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ATIVIDADE ANTIFÚNGICA DOS FENILPROPENOS EUGENOL E ISOEUGENOL
SOBRE CEPAS DE *Cryptococcus neoformans*

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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SOBRE CEPAS DE *Cryptococcus neoformans***

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RESUMO

PINHEIRO, L. S. **ATIVIDADE ANTIFÚNGICA DOS FENILPROPENOS EUGENOL E ISOEUGENOL SOBRE CEPAS DE *Cryptococcus neoformans***. 2017. 122f. Tese (Pós-graduação em Produtos Naturais e Sintéticos Bioativos, Farmacologia). UFPB/CCS – João Pessoa – PB.

Cryptococcus neoformans é uma levedura encapsulada de natureza predominante oportunista, com capacidade de infectar homem e animais. A criptococose afeta principalmente o sistema nervoso central (SNC), com evolução grave e fatal. Essa micose está relacionada a elevadas taxas de mortalidade, especialmente em indivíduos com o sistema imunológico comprometido, como os HIV positivos, transplantados e leucêmicos. O tratamento envolve um número limitado de agentes antifúngicos, com reconhecida toxicidade, resistência e elevados custos. Diante desse contexto, a busca por substâncias antifúngicas em produtos naturais têm sido uma alternativa promissora com grande atenção dos estudiosos quanto à utilização dos compostos fenilpropenos (eugenol e isoeugenol), devido ao amplo espectro de propriedades farmacológicas, incluindo a atividade antifúngica. Assim, o presente estudo teve como objetivo avaliar através de análise teórica, *in silico*, o potencial antimicrobiano desses fenilpropenos e investigar a atividade antifúngica *in vitro*, seus possíveis mecanismos de ação e o efeito da associação com antifúngico contra *C. neoformans*. A análise do potencial antimicrobiano dos fenilpropenos foi realizada *in silico* utilizando o software Pass® online. A concentração inibitória mínima (CIM), concentração fungicida mínima (CFM), o efeito do eugenol e isoeugenol sobre a parede celular (ensaio de sorbitol) e ligação ao ergosterol da membrana foram realizados pela técnica de microdiluição. Também foi avaliado o efeito da associação dos produtos testados com antifúngico padrão pelo método de *checkerboard*. No estudo *in silico*, eugenol e isoeugenol demonstraram uma maior propabilidade de serem ativos, expressa em porcentagem, em relação aos micro-organismos fúngicos. Os testes para a atividade antifúngica revelaram que os fenilpropenos estudados promoveram efeito antifúngico com CIM₅₀ de 16 µg/mL para as cepas de *C. neoformans*, assim como, ação fungicida por apresentarem CFM₅₀ 32 µg/mL. Na investigação do mecanismo de ação antifúngica foi evidenciado que os valores de CIM de eugenol e isoeugenol contra *C. neoformans* permaneceram inalterados na presença de sorbitol 0.8 M, sugerindo que estes compostos não atuam através da inibição da síntese da parede celular fúngica. Por outro lado, os valores de CIM dos fenilpropenos variaram (16 a 128 µg/mL) na presença de ergosterol (400 µg/mL) no ensaio sobre a membrana plasmática, mostrando que ambos os compostos formam complexos com ergosterol. As associações eugenol-anfotericina B e isoeugenol-anfotericina B foram antagonistas para as cepas testadas (LM 615 e INCQS 40221). Assim, esses fenilpropenos podem representar uma nova possibilidade entre os produtos com atividade antifúngica, pois mostraram promissora atividade por um mecanismo envolvendo a complexação com ergosterol presente na membrana de *C. neoformans*.

Palavras chave: eugenol, isoeugenol, antifúngicos, *Cryptococcus neoformans*, ergosterol

ABSTRACT

PINHEIRO, L. S. **ANTIFUNGAL ACTIVITY OF PHENYLPROPENES EUGENOL AND ISOEUGENOL AGAINST STRAINS OF *Cryptococcus neoformans***. 2017. 122f. **Tese** (Pós-graduação em Produtos Naturais e Sintéticos Bioativos, Farmacologia). UFPB/CCS – João Pessoa – PB.

Cryptococcus neoformans is an encapsulated yeast of predominantly opportunistic nature, capable of infecting man and animals. Cryptococcosis mainly affects the central nervous system (CNS), with severe and fatal evolution. This mycosis is related to high mortality rates, especially in individuals with compromised immune systems, such as HIV-positive, transplanted and leukemic. Treatment involves a limited number of antifungal agents, with recognized toxicity, resistance and high costs. In this context, the search for antifungal substances in natural products has been a promising alternative with great attention of the students on the use of phenylpropene compounds (eugenol and isoeugenol), due to the wide spectrum of pharmacological properties, including antifungal activity. The aim of the present study was to evaluate the *in silico* antimicrobial potential of these phenylpropenes and to investigate the *in vitro* antifungal activity, its possible mechanisms of action and the effect of the association with antifungal against *C. neoformans*. The analysis of the antimicrobial potential of phenylpropenes was performed *in silico* using the Pass® online software. The minimum inhibitory concentration (MIC), minimum fungicidal concentration (CFM), the effect of eugenol and isoeugenol on the cell wall (sorbitol assay) and membrane ergosterol binding were performed by the microdilution technique. The effect of the association of products tested with standard antifungal by the checkerboard method was also evaluated. In the *in silico* study, eugenol and isoeugenol demonstrated a greater propensity to be active, expressed as a percentage, in relation to fungal microorganisms. The tests for antifungal activity revealed that the phenylpropenes studied promoted antifungal effect with IC₅₀ of 16 µg/mL for the *C. neoformans* strains, as well as fungicidal action due to the presence of CFM₅₀ of 32 µg/mL. In the investigation of the mechanism of antifungal action it was evidenced that the MIC values of eugenol and isoeugenol against *C. neoformans* remained unchanged in the presence of 0.8 M sorbitol, suggesting that these compounds do not act through the inhibition of fungal cell wall synthesis. On the other hand, phenylpropene MIC values varied (16 to 128 µg / mL) in the presence of ergosterol (400 µg/mL) in the plasma membrane assay, showing that both compounds form complexes with ergosterol. The associations eugenol-amphotericin B and isoeugenol-amphotericin B were antagonists for the strains tested (LM 615 and INCQS 40221). Thus, these phenylpropenes may represent a new possibility among the products with antifungal activity, since they showed promising activity by a mechanism involving the ergosterol complexation present in the membrane of *C. neoformans*.

Keywords: eugenol, isoeugenol, antifungals, *Cryptococcus neoformans*, ergosterol

LISTA DE ABREVIATURAS E SIGLAS

Abreviaturas/Siglas	Descrição
5-FU.....	5-fluorouracil
AFLP.....	Polimorfismo da fração longa amplificada
AFLP1.....	Polimorfismo da fração longa amplificada de DNA 1, sorotipo A
AFLP3.....	Polimorfismo da fração longa amplificada de DNA 3, sorotipo AD
AFLP4.....	Polimorfismo da fração longa amplificada de DNA 4, sorotipo B
AFLP7.....	Polimorfismo da fração longa amplificada de DNA 7, sorotipo C
AFR1.....	Antifungal resistance 1
AIDS.....	Síndrome da imunodeficiência adquirida
ASD.....	Ágar Sabourad dextrose
<i>C. gattii</i>	<i>Cryptococcus gattii</i>
<i>C. neoformans</i>	<i>Cryptococcus neoformans</i>
CCS.....	Centro de Ciências da Saúde
CFM.....	Concentração fungicida mínima
CI ₅₀	Concentração inibitória para 50%
CIF.....	Concentração Inibitória Fracionada
CIM.....	Concentração inibitória mínima
CMRVS.....	Coleção de Micro-organismos de Referência em Vigilância Sanitária
CSD.....	Caldo Sabourad Dextrose
DCF.....	Departamento de Ciências Farmacêuticas

DL ₅₀	Dose letal para 50%
DMSO.....	Dimetilsulfóxido
DNA.....	Ácido desoxirribonucleico
FAO.....	Organização das Nações Unidas para a Agricultura e Alimentação
FDA.....	<i>Food and Drug Administration</i>
FIOCRUZ.....	Fundação Oswaldo Cruz
FUMP.....	Ácido 5-fluorouridílico
GRAS.....	Geralmente Reconhecido como Seguro
HE.....	Hematoxilina-eosina
HIV.....	Vírus da imunodeficiência humana
INCQS.....	Instituto Nacional de Controle de Qualidade em Saúde
LCR.....	Líquido cefalorraquidiano
OMS.....	Organização Mundial da Saúde
Pa.....	Probabilidade de ser ativo
PASS.....	Previsão do Espectro de Atividades para Substâncias
pH.....	Potencial hidrogeniônico
Pi.....	Probabilidade de ser inativo
PCR.....	Reação em cadeia de polimerase
PLB1.....	Fosfolipase do tipo B1
RFLP.....	<i>Restriction Fragment Length Polymorphism</i>
RNA.....	Ácido ribonucleico
<i>S. typhimurium</i>	<i>Salmonella typhimurium</i>
SNC.....	Sistema nervoso central
UFC/mL.....	Unidades formadoras de colônia por mililitro

UFPB..... Universidade Federal da Paraíba
UV..... Ultravioleta
VGI..... Variedade *gattii* tipo molecular I
VGIV..... Variedade *gattii* tipo molecular IV
VNI..... Variedade *neoformans* tipo molecular I
VNII..... Variedade *neoformans* tipo molecular II
VNIII..... Variedade *neoformans* tipo molecular III
VNIV..... Variedade *neoformans* tipo molecular IV

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Introdução



1 INTRODUÇÃO

As infecções fúngicas invasivas tem aumentado significativamente nas últimas décadas como resultado do crescente número de indivíduos imunodeficientes. Esses indivíduos são particularmente mais propensos a infecções causadas por patógenos fúngicos de natureza oportunista, embora indivíduos imunocompetentes também possam ser acometidos, em alguns casos (BUTTS; PALMER; ROGERS, 2017).

A prevalência dessas infecções é preocupante pois as elevadas taxas de morbidade e mortalidade estão associadas mesmo com a intervenção terapêutica, relacionadas aos agentes *Candida albicans*, *Aspergillus fumigatus* e *Cryptococcus neoformans* (LAI et al., 2008; PARK et al., 2009).

Cryptococcus neoformans é um dos principais agentes fúngicos causadores de infecções em pacientes imunodeficientes, principalmente em pacientes com Síndrome da Imunodeficiência Adquirida (AIDS), sendo responsável por 1/3 dos óbitos nesse grupo de indivíduos, superando as taxas de mortalidade causadas por tuberculose na África (DEL POETA; CASADEVALL, 2012; LANJEWAR, 2011; MCCARTHY et al., 2006; PARK et al., 2009). A criptococose é reconhecida frequentemente como micose sistêmica que afeta homem e animais, com envolvimento do sistema nervoso central (SNC), causando formas graves de meningite e meningoencefalite.

Apesar da eficácia do tratamento, muitos estudos indicam o crescente impacto dessa infecção e as limitações encontradas (pequena disponibilidade de drogas, resistência antifúngica, efeitos colaterais e alta toxicidade) com o uso excessivo dos antifúngicos, o que tem motivado a busca por drogas alternativas a partir de produtos naturais e a terapia combinada a antifúngicos sintéticos, na tentativa de melhorar o sucesso da resposta terapêutica (CHAMILOS; FERRIS et al., 2002; GULLO et al., 2013; KONTOYIANNIS, 2006; PERÉZ-PARRA et al., 2009; ZUZARTE et al., 2011).

Nesta perspectiva, a descoberta de drogas alternativas com eficácia e menor toxicidade ao tratamento dessa doença infecciosa é de grande importância. Os fitoconstituintes encontrados em plantas tem despertado o interesse de vários pesquisadores para a terapia antimicrobiana, pois tem se mostrado com potencial

para o tratamento de muitas doenças (DHAMA et al., 2014; EDEOGA; OKWU; MBAEBIE, 2005; TEPE et al., 2004; YADAV et al., 2016).

Estima-se que aproximadamente 80% da população mundial utiliza a medicina tradicional para seus cuidados de saúde, especialmente quanto ao uso de terapias que envolvem fitoterápicos (BAGETTA et al., 2010). Muitos fármacos incorporados em medicamentos da atualidade já foram utilizados inicialmente, na sua forma bruta, em práticas tradicionais ou populares que então sugeriram atividade biológica potencialmente útil (BILAL et al., 2017).

Devido às suas aplicações versáteis, os compostos antifúngicos derivados de plantas estão atraindo bastante interesse como alternativas naturais (AHMAD et al., 2010; KHAN et al., 2010). Eugenol e isoeugenol são fenilpropenos que apresentam uma variedade de atividades farmacológicas, incluindo atividade antimicrobiana contra *Fusarium verticillioides*, *Saccharomyces cerevisiae*, *Candida albicans*, *C. tropicalis*, *C. dubliniensis*, *C. krusei*, *Trichophyton rubrum*, *Aspergillus flavus* (BENNIS et al., 2004; BHATIA et al., 2012; DAMBOLENA et al., 2012; GALLUCCI et al., 2014; HE et al., 2007; PEREIRA; MENDES; LIMA, 2013; TRAJANO; LIMA; SOUZA, 2012). Embora estudos anteriores tenham demonstrado o forte potencial antifúngico destes fenilpropenos, pouco se sabe sobre os mecanismos de ação relacionados a atividade antifúngica.

Diante da importância clínica e alta incidência da criptococose, aumento do número de indivíduos imunocomprometidos, surgimento de cepas resistentes e toxicidade dos antifúngicos existentes, a busca por novos agentes antifúngicos e alternativas terapêuticas se faz necessário, sendo o eugenol e o isoeugenol excelentes candidatos para esse propósito. Assim, o objetivo desse estudo foi investigar a atividade antifúngica desses fenilpropenos contra cepas de *Cryptococcus neoformans*, seus possíveis mecanismos de ação e efeito da associação com antifúngicos.

Objetivos



2 OBJETIVOS

2.1 OBJETIVOS GERAIS

- Investigar a atividade antifúngica *in silico* e *in vitro* do eugenol e isoeugenol, os possíveis mecanismos de ação envolvidos com a morte celular e os efeitos da associação desses compostos com antifúngico padrão contra cepas de *Cryptococcus neoformans*

2.2 OBJETIVOS ESPECÍFICOS

- Avaliar as possíveis propriedades antimicrobianas dos fenilpropenos através análise *in silico*
- Determinar a Concentração Inibitória Mínima (CIM) dos fenilpropenos estudados frente as cepas de *C. neoformans*
- Determinar a Concentração Fungicida Mínima (CFM) dos fenilpropenos estudados frente as cepas de *C. neoformans*
- Investigar o possível mecanismo de ação do eugenol e isoeugenol sobre a parede celular fúngica (Ensaio com sorbitol)
- Investigar o possível mecanismo de ação do eugenol e isoeugenol sobre a membrana celular fúngica (Interação com ergosterol)
- Avaliar os possíveis efeitos da associação do eugenol e isoeugenol com o antifúngico anfotericina B (*Checkerboard*)

Fundamentação Teórica



3 FUNDAMENTAÇÃO TEÓRICA

3.1 ASPECTOS GERAIS DO *Cryptococcus neoformans*

Taxonomicamente, *Cryptococcus* pertence ao reino Fungi, filo *Basidiomycota*, ordem *Tremellales*, família *Tremellaceae* (ANTINORI, 2013; HIBBETT et al., 2007; KURTZMAN; FELL; BOEKHOUT, 2011). O gênero *Cryptococcus* compreende mais de 39 espécies (MA; MAY, 2009), e entre essas, encontram-se as leveduras patogênicas, os principais agentes etiológicos da criptococose, relacionadas às espécies *Cryptococcus neoformans* e *Cryptococcus gattii* (KANTARCIOGLU et al., 2007).

As leveduras encapsuladas, *C. neoformans* e *C. gattii*, são caracterizadas como patógenos importantes em humanos e animais, com capacidade de infectar hospedeiros imunodeficientes e imunocompetentes (HAGEN et al., 2015; HEITMAN et al., 2011; HEITMAN-INGROFF et al., 2015).

As diferenças entre os patógenos e hospedeiros, em conjunto com a variabilidade de sorotipos e diversos grupos de marcadores moleculares, tem estabelecido a diferenciação dessas espécies distintas, *C. neoformans* e *C. gattii* (IDNURM; LIN, 2015; KWON-CHUNG, 1976; 2002).

De acordo com a classificação atual, existem cinco sorotipos: A, D e AD na espécie *C. neoformans* e os sorotipos B e C na espécie *C. gattii*. *C. neoformans* é subdividido em duas variedades: variedade *grubii* (sorotipo A) e variedade *neoformans* (sorotipo D) (FRANZOT et al., 1999; KWON-CHUNG et al., 2002; 2011; KWON-CHUNG; VARMA, 2006).

Os tipos moleculares variam de VNI a VNIV para *C. neoformans* e VGI a VGIV para *C. gattii* com base em estudos moleculares realizados por métodos, incluindo PCR *fingerprinting* (com iniciador M13) e *Restriction Fragment Length Polymorphism* (RFLP) do gene *URA5* (HAGEN et al., 2010; MEYER et al., 1999; 2003). Os tipos moleculares VNI a VNIV também tem sido designados como AFLP1 a AFLP3 e os tipos VGI a VGIV como AFLP4 a AFLP7 com base nos estudos de tipagem molecular utilizando a análise por polimorfismo da fração longa amplificada (AFLP) (BOVERS; HAGEN; BOEKHOUT, 2008; BYRNES et al., 2010; 2011).

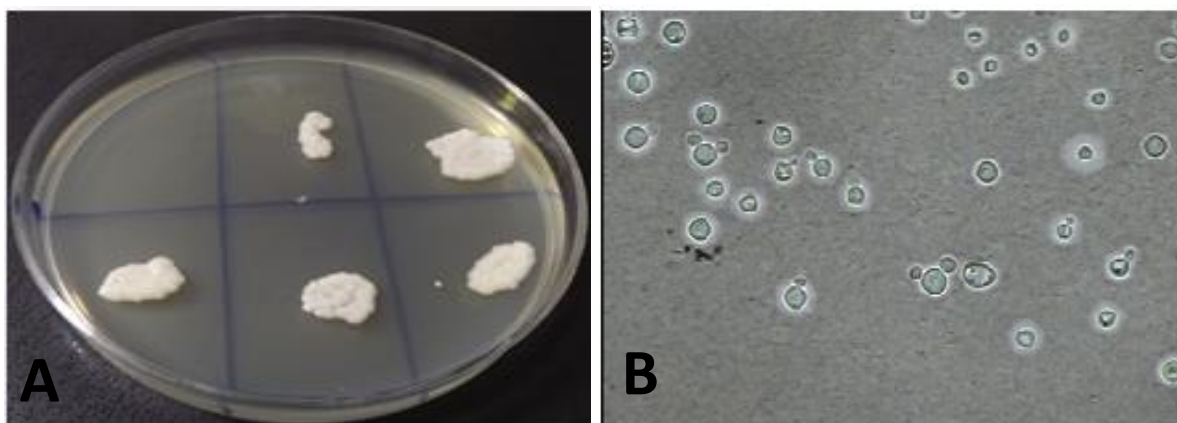
C. neoformans é considerado um patógeno oportunista que comumente causa infecções no SNC, principalmente em pacientes com HIV / AIDS e em outros

pacientes imunodeficientes, incluindo leucêmicos e transplantados (HEITMAN et al., 2011; NGAMSKULRUNGROJ et al., 2012). O fungo apresenta características saprófita e cosmopolita, podendo ser encontrado normalmente em solos contaminados com excremento de aves e vegetais em decomposição (HEITMAN; LIN, 2006; SORREL; ELLIS, 1997; STEENBERGEN; CASADEVALL, 2003).

A micromorfologia desse fungo compreende 3 a 8 μm de diâmetro, formado por brotamento único ou múltiplo, envolto por uma cápsula mucopolissacarídica característica, visualizada por microscopia ótica utilizando tinta nanquim ou microscopia de contraste de fase (CONSENSO EM CRIPTOCOCOSE, 2008; NEGRONI, 2012).

Macroscopicamente, suas colônias têm coloração branca a creme, brilhante, com textura mucoide, margem lisa e inteira, com crescimento após três dias de incubação em temperatura de 25 a 37 °C, utilizando-se para isso os meios como Agar Sabourad dextrose (ASD), sob condições aeróbias (CHAYAKULKEEREE; PERFECT, 2006; CONSENSO EM CRIPTOCOCOSE, 2008; NEGRONI, 2012).

Figura 1 – Características morfológicas do *Cryptococcus neoformans*



Fonte: PINHEIRO (2013).

A: Macromorfologia, colônias após 3 dias de crescimento em ASD a 35° C, apresentando coloração branca a creme, com textura brilhante.

B: Micromorfologia, presença de leveduras encapsuladas, com brotamento único ou múltiplo.

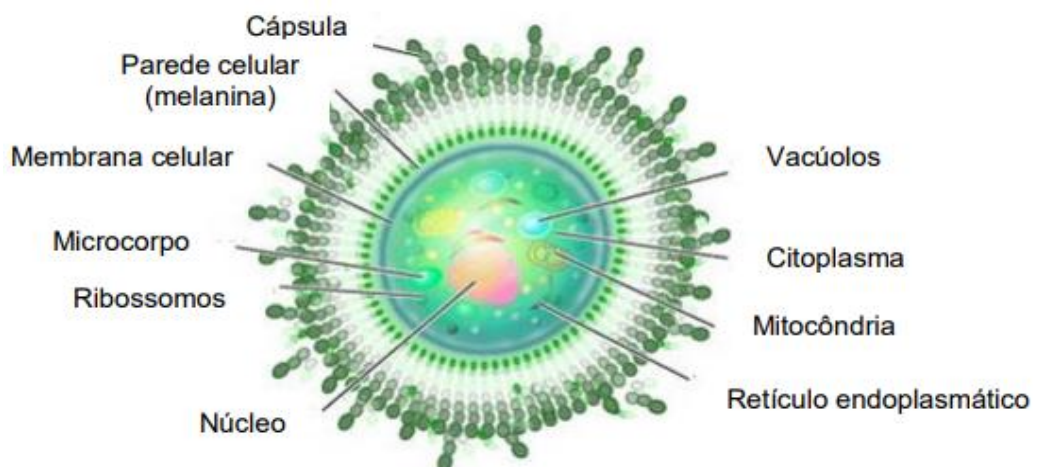
Uma propriedade interessante dessa levedura encapsulada está na capacidade de alternar o seu crescimento da forma leveduriforme para a forma filamentosa durante o ciclo sexual, embora não seja classificado como fungo

dimórfico, por conta da predominância da sua forma leveduriforme no ambiente e no ser humano (KOZUBOWSKI; HEITMAN, 2012).

O ciclo de vida é composto por fase assexuada e sexuada. Na fase sexuada coexistem as formas de basidiósporos e hifas (fase teleomorfa), e a espécie é denominada *Filobasidiella neoformans*, onde na fase assexuada existem as formas leveduriformes (fase anamorfa) e a espécie correspondente é denominada *C. neoformans* (KURTZMAN; FELL; BOEKHOUT, 2011; KWON-CHUNG, 1976).

C. neoformans (Figura 2) apresenta capacidade de resistir à imunidade do hospedeiro através de diversos fatores de virulência (MATSUMOTO et al., 2011), dentre os mais importantes, destacam-se a cápsula polissacarídica que circunda a parede celular e o diferencia de outros fungos patogênicos, a síntese de melanina, a secreção de urease e fosfolipase, e a capacidade de sobreviver à temperatura de 37 °C (COX et al., 2000, 2001; DOERING, 2009; GOMEZ; NOSANCHUK, 2003).

Figura 2 – Superfície celular de *C. neoformans*



Adaptado de <http://www.scq.ubc.ca/a-peach-of-a-pathogen-cryptococcus-neoformans/>

A cápsula, o principal fator de virulência, é constituída de material polissacarídico que permite a sobrevivência da levedura em diversos ambientes e no hospedeiro exibe capacidade fagocítica e imunossupressora, diferenciando os diversos sorotipos (BOSE et al., 2003; CHERNIAK, 1994; DOERING, 2009; LEITE, 2010).

A melanina que está presente na parede celular do fungo, é proveniente de substratos contendo dopamina e da ação de enzimas como a fenoloxidase. Como o cérebro é rico em substratos para a fenoloxidase, tais como a dopamina, esta seria uma possível explicação para o tropismo desta levedura pelo sistema nervoso (CONSENSO EM CRIPTOCOCOSE, 2008). Além disso, a melanina confere resistência ao estresse ambiental, protegendo o fungo da radiação ultravioleta (UV), dissecação e temperaturas extremas (LANGFELDER et al., 2003).

A urease tem a propriedade de catalisar a hidrólise da ureia em amônia e carbamato, em condições fisiológicas, promovendo aumento do pH. E essa alcalinização favorece a sobrevivência do fungo no fagolisossoma (COX et al., 2000). A fosfolipase do tipo B1 (PLB1) tem a propriedade de destruir os componentes da membrana celular, ocasionando a lise das células do hospedeiro infectado (COX et al., 2001; STEENBERGEN; CASADEVALL, 2003).

A temperatura é uma característica importante ao ambiente de sobrevivência do fungo, que cresce bem entre 25 e 37 °C, e sob elevadas condições de temperatura, tem seu crescimento inibido (CASADEVALL; PERFECT, 1998; MITCHELL; PERFECT, 1995). Essa capacidade de sobreviver e se replicar a 37 °C é denominada termotolerância, sendo muito importante para o fungo invadir o tecido e causar infecção (BUCHANAN; MURPHY, 1998).

3.2 CRIPTOCOCOSE

Embora a criptococose seja considerada uma doença micótica sistêmica, ela apresenta variações no espectro da doença, que vão desde infecções cutâneas autolimitantes a sistêmicas fatais (MITCHELL; PERFECT, 1995; PERFECT et al., 2010). Os fatores determinantes para que a micose se estabeleça estão relacionados ao estado imunológico do hospedeiro, o tamanho do inóculo fúngico e a virulência do micro-organismo (MITCHELL; PERFECT, 1995; NEGRONI, 2012).

A infecção cutânea é menos frequente e ocorre devido a inoculação direta na pele através de algum trauma (CHAYAKULKEEREE; PERFECT, 2006). Em hospedeiros imunocompetentes, a infecção cutânea primária é rara, ocorrendo em alguns casos, em áreas endêmicas, apresentando-se com evolução espontânea e benigna (CASADEVALL; MUKHERJEE; PERFECT, 1994; NEGRONI, 2012; NEUVILLE et al., 2003).

As lesões cutâneas aparecem na forma de pápulas, tumores, vesículas, placas, abscessos, celulite, púrpura, úlceras, bolhas ou inchaço subcutâneo (PAZARE, 2016). Essas lesões aparecem em 6 % dos pacientes com AIDS e são consideradas um sinal de alerta para criptococose disseminada (CHAYAKULKEEREE; PERFECT, 2006; NEUVILLE et al., 2003).

A infecção sistêmica apresenta o trato respiratório como porta usual de entrada para o organismo, por inalação de basidiósporos ou leveduras dissecadas encontradas em fontes ambientais (BOTTS; HULL, 2010; GILES et al., 2009; VELAGAPUDI et al., 2009). Após a invasão dos pulmões, o micro-organismo pode tornar-se na forma latente por longos períodos em indivíduos imunocompetentes até que um comprometimento imunológico apareça. A infecção pode então difundir-se via hematológica a qualquer parte do corpo, mais notavelmente para o SNC, causando meningite ou meningoencefalite, que se não tratada, é fatal (HAYNES et al., 2011; MICHEL; PERFECT, 1995; SRIKANTA et al., 2014; WAJID et al., 2005).

A criptococose acomete principalmente o SNC ou pulmões, e menos frequente, o sangue, a pele, olhos e a próstata (GOLDMAN et al., 2010; NEUVILLE et al., 2003). A meningoencefalite é o tipo de manifestação clínica mais comum, presente em 70 – 90% dos casos de criptococose em hospedeiros imunodeficientes (BICANIC; HARRISON, 2005; ESCANDON et al. 2012; SATISHCHANDRA et al., 2007).

No SNC, a infecção normalmente é caracterizada por cefaleia, febre, e com menor frequência, apresenta alterações mentais. Algumas complicações dessa infecção no SNC são referidas como hidrocefalia, déficits motores ou sensoriais, paralisia do nervo craniano, disfunção cerebelar, convulsões e demência (PAZARE, 2016).

Nos pulmões, a infecção manifesta-se em pacientes imunodeficientes, e nos hospedeiros imunocompetentes, torna-se latente assintomática ou as células fúngicas são eliminadas através do sistema imunológico (MAY et al., 2016). A criptococose pulmonar apresenta sinais de tosse, febre, mal-estar, falta de ar e dor pleurítica. O exame radiográfico do tórax pode apresentar infiltrados difusos, nódulos subpleurais solitários, massa, consolidação, adenopatia hilar e mediastinal, derrames pleurais (PAZARE, 2016).

A próstata é reconhecida como um reservatório para as leveduras durante o tratamento da criptococose. A infecção nesse local geralmente é assintomática e

não causa transmissão conjugal (MITCHELL; PERFECT, 1995). A infecção pode permanecer latente na glândula e após o tratamento antifúngico tornar-se fonte de reinfecção (NDIMBIE et al., 1994). Na criptococose ocular, as manifestações vão desde a paralisia ocular ao comprometimento da retina, com risco potencial de perda da visão (CRUMP et al., 1992).

A criptococose é considerada oportunista por causa da grande incidência em pacientes com deficiência imunidade celular, associado ao vírus HIV, transplante de órgãos sólidos, pacientes com neoplasias malignas, tratamento com corticosteroides e doenças linfoproliferativas (BADDLEY et al., 2011; MARTINS et al., 2011). Embora exista relação de infecção também em indivíduos imunocompetentes (CHAYAKULKEEREE; PERFECT, 2006).

A criptococose é uma das micoses com maior facilidade de diagnóstico, devido ao tropismo neurológico, presença de cápsula característica, abundância de elementos fúngicos nas lesões e no líquido, diagnóstico imunológico e coloração tecidual específica (CONSENSO EM CRIPTOCOCOSE, 2008; QUEIROZ et al., 2008). O diagnóstico pode ocorrer através do exame direto, cultura, diagnóstico molecular, radiológico e sorologia (SEVERO et al., 2009; SIDRIM et al., 2010).

Os materiais clínicos obtidos a partir de lavado brônquico, líquido cefalorraquidiano (LCR), urina, pus de abscesso, fragmentos de tecido, aspirados de medula óssea e de gânglios podem ser analisados por microscopia através do exame direto do fungo numa preparação com tinta da China / tinta nanquim (CONSENSO EM CRIPTOCOCOSE, 2008; QUEIROZ et al., 2008).

C. neoformans cresce em muitos meios de cultura, como exemplo: o ASD, Agar-sangue, Ágar infusão de cérebro-coração. A utilização de meios contendo compostos difenólicos resulta na coloração das colônias na tonalidade marrom, característico da presença de lacase produtora de melanina (BICANIC; HARRISSON, 2005).

O diagnóstico histopatológico pode ser realizado por biópsia de vários sítios orgânicos (pele, pulmão, fígado, linfonodo). A biópsia identifica as leveduras encapsuladas utilizando várias colorações: Hematoxilina-eosina (HE) que não cora a espessa cápsula de polissacarídeos, mas apresenta aspecto característico de “bolhas de sabão” pela visualização das leveduras aglomeradas; Mucicarmim de Mayer que visualiza a cápsula na cor magenta e Fontana-Masson, que identifica a

melanina na parede celular da levedura (CONSENSO EM CRIPTOCOCOSE, 2008; QUEIROZ et al., 2008).

O teste sorológico para o diagnóstico de criptococose é realizado através da pesquisa do antígeno capsular polissacarídeo pelos métodos de aglutinação em látex e Enzima Imuno Ensaio (MITCHELL; PERFECT, 1995). A pesquisa de anticorpos anti-*C. neoformans* não é recomendada para o diagnóstico da criptococose, uma vez que, os mesmos não são detectáveis na fase de infecção ativa e podem aparecer durante o tratamento e na fase de recuperação da doença (BICANIC; HARRISSON, 2005; CONSENSO EM CRIPTOCOCOSE, 2008).

3.3 EPIDEMIOLOGIA DA CRIPTOCOCOSE

A criptococose atingiu proporções globais nos últimos anos, associada ao aumento do número de pacientes com deficiente imunidade celular, resultado de terapias imunossupressoras ou doenças imunológicas hereditárias; assim como, devido as mudanças climáticas e a propagação mundial da infecção pelo HIV (LUNGRAN et al., 2014).

Em algumas regiões, a criptococose é considerada a segunda infecção fúngica grave mais frequente, onde cerca de 80 a 90% dos pacientes acometidos com essa micose são associados a AIDS (PFALLER et al., 2010; WALLACE et al., 2008).

C. neoformans é o agente etiológico mais frequente da criptococose, correspondendo a 80% das infecções (KWON-CHUNG et al., 2014). A variedade *grubii* (sorotipo A) é a mais comum entre os outros sorotipos encontrados em espécimes clínicos em todo mundo, relacionando-se com mais de 95% dos casos de criptococose e 98% das infecções em pacientes com AIDS (SIMWAMI et al., 2011). Dentre os tipos moleculares, VNI é mais predominante (63%), seguidos de VNII / VNIII (6%) e VNIV (5%), como causadores da criptococose em todo o mundo (MEYER et al., 2011).

A incidência da criptococose varia entre região e país devido a prevalência do HIV, fatores geográficos e étnicos diferentes (CHEN et al., 2000; FRIEDMAN et al., 2005; MCCARTHY et al., 2006; MIRZA et al., 2003). Anualmente, são estimados mais de 1 milhão de novas infecções e cerca de 700.000 mortes em todo o mundo,

relacionadas a criptococose (CHAYAKULKEEREE; PERFECT, 2006; PARK et al., 2009).

A meningite criptocócica é a apresentação clínica mais comum e tem sido relacionada como doença definidora da AIDS (ABADI et al., 1999; MAMIDI et al., 2002; WRIGHT; SCHNEIDER; BERGER, 1997). A África Subsaariana e o Sudeste Asiático são as regiões com maior registro da meningite criptocócica (88% dos casos mundiais) e apresentam mais de 90% das mortes associadas a essa doença, seguidos da América Latina, considerada a terceira região global em casos de meningites criptocócicas (PARK et al., 2009).

As taxas de mortalidade da meningite criptocócica variam a nível mundial com a região afetada, dependendo também de diversos fatores como o acesso e disponibilidade à terapia antirretroviral, aos antifúngicos, diagnóstico da doença e a pressão exercida no SNC (DESNOS-OLLIVIER et al., 2015; MORA et al., 2010).

Mesmo na era da terapia antirretroviral com consequente diminuição dos casos de meningite criptocócica nos países ocidentais, essa infecção continua sendo importante causa de morbidade e mortalidade em pacientes HIV positivos em regiões da África Subsaariana e Sudeste Asiático (HARRIS; LINDSLEY; HENCHAICHON, 2012; JARVIS; BOULLE; LOYSE, 2009; MIRZA et al., 2003; VAN; WALENKAMP; LIPOVSKY, 2000).

Na África Subsaariana, 50 a 70% dos pacientes com meningite criptocócica associada a HIV / AIDS não sobrevivem (HAKIM et al., 2000; HOLMES et al., 2003). Nos Estados Unidos e outros países desenvolvidos, as taxas de mortalidade diminuíram em pacientes com HIV / AIDS devido a disponibilidade terapêutica antirretroviral elevada, e estão em torno de 12% (COGLIATI, 2013). Em países da Europa estas taxas estão entre 6,5 a 32% (DESNOS-OLLIVIER et al., 2015; DROMER et al., 2007).

No Brasil, a criptococose por *C. neoformans* ocorre em todas as regiões, sendo considerada a terceira causa de infecção no sistema nervoso central em pacientes com a AIDS (PINTO JUNIOR et al., 2006). Uma pesquisa realizada nos anos de 1980 – 2002 revelou que dos 215.000 casos de AIDS notificados no Brasil, 6% foram diagnosticados com a criptococose (MINISTÉRIO DA SAÚDE, 2002). Os dados epidemiológicos acerca das mortes causadas por criptococose realizados em 2009, identificaram um aumento anual de 24,5 a 26,8% no Brasil (PRADO et al., 2009).

As estimativas de mortalidade quando a criptococose é associada a AIDS, podem chegar a 79% nesse grupo de pacientes (PAPPALARDO; MELHEM, 2003). Embora o tratamento antirretroviral seja fornecido gratuitamente pelo serviço de saúde pública, a mortalidade na primeira semana de admissão ainda é elevada (42 a 51%), relacionada à imunossupressão avançada que ocorre no início do tratamento do HIV, em algumas vezes, associada ao diagnóstico tardio (MORA et al., 2012; PAPPALARDO; PASCHOAL; MELHEM, 2007).

A doença é mais frequente em adultos, com alguns casos já relatados na literatura em crianças (PAPPALARDO; MELHEM, 2003). No Brasil, um estudo realizado entre os anos de 1972 a 1996, com pacientes em idade de 8 meses a 79 anos, foi analisado que a criptococose foi mais comum em um terço dos indivíduos em idade inferior a 15 anos (DARZÉ et al., 2000).

Um estudo realizado em 2008, a partir de dados sobre a criptococose no Brasil, revelou que a macrorregião Sul compreendeu os estados onde a criptococose por *C. neoformans* foi predominante, correspondendo aos estados de Mato Grosso do Sul, Minas Gerais, São Paulo, Rio de Janeiro, Paraná e Rio Grande do Sul. E os tipos moleculares mais frequentes, revelaram a prevalência de VNI (64%), VNII (5%), VNIV (3%) e VNIII (< 1%) (TRILLES et al., 2008).

Outros estudos tem revelado a incidência da criptococose em 6,6 casos por um milhão de pessoas na Austrália e 15,6 por 100.000 pessoas na Província Guateng – África do Sul (FANG et al., 2015). Na China, 8.769 casos de criptococose foram avaliados em uma revisão sistemática entre os anos de 1985 e 2010 (CHEN et al., 2012). Na Tailândia, entre os anos de 1994 a 1998, a meningite criptocócica apresentou maior prevalência do que as infecções causadas por bactérias e a tuberculose (BICANIC; HARRISON, 2005; CHARİYALERTSAK et al., 2001).

3.4 TERAPIA ANTIFÚNGICA DA CRIPTOCOCOSE

O tratamento da criptococose dispõe de poucas opções terapêuticas, limitadas principalmente, ao uso de medicamentos como a anfotericina B, 5-fluorocitosina e fluconazol, administrados em associação ou de forma individualizada (PERFECT; BICANIC, 2015). Nos casos de meningite criptocócica, o tratamento consiste em três fases: a terapia de indução com a utilização da anfotericina B combinada com 5-fluorocitosina, seguidos do uso de fluconazol durante as fases de

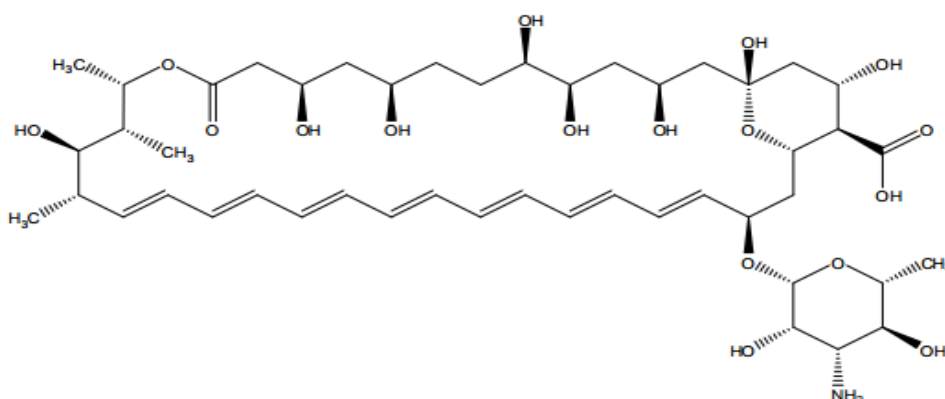
consolidação e manutenção até a restauração imunológica do hospedeiro (PERFECT et al., 2010).

A fase de indução, etapa inicial do tratamento, está relacionada a redução da carga fúngica e segundo a Organização Mundial da Saúde (OMS) e a Sociedade de Doenças Infecciosas da América, essa terapia combinada é recomendada como o "padrão-ouro", mas apresenta desafios substanciais em regiões com poucos recursos, pois além da toxicidade notável, não estão amplamente disponíveis nessas regiões onde a criptococose é mais prevalente (LOYSE et al., 2013; PERFECT et al., 2010; PERFECT; BICANIC, 2015).

A fase de consolidação é instituída após a fase de indução, quando o paciente apresenta melhora clínica (status mental, febre, cefaléia, sinais meníngeos e/ou negativação da cultura do LCR) e parâmetros laboratoriais normais. A fase de manutenção, também conhecida com supressão, ocorre logo após o tratamento efetivo e com a finalidade de evitar recaídas, principalmente em hospedeiros imunodeficientes (CONSENSO EM CRIPTOCOCOSE, 2008).

A anfotericina B (Figura 3) é um derivado poliênico pertencente à classe dos macrolídeos naturais que apresenta amplo espectro de atividade antifúngica, incluindo atividade contra fungos oportunistas e endêmicos (GROLL; KOLVE, 2004).

Figura 3 – Estrutura química da anfotericina B

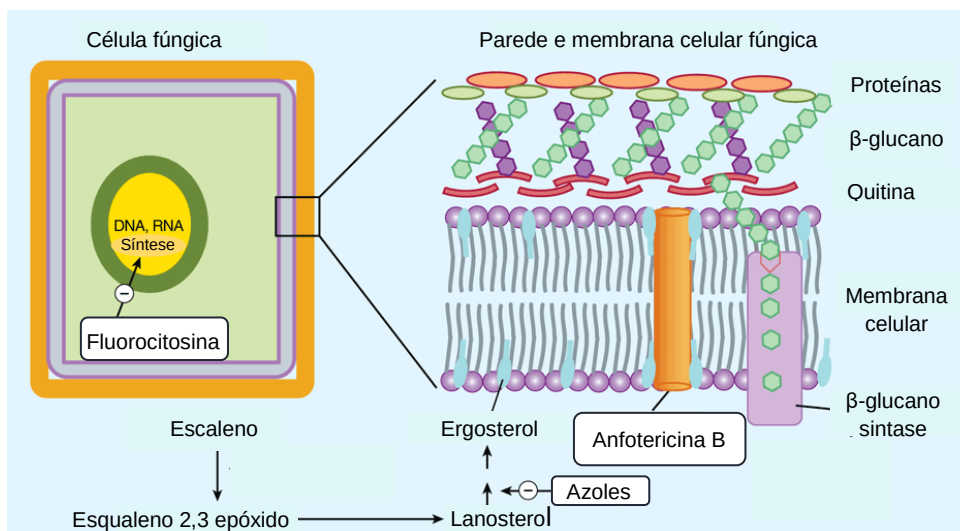


Fonte: BRAUTASET et al., 2011.

Esse fármaco apresenta mecanismo de ação nas células fúngicas por interação específica com o ergosterol da membrana, resultando na desorganização

da estrutura pela formação de poros aquosos (Figura 4). Esses defeitos promovem despolarização da membrana e aumento da permeabilidade aos prótons e cátions monovalentes, com consequente morte celular (GHANNOUM; RICE, 1999; GROLL; KOLVE, 2004; SANGLARD, 2002).

Figura 4 – Alvos para a ação de drogas antifúngicas no núcleo, parede celular, e membrana utilizados na clínica.



Adaptado de KATZUNG; MASTERS; TREVOR, 2009.

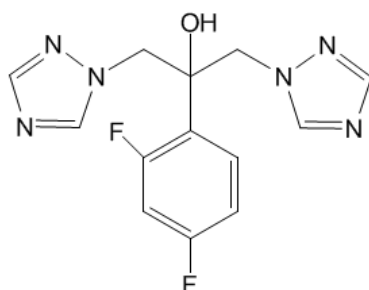
Contra *C. neoformans*, o efeito fungicida desse fármaco está relacionado a ligação ao ergosterol da membrana e indução da morte celular por estresse oxidativo; dependendo também, do tipo de cepa, da concentração utilizada e um bom veículo de condução (ANDERSON et al., 2014; ARAÚJO et al., 2005; BELENKY; CAMACHO; COLLINS, 2013; GRAY et al., 2012).

A toxicidade da anfotericina B, relacionada principalmente aos efeitos nefrotóxicos, são observados nos primeiros dias após o uso clínico, através de distúrbios eletrolíticos (HA et al., 2011; ODDS; BROWN; GOW, 2003; OSTROSKY-ZEICHNER et al., 2003). No entanto, o interesse clínico por esse fármaco é justificado devido à sua alta potência, amplo espectro de ação, efeito fungicida e raros aparecimentos de resistência entre os fungos patogênicos (AKINS, 2005; BRAUTASET et al., 2011; EGGIMAN; GARBINO; PITTET, 2003).

A resistência de *C. neoformans* a anfotericina B não é tão comum, mas já foi relatado que o uso clínico desse fármaco foi capaz de promover o isolamento de cepas mutantes com alterações ao nível dos esteróis da membrana (KELLY et al., 1994). A resistência a este antifúngico também ocorre em pacientes tratados previamente com os derivados azólicos, provavelmente através de uma alteração na membrana celular fúngica, já que o ergosterol é o alvo destes antifúngicos (GHANNOUM; RACE, 1999; PEREA; PATTERSON, 2002).

Os derivados azólicos constituem a maior classe de agentes antifúngicos, caracterizados por compostos sintéticos heterocíclicos constituídos com um anel pentagonal (MURRAY et al., 2014). O fluconazol (Figura 5) tem como alvo a biossíntese do ergosterol da membrana fúngica, através da inibição do citocromo P450 lanosterol 14- α -desmetilase (Figura 4), ocasionando a diminuição do ergosterol e acúmulo de precursores do esterol, principalmente os esteróis metilados, resultando assim numa membrana plasmática com estruturas e funções alteradas (GHANNOUM; RICE, 1999; SANGLARD, 2002).

Figura 5 – Estrutura química do fluconazol



Fonte: PASQUALOTTO; DENNING, 2008.

O fluconazol é um agente fungistático (e não fungicida) de uso sistêmico, menos efetivo na remoção dos patógenos, e apesar de apresentar boa penetração no LCR, não é recomendado na terapia inicial e como monoterapia na meningite criptocócica (MAY et al., 2016; PERFECT et al., 2010).

As alterações na membrana fúngica provocadas pelo fluconazol comprometem as funções de determinados sistemas enzimáticos ligados à

membrana, como a ATPase e as enzimas do sistema de transporte de elétrons, resultando na inibição do crescimento e replicação dos fungos (BENNETT, 2006; DODDS-ASHLEY, 2006; MOHR et al., 2008).

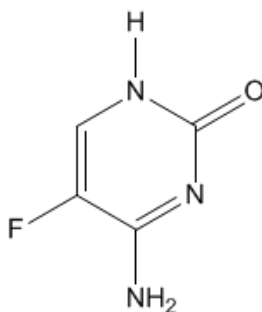
O uso prolongado do fluconazol durante a terapia da criptococose, principalmente em pacientes com AIDS, pode ocasionar a resistência do *Cryptococcus*, justificando o seu uso em intervalos de tempos definidos (FRIESE et al., 2001).

A resistência de algumas cepas de *C. neoformans* ao fluconazol tem sido demonstrado como efeito à pressão exercida pelo fármaco promovendo várias duplicações cromossômicas, como a encontrada no cromossomo 1, intimamente relacionada com os genes, *ERG11*, que é o alvo do fluconazol, e o *AFR1*, que é o transportador dos azoles pelo *C. neoformans* (SIONOV et al., 2010).

Embora o fluconazol seja bastante utilizado entre os compostos azólicos, outros derivados desse grupo, incluindo itraconazol, voriconazol e posaconazol, também tem sido utilizados na terapia da criptococose devido a resistência *in vitro* entre os isolados de *Candida* e *Cryptococcus* ao fluconazol (PERFECT et al., 2010). Esses fármacos atuam como o fluconazol, no bloqueio da via biossintética do ergosterol, inibindo a enzima 14- α -desmetilase, que é codificada pelo gene *CYP51* em *C. neoformans*, também chamado *ERG11* (REVANKAR et al., 2004).

A 5-fluorocitosina é uma pirimidina fluorada (Figura 6), que tem a capacidade de penetrar na célula fúngica por ação de uma permease, sendo transformada em 5-fluorouracil (5-FU) pela ação da enzima citosina desaminase. O 5-fluorouracil é convertido a ácido 5-fluorouridílico (FUMP) pela enzima UMP-pirofosforilase, o qual é fosforilado e incorporado ao RNA, ocasionando a inibição da síntese proteica. Outro possível mecanismo ocorre através da ação sobre a enzima timidilato sintase, inibindo a síntese do DNA e a divisão celular. Portanto, a fluorocitosina interfere no metabolismo das pirimidinas, DNA, RNA e síntese proteica (Figura 4) (GHANNOUM; RICE, 1999; NATH, 2005).

Figura 6 – Estrutura química da 5-fluorocitosina



Fonte: BENNETT, 2006.

A fluorocitosina é pouco utilizada na terapia antifúngica devido ao seu limitado espectro de ação e toxicidade, relatados por problemas gastrintestinais e hematológicos, devido ao rápido desenvolvimento de cepas resistentes, como também, ao alto custo que torna esse fármaco indisponível em regiões com poucos recursos (LOYSE et al., 2013; WILSON; DREW; PERFECT, 2009).

O aparecimento de cepas resistentes à fluorocitosina é causado principalmente em pacientes que receberam a monoterapia com fluorocitosina, o que tem sido recomendado o uso associado a anfotericina B, como combinação sinérgica importante no tratamento de infecções graves sistêmicas (RANGE; DALE; 2007).

Essa resistência já foi observada *in vitro* e na prática clínica como resultado de mutações únicas em pontos variados ao longo das vias de metabolismo do fármaco, uma vez que, a monoterapia leva a seleção rápida de *Cryptococcus* resistentes, ocasionando a falha no tratamento (MAY et al., 2016).

O uso da fluorocitosina também apresenta relatos de resistência como resultado da diminuição de sua incorporação à célula, ocasionada pela perda da atividade da permease, assim como, pode estar relacionada à perda da atividade da enzima responsável pela conversão do FUMP (GHANNOUM; RACE, 1999; WHITE; MARR; BOWDEN, 1998).

A terapia antifúngica tem se tornado um grande desafio devido ao desenvolvimento de cepas resistentes, a toxicidade e os elevados custos dos antifúngicos, interações medicamentosas, biodisponibilidade e disponibilidade insuficiente das drogas antifúngicas (CAVALEIRO et al., 2006; KHAN et al., 2010;

MARUI et al., 2010; SAAD, 2010). E, portanto, existe uma necessidade urgente de novas drogas, o que têm motivado a busca de agentes antifúngicos a partir dos produtos naturais, em especial, das plantas medicinais (BANSOD; RAI, 2008; ODDS; BROWN; GOW, 2003).

3.5 CONSIDERAÇÕES SOBRE PRODUTOS NATURAIS

Os produtos naturais têm sido reconhecidos tradicionalmente como fontes importantes de compostos terapêuticos utilizados contra doenças infecciosas e outras doenças (RODRIGUES, 2001). Os estudos que comprovam esses benefícios são essenciais para que o uso desses produtos seja baseado em sua atividade terapêutica, pois apresentam grande importância econômica e para a saúde (RAHMAN et al., 2004; YUNES; PEDROSA; CECHINEL-FILHO, 2001).

As plantas constituem uma fonte inesgotável de compostos naturais potencialmente ativos, produzidos a partir do seu metabolismo (CARDOSO; SHAN; SOUZA, 2001). Os metabólitos primários, incluindo os lipídeos, glicídeos, protédeos e nucleotídeos, são encontrados em todas as plantas e desempenham papéis essenciais à sobrevivência desses vegetais (BUCHANAN; GRUISSEM; JONES, 2000).

A partir do metabolismo primário, rotas biossintéticas diversas e frequentemente desconhecidas, originam os metabólitos secundários, que são encontrados em pequenas concentrações em grupos de plantas e essenciais à adaptação e perpetuação da espécie no ecossistema (POSER; MENTZ, 2004; SANTOS, 2004).

Os metabólitos secundários apresentam grande versatilidade de aplicações, incluindo propriedades antimicrobianas contra micro-organismos patogênicos, que são influenciadas de acordo com a estrutura e composição química (BILAL et al., 2017; HAYEK et al., 2013). Entre os metabólitos secundários, os principais grupos encontrados com atividade antimicrobiana são relacionados aos compostos fenólicos, quinonas, saponinas, flavonoides, taninos, cumarinas, terpenoides, alcalóides e óleos essenciais (CIOCAN; BARA, 2007; LAI; ROY, 2004; SEOW; YEO; YUK, 2014).

A eficácia desses fitoconstituintes (comumente chamados de fitoquímicos / fitobióticos) tem atraído o interesse de vários pesquisadores, o que tem tornado

esses produtos derivados de plantas medicinais, como fontes preferenciais para a pesquisa farmacológica e o desenvolvimento de drogas (BARIS et al., 2006; BILAL et al., 2017; BRASIL, 2006).

Está bem estabelecido que os óleos essenciais são produtos naturais concentrados com cheiros fortes, produzidos por plantas aromáticas como metabólitos secundários (NAZZARO et al., 2013) e exibem propriedades inseticidas, antioxidantes, anti-inflamatórias, antialérgicas e anticancerígenas, além de suas características antimicrobianas (LEE et al., 2011; SVOBODA; BROOKER; ZRUSTOVA, 2006). Assim, seus principais usos são identificados na microbiologia médica e clínica, nas indústrias alimentícias (conservantes e aromatizantes) e de perfumes (OROOJALIAN et al., 2010; SVOBODA; BROOKER; ZRUSTOVA, 2006).

Os fitoquímicos, como os óleos essenciais, apresentam um amplo espectro antimicrobiano contra o crescimento e a sobrevivência de bactérias, leveduras e bolores (CALLAWAY et al., 2011; YADAV et al., 2016). Essas características e a crescente necessidade por aditivos alimentares naturais levaram ao interesse no uso desses compostos como potenciais antimicrobianos alternativos (FISHER; PHILLIPS, 2008; SOLÓRZANO-SANTOS; MIRANDA-NOVALES, 2012).

As misturas complexas de compostos com baixo peso molecular que compõem os óleos essenciais apresentam grandes diferenças na atividade antimicrobiana (HYLDGAARD; MYGIND; MEYER, 2012). A maioria dos óleos essenciais é constituída de derivados terpenoides ou de fenilpropanoides (SIMÕES; SPITZER, 2010).

Os fenilpropanoides são compostos amplamente distribuídos na natureza, encontrados em uma grande variedade de plantas. Eles derivam do aminoácido fenilalanina, produzido através da via do chiquimato, e consistem em um esqueleto C6-C3 formado pelo grupo fenol aromático de seis carbonos e uma cadeia lateral propeno com três átomos de carbono do ácido cinâmico (DIXON et al., 2002; HYLDGAARD; MYGIND; MEYER, 2012; KFOURY et al., 2014).

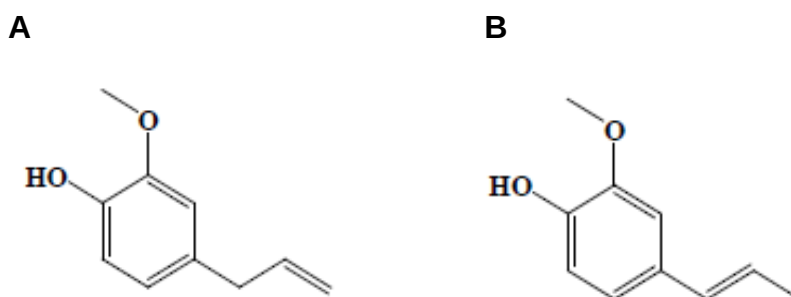
Os fenilpropenos são classificados como subfamília dos fenilpropanoides, compõem uma parte relativamente pequena dos óleos essenciais e contribuem para as propriedades de muitas especiarias e ervas (GANG et al., 2001; HYLDGAARD; MYGIND; MEYER, 2012). Eles têm atraído interesse científico, pois são constituintes de várias especiarias importantes, por exemplo, cravo (*Eugenia caryophyllata*), anis estrelado (*Illicium verum*) e ervas aromáticas, como manjerição doce (*Ocimum*

basilicum), erva-doce (*Foeniculum vulgare*) e louro (*Laurus nobilis*) (ATKINSON, 2016; IVANOVIĆ et al., 2010).

Previamente, a composição química do óleo essencial de *Laurus nobilis* foi analisada por cromatografia gasosa acoplada à espectrometria de massas, revelando o isoeugenol (57%) como principal constituinte majoritário (PINHEIRO et al., 2017a). O isoeugenol é um isômero de posição do composto eugenol (Figura 6), e geralmente, é uma mistura de isômeros Z e E (cis e trans, respectivamente).

A semelhança entre eugenol ($C_{10}H_{12}O_2$) e isoeugenol ($C_{10}H_{12}O_2$) garante algumas propriedades farmacológicas comuns (inseticida e analgésica), e apresenta diferenças responsáveis pelos aromas distintos e característicos destas moléculas (CORRÊA et al., 2016).

Figura 7 – Estrutura química do eugenol (A) e isoeugenol (B)



Fonte: PINHEIRO, 2017b.

A: eugenol; B: isoeugenol

3.5.1 Eugenol

O eugenol (2-metóxi-4-prop-2-en-1-il-fenol) é um fenilpropeno reconhecido como o principal constituinte (70 – 90%) e responsável pelo aroma marcante do óleo essencial de cravo (ATKINSON, 2016). Esse composto foi identificado em várias plantas aromáticas, como canela, manjeriço, manjeriço-doce, noz moscada, louro e cânfora (KAMATOU; VERMAAK; VILJOEN, 2012).

Comumente, o eugenol é adicionado à preparação de alimentos, bebidas e de enxaguantes bucais, devido as suas propriedades aromatizantes e antissépticas (BENDRE et al., 2016; DORMAN; DEANS, 2000; LEE et al., 2001). É também

empregado como ingrediente na indústria de cosméticos e perfumes. Na odontologia é utilizado em combinação com óxido de zinco para formar o cimento temporário dos dentes, além de ser utilizado como agente sedativo, fazer o capeamento da polpa e revestimento de cavidades (JADHAV et al., 2004; MARKOWITZ et al., 1992).

Eugenol é uma molécula extraordinariamente versátil e atraiu a atenção de muitos pesquisadores devido ao seu amplo espectro de atividades biológicas e farmacológicas, como antifúngicas, antiparasitárias, antioxidantes, anestésicas, anti-inflamatórias e analgésicas (DANIEL et al., 2009; GUENETTE et al., 2006; HIDALGO et al., 2009; MACHADO et al., 2011; SOUZA et al., 2014).

Eugenol é Geralmente Reconhecido como Seguro (GRAS) sob as condições de uso como aromatizante. A atividade antigenotóxica do eugenol foi investigada *in vitro* no teste de síntese de DNA não programada e através do ensaio de mutagenicidade com *Salmonella typhimurium*. O eugenol foi capaz de suprimir a mutagenicidade induzida por furilamida e aflatoxina B em *S. typhimurium* (CHOGO; CRANK, 1981). Além disso, não apresentou nenhum efeito no teste de síntese de DNA não programada (ROMPELBERG et al., 1996).

Efeitos antigenotóxicos também foram obtidos no teste de micronúcleo em medula óssea de camundongos Swiss. O pré-tratamento com eugenol resultou em efeitos protetores significativos contra a genotoxicidade induzida por procarbazona, ciclofosfamida e uretano (ABRAHAM, 2001). Estudos *in vitro* sobre o efeito do eugenol em eritrócitos humanos demonstraram que o composto apresentou mínima atividade hemolítica, e portanto, não foi considerado tóxico por não apresentar atividade hemolítica ($Cl_{50} < 200\mu\text{g/mL}$) nesta concentração (HEMAISWARYA; DOBLE, 2013).

A atividade citotóxica do eugenol tem sido avaliada em diversos estudos que demonstram a ação desse composto na indução da morte celular em vários tipos de células e tumores, incluindo carcinoma de cólon e cervical, câncer de próstata, mastócitos, adenocarcinoma de mama, polpa de dente humano, macrófagos murinos peritoneais, entre outros (AWUTI et al., 2012; GHOSH et al., 2009; HEMAISWARYA; DOBLE, 2013; JAGANATHAN; SUPRIYANTO, 2012; MAHAPATRA et al., 2011; SLAMENOVÁ et al., 2009).

Quando utilizado em baixas concentrações, o eugenol atua como agente antioxidante e anti-inflamatório, ao contrário do uso em altas concentrações que

apresenta comportamento pró-oxidante, aumentando a geração de radicais livres (ASHA et al., 2001; CHOGO; CRANK, 1981).

Além disso, o uso do eugenol em humanos é considerado não mutagênico, não carcinogênico, não citotóxico nos linfócitos e seguro pela FDA (*Food and Drug Administration*) dos Estados Unidos (SATOOKA; KUBO, 2009). O limite permitido para ingestão diária é de 2,5 mg/kg de peso corporal, segundo os comitês de aditivos alimentares da FAO (Organização das Nações Unidas para a Agricultura e Alimentação) e da OMS (JAGANATHAN; SUPRIYANTO, 2012; OPDYKE, 1975).

Em um estudo de 24 h sobre a toxicidade aguda oral em dose única em ratos, a DL₅₀ do óleo essencial de cravo (89,6% de eugenol) e do eugenol foi superior a 2.000 mg/kg. No período de 14 dias não foram observadas alterações significativas, assim como, não houve mudanças no consumo de alimentos e água (SANTIN et al., 2011).

3.5.2 Isoeugenol

O isoeugenol (2-methoxy-4-prop-1-en-1-il-fenol) é um dos vários compostos fenilpropenos estruturalmente semelhantes, produzidos pelas plantas. Esse isômero do eugenol pode ser extraído de várias plantas ou flores, incluindo cravo, cálamo, cananga do Japão (ylang-ylang), manjerição, noz-moscada, junquilha, sândalo, gardênia, petúnia, e ainda ser sintetizado por isomerização do eugenol, que ocorre naturalmente na folha de louro, cravo, pimenta e canela (CERVENY et al., 1987; DEMOLE; DEMOLE; ENGGIST, 1976; GENNARO, 1985; HATTORI; MURAKI; YOSHIDA, 1978; OPDYKE, 1975).

O isoeugenol é utilizado como molécula chave para a síntese de outros compostos orgânicos (XIE; KAPTEYN; GAN, 2008). E assim como o eugenol, é comumente incorporado na preparação de alimentos e perfumes, artigos de higiene, incluindo cremes, sabões e detergentes, em algumas formulações pesticidas como atraente de insetos, e ainda apresenta uma variedade de propriedades bioativas, incluindo antioxidantes, antimicrobiana, analgésica e anti-inflamatória (ABOURASHED et al., 2008; ATSUMI; FUJISAWA; TONOSAKI, 2005; BENDRE et al., 2016; LI et al., 2006; OPDYKE, 1975).

Isoeugenol possui o status de GRAS pela FDA para a utilização em alimentos como substância aromatizante e adjuvante (CRF, 1990). O uso desse

composto como constituinte ativo no AQUI-S® (anestésico) tem sido reconhecido na aquicultura para a colheita de peixes e mariscos em várias regiões, incluindo Austrália, Chile, Ilhas Faroé, Coreia e Nova Zelândia (SCHNICK, 1999; 2005).

Em estudos de toxicidade aguda, a DL₅₀ do isoeugenol foi de 1.560 mg/kg de peso corporal, quando administrado por via oral em ratos (TAYLOR; JENNER; JONES, 1964). O consumo de 500 mg/kg de peso corporal na dieta de ratos machos e fêmeas, durante 16 semanas, não ocasionou mudanças no peso corporal, consumo de alimentos, avaliação hematológica ou peso dos órgãos (HAGAN et al., 1967).

Isoeugenol não foi mutagênico em nenhum dos testes independentes, utilizando *S. typhimurium* ou *Escherichia coli* WP2, com ou sem ativação metabólica exógena de enzimas hepáticas S9 (FLORIN et al., 1980; SEKIZAWA; SHIBAMOTO, 1982). Além disso, nenhuma indução da síntese de DNA não programada foi observada após a utilização de isoeugenol a 164 µg/mL (BURKEY et al., 2000).

No ensaio de micronúcleo, amostras de sangue periférico de camundongos B6C3F1 machos, tratados com isoeugenol (37,5 a 600 mg/kg) por gavagem durante 3 meses, não aumentaram a incidência de eritrócitos policromáticos micronucleados e eritrócitos normocromáticos micronucleados, indicando ausência de toxicidade relacionada à exposição à medula óssea (NTP, 2010).

Bhatia et al. (2012) avaliaram a atividade citotóxica do isoeugenol e um antifúngico convencional (fluconazol) contra glóbulos vermelhos humanos. O fluconazol foi significativamente mais citotóxico, pois induziu um aumento da hemólise de 10% (10 µg/mL) para 82% (600 µg/mL) em relação ao isoeugenol que apresentou baixa atividade citotóxica (4 a 22%).

A dose máxima diária permitida de isoeugenol na dieta humana foi calculada como 4,3 mg/kg para indivíduos entre 2 a 65 anos, ou 0,19 mg/kg de peso corporal/dia para indivíduos com idade entre 12 a 23 meses (Federação das Sociedades Americanas para a Biologia Experimental, 1973).

Devido ao alto potencial de exposição humana ao isoeugenol e a semelhança estrutural com alilbenzenos (estragol e safrol) conhecidos como carcinogênicos, estudos de carcinogenicidade do isoeugenol tem sido realizados para avaliar seus efeitos. O Programa Nacional de Toxicologia (2010) dos Estados Unidos desenvolveu estudos de carcinogenicidade do isoeugenol, utilizando ratos F344/N e camundongos B6C3F1 (ambos, machos e fêmeas) nas doses de isoeugenol a 75,

150 ou 300 mg/kg de peso corporal, presentes no óleo de milho e administrados por gavagem durante 2 anos.

Foi observado evidência equívoca (achado incerto) de atividade cancerígena do isoeugenol em ratos machos com base em raras ocorrências de aumento da incidência de timoma e carcinoma de glândula mamária, e em camundongos fêmeas, com base no aumento das incidências de sarcoma histiocítico. Não houve evidência de atividade cancerígena do isoeugenol em ratos fêmeas, enquanto que, camundongos machos apresentaram aumento da incidência de adenoma e/ou carcinoma hepatocelular (NTP, 2010).

Nos últimos anos, o isoeugenol tem sido amplamente estudado como um agente antifúngico promissor (BHATIA et al., 2012; HYLDGAARD et al., 2015). E suas propriedades antimicrobianas contra leveduras, bolores e bactérias foram equivalentes e em alguns estudos mais efetivas do que o seu isômero eugenol (LAEKEMAN et al., 1990).

Tanto eugenol como isoeugenol são moléculas de valor comercial e devido as suas baixas toxicidades abordadas na literatura e ao perfil ativo de várias propriedades farmacológicas, permite vislumbrar a análise potencial ao desenvolvimento de novos medicamentos com possível propriedade antifúngica, avaliando-se a abordagem desses estudos sobre o modo de ação antifúngica e os estudos de associação destes compostos com antifúngicos convencionais.

Considerando a importância clínica e epidemiológica dispensada à criptococose, e as dificuldades no seu tratamento, bem como o aumento da resistência aos antifúngicos e a elevada toxicidade dos antifúngicos utilizados no tratamento dessa micose, torna-se necessário o estudo de novos fármacos naturais e/ou sintéticos com propriedades antifúngicas.

As investigações do presente estudo se mostram relevantes, uma vez que sugerem possíveis mecanismos de ação antifúngica, além de confirmar seus efeitos inibitórios sobre *C. neoformans* e seus efeitos quando utilizados em associação com antifúngicos da terapia convencional.

Material e Métodos



4 MATERIAL E MÉTODOS

4.1 LOCAL DO TRABALHO

Os ensaios para a análise da atividade antifúngica dos fenilpropenos eugenol e isoeugenol foram realizados no Laboratório de Micologia do Departamento de Ciências Farmacêuticas (DCF) no Centro de Ciências da Saúde (CCS) da Universidade Federal da Paraíba (UFPB).

4.2 CEPAS FÚNGICAS

Foram selecionadas para a execução deste trabalho dez cepas de *C. neoformans*: FGF10, LM 6109, LM 309, LM 230, LM 1401, LM 3, LM 119, FCF 5, LM 120 e LM 615, previamente isoladas e identificadas, obtidas da coleção do Laboratório de Micologia da Universidade Federal da Paraíba. A cepa INCQS 40221 foi adquirida da Coleção de Micro-organismos de Referência em Vigilância Sanitária (CMRVS) do Instituto Nacional de Controle de Qualidade em Saúde (INCQS), Fundação Oswaldo Cruz (FIOCRUZ). Todas as cepas foram mantidas em tubos de ensaio contendo ASD sob refrigeração (4 °C) e em estufa (35 °C $\pm$ 2 °C) no Laboratório de Micologia.

4.3 INÓCULO

A preparação do inóculo foi realizada a partir de culturas recentes de cada uma das cepas selecionadas de *C. neoformans*, previamente cultivadas em tubos estéreis contendo ASD inclinado e incubados a 35 °C $\pm$ 2 °C por 24 - 72 horas para atingirem crescimento satisfatório. As colônias recentes foram então submetidas a raspagens (cerca de 3 a 5 colônias) com o auxílio de uma alça de platina em “L” e cobertas com 5 mL de solução salina estéril (8,5 g/L NaCl, salina a 0,85%). A suspensão resultante foi agitada em Vortex (Fanem) durante 2 minutos, comparada e ajustada à suspensão de sulfato de bário do tubo 0,5 da escala de MacFarland, a qual corresponde a um inóculo de aproximadamente 10⁶ unidades formadoras de

colônias/mL (UFC/mL) (CLEELAND; SQUIRES, 1991; HADACEK; GREGER, 2000; ODDS, 1989).

4.4 MEIOS DE CULTURA

Os meios de cultura utilizados foram o ASD (Difco Lab., USA) e caldo Sabouraud dextrose – CSD (Difco Lab., USA), preparados conforme as instruções do fabricante, sendo solubilizados em água destilada e previamente esterilizados em autoclave a 121°C, 1 atm, por 15 minutos.

4.5 FITOCONSTITUINTES, ANTIFÚNGICOS E SUBSTÂNCIAS

Fenilpropenos (eugenol e isoeugenol), o antifúngico anfotericina B, sorbitol e ergosterol foram adquiridos na Sigma-Aldrich® (São-Paulo). As soluções foram preparadas no momento da execução dos testes, dissolvendo-os primeiramente em dimetilsulfóxido (DMSO) a 5% e Tween 80 (2%) com água destilada estéril para obtenção das concentrações desejadas.

4.6 METODOLOGIA

4.6.1 Ensaio da atividade antimicrobiana

4.6.1.1 Ensaio *in silico* - Pass on line

O potencial biológico dos fenilpropenos (eugenol e isoeugenol) como agente antimicrobiano foi avaliado através do software Previsão do Espectro de Atividade para Substâncias (PASS) on line (<http://www.way2drug.com/passonline>). Este programa presume a atividade biológica de moléculas orgânicas, incluindo compostos fitoquímicos, utilizando a relação estrutura-atividade. As atividades biológicas encontradas são relacionadas as interações dos compostos com as entidades biológicas. A atividade de predição é obtida através dos 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 (GOEL et al., 2011; SRINIVAS et al.,

2014). Quando um composto tem valores de P_a maiores do que os valores de P_i pode ser considerado como ativo para tais atividades farmacológicas (GOEL et al., 2011; KHURANA et al., 2011).

4.6.2 Atividade antifúngica *in vitro* sem alvos específicos

4.6.2.1 Determinação da Concentração Inibitória Mínima (CIM)

A determinação da CIM dos fitoconstituintes, bem como, do antifúngico anfotericina B, foi realizada através da técnica de microdiluição em caldo, utilizando-se placas estéreis contendo 96 poços (Alamar, Diadema, SP, Brasil) com fundo em formato de “U” e tampa (CLEELAND; SQUIRES, 1991; ELOFF, 1998; HADACEK, GREGER, 2000). Inicialmente foram adicionados aos poços 100 μ L de CSD duplamente concentrado, em seguida, dispensados 100 μ L da suspensão de cada produto a ser testado, também duplamente concentrado, nas cavidades da primeira linha da placa. E por meio de uma diluição seriada a uma razão de dois, foram obtidas as diferentes concentrações de 1024 até 1 μ g/mL. Foi adicionado também, 10 μ L de cada suspensão do micro-organismo ensaiado, preparado na escala 0,5 de McFarland (1×10^6 UFC/mL).

Um controle de micro-organismo foi realizado colocando-se nas cavidades 100 μ L do mesmo CSD duplamente concentrado, 100 μ L de água destilada estéril e 10 μ L do inóculo de cada espécie, assim como, um controle estéril foi realizado colocando-se 200 μ L do mesmo CSD duplamente concentrado sem a adição da suspensão fúngica. Para verificar a ausência de interferência nos resultados pelos solventes utilizados na preparação da emulsão, no caso DMSO e o Tween 80, foi utilizado um controle no qual foi colocado nas cavidades 100 μ L do caldo duplamente concentrado, 50 μ L de DMSO (5%), 50 μ L de Tween 80 (2%) e 10 μ L da suspensão fúngica.

Os experimentos foram realizados em duplicata. O sistema foi assepticamente fechado e incubado em estufa a 35 ± 2 °C por 72 horas (SAHIN et al., 2004; VILJOEN et al., 2003). A CIM para os produtos foi determinada por meio de observação visual, uma vez que o crescimento destes micro-organismos nos poços da placa de microdiluição ocorre através da formação dos chamados botões de crescimento (aglomerado de células). E desta forma, foi considerado como CIM a

menor concentração capaz de provocar uma inibição visual total do crescimento da cepa ensaiada ao final do período de incubação (ESPINEL-INGROFF et al., 1991; 1992).

4.6.2.2 Determinação da Concentração Fungicida Mínima (CFM)

Após leitura da CIM, alíquotas de 10 µL do sobrenadante das cavidades onde foi observada completa inibição do crescimento fúngico nas placas de microdiluição e as outras duas concentrações imediatamente maiores, foram semeadas em placas de ASD (DISPOPETRI/ Indústria brasileira/ 90 x 15 mm), sem adição de antifúngico. Após a inoculação, as placas foram incubadas a 35 ± 2 °C por 72 horas, e a CFM foi obtida como a menor concentração dos produtos testados com capacidade de inibir o crescimento visível na placa de ASD, ou a concentração em que o crescimento seja inferior a 3 UFCs (ERNST et al., 1999; KLEPSEK et al., 1998). O ensaio foi realizado em duplicata.

4.6.3 Atividade antifúngica *in vitro* com alvos específicos

4.6.3.1 Ação dos fenilpropenos na parede celular fúngica

4.6.3.1.1 Ensaio com sorbitol

Para avaliar se os fenilpropenos utilizados no estudo (eugenol e isoeugenol) estariam agindo ao nível da parede celular fúngica, foi utilizado um ensaio com o protetor osmótico, sorbitol, considerado estabilizador de protoplastos de fungos. E caso exista atuação dos produtos sob a parede celular, haverá lise de suas células na ausência do estabilizador osmótico, no entanto, haverá crescimento na sua presença, utilizando-se para isso a comparação das CIM's dos produtos antifúngicos na ausência e na presença do sorbitol a 0,8 M.

A determinação da CIM, na presença do sorbitol (0,8 M), foi realizada pelo método de microdiluição, utilizando placas de microtitulação, em duplicata, semelhante ao item 4.6.2.1. Em cada orifício da placa, foram adicionados 100 µL do meio líquido CSD previamente adicionado de sorbitol (PM = 182,17), ambos duplamente concentrados. Em seguida, 100 µL da solução de cada fenilpropeno,

também duplamente concentrado, foram dispensados em cada cavidade da primeira linha da placa. E por meio de uma diluição seriada a uma razão de dois, foram obtidas concentrações dos produtos testados (eugenol e isoeugenol), e no caso do sorbitol, uma concentração final de 0,8 M em cada cavidade. Posteriormente, também foram adicionados 10 µL do inóculo das cepas de *C. neoformans* nas cavidades, onde cada coluna da placa refere-se a uma cepa fúngica, especificamente.

Paralelamente ao ensaio, um controle de microrganismo foi realizado colocando-se nas cavidades de uma placa, 100 µL do mesmo CSD e sorbitol (0,8 M) também duplamente concentrados, 100 µL de água destilada estéril e 10 µL do inóculo de cada cepa. Um controle de esterilidade também foi realizado, onde foi colocado 200 µL do CSD em um orifício sem a suspensão os fungos. As placas foram assepticamente fechadas e incubadas a 35 ± 2 °C por 72 horas para ser realizada a leitura (FROST et al., 1995; ZACCHINO et al., 2001).

4.6.3.2 Ação dos fenilpropenos na membrana celular fúngica

4.6.3.2.1 Ensaio de ligação ao ergosterol

Alguns antifúngicos utilizados na prática clínica interagem com o ergosterol da membrana celular fúngica, ocasionando a ruptura dessa estrutura e o extravasamento do conteúdo intracelular. Para avaliar se cada um dos fenilpropenos utilizados nesse estudo ligam-se a esse esterol da membrana fúngica, foi realizada a avaliação da CIM dos eugenol e isoeugenol, na presença e na ausência de ergosterol exógeno. Uma vez que, a presença do ergosterol exógeno serve como impedimento à ligação dos produtos teste com o ergosterol da membrana fúngica e como consequência, a CIM desses produtos tende a aumentar na presença do ergosterol exógeno em relação ao ensaio controle. Contudo, caso a CIM dos produtos permaneça inalterada na presença de diferentes concentrações exógenas de ergosterol, sugere-se que este composto não esteja ligando-se ao ergosterol da membrana.

A avaliação da CIM dos produtos contra cepas de *C. neoformans* foi realizada por microdiluição e em duplicata, semelhante ao protocolo exposto no item 4.6.2.1. O meio de cultura (CSD) foi utilizado na ausência e na presença de 400 µg/mL de

ergosterol (Sigma-Aldrich®). Um controle de micro-organismo foi realizado colocando-se nas cavidades 100 µL do mesmo CSD e ergosterol nas mesmas concentrações e 10 µL do inóculo de cada espécie. Um controle de esterilidade também foi realizado, colocando-se 200 µL do CSD em um orifício sem a suspensão dos fungos. Por último, foi realizado o mesmo procedimento com a anfotericina B, cuja interação com ergosterol já é conhecida, para servir de controle positivo dos resultados. As placas foram seladas e incubadas a 35 ± 2 °C por 24 - 72 horas para ser realizada a leitura (ESCALANTE et al., 2008).

4.6.4 Estudo de associação dos fenilpropenos com antifúngico padrão

4.6.4.1 Teste de sinergismo – Método de *Checkerboard*

O efeito combinado do eugenol e isoeugenol testados com antifúngico padrão (anfotericina B) foi determinado a partir do método de *checkerboard* para derivação do índice da Concentração Inibitória Fracionada (CIF). As soluções dos produtos testados foram utilizadas em concentrações determinadas a partir de suas respectivas CIM. Inicialmente, 100 µL de caldo Sabouraud dextrose foram adicionados aos poços da microplaca estéril contendo 96 cavidades, com fundo em forma de “U” (Alamar, Diadema, SP, Brazil). Em seguida, 50 µL de cada produto testado e do antifúngico padrão, em diversas concentrações ($CIM \div 16$, $CIM \div 8$, $CIM \div 4$, $CIM \div 2$, CIM, $CIM \times 2$, $CIM \times 4$ e $CIM \times 8$) foram adicionados no sentido vertical (antifúngico padrão) e horizontal (produtos teste) da microplaca. Por fim, foi adicionado 10 µL da suspensão fúngica. O ensaio foi realizado em duplicata, sendo as microplacas incubadas a 35 ± 2 °C por 72 horas (DUTTA et al., 2004; ELIOPOULOS; MOELLERING, 1991).

O CIF foi calculado através da soma do $CIF^A + CIF^B$, onde A representa os fitoconstituintes testados e B o antifúngico padrão. 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 ($CIF \leq 0,5$), aditivo ($> 0,5$ e < 1), indiferente (≥ 1 e < 4) e antagonismo ($ICIF \geq 4,0$) (LEWIS et al., 2002).

Resultados e Discussão



5.1 Analysis *in silico* and *in vitro* of the antimicrobials properties of the eugenol and isoeugenol

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Analysis *in silico* and *in vitro* of the antimicrobials properties of the eugenol and isoeugenol

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Abstract

Phenylpropenes are known to exhibit a range of pharmacological activities, including antimicrobial activities. The aim of this study was evaluated antimicrobial effect of the phenylpropenes eugenol and isoeugenol. The program PASS was used to calculate the possible antimicrobial activity *in silico*. The antifungal activity of the phenylpropenes was evaluated through at the broth microdilution technique. The eugenol and isoeugenol showed higher "Pa" values for antifungal activity, 0.473 and 0.492, respectively. The inhibitory effect on *Cryptococcus neoformans* growth was in concentration minimal of 16 µg / mL for both tested compounds. *In silico* analysis indicated that both eugenol as isoeugenol are more likely to have an antifungal effect. This effect was evidenced by the strong anti-cryptococcal activity of the products, which may suggest a continuity of studies to elucidate the mechanism of antifungal action against *C. neoformans*.

Keywords: Phenylpropenes, Eugenol, Ioeugenol, Antimicrobials, *In silico*

Introduction

Phenylpropanoids constitute a large family of natural products with different structural classes and biological functions. They derive from the amino acid 1-phenylalanine and consist of a C6-C3 skeleton formed by the six-carbon aromatic phenol group and a three-carbon propene tail of cinnamic acid.^{1,2}

Phenylpropenes are classified as a subfamily of phenylpropanoids, constitute a relatively small part of essential and contribute to the properties of many spices and herbs.³ Eugenol and isoeugenol are phenylpropenes that occur naturally in many species as a mixture of volatiles compounds, for example in the flowers of *Clarkia breweri* and flowers of *Petunia hybrid*.^{4,5} Other genres also produce eugenol and isoeugenol in variables percentages, such as: *Myristica* and *Cinnamomum*.^{6,7}

Eugenol and isoeugenol has been arouse the interest of many researchers due to the diversity of pharmacological activities associated with these molecules.⁸⁻¹¹ Studies have shown that their antimicrobial and antioxidant activities have attracted the use of these compounds in the pharmaceutical, dental, cosmetic and food industry.¹²

Consistent with this, we decided to investigate antimicrobial properties *in silico* and *in vitro* of the phenylpropenes eugenol and isoeugenol.

Methods

Passonline

The biological potential of phenylpropenes eugenol and isoeugenol as antimicrobial agent was evaluated by software Prediction of Activity Spectra for Substances (PASS) online (<http://www.way2drug.com/passonline>). This program presumes the biological activities of organic molecules, including phytochemical compounds, using the structure-activity relationship. The biological activities found relate to the interactions of the compounds with the biological entities. The prediction is analyzed as probable activity (Pa) and probable inactivity (Pi).¹³ When a compound has values of Pa greater than the values of Pi can be considered as active for a certain pharmacological action.¹³⁻¹⁴

Evaluated of the Minimum Inhibitory Concentration (MIC)

MIC of the phenylpropenes eugenol and isoeugenol were determined by the broth microdilution technique.¹⁵⁻¹⁷

The clinical strains of *C. neoformans* used in the trial (LM 6109, LM 309, LM 230 and LM 1401) belong to the collection of the Mycology Laboratory of the Federal University of Paraiba - Brazil and the strain INCQS 40221, acquired by the National Institute for Quality Control in Health, Oswaldo Cruz Foundation, Rio de Janeiro - Brazil. In the preparation for the suspension to be used, each the strain was seeded in sabourad dextrose agar, incubated at 35 °C for 24-72 h and after this period, colonies of this culture were suspended in sterile 0.85% NaCl to obtain the inoculum 10^6 count forming unit per ml corresponding to tube 0.5 of McFarland scale.

Were used in the assay 96 well plates. Initially 100 µL of sabourad dextrose broth (Difco Lab., USA) doubly concentrated was added to all wells. Then, 100 µL of the emulsion of eugenol and isoeugenol also doubly concentrated; solubilized in 5% dimethylsulfoxide (DMSO) and 2% Tween 80 (Sigma-Aldrich) prepared in sterile distilled water were dispensed in the wells of the first row of the plate. Next, serial dilutions were obtained to obtain concentrations varying between 1 and 1024 µg.mL⁻¹.

Finally, 10 µl of inoculum from each yeast were added to the wells, and the plates were incubated at 35°C for 24 - 72 h. The test was performed in duplicate. MIC was defined as the lowest concentration capable of visually inhibiting fungal growth seen in the wells.¹⁸

The same procedure was carried out with amphotericin B (positive control), DMSO (5%) and Tween 80 (2%), without drugs (negative control) to verify the absence of interference on fungal growth.

Results

The antimicrobials properties *in silico* of the phenylpropenes were predicted using PASS software. As can be seen in table 1, the predictions found for the study were evaluated and used flexibly. Eugenol and isoeugenol revealed higher values of Pa (0.473 and 0.492, respectively) for antifungal activity in relation to the other activities presented.

TABLE 1 – Results of PASS calculations for antimicrobials properties of eugenol and isoeugenol

Activity	Eugenol		Isoeugenol	
	Pa	Pi	Pa	Pi
Antifungal	0.473	0.035	0.492	0.032
Antibacterial	0.329	0.050	0.379	0.035
Anthelmintic	0.282	0.044	0.335	0.028
Antiprotozoal	0.203	0.102	0.268	0.061
Antiviral	0.171	0.133	0.199	0.097

Eugenol and isoeugenol were analyzed for their antifungal activity *in vitro* against strains of *C. neoformans* by broth microdilution technique. The MIC₅₀ (Minimum Inhibitory Concentration for 50% of strains tested) in both compounds studied were 16 µg / ml (Table 2). The positive control (amphotericin B) inhibited the growth of all strains tested at the concentration of 1 µg/ml. The negative control (no drugs) did not inhibit yeast growth in the culture medium and confirmed that there was no interference of DMSO or tween 80.

TABLE 2 – MIC values of the eugenol, isoeugenol and amphotericin B on strains of *C. neoformans*.

Strains	Eugenol (µg/ml)	Isougenol (µg/ml)	Amphotericin B (µg/ml)	Control Negative
INCQS 40221	16	16	1	-
LM 6109	16	16	1	-
LM 309	16	16	1	-
LM 230	16	32	1	-
LM 1401	32	64	1	-

(-) No inhibition

Discussion

In recent years, interest in natural products has increased because of the recognition of the structures of these compounds and their potential as new pharmaceutical agents.¹³ Several strategies are used in scientific research to explore

the antimicrobial properties of compounds. Methods using computer programs are considered important for the identification pharmacological of natural products.¹⁹

The PASS software presents wide utility in the research of the biological activities of compounds. This program analyzes the spectrum of activity as a result of the interaction of chemical compounds with biological entities. In clinical trials, the biological entity is the human organism, in pre-clinical trials the entity is the experimental animal type (*in vivo*) and/or experimental model (*in vitro*).²⁰

In this study, the phenylpropenes presented antifungal activity as the main effect obtained through *in silico* evaluation and this justified the *in vitro* research against *C. neoformans*.

The antifungal effect confirmed in the *in vitro* was characterized by the strong anti-cryptococcal activity of the products, according to the classification proposed for natural products, where MIC values below 500 µg / mL show strong antimicrobial activity.²³ Thus, both eugenol and isoeugenol had a strong antifungal effect against *C. neoformans* strains.

The *in silico* and *in vitro* antifungal activity obtained in this study is in agreement with previous studies that have already demonstrated the antifungal capacity of the phenylpropenes eugenol and isoeugenol against other fungi, such as *Candida albicans*, *Trichophyton rubrum*, *Aspergillus flavus*, *Fusarium verticillioides*.²⁴⁻

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Eugenol and isoeugenol are isomers with respect to aliphatic C = C bonding. Studies have demonstrated that the antimicrobial activity of phenolic compounds may present differences due to the structural and chemical variations of the compounds.²⁹

Our results showed that there are no significant differences between the compounds for the antimicrobial activity analyzed *in silico* and *in vitro*. The different location of the double bond in the side chain of these compounds was not able to confer different antifungal activity between eugenol and isoeugenol in our study.

Conclusions

The *in silico* analysis of the phenylpropenes eugenol and isoeugenol presented an important spectrum of antimicrobial activity with greater susceptibility to the antifungal effect. This effect was evidenced by the anti-cryptococcal activity *in vitro*. We can consider that both eugenol and isoeugenol presented similar activities

and therefore, the structural differences for the two isomers were not able to identify differences in the antifungal activities related to our study. However, further studies are needed to elucidate the mechanism of antifungal action and its therapeutic utility in the treatment of cryptococcosis.

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5.2 Antifungal activity of the eugenol against strains of *Cryptococcus neoformans*: interaction with ergosterol

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Antifungal activity of the eugenol against strains of *Cryptococcus neoformans*: interaction with ergosterol

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Abstract

Eugenol belongs to the class of phenylpropenes phenolic compounds and is considered one of the main constituents of several essential oils. This compound exhibits antimicrobial properties, which has awakened the interest of various researches to find alternatives for treatment of many serious diseases, such as those caused by *Cryptococcus neoformans*. This yeast is an opportunistic agent that causes cryptococcosis, a severe systemic mycosis. In this sense, the objectives of this study were to evaluate the antifungal activity of eugenol using Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (CFM) against strains of *C. neoformans*, and investigate the possible mechanism of antifungal action. This investigation consisted in analyzing the interference of eugenol in the cellular wall and plasma membrane of fungi. Eugenol presented antifungal activity with a MIC of 16 µg/ml and a CFM of 32 µg/ml, and also fungicidal effect in all strains tested. The mechanism of antifungal action of eugenol is not related to mechanisms of cell wall synthesis, but it has the ability to form complexes with ergosterol present in the

plasma membrane. These results lead to the recognition of eugenol as a potential antifungal agent against *C. neoformans*.

Keywords: eugenol, antifungal activity, *Cryptococcus neoformans*, ergosterol

Introduction

The incidence of opportunistic mycoses has increased at an alarming rate in recent years, especially due to the growing population of immunocompromised patients. The genus *Cryptococcus* comprises more than 70 identified yeasts, and the agents *Cryptococcus neoformans* and *Cryptococcus gattii* are recognized as responsible for the increased rates of morbidity and mortality related to cryptococcosis (Fang et al., 2015; Kurtzman et al., 2010).

Cryptococcus neoformans is an encapsulated yeast that has a wide distribution and can usually be found in soils contaminated with decomposing vegetables and bird droppings, mainly pigeons droppings (Heitman and Lin, 2006; Sorrel and Ellis, 1997; Steenbergen and Casadevall, 2003).

Cryptococcosis usually occurs after inhalation of this organism found in the environment. Initially, the disease causes pulmonary infection, which can develop into a disseminated infection and thus affect the central nervous system (CNS), causing meningitis, encephalitis or meningoencephalitis (Sorrel and Ellis, 1997; Goldman et al., 2000; Jong et al., 2008).

Cryptococcosis is considered an important global systemic mycosis that affects individuals with compromised immune systems, especially human immunodeficiency virus (HIV) positive individuals. Less common infection is caused in immunocompetent hosts (Lanjewar, 2011; Lemmer et al., 2004; Cogliati, 2013).

There is a limited number of effective antifungal drugs commercially available. In addition, other conditions such as fungistatic mechanism of action, high toxicity, numerous drug interactions, increased number of resistant yeasts, and insufficient bioavailability have made the therapeutic approach a great challenge (Pfaller, 2012; Denning and Hope, 2010; Rapp, 2004).

For all these reasons, currently, mycoses are considered a serious problem worldwide, requiring additional antifungal agents with therapeutic potential. Natural compounds, such as phenylpropenes, constitute a relatively small part of essential

oils, and have received much attention recently (Hyldgaard et al., 2012). They are recognized for their antimicrobial activities and are a promising tool for treating various pathogens (Pauli and Kubeczka, 2010).

Eugenol is a natural allylphenol with the chemical formula $C_{10}H_{12}O_2$, and its biological properties are related to the presence of phenolic group (Pereira et al., 2013). It is one of the main volatile compounds found in essential oils of *Cinnamomum zeylanicum*, *Ocimum basilicum*, *O. gratissium*, and also in the buds and leaves of *Eugenia caryophyllata* Thunb (Grayer et al., 1996; Nakamura et al., 2006).

Eugenol is an extraordinarily versatile molecule and has attracted attention of many researchers due to its wide spectrum of biological and pharmacological activities, such as antifungal, antiparasitic, antioxidant, anaesthetic, anti-inflammatory and analgesic (Souza et al., 2014; Machado et al., 2011; Hidalgo et al., 2009; Guenette et al., 2006; Daniel et al., 2009).

In this sense, the eugenol antifungal mode of action needs further investigation. So, the purpose of this study was to determine the antifungal activity of eugenol by analyzing the Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC), and also investigate its mode of action using sorbitol and ergosterol assays.

Materials and Methods

Chemicals

The eugenol, ergosterol, sorbitol and amphotericin B used in this study were purchased from Sigma-Aldrich, Brazil. The substances were solubilized in 5% dimethylsulfoxide (DMSO) and 2% Tween 80 (Sigma-Aldrich). Sterile distilled water was used to obtain solutions, which were mixed for 5 minutes using a Vortex (Fanem).

Microorganisms

Strains of *C. neoformans* (LM-615, LM-120, FCF-5, LM-3, LM-119, FGF-10), used for antifungal activity assays, belong to the archival collection of Laboratory of Mycology, Federal University of Paraiba – Brazil, and they were provided by Professor Dr. Edeltrudes de Oliveira Lima. There was a strain from National Institute

for Quality Control in Health, Oswaldo Cruz Foundation, Rio de Janeiro – Brazil, INCQS 40221. The inoculum preparation was performed using fresh cultures of each selected strains of *C. neoformans*, which were previously grown in tilted sterile tubes containing Sabouraud Dextrose Agar (SDA; Difco Lab., USA), and incubated at 35°C for 72 hours. Colonies of this culture were suspended in sterile NaCl 0.85% in order to provide a final concentration of approximately 10^6 colony forming unit per ml (CFU.ml⁻¹), adjusted to the turbidity of 0.5 according to the McFarland standard (Cleeland and Squires, 1991; Hadacek and Greger, 2000).

Determination of Minimum inhibitory concentration (MIC)

The MIC was performed using the broth microdilution technique, in 96 well plates (Alamar, Diadema, SP, Brazil). Initially, 100 µl of Sabouraud Dextrose Broth (SDB; Difco Lab., USA) and 100 µl of eugenol were added at concentrations of 1024 µg/ml (initial concentration) to 1 µg/ml (final concentration). Then, 10 µl aliquots of inoculum suspension for each of the strains were added to the wells. In parallel, a positive control with the standard antifungal (amphotericin B) and a negative control without drugs were made to confirm yeast viability and sensitivity to DMSO. Tween 80 was also included in the studies. It was performed a duplicate test, and the plates were incubated at 35°C for 72 hours. The results were analyzed by observation of turbidity in the medium that is the indicative of microorganism growth. The MIC is defined as the lowest concentration that produces visible inhibition of yeast growth (Cleeland and Squires, 1991; Eloff, 1998; Hadacek and Greger, 2000).

Determination of Minimum fungicidal concentration (MFC)

After determining the MIC, 10µl aliquots of each of the wells without fungal growth (MIC, MIC x2, MIC x4) were added on a plate containing SDA (Dispoptetri, 90 x 15 mm, Brazil). It was performed a duplicate test, and the plates were incubated at 35°C for 72 hours. The MFC was considered as the lowest concentration cultivated in plate with SDA in which growth was less than 3 CFU (inhibition of fungal growth to a limit of 3 CFUs) (Ernst et al., 1999; Klepser et al., 1998).

Effect of the Sorbitol Assay

For this assay, two representative strains were selected (LM 615 and INCQS 40221) to evaluate if the mechanism of eugenol antifungal activity was related to the yeast cell wall. The eugenol MIC was determined by using broth microdilution technique (Cleeland and Squires, 1991; Hadacek and Greger, 2000), in the absence and presence of 0.8 M of sorbitol. If higher values of MIC are observed for the medium with sorbitol, it means that the cell wall is one of the possible targets for the tested product (Frost et al., 1995). The test was performed in duplicate and the plates were incubated at 35°C for 72 hours.

Ergosterol Binding Assay

As in the sorbitol assay, two representative strains were selected (LM 615 and INCQS 40221). The MIC of eugenol against *C. neoformans* was determined by using broth microdilution technique (Cleeland and Squires, 1991; Hadacek and Greger, 2000), in the absence and presence of 400 µg/ml of exogenous ergosterol. This assay was made according to the method described by Escalante et al. (2008), just with some modifications, in order to evaluate if the eugenol binds to the fungal membrane sterols. Amphotericin B was used as control drug, since its interaction with ergosterol membrane is already known.

Results and Discussion

Despite all advances in medicine, anti-cryptococcosis therapy remains limited, leading to the emergence of problems such as resistance and toxicity, which makes the treatment ineffective and contributes to the high disease-related mortality rates.

Scientific investigations about the antifungal action mode of natural products have increased in recent years. A great number of studies have reported eugenol antimicrobial properties against yeasts, molds and bacteria (Bennis et al., 2004; Matan et al., 2014; Gill and Holley, 2004). However, the knowledge about the antifungal mechanism of eugenol still needs to be further analyzed (Hildggard et al., 2012).

In this study, the antifungal activity of eugenol against *C. neoformans* strains was evaluated using the microdilution technique for determination of MIC and

MFC. Eugenol showed MIC ranging from 16 to 32 µg/ml, and 50% of tested microorganisms was inhibited at the concentration of 16 µg/ml (Table 1).

TABLE 1 – MIC values of eugenol and amphotericin B on strains of *C. neoformans*.

Strains	Eugenol (µg/ml)	Amphotericin B (µg/ml)	Negative Control
INCQS 40221	16	1	-
LM 615	16	1	-
LM 120	16	1	-
LM 3	16	1	-
LM 119	32	1	-
FGF 10	32	1	-
FCF 5	32	1	-

(-) No inhibition

Positive control for the standard antifungal, amphotericin B, presented fungal growth inhibition with MIC value of 1 µg/ml for 100% of tested microorganisms. Therefore, all microorganisms were sensitive to amphotericin B when compared to previous studies that defined MIC values ≥ 2 µg/ml as being resistant (Nguyen and Yu, 1998; Lozano-Chiu et al. 1998). Negative control, without drugs, confirmed the fungal growth in broth and the absence of fungal growth inhibition by DMSO or Tween 80.

Essential oils, which have eugenol as the main constituent, have demonstrated promising antifungal activity. In study conducted by Nakamura et al. (2004), it was reported that the essential oil of *O. gratissimum* L. (eugenol - 67%) presented MIC of 750 µg/ml against *C. albicans*. Bin Jatan et al. (2008) analyzed the antifungal activity of the essential oil of *C. zeylanicum* Blume (eugenol - 92%) against genus *Candida*, and found a MIC ranging from 0.31 to 0.63 µg/µl.

The anti-yeast activity of eugenol against *C. neoformans*, *Candida albicans*, *C. dubliniensis*, *C. tropicalis* and *Saccharomyces cerevisiae*, was clearly demonstrated using the broth dilution technique (Boonchirdn and Flegel, 1982; Ahmad et al., 2010a; De Paula et al., 2014; Darvishi et al., 2013).

The eugenol used in this work showed antifungal activity in a lower concentration, in comparison with previous studies. Lemos et al. (2005) reported the growth inhibition of *C. neoformans* by eugenol with a MIC of 250 µg/ml for 50% of tested strains. Ahmad et al. (2010b) presented a MIC of 500 µg/ml against yeasts of the genus *Candida*, while De Paula et al. (2014) reported a MIC value of 375 µg/ml against *C. dubliniensis* and *C. tropicalis*.

According to the criteria established by Sartorato et al. (2004), natural products with MIC values lower than 500 µg/mL present strong antimicrobial power, while MIC between 600 and 1500 µg/mL represent moderate antimicrobial power, and values greater than 1500 µg/mL represent weak antimicrobial power. In this sense, it is possible to say that eugenol presented strong effect against *C. neoformans* strains (MIC₅₀ of 16 µg/mL).

The eugenol MFC ranged from 16 to 64 µg/ml, and 50% of microorganisms were inhibited at the concentration of 32 µg/ml. The MFC values were equal to twice the MIC values for the most of tested microorganisms, except for the INCQS 40221 and LM-119 strains, for which the MFC was the same of MIC (Table 2).

TABLE 2 – MFC values of eugenol on strains of *C. neoformans*.

Strains	Eugenol (µg/ml)	Negative Control
INCQS 40221	16	-
LM 615	32	-
LM 120	32	-
LM 3	32	-
LM 119	32	-
FGF 10	64	-
FCF 5	64	-

(-) No inhibition

Pinto et al. (2009) revealed that eugenol in concentrations of MIC presented fungicidal effect against *Candida* isolates and resulted from an extensive lesion of the cell membrane. Fontenelle et al. (2011) also demonstrated an important fungicidal activity of eugenol, which is equal to twice the MIC values for tested *Candida* strains.

According to the criteria established by Hafidh et al. (2011), values of 1 or 2 for the MFC/MIC ratio are considered as fungicidal effect, and values greater than 2 are considered fungistatic. So, in this study, the eugenol presented fungicidal effect against all strains of *C. neoformans*.

Based on the results of eugenol antifungal activity, the mechanism of action for this compound was determined using two isolated strains of *C. neoformans* (INCQS 40221 and LM 615), since both presented MIC values of 16 µg/ml (eugenol) and 1 µg/ml (amphotericin B).

The drugs used for mycoses treatment have mechanisms of antifungal action, mostly related to the cell wall and plasma membrane, mainly ergosterol and its biosynthesis (Odds; Brown; Gow, 2003). Thus, these kind of mechanisms of action were investigated for eugenol.

The sorbitol protection assay was used to evaluate if eugenol acts on the fungal cell wall, since this assay is largely used by researchers to study the mechanisms of action of various antibiotics against yeast and other fungi (Yamaguchi et al., 1982; Varona et al., 1983).

The cellular wall is an important structure for growth and viability of fungi, constituted by macromolecules such as chitin, glucans, mannans and other proteins. The mechanisms of synthesis of these cell wall constituents may be important antifungal targets (Frost et al., 1995; Reis et al., 1992).

As it can be seen in Table 3, MIC values did not change in the presence of sorbitol (0.8 M), which works as an osmotic protector for the fungal protoplasts stabilization. The test results are expressed as the mean of replicates.

TABLE 3 – Effect of eugenol against *C. neoformans* INCQS 40221 and LM 615 in absence and presence of sorbitol (0.8 M)

Strains	Eugenol (MIC µg/ml)	
	-sorbitol	+sorbitol
INCQS 40221	16	16
LM 615	16	16

The MIC values did not become higher in the presence of sorbitol. So, it is suggested that eugenol does not interfere in mechanisms that act on cell wall synthesis. In contrast with results presented in this study, Pereira et al. (2013) demonstrated that the antifungal mechanism of eugenol against *Trichophyton rubrum* was able to increase the MIC values in the presence of sorbitol, suggesting its antifungal activity on the cell wall.

The action of eugenol in the ergosterol of fungal cell membrane was also analyzed. Ergosterol is an exclusive sterol present in the fungal cell membrane, and it is essential for growth and functioning of membrane in yeast and filamentous fungi (Onyewu et al., 2003; Filho et al., 2016).

In this assay, ergosterol added to the medium has the property of forming complexes with products that bind to ergosterol of the fungal membrane and this interaction is capable of increasing MIC values. As it can be seen in Table 4, MIC values for eugenol against *C. neoformans* increased about 8 times in the presence of exogenous ergosterol (0.8 M). Amphotericin B is known for its interaction with ergosterol of fungal membrane, and it was used as positive control, causing an increase of 256 times the MIC values (Baran et al., 2009). The test results are expressed as the mean of the replicates.

TABLE 4 – MIC ($\mu\text{g/ml}$) of eugenol in absence and presence ergosterol (400 $\mu\text{g/ml}$) against *C. neoformans* INCQS 40221 and LM 615

Strains	Eugenol (MIC $\mu\text{g/ml}$)		Amphotericin B (MIC $\mu\text{g/ml}$)	
	-ergosterol	+ergosterol	-ergosterol	+ergosterol
INCQS 40221	16	128	1	256
LM 615	16	128	1	256

Compounds that exhibit high lipophilicity and low polarity are able to cross between the fatty acid chains present in the cell membrane and alter their fluidity and permeability (Sortino et al., 2007). Eugenol is known through its lipophilic properties which may possibly explain the ability of this compound to penetrate the membrane, form complexes with ergosterol and cause cell death in yeasts.

Conclusion

According to the results of this work, eugenol presents potential antifungal activity, fungicidal effect, and its antifungal mechanism of action is possibly related to its binding with ergosterol, present in the cellular membrane. The results found motivate the study of new drugs that can be used for treatment of opportunistic systemic mycoses such as cryptococcosis.

Conflict of interest

The authors declare no conflict of interest.

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5.3 Antifungal activity and mode of action of the isoeugenol against strains of *Cryptococcus neoformans*

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Antifungal activity and mode of action of the isoeugenol against strains of *Cryptococcus neoformans*

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Abstract

Several researches have directed the focus of study to the discovery of new and more effective antifungal agents against opportunistic pathogens such as *Cryptococcus neoformans*, an important causative agent of cryptococcosis in humans and animals. The isoeugenol is a phenylpropene which has recognized antimicrobial property. Thus, this study aimed to evaluate the antifungal activity isoeugenol and the mode of action against strains of *C. neoformans*. The antifungal activity of isoeugenol was evaluated by broth microdilution technique for determining the Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC). We also investigated the possible action of the isoeugenol on the cell wall (0.8 M sorbitol) and cell membrane (binding to ergosterol). Isoeugenol showed inhibitory effects on fungal growth in the tests, with MIC variations between 16 - 32 µg / ml and MFC between 16 - 64 µg / ml for all strains of *C. neoformans* analyzed. The possible mechanism of action of isoeugenol was evidenced by the binding of the compound to the ergosterol of fungal membrane. In conclusion, the isoeugenol showed promising antifungal activity against strains of *C. neoformans*.

Keywords: *Cryptococcus neoformans*, cryptococcosis, compound antifungal natural, isoeugenol

Introduction

The opportunistic pathogen *Cryptococcus neoformans* is an encapsulated yeast, clinically important because it causes about 1 million new infections and more than 700,000 deaths per year worldwide related cryptococcosis (Chayakulkeeree and Perfect, 2006; Park et al., 2009). This systemic mycosis is more common in patients with impaired cellular immunity, especially those with AIDS, and is considered as an AIDS-defining illness (Mamidi et al., 2002; Wright et al., 1997), but also occurs in non-HIV patients with impaired immunity, with solid organ transplantation, treatment with corticosteroids and lymphoproliferative disorders (Casadevall and Perfect, 1998; Martins et al., 2011).

The treatment of cryptococcosis is still a major challenge because only three classes of antifungal agents are used in the clinic: polyenes (amphotericin B), pyrimidine analog flucytosine (5-FC) and azoles (fluconazole), with known resistance and toxicity, which has contributed to the treatment failure (May et al., 2016).

There is an urgent need for new drugs, either through the discovery or development of existing molecules with therapeutic potential. In this context, studies have demonstrated the efficacy of compounds derived from aromatic plants against pathogenic fungi (Miron et al., 2014).

Isoeugenol (4-propenyl-2-methoxyphenol) belong to the class of organic compounds phenylpropenes, synthesized from the amino acid phenylalanine in plants (Hyltdgaard et al., 2012). Since antiquity, humans have used plant material containing phenylpropenes to preserve the flavor and your food, because of the antimicrobial properties (Koeduka et al., 2006). The phenylpropenes are constituents present in the essential oils, and the isoeugenol is an oily liquid, common in essential oils of nutmeg, cinnamon, clove, bay leaves and pimento (Badger et al., 2002; Hyltdgaard et al., 2015).

Several studies report that isoeugenol has a wide range of pharmacological activities such as anti-inflammatory, antioxidant, antimicrobial properties (Choi et al., 2007; Laekeman et al., 1990).

After evaluating the antifungal activity of a compound it is important to identify how the compound interacts with the cell to promote these effects (Hyltdgaard et al., 2012). For these reasons, the aim of this work was investigate the minimal inhibitory

concentration (MIC) and minimal fungicidal concentration (MFC) of isoeugenol, and to investigate its mode of action using sorbitol and ergosterol assays.

Materials and Methods

Chemicals

Isoeugenol, ergosterol, sorbitol and amphotericin B, used in this work were purchased from Sigma-Aldrich, Brazil. The substances were solubilized in 5% dimethylsulfoxide (DMSO), and 2% Tween 80 (Sigma-Aldrich). Sterile distilled water was used to obtain solutions, and the tubes mixed for 5 min using a Vortex (Fanem).

Microorganisms

Strains of *C. neoformans* used antifungal activity assays belong to the archival collection of Laboratory of Mycology, Federal University of Paraiba – Brazil, provided by Professor Dr. Edeltrudes de Oliveira Lima. They included clinical strains (LM-615, LM-120, FCF-5, LM-3, LM-119, FGF-10). The strain INCQS 40221, acquired by the National Institute for Quality Control in Health, Oswaldo Cruz Foundation, Rio de Janeiro – Brazil. The preparation of the inoculum were performed from fresh cultures of each of the selected strains of *C. neoformans*, previously grown in sterile tubes containing tilted Sabourad dextrose agar (SDA; Difco Lab., USA) and incubated at 35 °C for 72 hours. Colonies of this culture were suspended in sterile 0.85% NaCl to provide a final concentration of approximately 10^6 count forming unit per ml (CFU.ml⁻¹) adjusted according to the turbidity of 0.5 McFarland scale tube (Cleeland and Squires, 1991; Hadacek and Greger, 2000).

Determination of Minimum inhibitory concentration (MIC)

The MIC was performed using the broth microdilution technique, in 96 well plates sterile (Alamar, Diadema, SP, Brazil). Initially, 100 µL Sabourad dextrose broth (SDB; Difco Lab., USA) and 100 µl of isoeugenol were added at concentrations of 1024 µg/mL (initial concentration) to 1 µg/mL (final concentration). Then, 10 µL aliquots of the inoculum suspension for each one of the strains were added to the wells. In parallel, positive control with the standard antifungal (amphotericin B) was realized and negative control (without drugs) to confirm yeast viability and sensitivity to DMSO and Tween 80 were also included in the studies. The test was performed in

duplicate and the plates were incubated at 35 °C for 72 hours. The results were analyzed by observing turbidity of the medium; indicating growth of the microorganism. The MIC was defined as the lowest concentration that produced visible inhibition of yeast growth (Cleeland and Squires, 1991; Eloff, 1998; Hadacek and Greger, 2000, CLSI, 2002).

Determination of Minimum fungicidal concentration (MFC)

After determining the MIC, 10µl aliquots of each of the wells without fungal growth (MIC, MIC x2, MIC x4) was seeded on a plate containing SDA (Dispopenetri, 90 x 15 mm, Brazil). The test was performed in duplicate and the plates were incubated at 37 °C for 72 hours. The MFC was considered as the lowest concentration cultivated in plate with SDA in which growth was less than 3 CFU (inhibit fungal growth up to a limit of three CFUs) (Ernst et al., 1999; Klepser et al., 1998).

Sorbitol Assay Effect

In this assay, two representative strains were selected (LM 615, INCQS 40221) to evaluate if mechanism involved in the antifungal activity of the isoeugenol was related the yeast cell wall. The MIC of the isoeugenol was determined by using broth microdilution technique (Cleeland and Squires, 1991; Hadacek and Greger, 2000), in the absence and presence of 0.8 M of sorbitol. If the higher MIC values were observed in the medium with added sorbitol compared to the standard medium, implicate who the cell wall is one of the possible cell targets for the product tested (Frost et al., 1995). The test was performed in duplicate and the plates were incubated at 35 °C for 72 hours.

Ergosterol Binding Assay

As in assay of sorbitol, two representative strains were selected (LM 615, INCQS 40221). The MIC of the isoeugenol against *C. neoformans* was determined by using broth microdilution technique (Cleeland and Squires, 1991; Hadacek and Greger, 2000), in the absence and presence of 400 µg/ml exogenous ergosterol, in accordance to the method described by Escalante et al. (2008), with some modifications, to assess if the isoeugenol binds to the fungal membrane sterols. As

control drug, amphotericin B was used, whose interaction with membrane ergosterol is already known.

Results and Discussion

The cryptococcosis is the second most common serious mycosis in certain regions, and the high mortality rates associated with the disease occur due to inadequate treatment, especially in resource-limited settings (Brown et al., 2012; Espinel-Ingroff et al., 2015).

Research in the field of natural products have increased in recent years in an attempt to find compounds able to control the growth of fungi as an alternative to chemicals compounds (Pizzolitto et al., 2015). The most of these publications report the antimicrobial mode of action against bacteria, while little is known about the mechanisms involved in the inhibition of yeast (Hyltdgaard et al., 2012).

Isoeugenol has been extensively studied as a promising antifungal agent (Bhatia et al., 2012; Hyltdgaard et al., 2015). And its antimicrobial properties against yeast, molds and bacteria have been equivalent and in some studies greater than eugenol isomer (Laekeman et al., 1990).

In preset study, we determined the effect of isoeugenol on *C. neoformans* strains. As seen in Table 1, using the technique of microdilution, the MIC values of isoeugenol against *C. neoformans* strains varied from 16 - 32 µg/ml, the MIC₅₀ (Minimum Inhibitory Concentration for 50% of strains tested), were 16 µg/ml.

TABLE 1 – MIC values of the isoeugenol and amphotericin B on strains of *C. neoformans*

Strains	Isoeugenol (µg/ml)	Amphotericin B (µg/ml)	Negative Control
INCQS 40221	16	1	-
LM 615	16	1	-
LM 120	16	1	-
LM 3	16	1	-
LM 119	32	1	-
FGF 10	16	1	-
FCF 5	16	1	-

(-) No inhibition

The MIC values of the compound were lower than those found by Bhatia et al. (2012) against *Candida* strains, which they showed variations of MIC 100 - 250 µg/ml. Another study by Gallucci et al. (2014) showed antifungal activity of the isoeugenol against four different species of *Candida* isolates: *Candida albicans*, *Candida krusei*, *Candida tropicalis* and *Candida dubliniensis*.

Laekeman et al. (1990) demonstrated that isoeugenol inhibited the growth of *Trichophyton mentagrophytes*, *Aspergillus fumigatus*, *Aspergillus flavus*, and *Aspergillus niger* in a concentration of 100 µg/ml. Fujita and Kubo (2004) reported an MIC of 400 µg/ml against *Saccharomyces cerevisiae*.

According to criteria of Sartoratto et al. (2004), isoeugenol showed strong antifungal activity, as the values of MIC were lower than 500 µg/ml. The MIC of amphotericin B (positive control) was 1 µg/ml for all tested fungal strains. The results for the negative control (without drugs) confirmed the viability of the yeast, showing no fungal growth inhibition. Amphotericin B was used as a positive control because it is one of the commonly used antifungal drugs, especially in the induction phase of therapy cryptococcosis (Pappalardo et al., 2009). All tested strains were susceptible to amphotericin B, when compared to previous studies that consider MIC 2 µg/ml how tough (Pedroso et al., 2006).

The MFC of microorganisms according to the technique proposed by Klepser et al. (1998) and Ernst et al. (1999), ranged between 16 and 64 µg/ml. The MFC₅₀ (Minimum Fungicidal Concentration for 50% of strains tested), for isoeugenol were 32 µg/ml, coincided with the MIC x2 for most strains tested, except for the INCQS 40221) which was equal to the MIC (Table 2). The test results are expressed as the geometric mean of the replicates.

TABLE 2 – CFM values of isoeugenol on strains of *C. neoformans*

Strains	Isoeugenol (µg/ml)	Negative Control
INCQS 40221	16	-
LM 615	32	-
LM 120	32	-
LM 3	32	-
LM 119	64	-
FGF 10	32	-

FCF 5	32	-
(-) No inhibition		

Study of antifungal activity *in vitro* isoeugenol had fungicidal effect to all strains of *C. neoformans* studied considering the classification made by Hafidh et al. (2011), where MFC/MIC values when presenting items 1 or 2, indicate that the antifungal effect of natural compounds are fungicidal and rather than fungistatic. Our studies are in agreement with Fujita and Kubo (2004) showed fungicidal activity isoeugenol with MFC values coinciding with the MIC and MIC x2, or 1 and 2, respectively. On the other hand, isoeugenol was effective against *S. cerevisiae* with an MFC of 400 and 800 µg/ml in growing conditions aerobic and anaerobic, respectively (Kubo et al., 2008). In another study by Gallucci et al. (2014), the MFC of isoeugenol against investigated *Candida* isolates were 2.19 mg/ml for *C. albicans*, 2.64 mg/ml for *C. tropicalis*, much higher than the results obtained in our study.

The antifungal activity of the natural products have been proposed by many mechanisms that lead to membrane disruption, inhibition of cell division, interference in synthesis of cell wall components, interference in sphingolipid synthesis and interference in protein synthesis (Vicente et al., 2003).

The hydrophobic properties of the constituents of essential oils are important in determining their antifungal activities as they allow the compounds to penetrate the cell membrane and promote physical and chemical changes in the cell walls, cell membranes and cell organelles (Knobloch et al., 1987; Rasooli and Owlia, 2005). Due to the hydrophobic nature of isoeugenol, we investigated whether the inhibition of fungal growth occur through alterations in wall / cell membrane.

Two strains of *C. neoformans* (INCQS 40221 and LM 615) were employed to explore the mode of action of isoeugenol in inhibiting yeast growth, as both isolates had MIC values of 16 µg/ml (isoeugenol) and 1 µg/ml (amphotericin B).

To investigate whether the isoeugenol interacts with the fungal cell wall, was utilized the test with the osmotic protective sorbitol, used in stabilizing fungal protoplasts. The MIC of isoeugenol did not change in the presence of sorbitol (0.8 M) in the culture medium, at both the strains studied, after 72 hours of incubation (Table 3). The test results are expressed as the geometric mean of the replicates.

TABLE 3– Effect of isoeugenol against *C. neoformans* INCQS 40221 and LM 615 in absence and presence of sorbitol (0.8 M)

Strains	Isoeugenol (MIC µg/ml)	Isoeugenol (MIC µg/ml)
	-sorbitol	+sorbitol
INCQS 40221	16	16
LM 615	16	16

The fungal cell wall is considered one of the targets for new antifungals since the biochemical machinery for its synthesis is not present in mammalian cells (Vicente et al., 2003). Its dynamic structure is essential for the growth and viability of the micro-organism in a hypotonic environment, because it acts as a protective barrier, preventing osmotic bursting of the cells (Escalante et al., 2008).

Compounds that act on fungal cell wall may invert its effect in a medium containing the osmotic stabilizer sorbitol (Johann et al., 2010). Thus is expected that the MIC of a compound that interact with the cell wall will shift to a much higher value in the presence of the osmotic support. Our results showed that the antifungal properties of isoeugenol are not related to cell wall biosynthesis pathways, as the findings of the MIC values did not increase (Table 3) in presence of an osmotic protector (sorbitol), suggesting that this compound can exert their antifungal activity by other means than by inhibiting cell wall.

To determine the possible mechanism of interaction of isoeugenol with fungal cell membrane, ergosterol effect assay was realized in perspective of investigate the ability of isoeugenol to form complexes with ergosterol, as proposed by Escalante et al. (2008). In presence of 400 µg/mL ergosterol in the culture medium, MIC of the isoeugenol increased by three fold when tested against the INCQS 40221 and LM-615 strain (Table 4). The amphotericin B (positive control) increased by eight fold when tested against the INCQS 40221 and LM-615 strain. The test results are expressed as the geometric mean of the replicates.

TABLE 4– MIC ($\mu\text{g/ml}$) of isoeugenol in absence and presence ergosterol (400 $\mu\text{g/ml}$) against *C. neoformans* INCQS 40221 and LM 615

Strains	Isoeugenol (MIC $\mu\text{g/ml}$)		Amphotericin B (MIC $\mu\text{g/ml}$)	
	-ergosterol	+ergosterol	-ergosterol	+ergosterol
INCQS 40221	16	128	1	256
LM 615	16	128	1	256

The ergosterol is the main sterol of the fungi plasma membrane, and exerts the same function as cholesterol in the mammalian cell membranes (Bowman and Free, 2006). Such similarity may contribute to controlling the fluidity, asymmetry, integrity of the membrane by promoting proper functioning of enzymes bound to the structure. Compounds that act by binding to ergosterol can form rapidly a complex, preventing the cellular membrane from interacting with ergosterol, causing changes in membrane permeability, leakage of cytoplasmic contents and cell death (Miron et al., 2014; Peacock et al., 1993). Thus there is an MIC increase in the presence of exogenous ergosterol compared to the control (Escalante et al., 2008).

Our results showed that exist affinity of the isoeugenol with ergosterol because values MIC increased in the presence of exogenous ergosterol in the extracellular media (Table 4). Thus, our finding indicates that a possible mechanism of action antifungal of isoeugenol is due to their selective binding with ergosterol. The study of the antifungal mode of action of isoeugenol on species *C. neoformans* has not been reported in the literature, using the methodology of research, so this study is the first such report.

Although several studies have related the isoeugenol as promising antifungal agent, few studies have described the mode of action antifungal compound. Studies by Bhatia et al. (2012) reported the antifungal mechanism isoeugenol by targeting H^+ -ATPase located on the membranes of pathogenic *Candida* species. In a recent study by Gallucci et al. (2014), the antifungal activity of the isoeugenol was based on a non-specific interaction with the plasma and mitochondrial membrane in species of *Candida* isolates.

Isoeugenol is a small molecule, nonionic, possibly presents ability to locate at the lipid head group region and thus affect packing of lipids, due to the high structural similarity to the aromatic amino acid tyrosine or perill aldehyde, since both having this property (Hyldgaard et al., 2015). When a compound has antimicrobial mechanism of

action on the cell membrane, such interaction can lead to depolarization and increased permeability of the membrane, which can promote formation of pores or present fluidizing effect on membrane (Cristani et al., 2007; Trombetta et al., 2005).

Conclusions

The results obtained in this study show the pharmacological potential of isoeugenol antiyeast. The likely mechanism of action against strains of the *C. neoformans* not does involve interactions with the cell wall, but is likely mediated binding to membrane ergosterol. Therefore, this study presents the isoeugenol as a promising antifungal agent utilized to control etiological agents of systemic opportunistic mycoses, as *C. neoformans*.

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**5.4 Eugenol and isoeugenol in association with antifungal against
*Cryptococcus neoformans***

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Eugenol and isoeugenol in association with antifungal against *Cryptococcus neoformans*

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Abstract

The antifungal therapy combined is used in clinical practice of several mycoses as it may increase the efficacy of the treatment. The use of natural products (phytochemicals) in combination with conventional antifungal drugs has been related to beneficial effects, mainly synergistic effects. The aim of this study was to evaluate the effect of the combined use of eugenol / isoeugenol, compounds with recognized antimicrobial activity, in association with antifungal amphotericin B against strains of *Cryptococcus neoformans*. The combined antifungal effect were be determined from the Fraction Inhibitory Concentration index - checkerboard technique. The results obtained in this study showed that eugenol in combination with amphotericin B had antagonistic effect against the strains of *C. neoformans*, LM 615 and INCQS 40221 (FIC index 6.0 and 4.0), respectively. The combination of the isoeugenol and amphotericin B also showed antagonistic effects for both the LM 615 strain and INCQS 40221 (FIC index 6.0 and 5.0), respectively. This study contributed to the understanding of the antifungal effects of the association of phenylpropanoids (eugenol / isoeugenol) with amphotericin B. Further studies are needed to evaluate and compare the effects of the association of these phytochemicals with other conventional antifungal drugs used against *C. neoformans*.

Keywords: association, eugenol, isoeugenol, amphotericin B, *Cryptococcus neoformans*, antagonistic

Introduction

The use of antifungal drugs in combination is a common practice in the treatment of severe fungal infections. In cryptococcosis, the use of amphotericin B and 5-FC produces synergistic effects against *C. neoformans*, but presents serious challenges in terms of toxicity and acquired resistance¹.

Studies have indicated that the increasing impact of this infection has motivated the search for alternative drugs from natural products and the combination therapy with synthetic antifungals, in an attempt to improve the success of the therapeutic response^{2,3}.

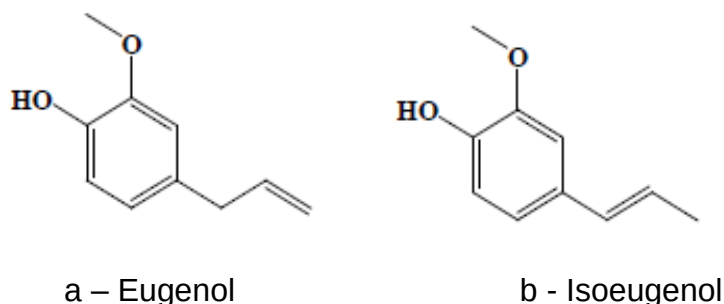
Essential oils and their products obtained, when used in combination with conventional antifungal drugs, may alter the activity of these antimicrobials, improving their performance, thereby reducing side effects and resistance^{4,5}.

Several structural compounds originate from the phenylpropanoid route⁶. The combination of phenolic phenylpropanoids (exhibiting strong antimicrobial activity) with other components has been related to the increase in the biological activity of these mixtures⁷.

Researches have demonstrated that the phenylpropanoids eugenol and isoeugenol when used in combination with pure substances (plant isolates) or with antifungal drugs exhibited synergistic activity *in vitro* against yeasts^{8,9}.

The eugenol and its isoeugenol isomer (Figure 1a and 1b) are used in scientific research for various purposes, such as the production of organic compounds, medicinal products, carbohydrates and natural products¹⁰. They are constituents in various aromatic plants and high antimicrobial activity has been reported to these compounds against a large variety of microorganisms, including yeasts¹¹.

Figure 1 – Chemical structure of eugenol (a) and isoeugenol (b)



Based on the above, the main objective of this study was observing the effects of combinations of eugenol and isoeugenol with amphotericin B used in clinical practice against yeasts encapsulated *C. neoformans*.

Material and Methods

Chemicals

Eugenol, isoeugenol and amphotericin B were purchased from Sigma-Aldrich® (Brazil). The substances were solubilized at the time of the tests dissolving them in Tween 80 (2%) and 5% dimethyl sulfoxide (DMSO) with sterile distilled water to obtain the desired concentrations. The tubes were mixed for 5 min using a Vortex (Fanem).

Fungal Strains

Strain of *C. neoformans* (LM 615) was provided by Professor Dr. Edeltrudes de Oliveira Lima; which belongs to the archival collection of Laboratory of Mycology, Federal University of Paraíba – Brazil. The strain INCQS 40221 was acquired by the National Institute for Quality Control in Health, Oswaldo Cruz Foundation, Rio de Janeiro - Brazil. In the preparation of the suspension to be used, each strain was seeded in Sabourad dextrose agar, incubated at 35 °C for 24-72 h and after this period, colonies of this culture were suspended in sterile 0.85% NaCl to obtain the inoculum 10^6 count forming unit per mL (1×10^6 cfu.mL⁻¹) corresponding to tube 0.5 of McFarland scale.

Determination of Minimum Inhibitory Concentration (MIC)

The determination of the MIC of eugenol and isoeugenol, as well as of the antifungal amphotericin B (positive control), was carried out using the broth microdilution technique using sterile 96-well plates (Alamar, Diadema, SP, Brazil)¹²⁻¹⁴. Initially, 100 μL of doubly concentrated Sabouraud Dextrose Broth (SDB; Difco Lab., USA) was then added to the wells. Then 100 μL of the suspension of each product being tested at the initial concentration of 2048 $\mu\text{g/mL}$ (also doubly concentrated) in the first row of the plate in order to obtain by means of a serial dilution of two different concentrations of 1024 to 1 $\mu\text{g/mL}$. Finally, 10 μL of each suspension of the tested microorganism prepared on the McFarland 0.5 scale ($1 \times 10^6 \text{ cfu.mL}^{-1}$) were also be added. In parallel, a negative control (without drugs) was made to confirm yeast viability and sensitivity to DMSO. Tween 80 was also included in the studies. It was performed a duplicate test, and the plates were incubated at 37°C for 72 hours.

The MIC for the products was determined by visual observation, since the growth of these microorganisms in the wells of the microdilution plate occurs through the formation of so-called growth buttons (cell agglomeration). In this way, the lowest concentration capable of causing a total visual inhibition of the growth of the strain tested at the end of the incubation period was considered as MIC^{15,16}.

Association study using checkerboard method

The combined effect of tested phytochemicals (eugenol and isoeugenol) with standard antifungal agent (amphotericin B) were be determined from the Fraction Inhibitory Concentration (FIC) index *checkerboard* technique. Solutions of the tested products were used in concentrations determined from their respective MICs. Initially, 100 μL of Sabouraud dextrose broth was added to the wells of the 96-well sterile microplate (Alamar, Diadema, SP, Brazil). Then, 50 μL of each product tested at various concentrations ($\text{MIC} \div 16$, $\text{MIC} \div 8$, $\text{MIC} \div 4$, $\text{MIC} \div 2$, MIC, $\text{MIC} \times 2$, $\text{MIC} \times 4$, $\text{MIC} \times 8$) were added vertically (standard antifungal) and horizontal (eugenol or isoeugenol) of the microplate. Finally, 10 μL of the fungal suspension was added. The assay was performed in duplicate, with the microplates incubated at 37 °C for 72 hours^{17,18}.

The FIC was calculated by adding the FICA + FICB, where A represents the phytochemicals tested and B the standard antifungal. The FICA, in turn, was

calculated through the combined MICA / MICA ratio alone, while the FICB = combined MICB / MICB alone. This index is interpreted as follows: synergism, antagonism, additive and indifference¹⁹.

RESULTS

In order to evaluate the possible combined use of the phenylpropanoids eugenol / isoeugenol with the standard antifungal in therapy of the cryptococcosis, the effect of the association of these phenolic compounds with amphotericin B against strains of *C. neoformans* was evaluated using the checkerboard technique for derivation of FIC Index, which classifies interactions as: synergic (≤ 0.5), additive (> 0.5 and < 1), indifferent (≥ 1 and < 4) or antagonistic (≥ 4.0)¹⁹.

The results of the analysis of the checkerboard assay are shown in Table 1. The interactions found for the LM 615 and INCQS 40221 strains were antagonistic both for eugenol and isoeugenol. The combination eugenol - amphotericin B showed FIC = 6.0 for strain LM 615 and FIC = 4.0 for strain INCQS 40221, while the interaction isoeugenol - amphotericin B showed FIC = 6.0 for strain LM 615 and FIC = 5.0 for strain INCQS 40221.

Table 1 - Minimum Inhibitory Concentration (MIC) of eugenol / isoeugenol and effect of combination with antifungal against *C. neoformans* (LM 615 and INCQS 40221).

Eugenol / Isoeugenol + Antifungal	<i>C. neoformans</i>		<i>C. neoformans</i>	
	LM 615		INCQS 40221	
	MIC ($\mu\text{g/mL}$)	Type of interaction	MIC ($\mu\text{g/mL}$)	Type of interaction
Eugenol	16		16	
Isoeugenol	16		16	
ANFB	1		1	
FIC* Index EU/ANFB	6.0	Antagonistic	4.0	Antagonistic
FIC*Index ISO/ANFB	6.0	Antagonistic	5.0	Antagonistic

*FIC: Fractional Inhibitory Concentration; EU: eugenol; ISO: isoeugenol; ANFB: amphotericin B.

DISCUSSION

Compounds derived from plants have already been proven through several studies as efficient antifungal. Studies of association between compounds obtained from the plants and antifungal used in clinical practice are still considered scarce, although some results are positive²⁰.

The use of combined drugs in low concentration may result in potentiation and greater efficacy of each drug, which can lead to better tolerability and safety, lower antifungal resistance, toxicity, lower dose and a higher possibility of cure²¹⁻²³.

Studies using the combination of natural compound, pedalitin, isolated from the plant *Pterogyne nitens*, when associated with amphotericin B, showed additive and synergistic effects against strains of *C. neoformans*²⁴. Suzano [25] also reported who natural substances isolated from *Serjania erecta* and *Eclipta alba*, when combined with amphotericin B, presented synergic activity for the resistant strains of *C. neoformans*.

Hamilton and Heliot [26] reported that the results of antifungal association between amphotericin B and flucytosine may present synergistic and antagonistic responses against strains of *C. neoformans*. These *in vitro* antifungal effects varying between synergism and antagonism, by the combined use of amphotericin B and flucytosine, may occur depending on the species used and the strains^{21,27}.

Our results showed antagonistic effects for the combination of the studied phenylpropanoids (eugenol / isoeugenol) with amphotericin B, which does not make this association advantageous for growth control of *C. neoformans*.

The antifungal effect antagonistic of the combination of the phenylpropanoids used with amphotericin B can be explained by the fact that possibly these natural compounds may be acting via mechanism of action along the plasma membrane, at the same site or at another site that may interfere with amphotericin B activity.

CONCLUSION

This study provides important information about the effects of the association between eugenol / isoeugenol and amphotericin B against strains of *C. neoformans*.

Since the antagonistic effects found need to be evaluated, if they may also be present in association of the phenylpropanoids with other antifungal agents conventionally used in cryptococcosis therapy.

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DECLARACION OF INTEREST

The authors declare that do not have any conflict of interest.

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Considerações Finais



6 CONSIDERAÇÕES FINAIS

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

- Os fenilpropenos eugenol e isoeugenol apresentam efeitos antimicrobianos *in silico*, sendo o efeito antifúngico o mais proeminente. Além disso, observa-se que não há diferenças entre os efeitos farmacológicos desses isômeros;
- Eugenol e isoeugenol apresentam forte atividade antifúngica *in vitro* contra cepas de *Cryptococcus neoformans*;
- As concentrações inibitórias mínimas e fungicidas para 100 % das cepas submetidas às ações de eugenol e isoeugenol demonstram a atividade fungicida dos fenilpropenos estudados;
- Eugenol e isoeugenol não atuam sobre a parede celular de *C. neoformans*;
- O mecanismo de ação antifúngica do eugenol e isoeugenol contra *C. neoformans* envolve a formação de complexos com o ergosterol presente na membrana plasmática;
- As associações eugenol – anfotericina B e isoeugenol – anfotericina B possuem efeitos antagônicos para as cepas testadas pelo método de *checkerboard*;
- Assim, as diferenças estruturais dos isômeros não promove diferenças na atividade antifúngica, possível mecanismo de ação e associação com antifúngico usado contra *C. neoformans*. E esses fenilpropenos (eugenol e isoeugenol) podem representar novas possibilidades entre os produtos com atividade antifúngica para a terapia da criptococose. Entretanto, são necessários mais estudos acerca de outros alvos na célula fúngica e assim, permitir o seu uso na terapêutica dessas infecções fúngicas.

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