2006).

Plants and their derivatives are known to be important in pharmacological research; this is due to their great potential as a source for a variety of biologically active ingredients used in drug development. Among these products we find the terpenes, a class of natural vegetable substances formed by combining five carbons and called isoprene (C_5H_8) (Bakalli *et al*., 2008).

Citral (3,7-dimethyl-2-6-octadienal) is a natural mixture of two geometric isomers: geranial (*trans*-citral) and neral (*cis*-citral), which are acyclic α,β-unsaturated monoterpene aldehydes that occur naturally in many essential citrus fruit oils and in other herbs or spices (Dudai *et al*., 1994; Tzortzakis, Economakis, 2007). Citral has become a flavoring substance of great importance, a heavily used raw material for

the pharmaceutical, food, perfume, and cosmetics industries (Dawson, 1994; Marques *et al*., 2013).

Various biological activities such as anti-inflammatory (Ponce-Monter *et al*., 2010; Ortiz *et al*., 2010), anti-tumor (Xia *et al*., 2013; Chaoki *et al*., 2009), bronchodilator (Mangprayool, Kupittayanant, Chudapongse, 2013), antiprotozoal (Armas *et al*., 2015), vasodilator (Lopes *et al*., 2013), spasmolytic (Ponce *et al*., 2010; Chitra, Mui, Ismail, 2011), sedative and relaxing motor (Vale *et al*., 2002) have been reported for citral. In addition, we find: antimicrobial activity as demonstrated through *Trichophyton mentagrophytes* hyphae growth inhibition, where it was observed that the cell membrane and organelles were irreversibly damaged by citral (Park *et al*., 2009), potent in vitro activity against *Candida* spp. (Leite *et al*., 2014; Sousa *et al*., 2016), antiviral activity against yellow fever virus (Gomes, Stashenko, Ocazionez, 2013), and *in vitro* herpes simplex virus type 1 (HSV-1) activity (Astani, Reichling, Schnitzler, 2010).

Given the above, the aim of this study was to determine the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of citral, and to investigate the mechanism of action against *C. carrionii* in its mycelia growth, conidial germination, cell wall formation, and interactions involving ergosterol.

MATERIAL AND METHODS

Microorganisms. *Cladophialophora carrionii* (URM 2871, LM 0212, CQ 02). The strain *C. carrionii* URM 2871 used in the antifungal assay was obtained from the Mycology Department fungal collection (URM), Biological Sciences Center, Federal University of Pernambuco, Brazil and *C. carrionii* strains (LM 0212 and CQ 02) were obtained from the Microorganisms Collection of the Mycology Laboratory, at the Department of Pharmaceutical Sciences, Health Sciences Center, Federal University of Paraíba, Brazil. The samples were maintained on Sabouraud Dextrose Agar - SDA (DIFCO®) at room temperature (28° to 30°C) and under refrigeration (4°C).

Stock inoculations (suspensions) of *C. carrionii* were prepared from 7-14 day old sabouraud dextrose agar (Difco Lab., USA); the cultures were grown at room temperature. Fungal colonies were covered with 5 mL of sterile saline solution (0.9%), the surface was gently agitated with vortexes, and fungal elements in saline

solution were transferred to sterile tubes. Inoculator was standardized at 0.5 tube of McFarland scale (10^6 CFU/mL). The final concentration confirmation was done by counting the microorganisms in a Neubauer chamber (Cleeland, Squires, 1991; Hadacek, Greger, 2000; Sahin *et al*., 2004).

Chemicals. The product tested was the monoterpene Citral, obtained from Sigma Aldrich, Brazil. Amphotericin B and Voriconazole were obtained from Sigma Aldrich, Brazil. The monoterpene was dissolved in Tween 80 (2%) and DMSO (dimethylsulfoxide). The antifungal standards were dissolved in DMSO, and sterile distilled water to obtain solutions of 2048 μg/mL for each antifungal. The concentration of DMSO did not exceed 0.5% in the assays.

Culture Media. To test the biological activity of the products, Sabouraud dextrose agar (SDA) purchased from Difco Laboratories (Detroit, MI, USA), and RPMI-1640-L-glutamine (without sodium bicarbonate) (Sigma-Aldrich, Sao Paulo, SP, Brazil) culture media were used. Both were prepared and used according to the manufacturers' instructions.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC). Broth microdilution assays were used to determine the MICs of monoterpene citral, Amphotericin B, and Voriconazol against *C. carrionii* (URM 2871, LM 0212 and CQ 02). RPMI-1640 was added to all the wells of 96-well plates. Two-fold serial dilutions of the agents were prepared to obtain concentrations varying between 4 μg/mL and 1024 μg/mL. Finally, 10 μL aliquots of the inoculate suspension were added to the wells, and the plates were incubated at 28^{o}C for 5 days. Negative controls (without drugs) were used to confirm conidia viability, and sensitivity controls (for DMSO and Tween 80) were also included in the studies. At 5 days, there were visual observations of fungal growth. The MIC was defined as the lowest concentration capable of visually inhibiting fungal growth by 100%. The results were expressed as the arithmetic mean of three experiments (Cleeland, Squires, 1991; Hadacek, Greger, 2000).

The MFC was determined by microdilution method to verify if the inhibition was reversible or permanent (Denning et al., 1992; Rasooli, Abyaneh, 2004). Aliquots of 20 µL (from the wells that did not show growth in the MIC procedure) were

transferred to 96-well plates previously prepared with 100 µL of RPMI-1640. The plates were aseptically sealed followed by mixing on a plate shaker (300 rpm) for 30 seconds, incubated at 28ºC and read at 5 days of incubation. Tests were performed in duplicate and the geometric mean values were calculated. MFC was defined as the lowest citral concentration in which no visible growth occurred when subcultured on the 96-well plates containing broth without antifungal products.

After determination of the MIC and MFC we selected 1 strain (*C. carrionii* URM 2871), to continue the citral antifungal activity study.

Effects on Mycelia Growth. Analyses of the interferences of citral, Voriconazole, and Amphotericin on *C. carrionii* URM 2871 mycelia growth were determined using poisoned substrate technique (dilution in solid medium), by daily measuring of radial mycelial growth on SDA, by adding products in an amount adjusted to provide final concentrations similar to the MIC, MIC x 2, and MIC x 4 previously found. For this, 2 mm plugs taken from a 10-day-old mold culture cultivated on SDA slants at 28 ºC were placed at the center of the sterile SDA Petri dishes containing the test drugs. At different intervals (0, 2, 4, 6, 8, 10, 12, and 14 days) of incubation at 28 ºC, the mold's radial mycelial growth was measured (mm) with calipers. The controls in this assay revealed the mold's radial growth on SDA without adding drugs. Each assay was performed twice and the results were expressed as the average of the two repetitions (Adan *et al*., 1998; Thyágara, Hosono, 1996; Daferera, Ziogas, Polission, 2003).

Conidial Germination Assay. Citral, Voriconazole, and Amphotericin B were tested to evaluate effects on the germination of *C. carrionii* URM 2871 fungal conidia. Flasks containing MIC, MIC x 2 and MIC x 4 of citral, voriconazole, amphotericin and a control with distilled water were used. In sterile test tubes, 500 μL of RPMI-1640 plus citral were evenly mixed with 500 μL of fungal conidia suspension and immediately incubated at 28ºC. Samples of the mixture were taken after 48 h of incubation for analysis. The whole experiment was performed in duplicate, where the number of conidia was determined in a Neubauer chamber, and the spore germination inhibition percentage at each time point was calculated by comparing the results obtained in the test experiments with the results of the control experiment.

The analysis was conducted under an optical microscope (Zeiss Primo Star) (Pereira, Mendes, Lima, 2013; Rana, Singh, Taneja, 1997).

Sorbitol Assay Effects. The assay was performed using medium with and without sorbitol (control), to evaluate possible mechanisms involved in the antifungal activity of the test product on the *C. carrionii* URM 2871 cell wall. The sorbitol was added to the culture medium in a final concentration of 0.8 M. The assay was performed by microdilution method in 96-well plates in a “U” (Alamar, Diadema, SP, Brazil) (Cleeland, Squires, 1991; Hadacek, Greger, 2000). The plates were sealed aseptically, incubated at 28 ºC, and readings were taken at 5 days. Based on the ability of sorbitol to act as a fungal cell wall osmotic protective agent, the higher MIC values observed in the medium with sorbitol added (as compared to the standard medium), suggest the cell wall as a possible target for the product tested (Leite *et al*., 2014; Liu *et al*., 2007; Frost *et al*., 1995). The assay was performed in duplicate and expressed as the geometric mean of the results.

Ergosterol Binding Assay. MIC Value Determination in the Presence of Ergosterol. To assess if the product binds to fungal membrane sterols, an experiment was performed according to the method described by Escalante *et al.* (2008), with some modifications. Ergosterol was prepared as described by Leite *et al*. (2014). The MIC of citral, against *C. carrionii* URM 2871 was determined by using broth microdilution techniques (Cleeland, Squires, 1991; Hadacek, Greger, 2000), in the presence and absence of exogenous ergosterol (Sigma-Aldrich, São Paulo, SP, Brazil) added to the assay medium, in different lines on the same microplate. Briefly, a solution of citral was diluted serially twice with RPMI-1640 (volume = 100 μL) containing ergosterol added at a concentration of 400 μg/mL. A volume yeast suspension 10 μL (0,5 McFarland) was added to each well. The same procedure was realized for Amphotericin B, whose interaction with membrane ergosterol is already known, which served as a control drug. The plates were sealed and incubated at 28ºC. The plates were read after 5 days of incubation, and the MIC was determined as the lowest concentration of test agent inhibiting visible growth. The assay was carried out in duplicate and the geometric mean of the values was calculated. The binding assay reflected the ability of the compound to bind with ergosterol.

Statistical Analysis. The results are expressed as mean ± S.E. Differences between the means were statistically compared using the Student's t-test. The values were considered significant with $p < 0.05$.

RESULTS AND DISCUSSION

The results for citral's antifungal activity against *C. carrionii* were determined using the MIC and MFC in broth microdilutions. The MIC of citral varied between 128 and 256 µg/mL. The MIC_{50} (Minimum Fungicidal Concentration for 50% of strains tested), inhibiting the growth of the tested fungal strain, was 128 µg/mL. Amphotericin B and Voriconazol retained a lesser MIC_{50} than the phytoconstituent at 16 µg/mL MIC (Table I).

The MFC of citral varied between 256 and 1024 µg/mL. The MFC_{50} (Minimum Fungicidal Concentration for 50% of strains tested) was 512 µg/mL. The MFC_{50} for Amphotericin B and voriconazole were 64 µg/mL and 16 µg/mL, respectively (Table I).

The results for the control (Tween 80 and DMSO) showed no fungal growth inhibition; fungal growth in the medium without drug added was detected (sterile control). In accordance with the above results, the *C. carrionii* URM 2871 strain was selected for further testing.

TABLE I. MIC and MFC of citral, amphotericin B and voriconazole against *C. carrionii.*

Microorganisms	Citral (µg/mL)		Amphotericin B (µg/mL)		Voriconazole (µg/mL)		Control strains*
	MIC	MFC	MIC	MFC	MIC	MFC	
C. carrionii URM 2871	128	512	16	64	16	16	+
C. carrionii LM 0212	256	1024	>1024	ND	>1024	ND	+
C. carrionii CQ 02	128	256	16	64	8	16	+

Note. * Microorganism growth in RPMI-1640, DMSO (5%), and Tween 80 (2%), without antifungal or monoterpenes. ND- Not determined.

In our previous studies, it was observed that *Melissa officinalis* essential oil (at 256 μg/mL), has antifungal activity, inhibiting the mycelial growth and conidial germination of *C. carrionii* (Menezes *et al*., 2015). *M. officinalis* essential oil is characterized by monoterpene compounds, and citral is the principal component present (Meftahizade, Sargsyan, Moradkani, 2010).

The antimicrobial activity of citral has been confirmed. More recently, studies have shown that citral exhibits antimicrobial effect against *C. sakazakii* strains, with MICs ranging from 0.27 to 0.54 mg/mL (Shi *et al*., 2016). Khan, and Ahmad (2013) showed that citral has effective antifungal activity against azole-resistant strains of *Aspergillus fumigatus* and *Trichophyton rubrum*. In recent studies, citral showed *in vitro* antifungal potential against strains of *Candida albicans* (Leite *et al*., 2014), and *C. tropicalis* (Sousa *et al*., 2016).

In the present study, citral showed activity against *C. carrionii* URM 2871.The product is therefore considered actively antifungal in accordance with the parameters defined by Sartoratto et al. (2004).

This study also verified citral's action against *C. carrionii* mycelial growth and spore germination. The effect of differing concentrations of the test drug (MIC, MIC x 2, MIC x 4) on mycelia growth was determined by measuring radial mycelial growth, and the results are shown in Figure 1. With respect to *C. carrionii*, it can be seen that citral at its MIC concentration (128 μg/mL) was not capable of inhibiting mycelial growth, but at MIC x 2 (256 μg/mL) and MIC x 4 (512 μg/mL) concentrations, normal mycelia growth was inhibited when compared to the control (mycelia diameter being 100 %). The control strains showed a constant rate of mycelial growth over the time evaluated, indicating good antifungal effect for citral.

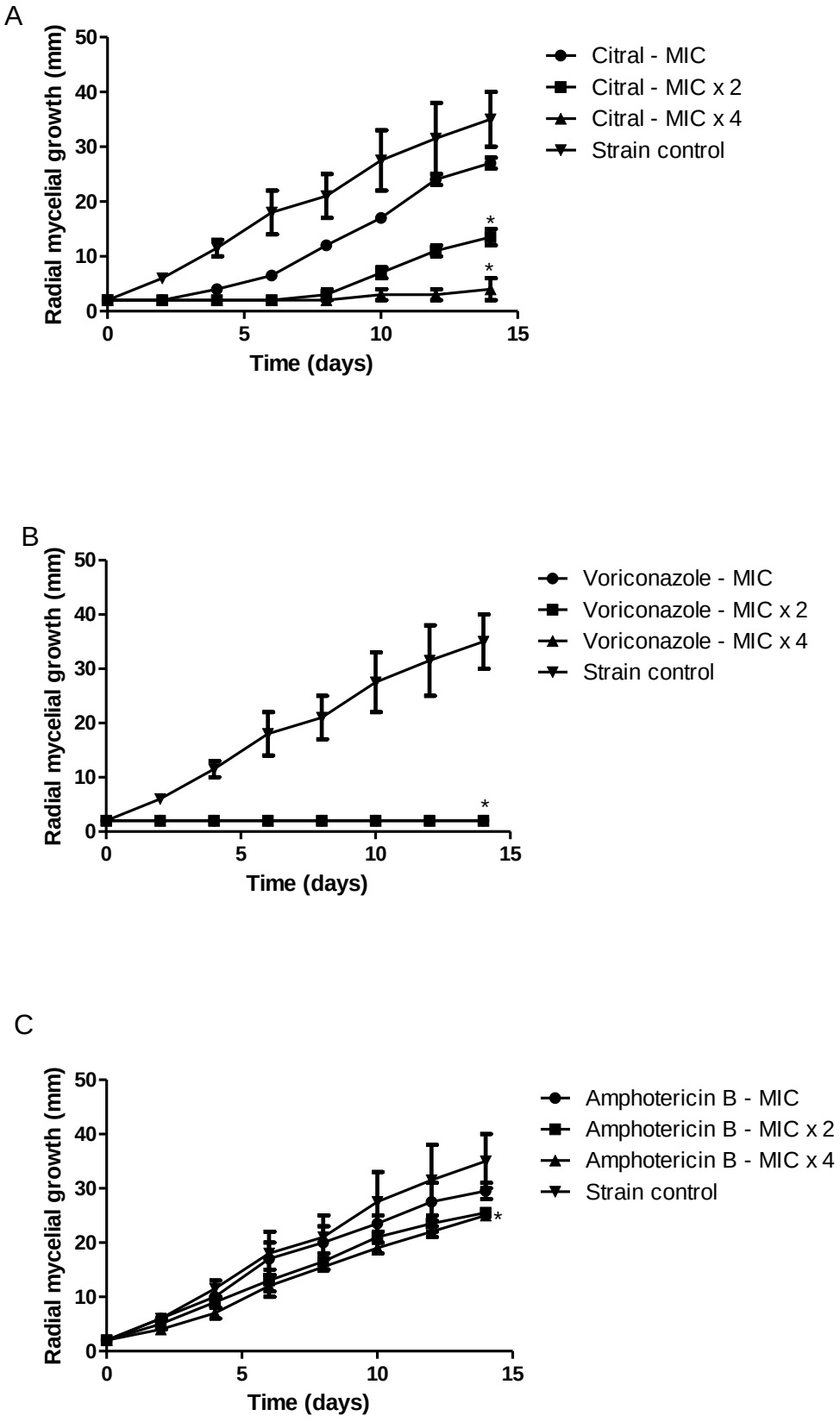

FIGURE 1. Radial mycelial growth produced by *C. carrionii* URM 2871 in the absence (control), and presence of citral (A), voriconazole (B) and amphotericin B (C). *$p < 0.05$ compared to control.

The voriconazole test against *C. carrionii* URM 2871 showed significant inhibition of mycelial growth at all concentrations tested (Fig. 1B). With amphotericin B, inhibition of mycelial growth was only observed at a higher concentration, MIC x 4 (64 µg/ml) (Fig. 1C).

The results show that citral at its MIC x 2 and MIC x 4 concentrations was more potent than amphotericin B at its respective MIC x 2 and MIC x 4 concentrations ($p < 0.05$).

However, the action was shown to be less potent ($p < 0.05$) when compared with respective MIC and MIC x 2 concentrations of voriconazole.

The production of hyphae and consequent mycelium formation are important virulence factors for fungal filaments. In an infection, longitudinal growth of the hyphae facilitates penetration into the inner layers of the skin, while lateral growth exacerbates the damage (Zurita, Hay, 1987; Gupta, Chaudhry, Elewski, 2003). Hyphae are more difficult to phagocytize and can induce apoptosis in macrophages, since they often form inside the macrophage after phagocytosis (Chotirmall *et al*., 2014).

These results corroborate the data obtained by certain researchers who have investigated the antifungal potential of citral in inhibiting the mycelial growth of pathogenic and non-pathogenic fungi (Zhou, Tao, Jia, 2014; Tao, OuYang, Jia, 2014).

A previous study reported that citral at concentrations of 50 and 100 µg/ml can inhibit *Trichophyton mentagrophytes* growth in PDA by 8,5 % and 100%, respectively (Park *et al*., 2009). These results also agree with those of Saddiq, Khayyat (2010) and Wuryatmo *et al*. (2003), who reported on the strong antifungal activity of citral.

More recently, OuYang *et al*. (2016) observed that citral inhibited the mycelial growth of *Penicillium digitatum*, with the minimum inhibitory concentration (MIC) of 1.78 mg/mL in a dose-dependent manner.

Given the importance of mycelial growth to the development of mycoses, the inhibition of *C. carrionii* mycelial growth caused by citral (as observed in this study), proving superior to Amphotericin B in its respective concentrations is a significant contribution to the search for new natural products with antifungal activity.

The study of the conidia germination has great implications for clinical practice, because it is possible to develop new therapeutic approaches that block the infection at its onset (Osherov, May, 2001). From this perspective, the effect of citral on the germination of *C. carrionii* URM 2871 conidia was investigated. The effects of different concentrations (MIC, MIC x 2 and MIC x 4) of citral, voriconazole and amphotericin B on the germination of conidia are shown in Figure 2.

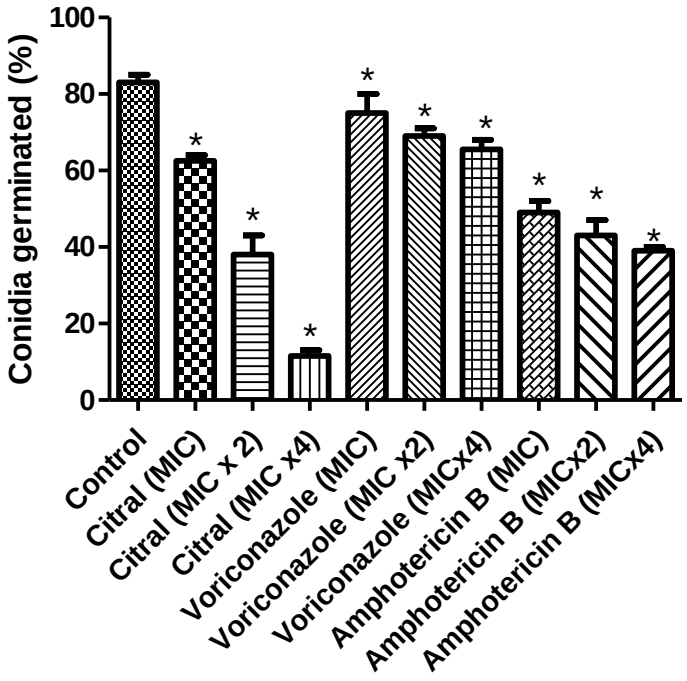

FIGURE 2. *C. carrionii* URM 2871 conidial germination percentage in the absence (control) and presence of citral, voriconazole and amphotericin B. **p< 0.05 compared to control.*

In the three test concentrations (MIC, MIC x 2 and MIC x 4), citral displayed significant inhibitory action against *C. carrionii*, as compared to the control. The antifungals standards (voriconazole and amphotericin B) tested, also showed significant inhibition on conidia germination. However, the results show that Citral at its MIC x 4 concentration was more potent than Amphotericin B and Voriconazole at their respective MIC x 4 concentrations ($p < 0.05$).

These results confirm previously published work, such as that by LI *et al*. (2014), who showed the effect of citral on *Magnaporthe grisea*. In this study, it was

found that germination in a concentration-dependent manner was significantly inhibited by citral, and a similar trend was observed for mycelial growth, demonstrating the efficacy of citral for inhibition of pathogenic fungal growth.

Conidia represent the most common mode of asexual reproduction; they play an important role in natural fungal propagation and are structurally resistant (Trabulsi, Alterthum, 2004). It is important to note that the conidia are not merely quiescent cells; a basal level of RNA and protein synthesis is required for survival of the spores (Liu *et al*., 2007). Conidia are distributed in large quantities in the atmosphere, and some have the ability to cause disease in humans, animals, and plants (Zoppas, Valencia-Barrera, Fernandez-Gonzáles, 2011). Thus, it is important to quantitatively evaluate the power of a product to interfere with fungal spore germination (Chotirmall *et al*., 2014).

The great challenge when developing new antifungal drugs is in the similarity between fungal cells and human cells. Thus, the targets for a new antifungal's action must be unique or at least sufficiently different from the host (Cihlar, Kellogg, Broedel, 2002; Martinez-rossi, Perez, Rossi, 2008). Based on this, two important fungal structures become targets for detecting antifungal agents; the fungal cell wall and ergosterol present in the plasma membrane.

To investigate the action of the product on the fungal cell wall we performed an assay with sorbitol (Table II), which has an osmoprotectant function. Sorbitol, an osmotic protective, is used to stabilize fungi protoplasts. Specific fungal cell wall inhibitors share a distinctive characteristic in that their antifungal effects are reversed in mediums containing sorbitol (Frost *et al*., 1995). Cells protected with sorbitol can grow in the presence of fungal cell wall inhibitors, whereas growth is inhibited in the absence of sorbitol. This effect is detected by increases in the MIC value as observed in medium with sorbitol as compared to the MIC value in medium without sorbitol (standard medium) (Frost *et al*., 1995; Svetaz *et al*., 2007). Osmotic destabilizing agents and cell wall disruptions lead to rearrangements of the cell wall, and allow the fungal cells to survive (CLSI, 2002).

In this work, the MIC values of citral in both experiments, (in mediums with and without sorbitol), were identical, suggesting that citral does not act by inhibiting fungal cell wall synthesis, but probably by affecting another target.

TABLE II: MIC values (μg/mL) against *C. carrionii* URM 2871 of drugs in the absence and presence of sorbitol (0.8 M) and ergosterol (400 μg/mL).

Drugs	Sorbitol		Ergosterol	
	Absence	Presence	Absence	Presence
Citral	128	128	128	2048
Amphotericin B[a]	-	-	16	2048

[a]Positive control. —: not tested.

These results are in agreement with those reported by Miron *et al*. (2014) who evaluated the antifungal activity of citral against seven opportunistic pathogenic yeasts, and four dermatophyte species; no changes were observed in the MICs of this monoterpene in the sorbitol protection assay.

Ergosterol is one of the principal sterol components in the fungal membrane and plays the same role in fungal membranes that cholesterol plays in the mammalian cell. Generally, a decrease in ergosterol content results in osmotic disturbances, with disruption of cell growth and proliferation (Tao, OuYang, Jia, 2014; Khan *et al*., 2010; Onyewu *et al*., 2003; Sun *et al*., 2011). Many drugs available for clinical use interact directly with ergosterol, causing damage to the fungal cell membrane (Valgus, 2003).

Considering possible fungal cell membrane interference of citral, the compound was tested to investigate its ability to form complexes with ergosterol (Table I). Whether the effects of citral on the fungal cell are due to ergosterol binding in the membrane can be verified if they interact directly. In the presence of exogenous ergosterol in the culture medium, decreased binding of the product to the ergosterol of the membrane occurs. Thus, the product's MIC tends to increase in the presence of exogenous ergosterol, needing a much higher concentration to interact with ergosterol in the fungal membrane (Leite *et al*., 2014; Sharma, Tripathi, 2006).

The MIC of citral against *C. carrionii* increased sixteen times in the presence of ergosterol at 400 μg/mL. Amphotericin B, the positive control that has a known interaction with ergosterol showed a 128-fold higher MIC in the presence of ergosterol.

These results suggest that the mechanism of citral's antifungal action involves direct interaction with ergosterol, which leads to the disruption of the fungal membrane and loss of intracellular contents.

The lipophilic nature of terpenoids enables them to preferentially enter the lipid membrane, which results in an increased membrane fluidity and eventually to an increase in membrane permeability (Khan *et al*., 2010; Burt, 2004).

According to Harris (2002), citral appears to act predominantly on the fungal cell membrane, affecting its structure, blocking its synthesis, and causing cell death; inhibiting spore germination, proliferation, and cellular respiration. The literature suggests that the antifungal activity of citral is due to its ability to form a charge transfer complex with fungal cell tryptophan, resulting in the death of the fungus (Kurita *et al*., 1981).

The action of citral on the cell membrane has been widely studied. In a recent study Tao *et al*. (2014) showed that citral considerably impaired ergosterol biosynthesis in *Penicillium italicum* cells, significantly decreasing lipid levels; suggesting that the plasma membrane may well be an important citral antifungal target.

Zhou *et al*. (2014) evaluated the antifungal activity of three volatile compounds: citral, octanal, and α- terpineol against *Geotrichum citri-aurantii*. It was found in the study that citral was able to significantly inhibit mycelial growth. Antifungal activity was attributed to cell membrane disruption and to the consequent loss of cellular components.

Another study also showed that citral at a concentration of 200 μg/mL irreversibly damaged cell organelles and the cell membrane of *Trichophyton mentagrophytes* (Park *et al*., 2009).

More recently OuYang *et al*. (2016) suggested that citral might exhibit its antifungal activity against *P. digitatum* by down-regulation of ergosterol biosynthesis.

The positive results of citral in the "Ergosterol Affinity Assay", and other reports on the subject strongly suggest that the mechanism of action of this monoterpene is related to ergosterol-binding and a subsequent destabilization of fungal cell membranes.

CONCLUSIONS

Based on the results, the present study demonstrates that citral has significant antifungal activity against *C. carrionii* and revealed that the product is capable of inhibiting both the mycelial growth and germination of conidia for the specie tested. The results also suggest that the action of citral affects the structure of the fungal cell membrane. The test product presents as a relevant and promising antifungal which may be considered as an alternative prototype for production of a new and future antifungal, thus contributing to the existing arsenal of products which have proven antifungal activity against *C. carrionii*. Investigations of this nature are important since they provide clearer expectations for future pharmacological studies, with a view to a better understanding of citral's mode of action, its toxicity, and possible therapeutic applications.

RESUMO

Cladophialophora carrionii é um dos agentes etiológicos mais frequentes de cromoblastomicose humana, uma doença cutânea crônica. Estas infecções são difíceis de tratar, levando a necessidade de novos tratamentos anti fúngicos. Citral é um monoterpeno com propriedades farmacológicas conhecidas, incluindo ação antimicrobiana. Este estudo teve como objetivo investigar a atividade antifúngica do citral contra *C. carrionii* URM 2871. A concentração inibitória mínima (CIM) e a concentração fungicida mínima (CFM) foram determinadas pelas técnicas de microdiluição em caldo. Citral foi testado para avaliar seus efeitos sobre o crescimento micelial e germinação de conídios de *C. carrionii*. Também investigou-se uma possível ação do citral na parede celular (0,8 M sorbitol) e membrana celular fúngica (interação do citral com o ergosterol). A CIM e CFM do citral foram, respectivamente, 128 μg / mL e 256 μg / mL. O estudo mostrou que o citral é capaz de inibir tanto o crescimento micelial e germinação de conídios de *C. carrionii*, e que a sua ação afeta a estrutura da membrana celular fúngica. O Citral mostrou, *in vitro,* potencial antifúngico contra *C. carrionii,* porém, são necessários mais estudos para descrever completamente os seus efeitos antes de ser utilizado no futuro, como um componente de novos antifúngicos.

Palavras-chaves: Monoterpeno, Citral, Antifúngico, *Cladophialophora carrionii*, Cromoblastomicose.

CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interests regarding the publication of this paper.

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5.4 Investigation on mechanism of antifungal activity of citral against *Cladosporium sphaerospermum*

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Investigation on mechanism of antifungal activity of citral against *Cladosporium sphaerospermum*

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ABSTRACT

Cladosporium sphaerospermum is a commonly distributed allergen. These are dematiaceous fungi commonly present as airborne contaminants. *C. sphaerospermum* is a very rare cause of human illness, but it can cause several different types of infections, including skin, eyes, sinus, and brain infections. And monoterpenes have been proven to be promising as antifungal agents for treatments these infections. The objective of this work was to investigate the antifungal activity of the citral against *C. sphaerospermum*, through determination of minimal inhibitory concentration (MICs) and minimal fungicidal concentration (MFCs), effects on mycelial growth and germination of conidia and also investigated possible citral action on cell walls (0.8 M sorbitol) and cell membranes (interaction with ergosterol). The MIC of citral varied 128–256 µg/mL, but the MFC of citral varied 256–1024 µg/mL. The MIC_{50} and MFC_{50} of citral were, respectively, 128 μg/mL and 256 μg/mL The results also showed that citral significantly inhibited mycelial development and germination of conidia. Investigation of the mechanism of antifungal action showed that citral Interact with ergosterol. These data indicate that monoterpene citral possess strong antifungal activity, which can be related to their interaction with ergosterol, supporting the possible use of these products in the treatment of mycosis by dematiaceous fungi.

Keywords: Citral, antifungal activity, *Cladosporium*, *C. sphaerospermum*.

1. Introduction

Nosocomial infections caused by fungi dematiaceous have increased greatly in recent years, mainly due to the risisng number of immunocompromised patients [1].

Cladosporium spp. are dematiaceous fungi usually identified as common airborne contaminants occupying a wide variety of habitats [2].

C. sphaerospermum is an important pathogen, being very harmful to crops [3]. For humans and animals, not all pathogenic strains are, however, some strains can occasionally cause skin and brain phaeohyphomycosis regardless of the immune status of the host [4-6].

This species is one of the most widely distributed allergens causing serious problems in patients with respiratory tract disease [7,8]. May cause allergic airways diseases, pulmonary emphysema, and intrabronchial lesions [9,10].

The resistence of microbes to antimicrobial agents has potentially serious implications for the management of infections [11]. However, the available antifungal therapeutic arsenal is limited, and the developmente of new drugs has been slow. Therefore, the search for alternative drugs with low resistance rates and fewer side effects remains a major challenge. Plants produce a variety of medicinal components that can inhibit pathogen growth. A considerable number of studies of medicinal plants and alternative compounds, susch as secondary metabolites, phenolic compounds, essential oils and extracts, have been performed [12].

Terpenes are compounds found in essential oils from several aromatic plant and form structurally and funcionally different classes. Terpenes can be classified according to their number of isoprene units: monoterpenes (C_{10}), the most representative molecules, and sesquiterpenes (C_{15}), but there are also hemiterpenes (C_5), diterpenes (C_{20}), triterpenes (C_{30}), and tetraterpenes (C_{40}) [13].

Citral (3,7-dimethyl-2,6-octadienal) is a natural mixture of two acyclic monoterpene aldehyde geometric isomers, geranial (trans-citral or citral A), and neral (cis-citral or citral B). It is present in the essential oil of many plants including lemon and orange species [14,15]. Citral presents different pharmacological properties, such as: anti-inflammatory [16], anti-tumor [17,18], bronchodilator [19], antiprotozoal [20] and antimicrobial [21,22] effects. The antifungal activity exerted by citral against molds and yeasts has already been demonstrated in varied conditions [23-25].

Although there are many reports on the antimicrobial properties of citral, there are few studies on its antifungal modes of action against strains of *C. sphaerospermum*. Given the above, the aim of this study was to determine the minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) and to investigate the action mechanism of citral against *C. sphaerospermum* in its mycelial growth, conidial germination, cell wall formation, and ergosterol interactions.

2. Material and Methods

2.1. Microorganisms. *Cladosporium sphaerospermum* (URM 5962, URM 5455, URM 5350, URM 6120) strains used in the antifungal assay were obtained from the Mycology Department fungal collection (URM), Biological Sciences Center, Federal University of Pernambuco, Brazil. The samples were maintained on Sabouraud Dextrose Agar - SDA (DIFCO®) at room temperature (28 °C) and under refrigeration (4 °C).

Stock inoculations (suspensions) of *C. sphaerospermum* were prepared from 7-14 day old sabouraud dextrose agar (Difco Lab., USA); the cultures were grown at room temperature. Fungal colonies were covered with 5 mL of sterile saline solution (0.9%), the surface was gently agitated with vortexes, and fungal elements in saline solution were transferred to sterile tubes. Inoculator was standardized at 0.5 tube of McFarland scale (10^6 CFU/mL). The final concentration confirmation was done by counting the microorganisms in a Neubauer chamber [26-28].

2.2. Chemicals. The product tested was the monoterpene Citral, obtained from Sigma Aldrich, Brazil. Amphotericin B and Voriconazole were obtained from Sigma Aldrich, Brazil. The monoterpene was dissolved in Tween 80 (2%) and DMSO (dimethylsulfoxide). The antifungal standards were dissolved in DMSO, and sterile distilled water to obtain solutions of 2048 μg/mL for each antifungal. The concentration of DMSO did not exceed 0.5% in the assays.

2.3. Culture Media. To test the biological activity of the products, Sabouraud dextrose agar (SDA) purchased from Difco Laboratories (Detroit, MI, USA), and RPMI-1640-L-glutamine (without sodium bicarbonate) (Sigma-Aldrich, Sao Paulo, SP, Brazil) culture media were used. Both were prepared and used according to the manufacturers' instructions.

2.4. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC). Broth microdilution assays were used to determine the MICs of monoterpene citral, Amphotericin B, and Voriconazol against *C. sphaerospermum* (URM 5962, URM 5455, URM 5350, URM 6120). RPMI-1640 was added to all the wells of 96-well plates. Two-fold serial dilutions of the agents were

prepared to obtain concentrations varying between 4 μg/mL and 1024 μg/mL. Finally, 10 μL aliquots of the inoculate suspension were added to the wells, and the plates were incubated at 28 ^{o}C for 5 days. Negative controls (without drugs) were used to confirm conidia viability, and sensitivity controls (for DMSO and Tween 80) were also included in the studies. At 5 days, there were visual observations of fungal growth. The MIC was defined as the lowest concentration capable of visually inhibiting fungal growth by 100%. The results were expressed as the arithmetic mean of three experiments [26, 27].

The MFC was determined by microdilution method to verify if the inhibition was reversible or permanent [29, 30]. Aliquots of 20 µL (from the wells that did not show growth in the MIC procedure) were transferred to 96-well plates previously prepared with 100 µL of RPMI-1640. The plates were aseptically sealed followed by mixing on a plate shaker (300 rpm) for 30 seconds, incubated at 28 ºC and read at 5 days of incubation. Tests were performed in duplicate and the geometric mean values were calculated. MFC was defined as the lowest citral concentration in which no visible growth occurred when subcultured on the 96-well plates containing broth without antifungal products.

After determination of MIC and MFC we selected 1 strain (*C. sphaerospermum* URM 6120), to continue the study of antifungal activity of citral.

2.5. Effects on Mycelia Growth. Analyses of the interferences of citral, Voriconazole, and Amphotericin on *C. sphaerospermum* URM 6120 mycelia growth were determined using poisoned substrate technique (dilution in solid medium), by daily measuring of radial mycelial growth on SDA, by adding products in an amount adjusted to provide final concentrations similar to the MIC, MIC x 2, and MIC x 4 previously found. For this, 2 mm plugs taken from a 10-day-old mold culture cultivated on SDA slants at 28 ºC were placed at the center of the sterile SDA Petri dishes containing the test drugs. At different intervals (0, 2, 4, 6, 8, 10, 12, and 14 days) of incubation at 28 ºC, the mold's radial mycelial growth was measured (mm) with calipers. The controls in this assay revealed the mold's radial growth on SDA without adding drugs. Each assay was performed twice and the results were expressed as the average of the two repetitions [31-33].

2.6. Conidial Germination Assay. Citral, Voriconazole, and Amphotericin B were tested to evaluate effects on the germination of *C. sphaerospermum* URM 6120 fungal conidia. Flasks containing MIC, MIC x 2 and MIC x 4 of citral, voriconazole, amphotericin and a control with distilled water were used. In sterile test tubes, 500 μL of RPMI-1640 plus citral were evenly mixed with 500 μL of fungal conidia suspension and immediately incubated at 28 ºC. Samples of the mixture were taken after 48 h of incubation for analysis. The whole experiment was performed in duplicate, where the number of conidia was determined in a Neubauer chamber, and the spore germination inhibition percentage at each time point was calculated by comparing the results obtained in the test experiments with the results of the control experiment. The analysis was conducted under an optical microscope (Zeiss Primo Star) [34, 35].

2.7. Sorbitol Assay Effects. The assay was performed using medium with and without sorbitol (control), to evaluate possible mechanisms involved in the antifungal activity of the test product on the *C. sphaerospermum* URM 6120 cell wall. The sorbitol was added to the culture medium in a final concentration of 0.8 M. The assay was performed by microdilution method in 96-well plates in a “U” (Alamar, Diadema, SP, Brazil) [26, 27]. The plates were sealed aseptically, incubated at 28 ºC, and readings were taken at 5 days. Based on the ability of sorbitol to act as a fungal cell wall osmotic protective agent, the higher MIC values observed in the medium with sorbitol added (as compared to the standard medium), suggest the cell wall as a possible target for the product tested [24, 36, 37]. The assay was performed in duplicate and expressed as the geometric mean of the results.

2.8. Ergosterol Binding Assay: MIC Value Determination in the Presence of Ergosterol. To assess if the product binds to fungal membrane sterols, an experiment was performed according to the method described by Escalante et al. [38], with some modifications. Ergosterol was prepared as described by Leite et al. [24]. The MIC of citral, against *C. sphaerospermum* URM 6120 was determined by using broth microdilution techniques [26, 27], in the presence and absence of exogenous ergosterol (Sigma-Aldrich, São Paulo, SP, Brazil) added to the assay medium, in different lines on the same microplate. Briefly, a solution of citral was diluted serially twice with RPMI-1640 (volume = 100 μL) containing ergosterol added at a concentration of 400 μg/mL. A volume yeast suspension 10 μL (0,5 McFarland) was

added to each well. The same procedure was realized for Amphotericin B, whose interaction with membrane ergosterol is already known, which served as a control drug. The plates were sealed and incubated at 28 ºC. The plates were read after 5 days of incubation, and the MIC was determined as the lowest concentration of test agent inhibiting visible growth. The assay was carried out in duplicate and the geometric mean of the values was calculated. The binding assay reflected the ability of the compound to bind with ergosterol.

2.9. Statistical Analysis. The results are expressed as mean ± S.E. Differences between the means were statistically compared using the Student's t-test. The values were considered significant with $p < 0.05$.

3. Results and Discussion

The results obtained reinforce the importance and necessity of research on the potential use of products naturals as a new therapeutic alternative in the treatment of fungal infections, due to emerging drug resistance, mainly related to dermatiaceous fungi.

The results for citral's antifungal activity against *C. sphaerospermum* were determined using the MIC and MFC in broth microdilutions. The MIC of citral varied between 128 and 256 µg/mL. The MIC_{50} (Minimum Fungicidal Concentration for 50 % of strains tested) was 128 µg/mL, inhibiting the growth of tested fungal strain. amphotericin B and voriconazol retained a lesser MIC_{50} than the phytoconstituent at 16 µg/mL MIC. Homever the strain *C. sphaerospermum* URM 6120, presented the MIC >1024 µg/mL for Amphotericin B (Table 1).

Table 1. MIC and MFC of citral, amphotericin B and voriconazole against *C. sphaerospermum.*

Microorganisms	Citral (µg/mL)		Amphotericin B (µg/mL)		Voriconazole (µg/mL)		Control strains*
	MIC	MFC	MIC	MFC	MIC	MFC	
C. sphaerospermum URM 5962	128	256	8	16	32	64	+
C. sphaerospermum URM 5455	256	512	16	32	16	32	+
C. sphaerospermum URM 5350	128	256	64	128	16	64	+
C. sphaerospermum URM 6120	256	1024	>1024	ND	16	32	+

Note. * microorganism growth in RPMI-1640, DMSO (5%), and Tween 80 (2%), without antifungal or monoterpenes. ND- Not determined.

According to the criteria proposed por Satoratto et al. [39], since products naturals with a MIC between 50 and 500 µg/mL are considered to have strong antimicrobial activity, while MICs between 500 and 1,500 µg/mL and over 1,500 µg/mL indicate moderate and weak activity, respectively. These results indicate that *citral* showed strong antifungal activity against strains of *C. spahaerospermum*.

After determination of the MIC, the fungicidal effect of the products was investigated. The MFC of citral varied between 256 and 1024 µg/mL. The MFC_{50} (Minimum Fungicidal Concentration for 50 % of strains tested) was 256 µg/mL. The MFC_{50} for amphotericin B and voriconazole was 32 µg/mL (Table 1).

According Siddiqui et al. [40] the MFC/MIC ratio is used to specify the nature of the antimicrobial effect against a particular pathogen. The ratio MFC/MIC was calculated in order to determine if the compound had a fungistatic (MFC/MIC ≤ 4) or fungicidal (MFC/MIC > 4) activity.

In the present study, the MFC of the citral was found to be two or four folds higher than the corresponding MIC results. The MFC/MIC ratios of citral were ≤ 4; this suggests that citral has a fungicidal effect against the strains tested.

The results for the control (Tween 80 and DMSO) showed no fungal growth inhibition; fungal growth in the medium without added drug was detected. In accordance with the above results, the strain *C. sphaerospermum* URM 6120 was selected for further testing.

Earlier studies demonstrated that the citral display a wide spectrum of antifungic activity. Garcia et al. [41] demonstrated the activity of citral against the fungi *Colletotrichum musae, Colletotrichum gloeosporioides* and *Fusarium subglutinans*. Citral is active against of methicillin-resistant *Staphylococcus aureus, Penicillium italicum* and *Rhizopus stolonifer* [15].

Citral showed strong inhibition on *Geotrichum citri-aurantii* with MIC and MFC of 0.50 µL/mL and 1.00 µL/mL [23]. Zheng et al. [42] demonstrated the antimicrobial activity of citral front of fungal strains of *Penicillium digitatum*.

Recently, our research group showed that the citral is *in vitro* antifungal potential against strains of *Candida albicans* [24] and *Candida tropicalis* [25].

After determination of MIC and MFC we selected strain *C. sphaerospermum* URM 6120 that It was resistant to amphotericin B, to continue the study of antifungal activity of citral.

Macromolecules whose functionality is related to growth, survival, virulence or cellular morphogenesis are pointed out as promising targets for new antifungal agents [43]. Thus, taking into consideration the promising antifungal activity of citral, the effect of different concentrations of that substance on mycelial growth and the germination of conidia of *C. sphaerospermum* URM 6120 was investigated.

The effect of differing concentrations of the test drug (MIC, MIC x 2 and MIC x 4) on mycelia growth was determined measuring of the radial mycelial growth, and the results are shown in Figure 1. With to effects on *C. sphaerospermum* URM 6120, it can be seen that citral in MIC concentrations of (256 μg/mL), MIC x 2 (512 μg/mL) and MIC x 4 (1024 μg/mL) inhibited normal mycelia growth ($p < 0.05$) when compared to the control (mycelia diameter being 100 %). Amphotericina B did not showed activity in all concentrations tested (Fig. 1C). However the voriconazole test on *C. sphaerospermum* URM 6120 showed significant inhibition of mycelial growth at all concentrations tested (Fig. 1B). Strain controls showed a constant rate of mycelial growth over the time evaluated, indicating good antifungal effect for the citral.

These results suggested that the substance evaluated inhibited normal mycelial development of *C. sphaerospermum* at all concentrations tested. And that

citral at its MIC, MIC x 2 and MIC x 4 concentrations was more potent when compared to amphotericin B at its respective MIC, MIC x 2 and MIC x 4 concentrations ($p < 0.05$).

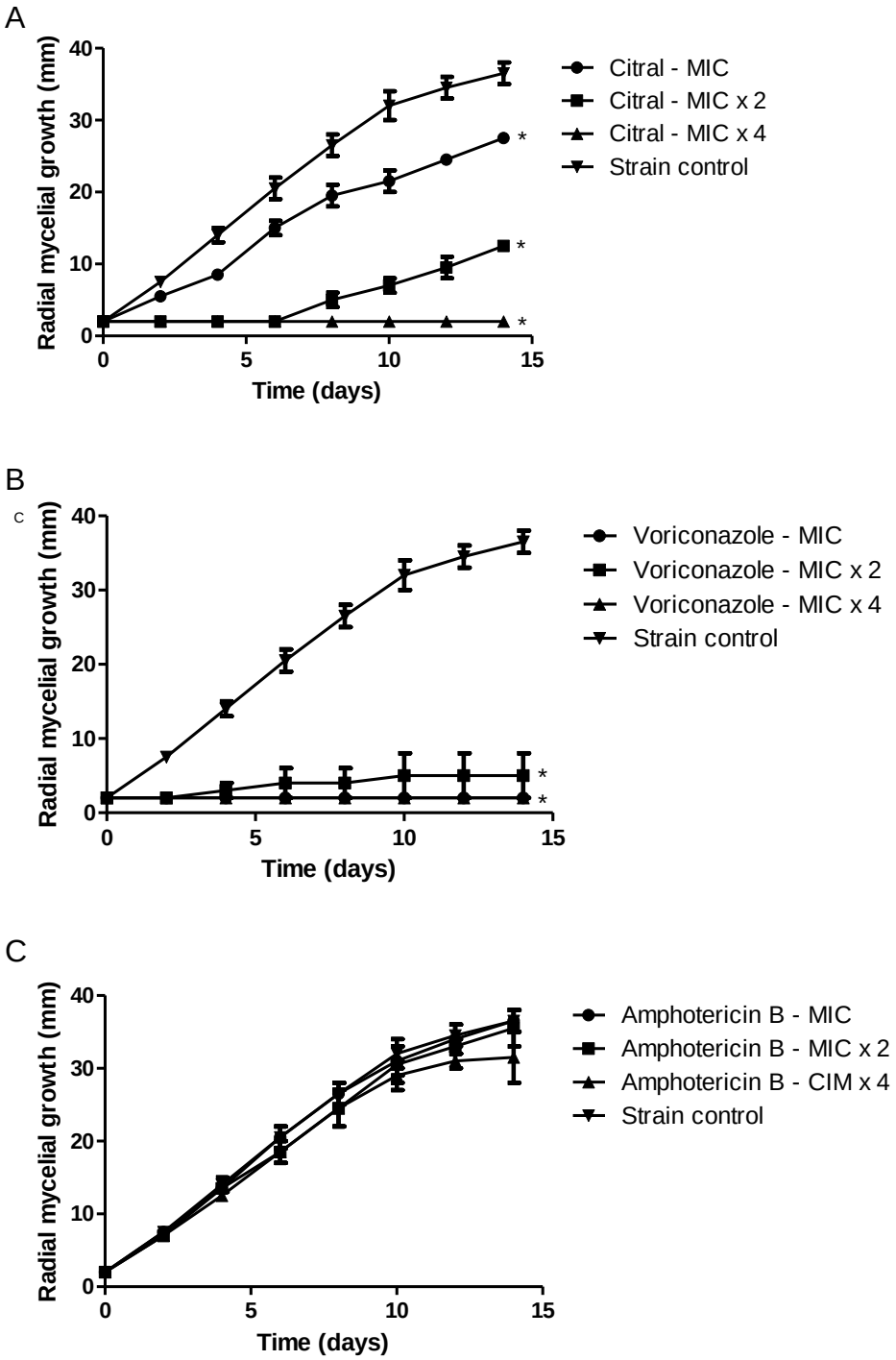

FIGURE 1. Radial mycelial growth produced by *C. sphaerospermum* URM 6120 in the absence (control) and presence citral (A), voriconazole (B) and amphotericin B (C). **p < 0.05 compared to control.*

These results corroborate the data obtained by some researchers who have investigated the antifungal potential of citral in inhibiting the mycelial growth of pathogenic and non-pathogenic fungi [23, 44].

The results reported to date can be considered of great relevance, due to the importance of mycelial growth in the development of infection fungal. Good fungal growth of *Cladosporium* species, similarly to other filamentous fungi, produce hyphae which can penetrate the innermost skin layer and aggravate the damage in the host [45, 46]. Therefore some researchers are investigating the products naturals potential in inhibiting mycelial growth of pathogenic fungi due to their importance in the mycosis development [47, 48].

The results obtained in this study agree with those of Zhou et al. [23] that evaluated the antifungal activity of three volatile compounds: citral, octanal, and α-terpineol against *Geotrichum citri-aurantii*. It was found that citral in the study was able to significantly inhibit mycelial growth.

More recently, Ouyang et al. [49] observed that citral dose-dependently inhibited the mycelial growth of *Penicillium digitatum*, with the minimum inhibitory concentration (MIC) of 1.78 mg/mL.

Thus, the study of the germination of conidia has great implications in clinical practice, because it is possible to develop new therapeutic approaches that block the infection at its onset [50]. In this perspective, the effect of the citral on the germination of the conidia of *C. sphaerospermum* URM 6120 was investigated. The effects of different concentrations (MIC, MIC x 2 and MIC x 4) of citral, voriconazole and amphotericin B on the germination of conidia are shown in Figure 2.

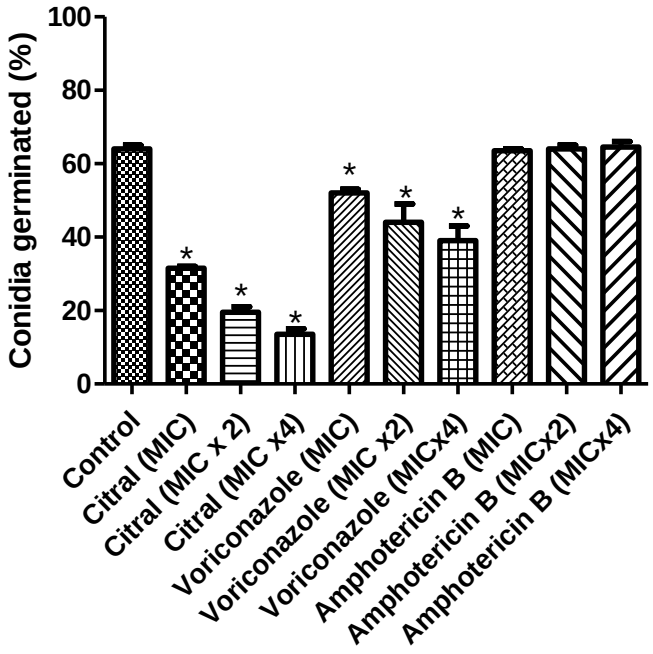

Figure 2. Percentage of conidial germination of *C. sphaerospermum* URM 6120 in the absence (control) and presence of citral, voriconazole and amphotericin B. *$p < 0.05$ compared to control.

At all concentrations tested (MIC, MIC x 2 and MIC x 4), the citral displayed significant inhibitory action against *C. sphaerospermum*, as compared to the control. The antifungal voriconazole tested, also showed significant inhibition on conidia germination. However, the results show that citral at its MIC, MIC x 2 and MIC x 4 concentrations was more potent when compared to voriconazole at their respective concentrations ($p < 0.05$). Amphotericin B not showed inhibition on conidia germination in concentrations tested (Fig. 2).

Conidia represent the most common mode of asexual reproduction, they play an important role in natural fungal propagation and are structurally resistant [51]. Fungal spores are distributed in large amounts in the outdoor air, and some of them may cause diseases in human beings, animals, and plants. Among several taxa, *Cladosporium* spp is one of the most ubiquitous and most widely distributed, being found in high concentrations in the air. *C. sphaerospermum* spores have been classified as important allergens, and are, therefore, important to the study of allergies [52]. Thus, it is deemed important to quantitatively evaluate the power of a product to interfere with fungal spore germination [53].

The antifungal potential of essential oils in inhibiting the germination of conidia has been extensively studied. It has been reported that the essential oil of *C. winterianus* has a strong inhibitory effect on the germination of conidia of *Trichophyton mentagrophytes* [47]. The essential oil of *C. zeylanicum* was shown to inhibit the germination of the conidia of *A. fumigatus, A. flavus* and *A. niger* [54]. Recently, our research group showed that the essential oil of *Melissa officinalis* inhibited the germination of the conidia of *Cladophialophora carrionii* [55].

Antifungal activities of 15 different plant essential oils or its components were evaluated during conidial germination and mycelial growth of *C. gloeosporioides*. It was found that citral in the study was able to significantly inhibit conidial germination [56].

The results obtained in this study corroborate those observed in previous studies, thus revealing the antifungal potential of the citral in blocking the infection induced by *C. sphaerospermum* soon after onset, since they significantly inhibit the germination of conidia.

The antifungal mechanism of volatile compounds has been attributed to its capacity to disturb the cellular membrane, interfere with the cellular metabolism, react with active sites of enzymes, or act as H^+ carriers [57, 58].

Several targets including cell wall, cell membrane, mitochondrion, and genetic material, have been proposed to account for the antifungal activity of essential oils or their volatile compositions [42, 59-61]. The cell wall is an extracellular layer outside the cell membrane which protects the cell against mechanical damage, osmotic strength and determines the cell shape.

To investigate the action of the product on the fungal cell wall we performed an assay with sorbitol (Table 2), which has an osmoprotectant function. Sorbitol, an osmotic protective is used to stabilize the yeast protoplasts. Specific fungal cell wall inhibitors share a distinctive characteristic where their antifungal effects are reversed in mediums containing sorbitol [37]. Cells protected with sorbitol can grow in the presence of fungal cell wall inhibitors, whereas growth would be inhibited in the absence of sorbitol. This effect is detected by increases in the MIC value as observed in medium with sorbitol as compared to the MIC value in medium without sorbitol (standard medium) [37, 64]. Osmotic destabilizing agents and disrupting the cell wall lead to rearrangements of the cell wall and allow the fungal cells to survive [65].

In this paper, the MIC values of citral in both experiments, in mediums with and without sorbitol, were identical, suggesting that citral does not act by inhibiting fungal cell wall synthesis, but probably by affecting another target in *C. sphaerospermum* URM 6120 (Table 2).

Table 2: MIC values (μg/mL) of drugs in the absence and presence of sorbitol (0.8 M) and ergosterol (400 μg/mL) against *C. sphaerospermum* URM 6120.

Drugs	Sorbitol		Ergosterol	
	Absence	Presence	Absence	Presence
Citral	256	256	256	2048
Amphotericin B[a]	-	-	>1024	>2048

[a]Positive control. —: not tested.

This is the first study to demonstrate the action of citral on the cell wall of *C. sphaerospermum* using sorbitol tests. The results are in agreement with those reported by Miron et al. [66] who no changes were observed in the MIC of citral in the sorbitol protection assay against *T. asahii* and with those reported by Leite et al. [24] and Sousa et al. [25] who have shown that citral does not act on the cell wall of *C. albicans* and *C. tropicalis*.

Since it appears that citral does not act at the level of the fungal cell wall, another possibility investigated was that it might act at the level of the cell membrane.

Considering the lipophilic nature of terpenes, as well as the interaction of these products with biological membranes, it was decided to investigate the participation of membrane sterols in the antifungal effect exerted by citral. Ergosterol is the principal sterol present in yeasts and filamentous fungi, where it is necessary for the growth and normal function of the fungal cell membrane. Besides controlling the fluidity, asymmetry and integrity of the membrane, ergosterol contributes to the proper functioning of enzymes bound to the membrane [67]. The majority of existing drugs for the treatment of fungal infections target the cell wall or plasma membrane directly or indirectly, particularly ergosterol and its biosynthesis [43, 67].

Considering this possible fungal cell membrane interference of citral, the compound was tested to investigate its ability to form complexes with ergosterol. This method is based on the exposure of a test compound to exogenous ergosterol,

where an affinity for sterol will lead to the rapid formation of a complex, thereby impeding complexation with sterols of the membrane and resulting in an increase in MIC [38]. The MICs of citral against *C. sphaerospermum* URM 6120 increased eight times in the presence of 400 µg/mL ergosterol. Amphotericin B, the positive control that has a known interaction with ergosterol, showed a higher MIC in the presence of this sterol (Table 2).

The mechanism of action of monoterpenes has not been completely clarified. Some studies showed the breakdown of cytoplasmic and organelle membranes exposed to certain volatile oils. The loss of membrane integrity can cause changes in membrane function leading to the antifungal activity [68-70]. Despite these findings, it is not known how volatile oils damage the membranes. The discovery of the mechanism of action can help maximize the effect of natural products, either by concentration of active ingredients or formulation optimization.

In this study, citral showed an affinity for ergosterol relating their mechanism of action to cell membrane destabilization. Recent studies have shown that citral inhibits ergosterol biosynthesis in *C. albicans* [71] and *Penicillium italicum* [72]. Ergosterol biosynthesis inhibition has also been observed for citral at 200 µg/mL in *Aspergillus ochraceus* [73].

Miron et al. [66] evaluated the antifungal activity of geraniol, nerol, citral, neral and geranial against seven opportunistic pathogenic yeasts and four dermatophyte species. It was found that citral in the study showed an affinity for ergosterol relating their mechanism of action to cell membrane destabilization.

4. Conclusions

On the basis of the data presented, the monoterpene citral has promising fungicidal activity, whereby is capable of inhibiting an infection at its onset. Such activities can be related to an interaction with ergosterol, a sterol present in the cell membrane of *C. sphaerospermum*, which plays an important role in the mycelial growth and germination of conidia of these fungi. Therefore, this monoterpene, may represent new alternative therapeutic agents in the treatment of mycosis by dematiaceous fungi. However, there is a need for more studies aimed at correlating their potent antifungal activity *in vitro* and *in vivo* and proving their safety for clinical application.

Conflict of interests

The authors declare that there are no conflicts of interests regarding the publication of this paper.

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5.5 Citral: Antifungal activity and Mode of Action, against *Cladosporium oxysporum*

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Citral: Antifungal activity and Mode of Action, against *Cladosporium oxysporum*

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ABSTRACT

Dematiaceous fungi are a heterogeneous group of fungi with dark colonies and pigmented fungal elements. The spectrum of diseases associated with dematiaceous fungi ranges from superficial skin and soft tissue infections to disseminated sepsis with high mortality. Therefore, it is necessary to study molecules with an antifungal action against these fungis. Attention has been drawn to the antimicrobial activity of aromatic compounds because of their promising biological properties. Citral is a monoterpene with known pharmacological properties, including antimicrobial action. Therefore, we investigated the antifungal activity of citral against strains of *C. oxysporum* which involved determining its minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC) and effects on mycelial growth and conidial germination. The effects of citral on the cell wall (sorbitol protect effect) and the cell membrane (citral to ergosterol binding) were investigated. Citral inhibited the growth of 50% of *C. oxysporum* strains employed in this study at an MIC 128 µg/ml, as well as mycelial growth and conidia germination. The results of these studies on the mechanisms of action suggested that citral exerts antifungal effects on the cell membrane of *C. oxysporum*. Finally, our studies support the potential use of the citral as an antifungal agent against dematiaceous fungi especially *C. oxysporum.*

1. Introdution

Dematiaceous fungi (black fungi) are a heterogeneous group of fungi present in diverse environments worldwide. Many species in this group are known to cause allergic reactions and potentially fatal diseases in humans and animals, especially in tropical and subtropical climates [1].

Cladosporium is mainly known as a ubiquitous environmental saprobic fungus or plant endophyte, and to date, just a few species have been documented as etiologic agents in vertebrate hosts, including humans. They are among the most important allergenic fungi linked to allergic rhinitis and respiratory arrest in asthmatic patients [2,3]. Some species are described as a cause of opportunistic phaeohyphomycosis, including subcutaneous and deep infections in humans and animals [4,5].

Cladosporium oxysporum is a common saprophyte frequently grows on various substrata [6]. This species is often found as culture contaminants [7] and cited in cases of cutaneous and subcutaneous disease [8,9].

The increased incidence of these fungal infections, especially dangerous hospital-acquired infections and infections in immunocompromised patients, has accentuated the need for new antifungal treatments [10].

There exists a clear demand for additional antifungals with therapeutic potential. In this context, attention has focused on the antifungal activity of aromatic plants and their constituents due to their potential biological properties [11].

Studies of plant species have been conducted to evaluate the characteristics of natural drug products, including their sustainability, affordability, and antimicrobial activity [12].

Citral ($C_{10}H_{16}O$) is one of the most common flavor compounds found in citrus oils, which has been already widely used in foods and beverages (e.g., soft drinks and desserts) [13]. Citral is a monoterpenoid aldehyde [14] often present in the form of stereoisomers geranial and neral [15] that has been identified in the leaves and fruits of several plant species including myrtle trees, basil, lemon, lime, lemongrass, orange, and bergamot [14,16]. A number of experimental and clinical studies have shown that citral has favorable anti-inflammatory [17], antitumoral [18] effects, and there is increasing evidence that citral acts as a fungicidal and bactericidal agent [19,20].

Although citral has been reported to be effective against a variety of microbial species, there have been no reports on its antimicrobial activity against *C*. *oxysporum* and possible mode of action. To fill this gap, the aim of the present study was to determine antifungal effect and mode of action of citral against *C*. *oxysporum.*

2. Material and Methods

2.1. Microorganisms. *Cladosporium oxysporum* (URM 5234, URM 6056 and URM 5412) strains used in the antifungal assay were obtained from the Mycology Department fungal collection (URM), Biological Sciences Center, Federal University of Pernambuco, Brazil. The samples were maintained on Sabouraud Dextrose Agar - SDA (DIFCO®) at room temperature (28°C) and under refrigeration (4°C).

Stock inoculations (suspensions) of *C. oxysporum* were prepared from 7-14 day old sabouraud dextrose agar (Difco Lab., USA); the cultures were grown at room temperature. Fungal colonies were covered with 5 mL of sterile saline solution (0.9%), the surface was gently agitated with vortexes, and fungal elements in saline solution were transferred to sterile tubes. Inoculator was standardized at 0.5 tube of McFarland scale (10^6 CFU/mL). The final concentration confirmation was done by counting the microorganisms in a Neubauer chamber [21-23].

2.2. Chemicals. The product tested was the monoterpene Citral, obtained from Sigma Aldrich, Brazil. Amphotericin B and voriconazole were obtained from Sigma Aldrich, Brazil. The monoterpene was dissolved in Tween 80 (2 %) and DMSO (dimethylsulfoxide). The antifungal standards were dissolved in DMSO, and sterile distilled water to obtain solutions of 2048 μg/mL for each antifungal. The concentration of DMSO did not exceed 0.5% in the assays.

2.3. Culture Media. To test the biological activity of the products, Sabouraud dextrose agar (SDA) purchased from Difco Laboratories (Detroit, MI, USA), and RPMI-1640-L-glutamine (without sodium bicarbonate) (Sigma-Aldrich, Sao Paulo, SP, Brazil) culture media were used. Both were prepared and used according to the manufacturers' instructions.

2.4. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC). Broth microdilution assays were used to determine the MICs of monoterpene citral, amphotericin B, and voriconazol against *C. oxysporum* (URM 5234, URM 6056 and URM 5412). RPMI-1640 was added to all the wells of 96-well plates. Two-fold serial dilutions of the agents were prepared to obtain concentrations varying between 4 μg/mL and 1024 μg/mL. Finally, 10 μL

aliquots of the inoculate suspension were added to the wells, and the plates were incubated at 28 ^{o}C for 5 days. Negative controls (without drugs) were used to confirm conidia viability, and sensitivity controls (for DMSO and Tween 80) were also included in the studies. At 5 days, there were visual observations of fungal growth. The MIC was defined as the lowest concentration capable of visually inhibiting fungal growth by 100%. The results were expressed as the arithmetic mean of three experiments [21,22].

The MFC was determined by microdilution method to verify if the inhibition was reversible or permanent [24,25]. Aliquots of 20 µL (from the wells that did not show growth in the MIC procedure) were transferred to 96-well plates previously prepared with 100 µL of RPMI-1640. The plates were aseptically sealed followed by mixing on a plate shaker (300 rpm) for 30 seconds, incubated at 28ºC and read at 5 days of incubation. Tests were performed in duplicate and the geometric mean values were calculated. MFC was defined as the lowest citral concentration in which no visible growth occurred when subcultured on the 96-well plates containing broth without antifungal products.

After determination of the MIC and MFC we selected 1 strain (*C. oxysporum* URM 5234), to continue the citral antifungal activity study.

2.5. Effects on Mycelia Growth. Analyses of the interferences of citral, voriconazole, and amphotericin on *C. oxysporum* URM 5234 mycelia growth were determined using poisoned substrate technique (dilution in solid medium), by daily measuring of radial mycelial growth on SDA, by adding products in an amount adjusted to provide final concentrations similar to the MIC, MIC x 2, and MIC x 4 previously found. For this, 2 mm plugs taken from a 10-day-old mold culture cultivated on SDA slants at 28 ºC were placed at the center of the sterile SDA Petri dishes containing the test drugs. At different intervals (0, 2, 4, 6, 8, 10, 12, and 14 days) of incubation at 28 ºC, the mold's radial mycelial growth was measured (mm) with calipers. The controls in this assay revealed the mold's radial growth on SDA without adding drugs. Each assay was performed twice and the results were expressed as the average of the two repetitions [26-28].

2.6. Conidial Germination Assay. Citral, voriconazole, and amphotericin B were tested to evaluate effects on the germination of *C. oxysporum* URM 5234 fungal

conidia. Flasks containing MIC, MIC x 2 and MIC x 4 of citral, voriconazole, amphotericin and a control with distilled water were used. In sterile test tubes, 500 μL of RPMI-1640 plus citral were evenly mixed with 500 μL of fungal conidia suspension and immediately incubated at 28ºC. Samples of the mixture were taken after 48 h of incubation for analysis. The whole experiment was performed in duplicate, where the number of conidia was determined in a Neubauer chamber, and the spore germination inhibition percentage at each time point was calculated by comparing the results obtained in the test experiments with the results of the control experiment. The analysis was conducted under an optical microscope (Zeiss Primo Star) [29,30].

2.7. Sorbitol Assay Effects. The assay was performed using medium with and without sorbitol (control), to evaluate possible mechanisms involved in the antifungal activity of the test product on the *C. oxysporum* URM 5234 cell wall. The sorbitol was added to the culture medium in a final concentration of 0.8 M. The assay was performed by microdilution method in 96-well plates in a “U” (Alamar, Diadema, SP, Brazil) [21,22]. The plates were sealed aseptically, incubated at 28 ºC, and readings were taken at 5 days. Based on the ability of sorbitol to act as a fungal cell wall osmotic protective agent, the higher MIC values observed in the medium with sorbitol added (as compared to the standard medium), suggest the cell wall as a possible target for the product tested [19,31,32]. The assay was performed in duplicate and expressed as the geometric mean of the results.

2.8. Ergosterol Binding Assay. MIC Value Determination in the Presence of Ergosterol. To assess if the product binds to fungal membrane sterols, an experiment was performed according to the method described by Escalante et al. [33], with some modifications. Ergosterol was prepared as described by Leite et al. [19]. The MIC of citral, against *C. oxysporum* URM 5234 was determined by using broth microdilution techniques [21,22], in the presence and absence of exogenous ergosterol (Sigma-Aldrich, São Paulo, SP, Brazil) added to the assay medium, in different lines on the same microplate. Briefly, a solution of citral was diluted serially twice with RPMI-1640 (volume = 100 μL) containing ergosterol added at a concentration of 400 μg/mL. A volume yeast suspension 10 μL (0,5 McFarland) was added to each well. The same procedure was realized for Amphotericin B, whose interaction with membrane ergosterol is already known, which served as a control drug. The plates were sealed

and incubated at 28 ºC. The plates were read after 5 days of incubation, and the MIC was determined as the lowest concentration of test agent inhibiting visible growth. The assay was carried out in duplicate and the geometric mean of the values was calculated. The binding assay reflected the ability of the compound to bind with ergosterol.

2.9. Statistical Analysis. The results are expressed as mean ± S.E. Differences between the means were statistically compared using the Student's t-test. The values were considered significant with $p < 0.05$.

3. Results and Discussion

The results for citral's antifungal activity against *C. oxysporum* were determined using the MIC and MFC in broth microdilutions. MICs values of citral against all *C. oxysporum* strains was 128 µg/mL. The MIC_{50} (Minimum Fungicidal Concentration for 50% of strains tested) was 128 µg/mL, inhibiting the growth of tested fungal strain. Amphotericin B and voriconazol retained a lesser MIC_{50} than the phytoconstituent at 16 µg/mL MIC (Table 1).

The MFC of citral was 256 µg/mL for all strains tested. The MFC_{50} (Minimum Fungicidal Concentration for 50% of strains tested) was 256 µg/mL. The MFC_{50} for Amphotericin B and voriconazole was 64 µg/mL (Table 1).

The results for the control (Tween 80 and DMSO) showed no fungal growth inhibition; fungal growth in the medium without added drug was detected, confirming the viability of the fungi.

Table 1. MIC and MFC of citral, amphotericin B and voriconazole against *C. oxysporum.*

Microorganisms	Citral (µg/mL)		Amphotericin B (µg/mL)		Voriconazole (µg/mL)		Control strains*
	MIC	MFC	MIC	MFC	MIC	MFC	
C. oxysporum URM 5234	128	256	16	64	16	64	+
C. oxysporum URM 6056	128	256	>1024	ND	>1024	ND	+
C. oxysporum URM 5412	128	256	16	32	8	32	+

Note. * microorganism growth in RPMI-1640, DMSO (5%), and Tween 80 (2%), without antifungal or monoterpenes. ND- Not determined.

According to criteria proposed by Sartoratto et al. [34], citral showed strong antifungal activity against *C. oxysporum* as the MIC values of this monoterpene was lower than 500 µg/mL. Amphotericin B and voriconazole were used as an positive control because they are commonly used antifungal drugs for the treatment of infections caused by dematiaceous fungi [35,36]. In the literature, citral has been found to be active against yeast and filamentous fungal species [37-39].

Citral, one of the volatile constituents in plant essential oils, has been demonstrated to have strong antifungal activity against *P. digitatum*, *P. italicum*, and *G. citri-aurantii* [40-42].

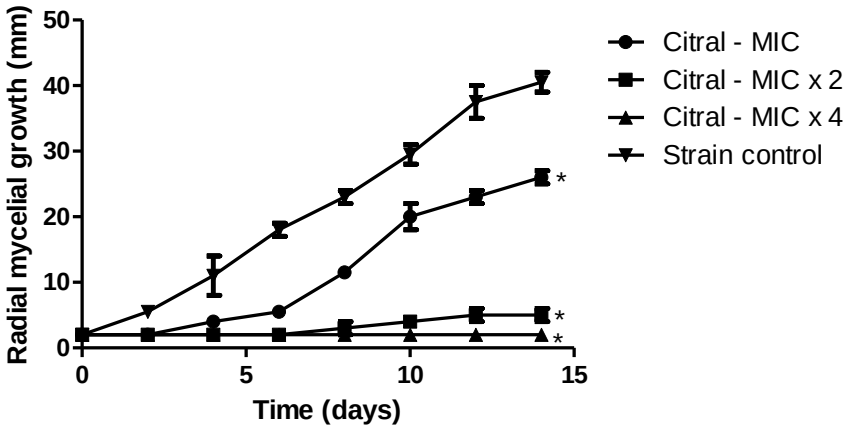
In accordance with the above results, the strain *C. oxysporum* URM 5234 was employed to explore the effects of citral, amphotericin B and voriconazole on mycelial growth, conidial germination, on the cell wall (sorbitol protect effect) and the cell membrane (interaction with ergosterol).

The fungi mycelium is (on the whole) the hyphae, and fungal filaments, or segments of filamentous mycelium. Mode of growth can also be na important factor contributing to the virulence of potentially pathogenic fungi, as both biofilm formation and tissue invasion have been shown to contribute to pathogenesis [43].

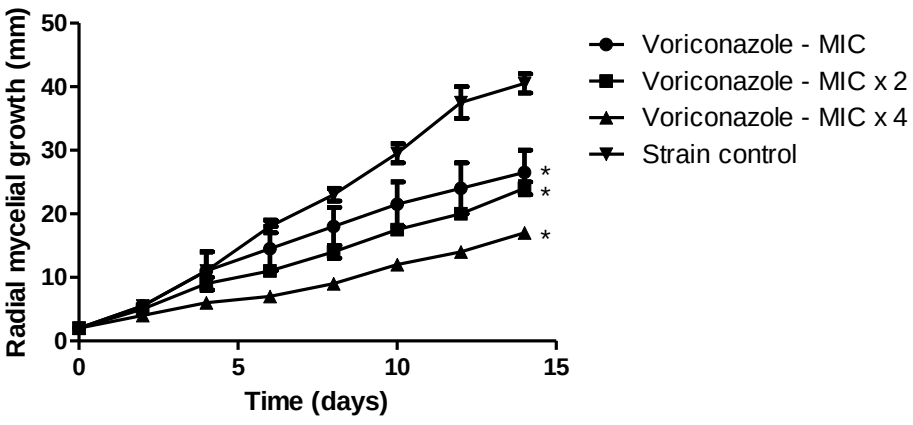
Therefore some researchers are investigating the products naturals potential in inhibiting mycelial growth of pathogenic fungi due to their importance in the mycosis development [44,45].

The effect of MIC, MIC x 2 and MIC x 4 of the drugs on the mycelial growth was determined by measuring the radial mycelial growth of *C. oxysporum* URM 5234 (Fig. 1). As seen in Figure 1A, citral all tested concentrations, especially at MIC x 2 and MIC x 4, inhibited the mycelial growth of *C. oxysporum* URM 5234 ($p < 0.05$) as compared with the control (mycelia diameter being 100%). Similar effects were noted with voriconazole in that the drug effectively inhibited the mycelial growth, in all concentrations tested (Fig. 1B). Amphotericin B did not show capability to develop a significant inhibitory effect on the mould mycelia growth along 14 days of exposure (Fig. 1C). In general, the mould strain when exposed to amphotericin B developed a progressive increasing in their mycelial growth showing a growth profiles similar to the ones found in the control assay.

A



B



C

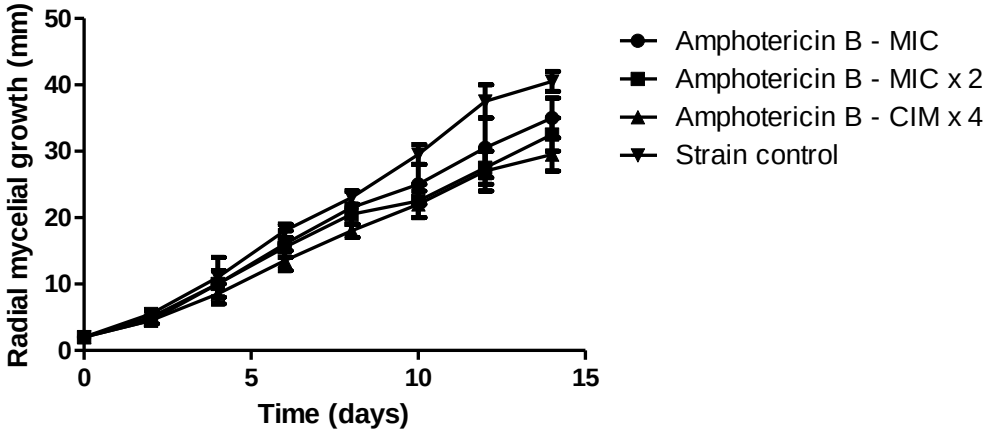


Figure 1. Radial mycelial growth produced by *C. oxysporum* URM 5234 in the absence (control) and presence citral (A), voriconazole (B) and amphotericin B (C). **p < 0.05 compared to control.*

In this study, citral it was capable of inhibiting the mycelial growth. In filamentous fungal, hyphal production is important because they penetrate into the deeper layers of the epidermis. This is of particular importance since the outer tissue layers are constantly being lost [46].

These results confirm previously published work, such as that by Ouyang et al. [47] showed that the citral exhibit its antifungal activity against the mycelial growth of *P. digitatum*. Li et al. [37] showed the effect of citral on *Magnaporthe grisea*, in this study, it was found that mycelial growth was significantly inhibited by citral in a concentration-dependent manner, concerning the efficacy of citral for anhibition of pathogenic fungi growth.

Thus, the study of the germination of conidia has great implications in clinical practice, because it is possible to develop new therapeutic approaches that block the infection at its onset [48]. In this perspective, the effect of the citral on the germination of the conidia of *C. oxysporum* was investigated. The percentage of germinated conidia of C. oxysporum URM 5234 are recorded in Figure 2. At their MICs, the drugs significantly inhibited conidial germination ($p < 0.05$).

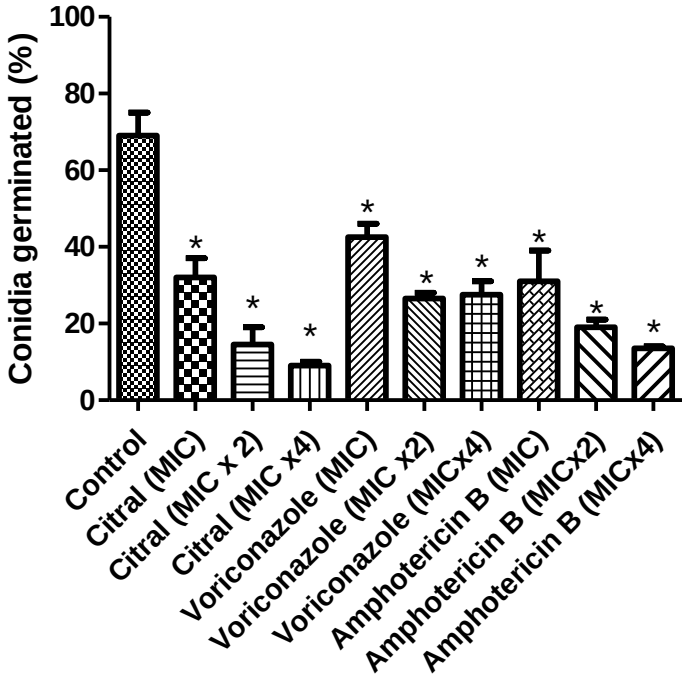


Figure 2. Percentage of conidial germination of *C. oxysporum* URM 5234 in the absence (control) and presence of citral, voriconazole and amphotericin B. **p < 0.05* compared to control.

The asexual spore, or conidium, is critical in the life cycle of many fungi because it is the primary means for dispersion and serves as a `safe house' for the fungal genome in adverse environmental conditions [48]. The study of conidial germination, in addition to being a scientific puzzle of great interest, has far-reaching practical implications. Intensive monoculture and inbreeding have greatly increased the incidence and severity of fungal infections in crops [49]. Often fatal fungal infections in immunodeficient patients have also increased markedly during the last decade [50]. In almost all cases in both plants and animals, fungal infection is initiated by contact of the host with airborne conidia, which begin the infective process by undergoing conidial germination. By achieving a molecular understanding of this process, it may be possible to develop novel therapeutic approaches that block infection at its outset.

The results showed in this study are agree with those of Neri, Mari and Brigati [51], wh reported that citral can inhibited the germination of the conidia of *Penicilium expansum* and Garcia et al. [52] observed that citral inhibited the germination of the conidia of *Colletotrichum musae, Colletotrichum gloeosporioides* and *Fusarium subglutinans*.

Several targets including cell wall, cell membrane, mitochondrion, and genetic material, have been proposed to account for the antifungal activity of essential oils or their volatile compositions [53-57].

The fungal cell wall is a dynamic structure that protects fungal protoplasts from external osmotic shocks and defi nes fungal morphogenesis. Thus, changes in the organization or functional disruption of the cell wall induced by antifungal agents are involved in fungal death [34,58].

The sorbitol protection assay was performed to further explore the mode of action of citral on the integrity of the fungal cell wall. Drugs that act on the cell wall cause lysis of fungal cells in the absence of an osmotic stabilizer (sorbitol), but their growth can continue in the presence of sorbitol [33]. This effect is detected by increases in the MIC value as observed in medium with sorbitol (0.8 M) as compared to the MIC value in medium without sorbitol (standard medium). This assay is known as a broad spectrum screen that can find not only agents that directly interfere in cell wall synthesis and assembly but also regulatory mechanisms involved in this process, including signal transduction pathways [32,59].

The results of the sorbitol protection assay are presented in Table 2. The citral MIC remained unchanged, suggesting that the citral does not act by inhibiting fungal cell wall synthesis, but probably by affecting another target in *C. oxysporum* URM 5234.

Table 2: MIC values (μg/mL) of drugs in the absence and presence of sorbitol (0.8 M) and ergosterol (400 μg/mL) against *C. oxysporum* URM 5234.

Drugs	Sorbitol		Ergosterol	
	Absence	Presence	Absence	Presence
Citral	128	128	128	2048
Amphotericin B[a]	-	-	16	2048

[a]Positive control. —: not tested.

This is the first study to investigated the action of citral on the cell wall of *C. oxysporum* under sorbitol testing, which complicates comparison with other investigations. However, the results confirm earlier studies for other microorganisms, Lima et al. [60] and Leite et al. [19] confirmed that antifungal activity of the citral

against *C. albicans* was not reversed in the presence of an osmotic support. Sousa et al. [39] reported that citral does not act on the cell wall of *C. tropicalis*.This would suggest that inhibiting fungal cell wall synthesis or assembly is not altered when the chemical structure of citral is maintained.

It is reported that the antimicrobial mechanism of cyclic hydrocarbons, such as citral, is related to its lipophilic character in that they increase the fluidity and permeability of the cell membrane of microorganisms. In fact, these compounds interfere with ion transport, unbalancing osmotic conditions in the membrane and making its associated proteins ineffi cient. In any case, this can lead to inhibition of microbial growth, and death or cell lysis [61].

The fungal cell membrane is a dynamic structure composed of a lipid bilayer where enzymes and transport proteins are embedded. Ergosterol is the main sterol component in the plasma membrane of fungi and plays the same role in the fungal membranes that cholesterol does in mammalian cell membranes Therefore, these two sterols seem to exhibit qualitatively similar properties [58].

To explore the possible mechanism of interaction of citral with fungal cell membrane, we studied the ability of the compound to form complexes with ergosterol. If the activity of citral is caused by binding to ergosterol, the exogenous ergosterol would prevent the binding to the fungal membrane's ergosterol. Consequently, it would cause an increase in MIC of citral in the presence of exogenous ergosterol with respect to the control experiment [62, 33]. Thus, the effect of exogenous ergosterol on the MIC of citral and amphotericin B was determined.

Results showed (Table 2) that the MIC values of citral against *C. oxysporum* URM 5234 was 2048 μg/mL in medium with additional 400 µg/mL ergosterol, increased sixteen times in the presence of this sterol, suggesting that the citral act by binding to membrane ergosterol. Regarding amphotericin B, 128 x MIC was observed in the presence of ergosterol.

Confirming our results, in others studies citral was found to destroy the membrane permeability and integrity of *P. italicum* and *G. citri-aurantii* by causing significant losses in total lipids or ergosterol contents [63, 38].

According to Rajput, Karuppayil [64] the mechanism of anti-*Candida* activity of citral appears to be associated with damage in the membrane integrity, through inhibition of ergosterol biosynthesis.

Recents studies Ouyang et al. [47] suggest that citral could exhibit its antifungal activity against the mycelial growth of *P. digitatum* by disrupting ergosterol biosynthesis.

Conclusion

This study support the view that citral exerts its antifungal activity on the cell membrane of *C. oxysporum* by binding to membrane ergosterol. Thus, our results may serve as a guide for future in vivo studies of clinical use of citral in treating fungi infections by demtiaceous fungi.

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5.6 Anti-*Cladosporium cladosporioides* effectiveness of citral and investigation of mode of action

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Anti-*Cladosporium cladosporioides* effectiveness of citral and investigation of mode of action

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ABSTRACT

The dematiaceous fungus Cladosporium cladosporioides is a widely distributed saprophyte that occasionally infects the lung, skin, eye and brain in both immunocompetent or immunosuppressed hosts. This study aimed to investigate the antifungal activity of citral against *C. cladosporioides* (INCQS 40188). The minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) were determined by the broth microdilution techniques. Citral was tested to evaluate its effects on mycelia growth and germination of fungal conidia of *C. carrionii.* We also investigated possible citral action on cell walls (0.8 M sorbitol) and cell membranes (citral to ergosterol binding). The results showed that citral inhibited the growth of *C. cladosporioides* at an MIC 64 µg/mL and MFC was 128 µg/ml, as well as mycelial growth and conidia germination. Investigation of the mechanism of antifungal action showed that the MIC of citral did not increase when sorbitol was added to the medium, suggesting that citral does not act on the cell wall. However, citral interact with ergosterol, suggesting that citral exerts antifungal effects on the cell membrane of *C. cladosporioides*. In the study *in silico*, citral showed good oral bioavailability. Thus, the monoterpene is presented as a promising antifungal agent, in particular in cases of mycosis caused by *C. cladoporioides.*

1. Introduction

The genus Cladosporium is one of the most common dematiaceous fungi that inhabits soil environments, certain animal feces, vegetables, rotten wood, nestles, omnivorous animals, mice and decayed fruits and food (Bensh et al., 2010).

Cladosporium cladosporioides is an opportunistic fungus that causes a variety of clinical infections in immunocompromised and immunocompetent humans and

animals. The clinical manifestations vary depending on the immunity of the hosts, as well as entries to the host (Shi et al., 2016). Can cause a wide spectrum of infection clinically in the skin (Nath et al., 2013; Bordoloi et al., 2015; Zhou et al., 2016), cornea (Chew et al., 2009), brain (Kantarcioglu, Yucel, De Hoog, 2002; Shimizu et al., 1982), lung (Kwon-Chung et al., 1995), hair (Zeller et al., 2015), gingiva (Pepe, Bertolotto, 1991) and onychomycosis (Shi et al., 2016). Also its airborne spores are relevant allergens, which may harm patients with asthma (Giri et al., 2013).

Fungal infections present a unique problem because both the mammalian host and the invading fungi are eukaryotic, making it difficult to develop a specific antifungal aimed only at the pathogen (Khan, Ahmad, 2013). Most of the antifungals have severe hepatotoxicity, nephrotoxicity and in addition human pathogenic fungi have also developed resistance (Cannon et al. 2009; Odds et al., 2003; Gupta, Thomas, 2003).

From this perspective, there is a growing demand for new antifungal agents that are more effective and less toxic than those already in use. This is the major factor that initate an intensive search among various sources, including natural products (Rajput, Karuppayil, 2013; Khan, Ahmad, 2013).

Antifungal compounds of natural origin, such as terpenes, have received much attention in recent times. They are a promising therapeutic tool for treating fungal infections and are known for their antimicrobial properties. Terpenes are a class of natural substances of vegetable origin formed by the condensation of isoprene units (C_5H_8) and are classified as monoterpenes (C_{10}), the most representative molecules, and sesquiterpenes (C_{15}). A terpene containing oxygen is called a terpenoid (Bakkali et al., 2008).

Citral (3,7-dimethyl-2-6-octadienal) is a natural mixture of two geometric isomers: geranial (trans-citral) and neral (cis-citral), which are acyclic α,β-unsaturated monoterpene aldehydes that occur naturally in many essential citrus fruit oils and in other herbs or spices, such as *Cymbopogon citratus* (Korenblum et al., 2013), *Lippia alba* (Tomazoni et al., 2016), *Melissa officinalis* (Pourghanbari et al., 2016).

Citral has been extensively studied due to its broad range of pharmacological activities such as its antiinflammatory, antioxidant, antitumor, antimicrobial properties, etc. (Song et al., 2016; Shi et al., 2016; Xia et al., 2013; Adukwu et al., 2016). Previous studies have reported that citral shows antifungal activity against *Penicillium digitatum* (Ouyang et al., 2016), *Penicillium italicum* (Tao et al., 2014), *Candida*

albicans (Leite et al., 2014), *Candida tropicalis* (Sousa et al., 2016). However, knowledge about the antifungal activity of this monoterpene against dematiaceous fungi is very limited.

This study seeks to contribute to the literature by providing data for the development of new antifungal drugs. The study therefore aims to investigate the antifungal activity of citral against *C. cladosporioides*. For this purpose, the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of citral were determined, along with its effects on mycelial growth, conidial germination, the fungal cell wall, and the fungal cell membrane.

2. **Material and Methods**

2.1. Microorganisms. *Cladosporium cladosporioides* (INCQS 40188). The strain *C. cladosporioides* (INCQS 40188) used in the antifungal assay was obtained from the collection of Sanitary Surveillance Reference Microorganisms, National Institute of Health Quality Control (INCQS), Oswaldo Cruz Institute (OCI), Brazil. The samples were maintained on Sabouraud Dextrose Agar - SDA (DIFCO®) at room temperature (28° to 30°C) and under refrigeration (4°C).

Stock inoculation (suspension) of *C. cladosporioides* was prepared from 7-14 day old sabouraud dextrose agar (Difco Lab., USA); the culture was grown at room temperature. Fungal colonie was covered with 5 mL of sterile saline solution (0.9%), the surface was gently agitated with vortexes, and fungal elements in saline solution were transferred to sterile tubes. Inoculator was standardized at 0.5 tube of McFarland scale (10^6 CFU/mL). The final concentration confirmation was done by counting the microorganisms in a Neubauer chamber (Cleeland, Squires, 1991; Hadacek, Greger, 2000; Sahin *et al*., 2004).

2.2. Chemicals. The product tested was the monoterpene citral, obtained from Sigma Aldrich, Brazil. Amphotericin B and voriconazole were obtained from Sigma Aldrich, Brazil. The monoterpene was dissolved in Tween 80 (2%) and DMSO (dimethylsulfoxide). The antifungal standards were dissolved in DMSO, and sterile distilled water to obtain solutions of 2048 μg/mL for each antifungal. The concentration of DMSO did not exceed 0.5% in the assays.

2.3. Culture Media. To test the biological activity of the products, Sabouraud dextrose agar (SDA) purchased from Difco Laboratories (Detroit, MI, USA), and RPMI-1640-L-glutamine (without sodium bicarbonate) (Sigma-Aldrich, Sao Paulo, SP, Brazil) culture media were used. Both were prepared and used according to the manufacturers' instructions.

2.4. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC). Broth microdilution assays were used to determine the MICs of monoterpene citral, amphotericin B, and voriconazol against *C. cladosporioides* (INCQS 40188). RPMI-1640 was added to all the wells of 96-well plates. Two-fold serial dilutions of the agents were prepared to obtain concentrations varying between 4 μg/mL and 1024 μg/mL. Finally, 10 μL aliquots of the inoculate suspension were added to the wells, and the plates were incubated at 28^{o}C for 5 days. Negative controls (without drugs) were used to confirm conidia viability, and sensitivity controls (for DMSO and Tween 80) were also included in the studies. At 5 days, there were visual observations of fungal growth. The MIC was defined as the lowest concentration capable of visually inhibiting fungal growth by 100 %. The results were expressed as the arithmetic mean of three experiments (Cleeland, Squires, 1991; Hadacek, Greger, 2000).

The MFC was determined by microdilution method to verify if the inhibition was reversible or permanent (Denning et al., 1992; Rasooli, Abyaneh, 2004). Aliquots of 20 µL (from the wells that did not show growth in the MIC procedure) were transferred to 96-well plates previously prepared with 100 µL of RPMI-1640. The plates were aseptically sealed followed by mixing on a plate shaker (300 rpm) for 30 seconds, incubated at 28ºC and read at 5 days of incubation. Tests were performed in duplicate and the geometric mean values were calculated. MFC was defined as the lowest citral concentration in which no visible growth occurred when subcultured on the 96-well plates containing broth without antifungal products.

2.5. Effects on Mycelia Growth. Analyses of the interferences of citral, voriconazole, and amphotericin on *C. cladosporioides* (INCQS 40188) mycelia growth were determined using poisoned substrate technique (dilution in solid medium), by daily measuring of radial mycelial growth on SDA, by adding products in an amount

adjusted to provide final concentrations similar to the MIC, MIC x 2, and MIC x 4 previously found. For this, 2 mm plugs taken from a 10-day-old mold culture cultivated on SDA slants at 28 ºC were placed at the center of the sterile SDA Petri dishes containing the test drugs. At different intervals (0, 2, 4, 6, 8, 10, 12, and 14 days) of incubation at 28 ºC, the mold's radial mycelial growth was measured (mm) with calipers. The controls in this assay revealed the mold's radial growth on SDA without adding drugs. Each assay was performed twice and the results were expressed as the average of the two repetitions (Adan *et al*., 1998; Thyágara, Hosono, 1996; Daferera, Ziogas, Polission, 2003).

2.6. Conidial Germination Assay. Citral, voriconazole, and amphotericin B were tested to evaluate effects on the germination of *C. cladosporioides* (INCQS 40188) fungal conidia. Flasks containing MIC, MIC x 2 and MIC x 4 of citral, voriconazole, amphotericin and a control with distilled water were used. In sterile test tubes, 500 μL of RPMI-1640 plus citral were evenly mixed with 500 μL of fungal conidia suspension and immediately incubated at 28ºC. Samples of the mixture were taken after 48 h of incubation for analysis. The whole experiment was performed in duplicate, where the number of conidia was determined in a Neubauer chamber, and the spore germination inhibition percentage at each time point was calculated by comparing the results obtained in the test experiments with the results of the control experiment. The analysis was conducted under an optical microscope (Zeiss Primo Star) (Pereira, Mendes, Lima, 2013; Rana, Singh, Taneja, 1997).

2.7. Sorbitol Assay Effects. The assay was performed using medium with and without sorbitol (control), to evaluate possible mechanisms involved in the antifungal activity of the test product on the *C. cladosporioides* (INCQS 40188) cell wall. The sorbitol was added to the culture medium in a final concentration of 0.8 M. The assay was performed by microdilution method in 96-well plates in a "U" (Alamar, Diadema, SP, Brazil) (Cleeland, Squires, 1991; Hadacek, Greger, 2000). The plates were sealed aseptically, incubated at 28 ºC, and readings were taken at 5 days. Based on the ability of sorbitol to act as a fungal cell wall osmotic protective agent, the higher MIC values observed in the medium with sorbitol added (as compared to the standard medium), suggest the cell wall as a possible target for the product tested (Leite et al.,

2014; Liu et al., 2007; Frost et al., 1995). The assay was performed in duplicate and expressed as the geometric mean of the results.

2.8. Ergosterol Binding Assay: MIC Value Determination in the Presence of Ergosterol. To assess if the product binds to fungal membrane sterols, an experiment was performed according to the method described by Escalante et al. (2008), with some modifications. Ergosterol was prepared as described by Leite et al. (2014). The MIC of citral, against *C. cladosporioides* (INCQS 40188) was determined by using broth microdilution techniques (Cleeland, Squires, 1991; Hadacek, Greger, 2000), in the presence and absence of exogenous ergosterol (Sigma-Aldrich, São Paulo, SP, Brazil) added to the assay medium, in different lines on the same microplate. Briefly, a solution of citral was diluted serially twice with RPMI-1640 (volume = 100 μL) containing ergosterol added at a concentration of 400 μg/mL. A volume yeast suspension 10 μL (0,5 McFarland) was added to each well. The same procedure was realized for Amphotericin B, whose interaction with membrane ergosterol is already known, which served as a control drug. The plates were sealed and incubated at 28ºC. The plates were read after 5 days of incubation, and the MIC was determined as the lowest concentration of test agent inhibiting visible growth. The assay was carried out in duplicate and the geometric mean of the values was calculated. The binding assay reflected the ability of the compound to bind with ergosterol.

2.9 *In silico* tests. As the citral was planned to be administered orally and hence must be capable of being absorbed into the tract gastrointestinal, he was evaluated according to the "Rule of Five" Lipinski, which states that at least three of four requirements must be provided so that the compound has a good oral bioavailability theoretical. Thus, compounds to be absorbed must possess molecular weight 500 daltons (Da), octanol / water calculated (ClogP) 5, the number of acceptors partition hydrogen bonding (nALH) 10 and the number of hydrogen bond donor groups (nDLH) 5 (Linpinski, 2001). In silico analysis was performed with the software Osisris (http://www.organic-chemistry.org/prog/peo/).

2.10. Statistical Analysis. The results are expressed as mean ± S.E. Differences between the means were statistically compared using the Student’s t-test. The values were considered significant with $p < 0.05$.

3. Results and Discussion

The results for citral's antifungal activity against *C. cladosporioides* (INCQS 40188) were determined using the MIC and MFC in broth microdilutions. The MIC of citral was 64 μg/mL, inhibiting the growth of tested fungal strain. Amphotericin B and voriconazol retained a lesser MIC than the phytoconstituent at 16 μg/mL MIC. The results for the control (Tween 80 and DMSO) showed no fungal growth inhibition; fungal growth in the medium without added drug was detected (strain control).

The MFC of citral against this microorganism was 128 μg/mL. The MFC of voriconazole almost entirely coincided with the MIC (16 μg/mL), However, the MFC of amphotericin B was 64 μg/mL.

According Siddiqui et al. (2013) the MFC/MIC ratio is used to specify the nature of the antimicrobial effect against a particular pathogen. The ratio MFC/MIC was calculated in order to determine if the compound had a fungistatic (MFC/MIC ≤ 4) or fungicidal (MFC/MIC > 4) activity.

In the present study, the MFC of the citral was found to be two folds higher than the corresponding MIC result. The MFC/MIC ratios of citral were ≤ 4; this suggests that citral has a fungicidal effect against the strain tested.

The results for the control (Tween 80 and DMSO) showed no fungal growth inhibition; fungal growth in the medium without added drug was detected (sterile control).

In the present study, citral showed potential antifungal activity against *C. cladosporioides* confirming the results obtained in previous studies. According Tomazoni et al. (2016) the chemotype citral, showing significant inhibition against *Alternaria solani*, an dematiaceou fungi, starting at the 0.5 µL mL-1 concentration. Garcia et al. (2008) demonstrated the activity of citral against the fungi *Colletotrichum musae, Colletotrichum gloeosporioides* and *Fusarium subglutinans*.

Recently, our research group showed that the citral is *in vitro* antifungal potential against strains of *C. albicans* (Leite et al., 2014) and *C. tropicalis* (Sousa et al., 2016).

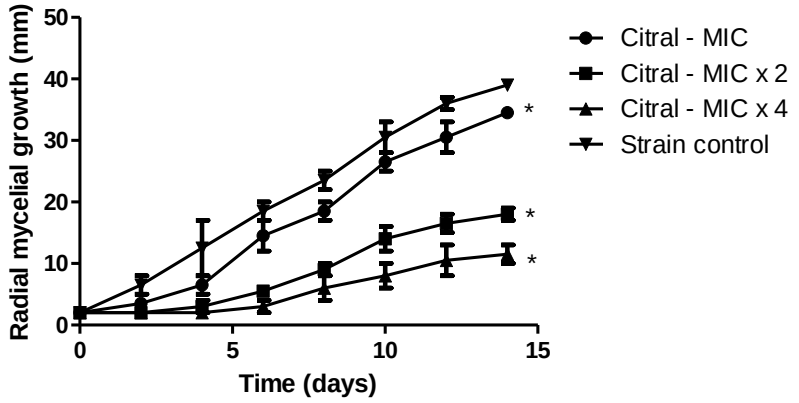
The fungi mycelium is (on the whole) the hyphae, and fungal filaments, or segments of filamentous mycelium. Mode of growth can also be na important factor contributing to the virulence of potentially pathogenic fungi, as both biofilm formation

and tissue invasion have been shown to contribute to pathogenesis (Powers-Fletcher et al., 2016).

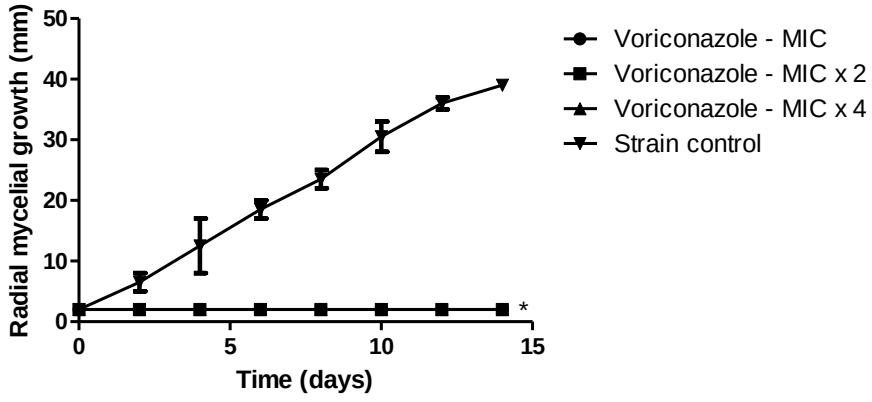
This study also verified citral's action against *C. cladosporioides* mycelial growth and spore germination. The effect of differing concentrations of the test drug (MIC, MIC x 2, MIC x 4) on mycelia growth was determined by measuring radial mycelial growth, and the results are shown in Figure 1.

With respect to *C. cladosporioides*, it can be seen that citral in MIC concentrations of (64 μg/mL), MIC × 2 (128 μg/mL) and MIC x 4 (256 μg/mL), inhibited the mycelial growth ($p < 0.05$) as compared with the control (mycelia diameter being 100 %). The control strains showed a constant rate of mycelial growth over the time evaluated, indicating good antifungal effect for citral. Similar effects were noted with Amphotericin B in that the drug effectively inhibited the mycelial growth, in all concentrations tested (Fig. 1C). The voriconazole test against *C. cladosporioides* INCQS 40188 showed significant inhibition of mycelial growth at all concentrations tested (Fig. 1B).

A



B



C

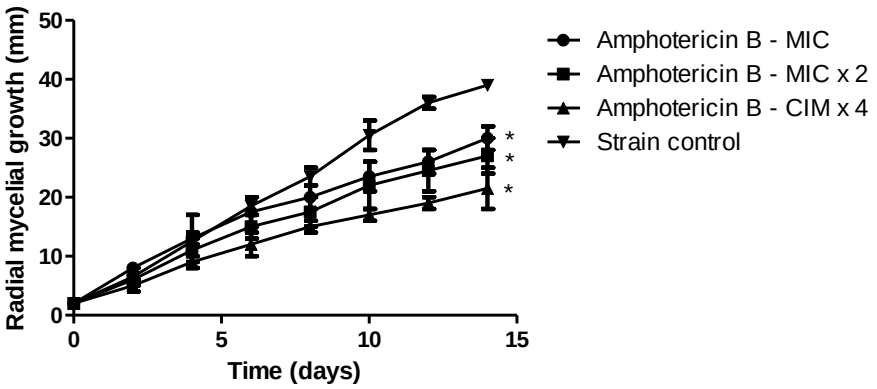


Figure 1. Radial mycelial growth produced by *C. cladosporioides* (INCQS 40188) in the absence (control), and presence of citral (A), voriconazole (B) and amphotericin B (C). * *p < 0.05 compared to control*

The analysis on the natural products effects on fungal growth in function of time used in this work has stood out among researchers around the world. Good fungal growth of *Cladosporium* species, similarly to other filamentous fungi, produce hyphae which can penetrate the innermost skin layer and aggravate the damage in the host (Gupta et al., 2003; Zurita, Hay, 1987).

Therefore, some researchers are investigating the essential oils and compounds potential in inhibiting mycelial growth of pathogenic fungi due to their importance in the mycosis development (Pereira et al., 2011; Menezes et al., 2015).

This study revealed that all tested citral concentrations managed to inhibit the mycelium development. These results also agree with those of Saddiq, Khayyat (2010), Fan et al. (2014) and Ouyang et al. (2016), who reported on the strong antifungal activity of citral.

Given the importance of mycelial growth in the development of mycoses, the inhibition of *C. cladosporioides* mycelial growth caused by citral as observed in this study is a significant contribution to the search for new natural products with antifungal activity.

The study of the germination of conidia has great implications in clinical practice, because it is possible to develop new therapeutic approaches that block the infection at its onset (Osherov, May, 2001). In this perspective, the effect of the citral on the germination of the conidia of *C. cladosporioides* INCQS 40188 was investigated. The effects of different concentrations (MIC, MIC x 2 and MIC x 4) of citral, voriconazole and amphotericin B on the germination of conidia are shown in Figure 2.

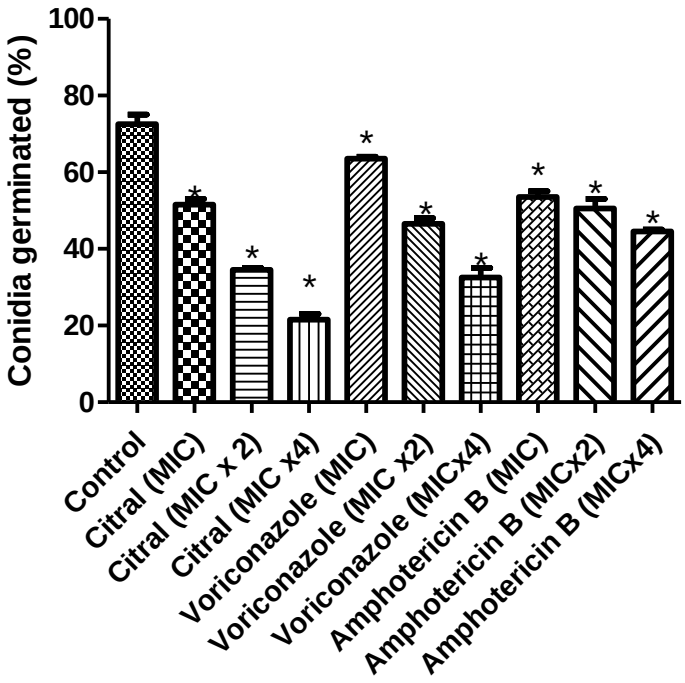


Figure 2. *C. cladosporioides* (INCQS 40188) conidial germination percentage in the absence (control) and presence of citral, voriconazole and amphotericin B. ** p < 0.05 compared to control.*

In this study, citral at all concentrations tested (MIC, MIC x 2 and MIC x 4) it was capable of inhibiting the conidia germination of *C. cladosporioides*. The antifungals standards (voriconazole and amphotericin B) tested, also showed significant inhibition on conidia germination (Figure 2).

These results confirm previously published work, such as that by Li et al. (2014), who showed the effect of citral on conidia germination of *Magnaporthe grisea*.

The mechanism of action of essential oils and terpenos may be occur due to several targets in the cell of the microorganism. The locations or mechanisms in the cell became sites of action for this natural products that damage the cytoplasmic membrane, the membrane proteins, leakage of cell contents, causing degradation of the cell wall, coagulation of cytoplasm and depletion of the proton motive force (Burt, 2004; Helander et al., 1998).

The Sorbitol Protection Assay was performed to test the effect of citral on the integrity of the fungal cell wall (Frost et al., 1995). In this assay, MIC determinations were conducted with and without 0.8 M sorbitol. Sorbitol is an osmotic protectant

used for stabilizing fungal protoplasts and it is expected that the MIC of a compound that damages the cell wall will shift to a much higher value in the presence of the osmotic support.

No variations in MIC was observed for citral in this assay (Table 1) suggesting that citral would not act by inhibiting control of cell wall synthesis or assembly mechanisms.

Table 1: MIC values (μg/mL) of drugs in the absence and presence of sorbitol (0.8 M) and ergosterol (400 μg/mL) against *C. cladosporioides* INCQS 40188.

Drugs	Sorbitol		Ergosterol	
	Absence	Presence	Absence	Presence
Citral	64	64	64	2048
Amphotericin B[a]	-	-	16	1024

[a]Positive control. —: not tested.

These results are in agreement with those reported by Miron *et al*. (2014) evaluated the antifungal activity of citral against seven opportunistic pathogenic yeasts and four dermatophyte species and no changes were observed in the MIC of this monoterpene in the sorbitol protection assay.

Lipophilic compounds such as terpenes, aromatics and phenols can interact with components of cytoplasmic membrane, it may enhance the membrane permeability and loss of integrity which can be toxic to the microorganism cell (Sikkema et al., 1995).

Ergosterol is the main lipid steroid of fungal cell membrane. This sterol has different functions in the fungus, such as bioregulator of membrane fluidity, membrane integrity, growth and membrane-bound enzymes (Khan et al., 2010; Luppeti et al., 2002). When antifungal agents bind to the membrane ergosterol, they lead to fungal membrane disruption and causing the loss of the intracellular content (Gungi, Arima, Beppu, 1983). Therefore, in the search for new antifungals, their effects on ergosterol are investigated (Martinez-Rossi, Peres, Rossi, 2008). The ability of citral to form complexes with ergosterol was evaluated from the perspective of investigating its action on the fungal cell membrane (ergosterol effect assay). The drug will bind to exogenous ergosterol, avoiding its ergosterol membrane binding to the membrane. If the activity of citral is caused by binding to ergosterol, the

exogenous ergosterol would prevent the binding to the fungal membrane's ergosterol. Consequently, it would cause an increase in MIC of citral in the presence of exogenous ergosterol with respect to the control experiment (Escalante et al., 2008; Lunde, Kubo, 2000). In this assay, amphotericin B was used as positive control. Thus, the effect of exogenous ergosterol on the MIC of citral and amphotericin B were determined.

The MICs of citral against *C. cladosporioides* (INCQS 40188) increased 32 times in the presence of 400 µg/mL ergosterol (Table 1). Thus, our findings indicates that the mechanism of action of citral involve complexation with ergosterol. Amphotericin B, the positive control that has a known interaction with ergosterol, in that its MIC increased 64 times in the presence of this sterol (Table 1).

Several studies have reported the action of citral on fungal cell membranes. Zhou et al. (2014) evaluated the antifungal activity of three volatile compounds: citral, octanal, and α- terpineol against *Geotrichum citri-aurantii*. It was found in the study that citral was able to significantly inhibit mycelial growth. Antifungal activity was attributed to cell membrane disruption and to the consequent loss of cellular components.

According to Rajput, Karuppayil (2013) the mechanism of anti-*Candida* activity of citral appears to be associated with damage in the membrane integrity, through inhibition of ergosterol biosynthesis.

Tao et al. (2014) showed that citral considerably impaired ergosterol biosynthesis in *Penicillium italicum* cells, significantly decreasing lipid levels; suggesting that the plasma membrane may well be an important citral antifungal target.

Another study also showed that citral at a concentration of 200 μg/mL irreversibly damaged cell organelles and the cell membrane of *Trichophyton mentagrophytes* (Park et al., 2009).

The positive results of citral on the affinity ergosterol assay and the other reports on the subject strongly suggest that the mechanism of action of this monoterpene is related to ergosterol-binding and a subsequent destabilization of fungal cell membranes.

Computer-assisted prediction models, so-called predictive tools, play an essential role in the proposed repertoire of alternative methods besides *in vitro* models. Hence, these tools are used to study both existing and hypothetical

compounds, which are fast, reproducible and are typically based on human bio-regulators (Srinivas et al., 2014; Angelo, Max, Markus, 2006).

The results, in silico study, show that the citral followed the "Rule of Five" Lipinski which requires that the compound must possess at least three of four requisites (nDLH ≤ 5, nALH ≤ 10, Da < 500 e cLogP < 5), thus the Citral may be active drug in humans by the oral route of administration (Lipinski, 2001) (Table 2).

Table 2- Theoretical analysis of the physico-chemical properties of citral involved in oral bioavailability of drugs following the "Rule of Five" Lipinski.

Substance	nDLH	nALH	Da	cLogP
Citral	0	1	152.24	3.65
Standard of "Rule of Five" Lipinski	≤ 5	≤ 10	< 500	< 5

4. Conclusion

From the results obtained, it was concluded that citral showed excellent antifungal activity against *C. cladosporioides* and revealed that the product concentrations is capable of inhibiting both the mycelial growth and germination of conidia for the specie tested. The results also suggest that the action of citral affects the structure of the fungal cell membrane. In the study *in silico*, citral showed good oral bioavailability. This information is important for future pharmacological applications of citral with the prospect of developing a new, safe, and effective antifungal for the treatment of opportunistic mycoses. However, we suggest further tests and clinical studies to correlate the potent in vitro - in vivo antifungal activity, thus confirming the efficacy and safety of the compound for later clinical application.

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5.7 Evaluation of Antifungal Activity of citral associated with antifungals Amphotericin B and Voriconazole, against *Cladophialophora carrionii* and *Cladosporium spp*

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Evaluation of Antifungal Activity of citral associated with antifungals Amphotericin B and Voriconazole, against *Cladophialophora carrionii* and *Cladosporium spp*

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ABSTRACT

Combining antifungal drugs may improve therapeutic response. The potential benefits of using therapeutic combinations include a broader spectrum of efficacy, improved cure rates, safety, and tolerability, reduction of resistance to antifungal drugs, dose reduction,and thus reduced toxicity. The research aimed to study the antifungal properties of citral associated with synthetic antifungal (Amphotericin B and Voriconazole) against strains of *Cladophialophora carrioni* and *Cladosporium* spp. The parameters used for this purpose were based on the determination of Fractional Inhibitory Concentration Index (Method of association – Checkerboard). The study shows that citral in combination with voriconazole an indifferent effect, and resulting in a FIC index varied between 1.5-3.0. However, the combination of the citral and amphotericin B showed FIC index varied between 6.0-8.0, for four species tested, obtained antagonistic effects. This study contributes to understanding the citral's antifungal activity associated with antifungals agents against dematiaceous fungi. However more studies are needed to investigate whether citral interations with drugs.

Keywords: Monoterpene, Citral, Antifungal Agents, *Cladophialophora carrionii*, *Cladosporium* spp. Checkerboard.

1. Introduction

Fungal participation in the aetiology of infections has increased considerably [1,2]. However, as medical technology has improved, the survival of patients with severe and life-threatening illnesses has led to a rapid increase in the

immunosuppressed population [3]. These changes are correlated with a substantial increase in the rate of invasive fungal infections. Moreover, drug-resistant strains are emerging, and the number of infections by intrinsically drug-resistant species has increased rapidly [4].

Synergistic drug combination has been proved to be a valid and pragmatic strategy to seek drugs with novel mode of actions. It can potentially reduce the dose of single drug usage with increased drug-efficacy, and subsequently lower the drug toxicity. The practice of targeting 2 or more drug targets simultaneously is consistent with the philosophy that a disease is a systematic and complicated outcome caused by multi-effects. Furthermore, the development of drug resistance can be slowed down by the multi-target strategy [5,6].

Despite the constant introduction of new and effective synthetic drugs to the market, medicinal plants, which are the historical basis of therapeutic health care, represent an alternative that is economical, accessible, and applicable to various pathologies, particularly in developing countries [7]. Therefore, parallel to the development of synthetic drugs, substantial attention has focused on natural products with antifungal properties, which has stimulated the search for therapeutic alternatives [8,9].

In addition to their inherent antimicrobial properties, natural products and their derivatives may alter the effects of standard antifungal agents (those used in clinical practice). The use of two or more antifungal combinations can lead to a reduction in the required drug dosages and decrease the normally produced adverse event profile [10,11].

Amongst these products we find the terpenes, a class of naturalsubstances of vegetable origin formed by combining five carbons called isoprene (C_5H_8). Terpenes can be classified according to their number of isoprene units: monoterpenes (C10), the most representative molecules, and sesquiterpenes (C15), but there are also hemiterpenes (C5), diterpenes (C20), triterpenes (C30), and tetraterpenes (C40) [12].

Citral is a lemon scented acyclic monoterpene aldehyde consists of a racemic mixture of two isomers geranial (*trans*-citral or citral A) and neral (*cis*-citral or citral B). Citral possesses many significant bioactivities such as, antimicrobial, anti-inflammatory, antiparasitic, allelopathic and mosquito repellent. Citral is most valuable monoterpene in flavors, fragrances, cosmetics, perfumery and pharmaceuticals [13].

Given the above, the aim of this study was to determine the antifungal properties of citral associated with synthetic antifungal (Amphotericin B and Voriconazole) against strains of *Cladophialophora carrioni* and *Cladosporium spp.*

2. Material and Methods

2.1. Microorganisms. *Cladophialophora carrionii* URM 2871, *Cladosporium oxysporum* URM 5234, *Cladosporium spherospermum* URM 6120 and *Cladosporium cladosporioides* (INCQS 40188) strains used in the antifungal assay were obtained from the Biological Sciences Center – Mycology Department fungal collection (URM), Federal University, Pernambuco (Brazil) and the collection of Sanitary Surveillance Reference Microorganisms, National Institute of Health Quality Control (INCQS), Oswaldo Cruz Institute (OCI). The sample was maintained on Sabouraud Dextrose Agar - SDA (DIFCO®) at room temperature (28 °C) and under refrigeration (4°C).

Stock inoculators (suspensions) of strains tested were prepared from 7-14-day old potato dextrose agar (Difco Lab., USA), the cultures grown at room temperature. Fungal colonies were covered with 5 mL of sterile saline solution (0,9%), the surface was gently agitated with vortexes, and fungal elements with saline solution were transferred to sterile tubes. Inoculator was standardized at 0.5 tube of McFarland scale (10^6 CFU/mL). The final concentration confirmation was done by counting the microorganisms in a Neubauer chamber [14-16].

2.2. Chemicals. The product tested was the monoterpeno Citral, obtained from Sigma Aldrich, Brazil. Amphotericin B and Voriconazole were obtained from Sigma Aldrich, Brazil. The monoterpene was dissolved in Tween 80 (2%) and DMSO (dimethylsulfoxide). The antifungal standard were dissolved in DMSO, and sterile distilled water was used to obtain solutions of 2048 μg/mL for each antifungals. The concentration of DMSO did not exceed 0.5% in the assays.

2.3. Culture Media. To test the biological activity of the products, RPMI-1640-L-glutamine (without sodium bicarbonate) (Sigma-Aldrich, Sao Paulo, SP, Brazil) culture media was used. They were prepared and used according to the manufacturers instructions.

2.4. Checkerboard Assay. A checkerboard microtiter test was performed to evaluate the interaction of citral with the antifungal drugs (voriconazole and Amphotericin B) against *C. carrionii* URM 2871, *C. oxysporum* URM 5234, *C. sphaerospermum* URM 6120 and *C. cladosporioides* (INCQS 40188). A series of 2-fold dilutions, in eight for citral and each antifungal drug, were made in RPMI-1640 to obtain four times the final concentration being achieved in the microtiter well. Furthermore, 50 μL of each dilution of citral was added to the 96-well microtiter plates in the vertical direction, while 50 μL of each dilution of antifungal drugs was added in the horizontal direction, so that various combinations of citral and antifungal drugs could be achieved. In addition, 10 μL of inoculum from the spore suspension (1.5×10^5 CFU/mL) was added to each well, and the plates were incubated at 28 ºC for 5 days. In order to evaluate the activity of the combinations of drugs, fractional inhibitory concentration (FIC) indices were calculated as $FIC^A + FIC^B$, where FIC^A and FIC^B represent the minimum concentrations inhibiting the fungal growth for drugs A and B, respectively: $FIC^A = MIC^A$ combination/MIC^A alone and $FIC^B = MIC^B$ combination/MIC^B alone. A mean FIC index was calculated based on the following equation: FIC index = $FIC^A + FIC^B$. In addition, the interpretation was made as follows: synergistic (<0.5), additivity (0.5–1.0), indifferent (>1), or antagonistic (>4) [17,18].

3. Results and Discussion

Several reports have been made concerning different antifungal combinations assayed *in vitro* and applied in the clinic [10,19,20], and with other plant derivatives [21].

This study evaluated the effect of citral in association with the antifungals amphotericin B and voriconazole against *C. carrionii and Cladosporium spp.* strains, using the checkerboard technique. The results are shown in Tables 1 and 2.

Table 1: MIC of Antifungal drugs and effect of combination with citral, against *C. carrionii* URM 2871 e *C. oxysporum* URM 5234.

Citral + Antifungal	*C. carrionii* URM 2817		*C. oxysporum* URM 5234	
	MIC* (µg/mL)	FIC* index (type of interaction)	MIC* (µg/mL)	FIC* index (type of interaction)
Citral	128		256	
Amphotericin B	16		16	
Voriconazole	16		16	
Citral/ Amphotericin B	256/64	6,0 (antagonistic)	256/64	6,0 (antagonistic)
Citral/Voriconazole	128/8	1,5 (indifferent)	256/16	2,0 (indifferent)

**MIC, minimal inhibitory concentration; *FIC, fractional inhibitory concentration*

Table 2: MIC of Antifungal drugs and effect of combination with citral, against *C. sphaerospermum* URM 6120 e *C. cladosporidioides* INCQS 40188.

Citral + Antifungal	*C. sphaerospermum URM 6120*		*C. cladosporidioides* INCQS 40188	
	MIC* (µg/mL)	FIC* index (type of interaction)	MIC* (µg/mL)	FIC* index (type of interaction)
Citral	256		64	
Amphotericin B	>1024		16	
Voriconazole	16		16	

Citral/ Amphotericin B	-	-	256/64	8,0 (antagonistic)
Citral/Voriconazole	256/16	2,0 (indifferent)	128/16	3,0 (indifferent)

**MIC, minimal inhibitory concentration; *FIC, fractional inhibitory concentration*

As can be seen, antagonistic effects were observed for the combinations of citral with Amphotericin B, resulting in a fractional inhibitory concentration (FIC) index varied between 6,0-8,0 against respective species tested. However, the combination of the citral and voriconazole showed FIC index varied between 1,5 -3,0, for four species tested, obtained indifferent effects. The strain of *C. sphaerospermum* URM 6120 was not evaluated with the citral-Amphotericin B combination because the MIC of Amphotericin B was greater than 1024 µg/mL.

Sousa et al. [22] evaluated the effect of citral in association with the antifungal fluconazole, and amphotericin B against *C. tropicalis* strains, using the checkerboard technique. It was found that the citral-amphotericin B combination was for indifferent (FICI = 1.0) for the *C. tropicalis* ATCC strain. In *C. albicans* strains, previous studies have shown for citral-amphotericin B; effects ranging from indifferent to synergistic [23].

The mechanism of action of monoterpenes has not been completely clarified. Some studies showed the breakdown of cytoplasmic and organelle membranes exposed to certain volatile oils. The loss of membrane integrity can cause changes in membrane function leading to the antifungal activity [24-26].

The action of citral on the cell membrane has been widely studied. In a recent study Tao et al. [27] showed that citral considerably impaired ergosterol biosynthesis in cells of *Penicillium italicum*, significantly decreasing lipid levls, suggesting that the plasma membrane may well be an important citral antifungal target. More recently OuYang et al. [28] suggests that citral could exhibit its antifungal activity against *P. digitatum* by the down-regulation of ergosterol biosynthesis.

These studies suggest that the mechanism of the antifungal action of the citral involves a direct interaction with ergosterol, which leads to the disruption of the fungal membrane and loss of intracellular contents [29].

Amphotericin B is one of the most potent antifungals, demonstrating activity against an array of yeast and filamentous fungal pathogens. Amphotericin B exerts

its activity through hydrophobic interactions with cell membrane ergosterol, subsequently disrupting membrane function. Pores formation allows the efflux of potassium, leading to cell death [30].

The voriconazole is antifungal azole drug class. This agents impair ergosterol synthesis by inhibiting C14-a sterol demethylase. Cell membrane integrity is disrupted by the accumulation of sterol precursors and the reduction of ergosterol [31,32].

The antagonistic and indifferent effects observed in this study when the citral was associated with amphotericin B and voriconazole, respectively, can be explained by the monoterpene, although action also interacting with the ergosterol of fungal membrane, as well as the antifungals possibly acts in different pharmacological conditions, blocking or otherwise interfering with the final effect of fungal growth inhibition.

According to published reports, the effect of combining amphotericin B and flucytosine, for example, has varied between synergism and antagonism, and also changes according to the species, and even which strain is tested [33,34].

The focus of this evaluation is of the efficacy of combination antifungal drugs with respect to the extent or rate of death of the fungal pathogen, although other potential interactions (such as pharmacokinetic drug interactions), may well impact efficiency when agents are used together, increased penetration of the antifungal agent provided by the action of the another cell membrane antifungal; inhibition of protein carriers; simultaneous inhibition of different cellular targets [34].

4. Conclusion

This study represents an advance in our understanding of citral's antifungal activity associated with antifungals agents against dematiaceous fungi. However, more studies are needed to investigate whether citral interations with others drugs.

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6. Considerações Finais

De acordo com os resultados obtidos neste trabalho, pode-se concluir que:

- Dentre os terpenos testados na triagem microbiológica, o citral apresentou melhor potencial antifúngico.
- Os estudos *in silico*, mostraram que o citral apresenta importantes atividades farmacológicas e uma boa disponibilidade oral teórica;
- O citral apresenta potente atividade antifúngica contra cepas de *C. carrionii e Cladosporium spp* ;
- Todas as cepas estudadas foram resistentes ao antifúngico Intraconazol (1024 μg/mL) (Dados não mostrados);
- As concentrações inibitórias mínimas e fungicidas para 100 % das cepas submetidas à ação do citral demonstraram a atividade fungicida do monoterpeno estudado;
- O citral interferiu na cinética do crescimento micelial radial das cepas fúngicas estudadas, apresentando efeito fungicida dependente de concentração;
- O citral foi capaz de inibir a germinação de conídios de *C. carrionii e Cladosporium* spp;
- Nos testes realizados o citral não atuou sobre a parede celular de *C. carrionii e Cladosporium* spp;
- O mecanismo pelo qual o citral promove atividade antifúngica contra *C. carrionii e Cladosporium* spp envolve a sua interação com o ergosterol presente na membrana plasmática fúngica;
- A associação citral-voriconazol foi indiferente para todas as cepas testadas, enquanto a associação citral-anfotericina B apresentou efeito antagônico pelo método de *checkerboard*;
- Assim, o citral pode representar uma nova possibilidade entre os produtos com atividade antifúngica contra fungos dematiáceos, especialmente pertencente aos gêneros *Cladophialophora* e *Cladosporium*. Entretanto são

necessários mais estudos acerca do seu mecanismo de ação, para assim permitir o seu uso na terapêutica das infecções antifúngicas.

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