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FACULDADE DE CIÊNCIAS DA SAÚDE
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Potencial farmacológico de novos complexos de Co(II) baseados em ligantes de tiocarbamoil-pirazolina como agentes antifúngicos

BIANCA BONI DIAS

**Dourados - MS
2020**

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Orientador: Prof^a. Dr^a. Kelly Mari Pires de Oliveira
Co-orientador: Dr^a. Fabiana Gomes da Silva Dantas

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DEDICATÓRIA

Dedico este trabalho aos meus pais, meu irmão e meu namorado por todo o amor, paciência e apoio que tiveram comigo.

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*“ Disse a flor para o pequeno príncipe:
É preciso que eu suporte duas ou
três larvas se quiser conhecer
as borboletas.”*

Antoine de Saint-Exupéry – O Pequeno Príncipe

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LISTA DE SIGLAS E SÍMBOLOS

| | |
|-------------------|---|
| ANVISA | Agência Nacional de Vigilância Sanitária |
| ATCC | <i>American Type Culture Collection</i> (Coleção de microorganismos Norte-Americana) |
| Br | Bromo |
| CIF | Concentração inibitória fracionária |
| CIM | Concentração inibitória mínima |
| CFM | Concentração fungicida mínima |
| Cl | Cloro |
| CLSI | <i>Clinical & Laboratory Standards Institute</i> (Instituto de normas Clínicas e Laboratoriais) |
| CO ₂ | Dióxido de carbono |
| CoCl ₂ | Cloreto de cobalto (II) |
| DMSO | Dimetilsulfóxido |
| DNA | Ácido desoxirribonucleico |
| EMA | <i>European Medicines Agency</i> (Agência Europeia de Medicamentos) |
| FDA | <i>Food and Drug Administration</i> (Administração de Alimentos e Medicamentos) |
| FLU | Fluconazol |
| H | Hora |
| HeLa | Henrietta Lacks |
| His+ | Histidina |
| HPV | Papilomavírus humano |
| ICIF | Índice de concentração inibitória fracionária |
| IFIs | Infecções fúngicas invasivas |
| IM | Índice de mutagenicidade |
| ITU | Infecção no trato urinário |
| M | Molaridade |
| MEV | Microscopia eletrônica de varredura |
| mL ⁻¹ | Mililitro |
| MTS | 3-(4,5-dimetiltiazol-2-il)-5-(3-carboximetoxifenil)-2-(4-sulfofenil)-2Htetrazólio |
| NDP | 4-nitro-ofenilenodiameno |

| | |
|-----|---|
| nm | Nanômetro |
| PBS | Tampão fosfato salino |
| pH | Potencial hidrogeniônico |
| RNA | Ácido ribonucleico |
| SUS | Sistema único de saúde |
| UFC | Unidade formadora de colônia |
| UTI | Unidade de terapia intensiva |
| XTT | (2,3-bis(2-methoxy-4-nitro-5-sulfohenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide)) |
| %VC | Porcentagem de viabilidade celular |
| % | Porcentagem |
| °C | Graus celsius |
| µg | Micrograma |
| µL | Microlitro |

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Ao vigésimo sétimo dia do mês de fevereiro do ano de 2020, às 14 horas, em sessão pública, realizou-se, no(a) Auditório da Faculdade de Ciências da Saúde – FCS, Universidade Federal da Grande Dourados - UFGRD II, a Defesa de Dissertação de Mestrado intitulada "Potencial farmacológico de novos complexos de CO(II) baseados em ligantes de tiocarbamoil-pirazolins como agentes antifúngicos." apresentada pelo(a) mestrando(a) **BLANCA BONI DIAS**, do Programa de Pós-Graduação Mestrado em Ciências da Saúde, à Banca Examinadora constituída pelos(as) professores(as) Dr^a. Kelly Mari Pires de Oliveira (Presidente/orientador(a)), Dr^a. Sílvia Aparecida Oesterreich/UFGRD (membro titular) e Dr. Lucas Pizzuti/UFGRD (membro titular). Iniciada sessão, a presidência deu a conhecer ao(à) candidato(a) e aos integrantes da Banca as normas a serem observadas na apresentação da Dissertação. Após o(a) candidato(a) ter apresentado a sua Dissertação, no tempo previsto de 20 até 30 minutos, os componentes da Banca Examinadora fizeram suas arguições, que foram intercaladas pela defesa do(a) candidato(a), no tempo previsto de até 240 minutos. Terminadas as arguições, a Banca Examinadora, em sessão secreta, passou ao julgamento, tendo sido o(a) candidato(a) considerado(a) Aprouada, fazendo jus ao título de **MESTRE EM CIÊNCIAS DA SAÚDE**. Nada mais havendo a tratar, lavrou-se a presente ata, que vai assinada pelos membros da Banca Examinadora.

Dourados, 27 de fevereiro de 2020.

Dr^a. Kelly Mari Pires de Oliveira

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Potencial farmacológico de novos complexos de Co(II) baseados em ligantes de tiocarbamoil-pirazolina como agentes antifúngicos.

RESUMO

Leveduras do gênero *Candida* têm sido por muitos anos um problema para a saúde pública, por estarem associadas a infecções superficiais e sistêmicas em humanos, e, nas últimas décadas, estarem desenvolvendo mecanismos de resistência e/ou diminuindo a sensibilidade aos antifúngicos usados em tratamentos convencionais. Diante da necessidade de alternativas, a busca por novas drogas antifúngicas é alvo de pesquisas atuais para tratamento de infecções fúngicas invasivas. Assim, o presente estudo teve como objetivo avaliar dois novos complexos de Co(II) com ligantes tiocarbamoil-pirazolina como possível droga para tratamento antifúngico. Para avaliação da atividade farmacológica foram realizados ensaios antifúngicos, mutagênicos e citotóxicos. A atividade antifúngica dos complexos foi avaliada pelo método de microdiluição em caldo, em microplaca de 96 poços, utilizando oito cepas padrão ATCC e seis isolados clínicos resistentes de *Candida glabrata* provenientes de amostras de urina. As leveduras foram testadas com concentrações entre 1,95 a 1000 $\mu\text{g mL}^{-1}$. A avaliação sinérgica dos complexos foi testada juntamente com o antifúngico fluconazol pelo método de *checkerboard*. Foram realizados ensaios para avaliação de inibição e destruição de biofilmes em concentrações de CIM, subCIM, 2,5xCIM, 5xCIM e 10xCIM e, posteriormente, microscopia eletrônica de varredura para a visualização da atividade antibiofilme. Para avaliação do mecanismo de ação dos complexos, foram realizados os ensaios de sorbitol e ergosterol. Para o teste de Ames foram utilizadas concentrações entre 50 a 5000 $\mu\text{g/placa}$ frente às linhagens TA98, TA100 e TA102 com e sem ativação metabólica. As mesmas concentrações foram utilizadas para o ensaio de citotoxicidade pelo método de MTS frente as células tumorais (HeLa e SiHa) e não-tumorais (Vero). Os complexos apresentaram atividade antifúngica em maior destaque para a *Candida glabrata* ATCC 2001 CIM de 15,62 $\mu\text{g mL}^{-1}$ com ação fungistática e fungicida. Dentre os isolados clínicos de *C. glabrata* a melhor ação biológica foi para o isolado 66, apresentando atividade de 3,90 $\mu\text{g mL}^{-1}$ para o complexo 1 e de 7,81 $\mu\text{g mL}^{-1}$ para o complexo 2. Para a *C. glabrata* ATCC 2001 os complexos apresentaram potencial aditivo em combinação com o fluconazol. Os complexos apresentaram um percentual de inibição de biofilme de até 90%. Os complexos não apresentaram potencial mutagênico e

citotóxico em concentrações inferiores a $1500 \mu\text{g mL}^{-1}$, sendo esse valor superior ao potencial antifúngico apresentado. Pelos testes de mecanismo de ação dos complexos foi possível avaliar que ambos apresentam atividade no ergosterol da levedura, sendo assim, os complexos interferem na membrana celular desses microrganismos. Dessa forma, os complexos podem ser promissores como novo medicamento para o tratamento antifúngico.

Palavras-chave: Atividade antimicrobiana, pirazolina, *Candida glabrata*, resistência microbiana, complexos de cobalto.

Pharmacological potential of new thiocarbamoyl pyrazoline ligand-based Co (II) complexes as antifungal agents

ABSTRACT

Yeasts of the genus *Candida* have been a public health problem for many years, as they are associated with superficial and systemic infections in humans, and, in the last decades, developing resistance lesions and / or decreasing the allocation to antifungals used in tests. In view of the need for alternatives, a search for new antifungal drugs is the subject of current research for the treatment of invasive fungal infections. Thus, the present study aimed to evaluate two new Co (II) complexes with thiocarbamoyl-pyrazoline ligands as a possible drug for antifungal treatment. To assess pharmacological activity, antifungal, mutagenic and cytotoxic tests were performed. An antifungal activity of the complexes was evaluated by the powder microdilution method, in a 96-well microplate, using eight standard ATCC heads and six resistant clinical tests of *Candida glabrata*, using urine tests. Yeasts were tested with a range from 1.95 to 1000 $\mu\text{g mL}^{-1}$. A synergistic assessment of the complexes was tested with antifungal fluconazole by the chess method. Tests were carried out to evaluate biofilm inhibition and destruction in CIM, subCIM, 2.5xCIM, 5xCIM and 10xCIM and, subsequently, scanning electron microscopy for antibiofilm activity responses. To evaluate the mechanism of action of the complexes, sorbitol and ergosterol tests were performed. For the Ames test, concentrations between 50 and 5000 $\mu\text{g / plate}$ were used against the strains TA98, TA100 and TA102 with and without metabolic activation. The same concentrations were used for the cytotoxicity assay by the MTS method against tumor cells (HeLa and SiHa) and non-tumor cells (Vero). The complexes showed antifungal activity in greater prominence for *Candida glabrata* ATCC 2001 CIM of 15.62 $\mu\text{g mL}^{-1}$ with fungistatic and fungicidal action. Among the clinical isolates of *C. glabrata*, the best biological action was for isolate 66, with activity of 3.90 $\mu\text{g mL}^{-1}$ for complex 1 and 7.81 $\mu\text{g mL}^{-1}$ for complex 2. For *C. glabrata* ATCC 2001 the complexes showed additive potential in combination with fluconazole. The complexes showed a percentage of biofilm inhibition of up to 90%. The complexes did not show mutagenic and cytotoxic potential at concentrations below 1500 $\mu\text{g mL}^{-1}$, this value being higher than the antifungal potential presented. By testing the mechanism of action of the complexes, it was possible to assess that both present activity in yeast ergosterol, thus,

the complexes interfere with the cell membrane of these microorganisms. Thus, the complexes can be promising as a new drug for antifungal treatment.

Keywords: Antimicron activity, pyrazoline, *Candida glabrata*, microbial resistance, cobalt complexes.

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1. INTRODUÇÃO

As infecções fúngicas ocasionadas por leveduras do gênero *Candida* aumentaram em níveis alarmantes nas últimas décadas, uma vez que, representam 80% das infecções fúngicas sistêmicas ocorridas em ambientes hospitalares e são responsáveis por 17% das infecções acometidas em unidade de terapia intensiva (UTI) (NETO et al., 2019; CANELA et al., 2017). As infecções fúngicas invasivas (IFIs) também elevam os índices de mortalidade e aumentam o tempo de permanência dos pacientes em hospitais, encarecendo o seu tratamento (PIMENTA et al., 2019; CANELA et al., 2017).

O gênero *Candida* abrange mais de 200 espécies de leveduras, das quais se destacam a *C. albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis*, *C. parapsilosis* e recentemente a *C. auris* (COLOMBO et al., 2017). *Candida albicans* lidera o ranking de agentes causadoras de IFIs, no entanto espécies de *Candida* não-*Candida albicans* também se destacam no cenário de infecções em indivíduos imunocomprometidos (JAIN et al., 2017; NUCCI et al., 2013). No Brasil, a infecção nosocomial se enquadra como um grave problema para saúde pública, pois essas infecções elevam o índice de mortalidade em pacientes hospitalizados (NUCCI et al., 2013).

Em pesquisa com isolados do gênero *Candida* provenientes de amostras de urina, a espécie *C. glabrata* apresentou isolados resistentes ou com dose dependência a antifúngicos, sendo um microrganismo causador de infecções fúngicas principalmente em UTI (LIMA et al., 2017). *Candida glabrata*, possui genoma haploide, característica que pode promover resistência secundária a equinocandinas e compostos azólicos (KIRAZ et al., 2010). Assim, a frequência de IFIs da mucosa e sistêmicas ocasionadas por *C. glabrata* tem aumentado exponencialmente nos últimos anos alavancando essa espécie para segunda posição em causadora de candidemias, atrás somente da *C. albicans* (RODRIGUES et al., 2014).

No cenário atual os tratamentos de infecções fúngicas estão limitados a três classes de antifúngicos como: os azóis, as equinocandinas e os polienos, somando um total de oito medicamentos aprovados para uso terapêutico (OSTROSKY-ZEICHNER et al., 2017). No entanto, os antifúngicos são ainda mais limitados no Brasil. Estão disponíveis para o tratamento Sistema Único de Saúde (SUS) para o tratamento de IFIs os antifúngicos, Itraconazol, Fluconazol, Anfotericina B, Análogos de Nucleosídeos (BRASIL, 2017).

Outro fato agravante no tratamento de IFIs, é que nos últimos anos as espécies de *Candida* apresentaram sensibilidade reduzida ou resistência intrínseca à classe dos compostos azólicos, principal fonte de tratamento no Brasil (LIMA et al., 2017; DOI et al., 2016). A capacidade de formação de biofilmes dessas espécies de *Candida* dificulta ainda mais o tratamento de IFI, pois aumenta a resistência às drogas antifúngicas utilizadas nos tratamentos convencionais (CAVALHEIRO et al., 2018). Desta forma, faz-se necessária a busca por novos antimicrobianos que tenham melhor biodisponibilidade e que proporcionem maior segurança e eficácia para o tratamento de IFIs.

Neste sentido, o pirazol, um composto aromático pentagonal, tem demonstrado atividades biológicas promissoras como antimicrobiana, antitumoral, antiviral, anti-inflamatória e outras (MURCIA et al., 2018; DAMLJANOVIC et al., 2009; BEKHIT et al., 2008). Moléculas orgânicas ligadas a íons metálicos podem sofrer mudanças em sua estrutura biológica e funções, podendo potencializar seus efeitos antimicrobianos dentre outras atividades farmacológicas (FERREIRA et al., 2013; PATIL et al., 2011).

Devido o aumento das infecções causadas por fungos e a limitação de fármacos convencionais para o tratamento, busca-se novos antifúngicos em potencial para tratamento de IFIs de modo sozinho ou em sinergia com drogas antifúngicas convencionais, e que demonstre baixa ou nenhuma reação indesejada, como toxicidade. Assim, dois novos complexos de cobalto (II) com ligantes tiocarbamoil-pirazolina foram sintetizados para avaliar a atividade antifúngica de espécies de *Candida* e isolados de *C. glabrata*, e também avaliar seus potenciais mutagênicos e citotóxicos.

2. REVISÃO DE LITERATURA

2.1 Infecções fúngicas

Diversos microrganismos vivem num sistema de comensalismo com o homem, dentre eles, algumas espécies do gênero *Candida* são leveduras características da microbiota de humanos e animais podendo colonizar desde a pele até mucosas dos tratos bucal, vaginal, digestivo e urinário (NEGRI et al., 2010). No entanto, cerca de 65% das espécies desse gênero não crescem à temperatura fisiológica de 37 °C do corpo humano, assim não se tornam agentes patogênicos nem conseguem manter comensalismo com o homem (CALDERONE, 2002).

Com o rápido avanço tecnológico e desenvolvimento de novas técnicas científicas e medicinais, alguns procedimentos cirúrgicos como transplantes de órgãos, transfusão de sangue e tratamentos para o vírus da imunodeficiência humana permitem uma vida mais prolongada aos pacientes. Em contrapartida, mesmo com esses avanços tais indivíduos podem ficar imunocomprometidos, os tornando mais suscetíveis a infecções fúngicas oportunistas (SANTOS et al., 2018; BINDER; LASS-FLÖRL, 2011). Outros fatores como estresse e má alimentação também podem favorecer as infecções oportunistas que possuem proliferação muito rápida, assim, tornando difícil o diagnóstico clínico, acarretando em elevados números de mortes em pacientes infectados (CORNELLY, 2008).

As infecções oportunistas, são as principais causadoras das infecções hospitalares em imunocomprometidos, sendo que espécies de *Candida* são os principais agentes etiológicos associados aos relatos (COLOMBO et al., 2007). Diversos fatores podem influenciar na incidência e gravidade dessas infecções fúngicas, dentre eles a doença base do indivíduo, pacientes imunocomprometidos, a exposição dos indivíduos a antibióticos de amplo espectro e, em alguns casos, até mesmo a localização geográfica de onde ocorreu a infecção (SANTOS et al., 2018; BINDER; LASS-FLÖRL, 2011).

Um dos mecanismos de patogenicidade das infecções fúngicas é a adesão as superfícies das células do hospedeiro. Essa adesão se inicia através dos componentes manoproteína, quitina e glucano que dão formato a parede celular e também fazem a interação microrganismo/meio ambiente (CALDERONI; FRONZI, 2001). As adesinas, proteínas da parede celular, fazem adesão do fungo aos receptores, fibrinogênio, fibronectina e laminina, localizados em tecidos celulares (YANG, 2003). Dessa maneira,

após a interação/adesão da levedura com as células do hospedeiro e/ou superfícies de aparelhos médicos há formação de biofilmes (CHANDRA et al., 2001) e posteriormente candidemia (OREN; PAUL, 2014).

2.2 Biofilme

Os biofilmes são conjuntos de microrganismos altamente organizados aderidos a uma superfície endógena ou exógena, englobadas por uma matriz extracelular e, quando formados, esses biofilmes adquirem maior tolerância a tratamentos antifúngicos, diminuindo a penetração dessas substâncias em sua matriz. Assim, aumentando o fator de virulência das espécies de *Candida* e conseqüentemente elevando o número de mortalidade causados pelo microrganismo (GUINEA, 2008; KARKOWSKA-KULETA et al., 2009; CHANDRA et al., 2001). A formação de um biofilme comumente segue basicamente cinco etapas: I – formação de células planctônicas; II – adesão do microrganismo a superfície endógena ou exógena; III – devido a ineficácia da defesa do hospedeiro, a levedura *Candida* se multiplica rapidamente, formando um biofilme complexo com uma matriz extracelular; IV – se a levedura *Candida* produzir hifas, há invasão dos tecidos; V – após a invasão, ocorre o quadro de candidemia e exposição ao sistema imune. No entanto, nem todos os biofilmes causam invasão ao tecido (CATE et al., 2009).

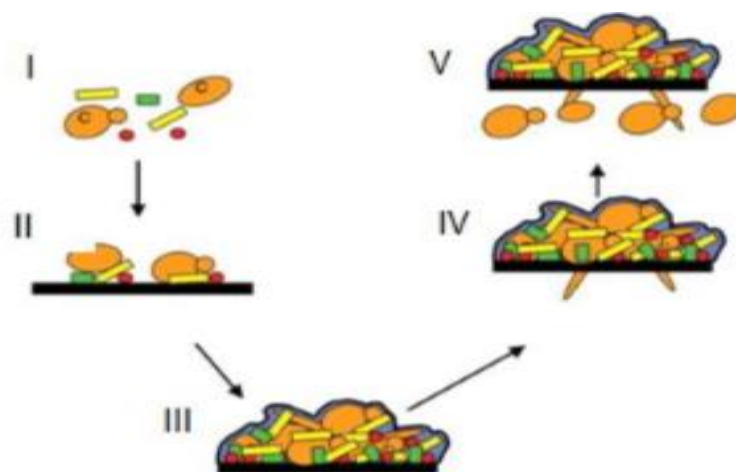


Figura 1. Esquema de formação e evolução de um biofilme formado por uma levedura *Candida* em cinco etapas (CATE et al., 2009).

Dentre as espécies do gênero *Candida*, a *C. albicans* é a espécie que apresenta maior prevalência em isolados de infecções invasivas, seguido da *C. glabrata*, logo em seguida da *C. parapsilosis*, *C. tropicalis* e *C. krusei* (PANIZO et al., 2009).

2.3 *Candida* spp

Taxonomicamente o gênero *Candida* está enquadrado no reino *Fungi*, divisão *Eumycota*, subdivisão *Deuteromycotina*, classe *Blastomycetes* e família *Cryptococcacea*. Espécies desse gênero possuem capacidade de desenvolvimento em ambientes aeróbios e anaeróbios. Podem ter reprodução assexuada ou sexuada (GIOLO et al., 2010).

Os microrganismos que compõem o gênero *Candida* possuem modo de crescimento variado. Na maioria das espécies há formação de pseudohifas, mas, no caso da *Candida albicans*, há formação de hifas filamentosas verdadeiras, denominadas polimórficas (LYON; RESENDE, 2006).

O gênero *Candida* engloba cerca de 200 espécies e cerca de 20 destas espécies são causadoras de infecções no homem. Estas leveduras são caracterizadas como patógenos oportunistas que desencadeiam aproximadamente 9% das septicemias adquiridas em tratamentos hospitalares e cerca de 80% das micoses invasivas em geral (PEMÁN, 2014; NEGRI et al., 2010).

O gênero *Candida* está entre os quatro grupos com maior incidência de agentes patogênicos decorrentes de infecções sanguíneas (OREN; PAUL, 2014). Cerca de 50% dos casos de infecção por *Candida* são ocasionadas por espécies de *Candida* não *Candida albicans*, sendo que a *C. glabrata* ocupa o segundo lugar no cenário de isolados das infecções fúngicas (SAMPAIO; PAIS, 2014).

2.4 *Candida glabrata*

De origem no gênero *Torulopsis glabrata*, por não ter capacidade de produção de hifas, atualmente a espécie é classificada como *Candida glabrata*. Diferentemente dos outros membros desse gênero, a *Candida glabrata* tem como característica crescimento monomórfico, sendo em forma de blastoconídios tanto em modo comensal como agente patogênico (RODRIGUES et al., 2014; SILVA et al., 2012).

Em meio não seletivo como *Sabouraud Dextrose Agar*, *C. glabrata* demonstra colônias de forma convexa, cor creme, pequena, brilhante e lisa e é a única espécie do

gênero *Candida* que não desenvolve pseudohifas em temperatura similar ou acima de 37 °C. Já em meios seletivos, como CHROMagar™ *Candida*, as espécies crescem com colorações específicas, obtendo uma identificação presuntiva onde a *C. glabrata* apresenta tonalidades branca a malva (RODRIGUES et al., 2014).

Outro teste para confirmação da *C. glabrata* é realizado a partir da degradação de trealose em duas moléculas de glicose, pois a enzima trealase que é responsável por essa hidrólise também é encontrada em outras espécies do gênero, porém não com a mesma eficácia e tempo (ZILLI, 2006).

As espécies *C. glabrata* e *C. albicans* apesar de possuírem algumas características similares e sistema de vida comensal semelhante, em análise sequencial genômica observa-se que são de ancestrais filogenéticos divergentes. No entanto, a *C. glabrata* está na segunda posição no quadro de infecções fúngicas invasivas, logo atrás da *C. albicans* (JANDRIC; SCHÜLLER, 2011).

Procedimentos invasivos, como passagem de sondas, cateteres entre outros podem facilitar a colonização ou infecção de espécies por *Candida* no indivíduo (CELEBI et al., 2007). Em especial, a *C. glabrata* é uma das agravantes em ambientes hospitalares, pois está presente em 15% dos casos de candidemia e infecções das mucosas, além de necessitarem de maior período de internação para tratamento (RODRIGUES et al., 2014).

Levesque e colaboradores (2015) demonstram em seus estudos que em 52 pacientes transplantados do fígado, 81% apresentaram alguma espécie do gênero *Candida* em sua microbiota, e em 47% eram da espécie *Candida glabrata*. A idade do hospedeiro pode influenciar na espécie colonizadora. Curiosamente a *C. glabrata* aumenta sua incidência em indivíduos de idade avançada e estado crítico de saúde, diferentemente da *C. parapsilosis* que atinge indivíduos mais jovens (OREN; PAUL, 2014; GUINEA, 2008).

Apesar de não possuir capacidade para formação de hifas filamentosas, a *Candida glabrata* é capaz de desenvolver biofilmes em tecidos celulares ou em ambientes abióticos através de adesão por multicamadas (SILVA et al., 2011). Quando em estado de biofilme maduro, a *C. glabrata* possui vantagens para sistemas de comensalismo ou patogênico aos hospedeiros, conferindo maior resistência aos tratamentos convencionais realizados e ainda diminui a competitividade em relação a outros microrganismos presentes (FONSECA et al., 2014; SILVA et al., 2010)

Candida glabrata tem como característica genoma haploide, diferentemente de *C. albicans* e outras espécies do gênero que possuem genoma diploide (FIDEL et al., 1999).

Essa característica genômica pode promover um sistema de resistência secundária as equinocandinas e aos compostos azólicos, devido a expressão constante do acúmulo sequencial de mecanismos de resistência adquirido (LEWIS et al., 2013; KIRAZ et al., 2010).

2.5 Terapias Antifúngicas

As células fúngicas apresentam grande similaridade fisiológica e bioquímica com as células humanas, limitando a gama de medicamentos antifúngicos para tratamentos de candidíases (ANTINORI et al., 2017). Existem quatro classificações de agentes antifúngicos, sendo elas: I) azóis: que atuam inibindo a biossíntese de ergosterol, ocasionando o acúmulo de substância tóxica acarretando estresse da membrana celular; II) polienos: atuam se ligando ao ergosterol, permitindo a passagem de substâncias através de poros formados na membrana celular; III) equinocandinas: atuam inibindo a biossíntese de β -(1,3)-D-glucano, danificando a integridade da parede celular; e IV) análogos de nucleosídeos: em sinergismo com o polieno Anfotericina B, atuam na inibição da síntese de DNA e RNA (COWEN; STEINBACH, 2008; MORIO et al., 2017).

Dessa maneira, os agentes antifúngicos possuem diferentes mecanismos de ação e atuam em diversos sítios da célula fúngica, como demonstrado na Figura 2.

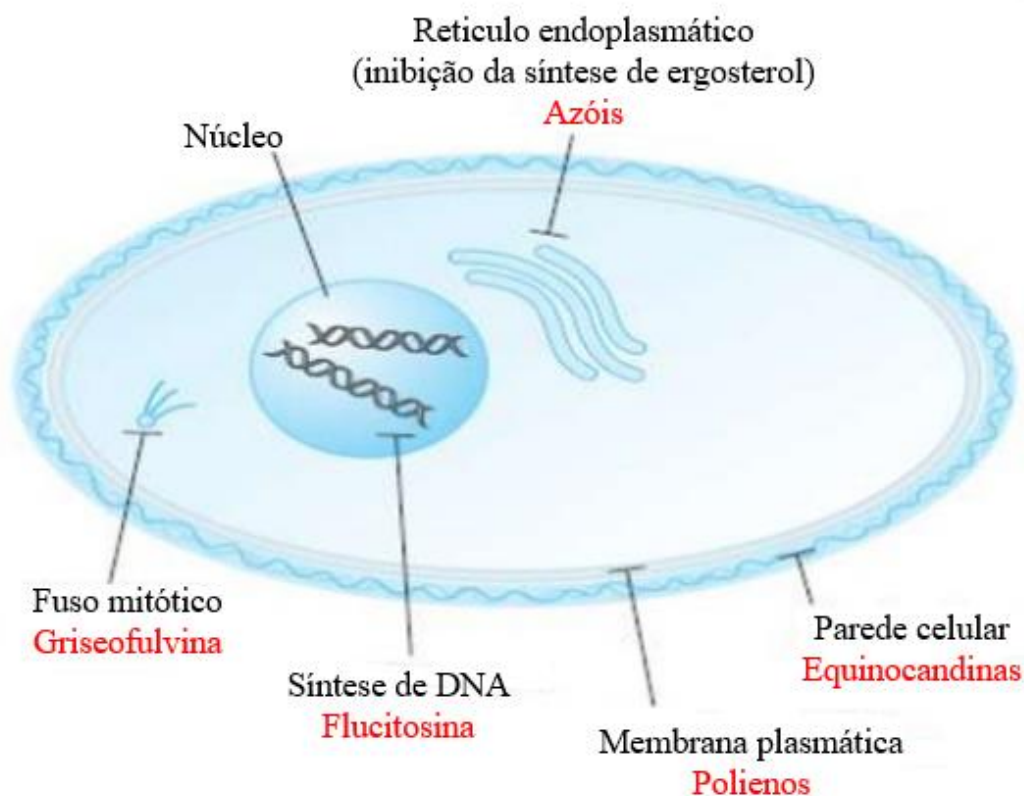


Figura 2. Imagem ilustrativa dos diversos sítios de ação utilizado por agentes antifúngicos. Adaptado de (MORIO et al., 2017).

Os azóis são compostos sintéticos desenvolvidos como paralelos ao uso do polieno Anfotericina B por terem efeitos menos tóxicos e ampla gama de ação a fungos patogênicos (SHERPPARD; LAMPİRIS, 2008). Nesta classe estão inclusos, fluconazol, itraconazol, voriconazol e posaconazol que possuem mecanismo de ação fungistática e inibem a biossíntese de ergosterol (NOBRE et al., 2002). Estes agentes antimicrobianos têm como alvo a enzima 14- α -demetilase, bloqueando a desmetilação do precursor lanosterol em ergosterol (MAERTENS, 2004). No entanto, possuem menor potencial antifúngico quando comparado a Anfotericina B (KATHIRAVAN et al., 2012).

Sabe-se que os microrganismos podem apresentar três mecanismos de defesa/resistência aos compostos azólicos, sendo eles: I) redução no meio intracelular ou acúmulo da substância antifúngica ou aumenta a excreção do agente antifúngico resultante da ação de compostos biossintetizados por genes de resistência antifúngica; II) modificação na cadeia estrutural da enzima 14- α -demetilase, diminuindo os sítios de ligação aos azóis; e III) aumento da produção da enzima 14- α -demetilase, reduzindo a eficácia dos compostos azólicos na célula fúngica (HEILMANN et al., 2010).

Os polienos são compostos antifúngicos que apresentam ação fungicida e incluem Nistatina e Anfotericina B. Essa classe de antifúngico atua na ligação ao ergosterol da membrana celular fúngica formando um complexo que permite o desenvolvimento de poros ou canais devido ao desequilíbrio osmótico ocasionado pela perda de íons, que resulta na perda de citoplasma e outros componentes intracelulares acarretando morte celular (SCORZONI et al., 2017). No entanto, essa classe tem uso restrito e limitado devido aos efeitos adversos resultante da alta toxicidade dos compostos (CAMPOY; ADRIO, 2017).

Poucos relatos sobre os polienos e a resistência de microrganismos aos mesmos são conhecidos, mas sabe-se que o mecanismo de resistência dos agentes patogênicos pode ser adquirido devido a uma alteração lipídica na membrana celular, trocando o ergosterol por outros lipídios formados (SANGLARD; ODDS, 2002).

As equinocandinas são fármacos que apresentam ação fungicida e tem efeitos colaterais reduzidos, dentre elas as mais conhecidas são, micafungina, caspofungina e anidulafungina. Estes antifúngicos atuam inibindo a síntese da (1→3)- β -D-glucana sintase, impedindo a formação de glucanas que são essenciais na síntese da parede celular fúngica, desestabilizando a função osmótica da célula (MARTINEZ, 2006). Essa redução da síntese de glucanas podem ainda interferir em outros metabolismos das células, como na menor produção de lanosterol e ergosterol e elevarem o nível de acúmulo de quitina (DOUGLAS et al., 1997). No entanto, as equinocandinas possuem menor espectro de ação quando comparada com azóis e polienos, devido uma gama inferior de agentes patogênicos dependerem desse polímero (CAMPOY; ADRIO, 2017).

São escassos os casos de microrganismos que desenvolvem mecanismos de defesa contra as equinocandinas, no entanto quando ocorre o fato é devido ao aumento na produção da proteína Sbep que faz parte da composição do complexo de Golgi (MOUDGAL et al., 2005). A síntese dessa proteína é controlada pelo gene GALI, que tem por função fisiológica regular o transporte de componentes para a parede celular fúngica (PARK et al., 2005). Ademais, pesquisas *in vitro* com equinocandinas demonstraram que isolados de *C. parapsilosis* obtiveram Concentração Inibitória Mínima (CIM) maiores que outras espécies de levedura *Candida* (PFALLER et al., 2002).

Os análogos de nucleosídeos ou pirimidinas fluoradas (5-fluorocitosina) apresentam mecanismo de ação a conversão de seus componentes em antimetabólico 5-fluorouracil no interior das células fúngicas, bloqueando a biossíntese de DNA (CAMPOY; ADRIO, 2017). Porém, somente a flucitosina não tem certa eficácia quando

o agente patogênico apresenta alguma resistência antifúngica, assim na maioria dos tratamentos está combinado com outro antifúngico (COELHO; CASADEVALL, 2016). Em tratamentos de candidíases invasivas, a flucitosina quando associada com outro antifúngico como Anfotericina B comumente demonstra bons resultados em tratamentos de peritonites (JOHNSON; PERFECT, 2010).

2.6 Compostos heterocíclicos

Os azóis, são conhecidos por possuírem potencial atividade antifúngica, por atuarem nos esteróis da membrana plasmática, atingindo o principal protetor da membrana e que está em grande abundância, o ergosterol. Dessa forma, causam inibição de sua síntese, sendo que o principal alvo molecular é a proteína Erg11p / Cyp51p do citocromo P-450, resultando na morte celular, por tornar sua membrana instável e permeável (CARRILLO-MUÑOZ et al., 2006).

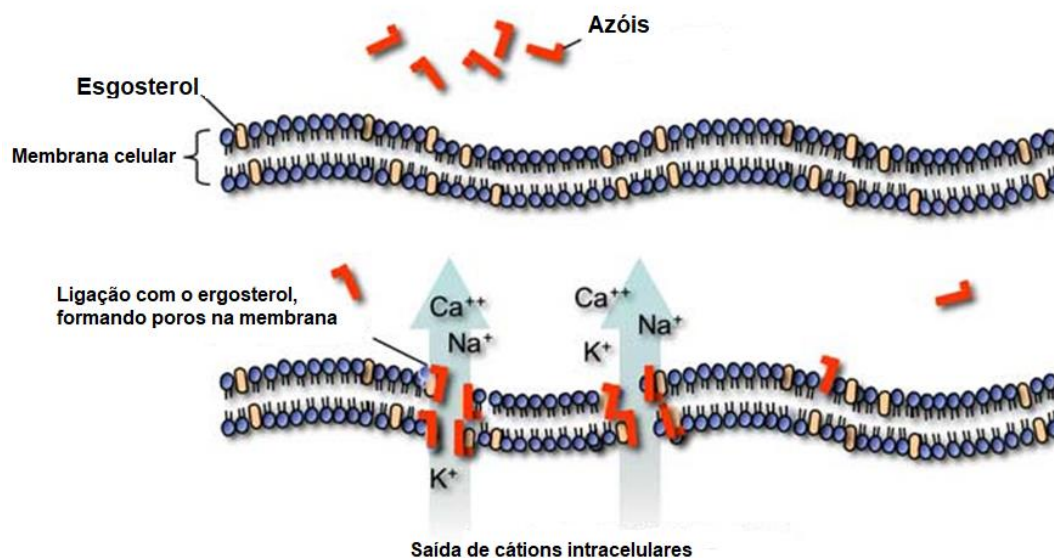


Figura 3. Mecanismo de ação dos azóis. Adaptado de (MOREIRA, 2010).

O pirazol, foi descrito pela primeira vez por Ludwig Knorr em 1883. São compostos orgânicos em forma de anel aromático heterocíclico, que possuem três átomos de carbono e dois de nitrogênio nas posições 1 e 2. É sabido que compostos que possuem núcleo pirazólico possuem atividades antimicrobiana, anti-hipertensiva, antitumoral, anti-inflamatória, antidepressivas, anticonvulsivantes e outras (VARGHESE et al., 2017).

Complexos metálicos são altamente reativos, dessa maneira podem apresentar potenciais atividades farmacológicas relacionados aos seus estados de oxidação. No entanto, essa alta reatividade pode apresentar efeitos não desejados, como toxicidade correspondente. Tais complexos metálicos quando coordenado com ligantes pirazolínicos podem ser uma possível ferramenta contra a resistência microbiana, ou podem aumentar a biodisponibilidade/potencializar de uma droga já utilizada em tratamentos tradicionais (DANTAS et al., 2018; BERALDO, 2011).

Quanto a ação do complexo metálico, a lipossolubilidade é um dos principais fatores de controle antimicrobiano, pois, a membrana que envolve os microrganismos permite apenas passagem de compostos lipossolúveis. Dessa forma, complexos que possuem compostos heterocíclicos (complexo central) realizam compartilhamento de elétrons com os grupos doadores, aumentando a lipossolubilidade do complexo tornando ele permeável para passagem pela membrana. Além da lise celular, os complexos também podem causar danos na respiração celular e bloqueiam a síntese de proteínas, causando diminuição no crescimento microbiano (SINGH et al., 2012).

Na literatura já existem relatos de complexos metálicos com atividades antifúngicas, como por exemplo diversos derivados de 1,3-tiazolidin-4-ona que apresentam atividades antifúngicas em concentrações inferiores aos fármacos convencionais utilizados como padrão (De Monte et al., 2016). Outro exemplo são dois novos complexos de cobre (II) com os ligantes 2-tiouracil (complexo I) e 6-metil-2-tiouracil (complexo II), onde o complexo de cobre (II) com o ligante 2-tiouracil (complexo I) apresentou atividades fungistática e fungicida em todos isolados do estudo, sugerindo que este ligante coordenado potencializou a atividade antifúngica do complexo estudado (Dantas et al., 2018).

Segundo estudos relatados na literatura, a síntese de determinados complexos de cumarina-pirazolinas demonstraram atividade antibacteriana mais potente que o fármaco convencional D-cicloserina contra as bactérias avaliadas. E para as leveduras, alguns complexos de cumarina-pirazolinas demonstraram equipotência ou melhor atividade antifúngica que o Miconazol, utilizado como padrão, como um composto com núcleo tiofeno (Chate et al., 2019). Num outro estudo com derivados de pirazolina e 4-tiazolidinona para avaliação de atividade antimicrobiana e alguns complexos apresentaram alta atividade para *E. coli*, *B. subtilis* e *S. aureus* (Thach et al., 2020).

3. OBJETIVOS

GERAL

Avaliar o potencial farmacológico de dois complexos de cobalto (II) com ligantes tiocarbamoil-pirazolinas.

ESPECÍFICOS

Determinar atividade antifúngica e antibiofilme dos complexos de cobalto com ligantes tiocarbamoil-pirazolina.

Avaliar a atividade dos complexos no sorbitol e no ergosterol na membrana plasmática.

Determinar a capacidade sinérgica dos complexos com o fluconazol.

Investigar atividades citotóxicas e mutagênicas dos complexos.

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5 APÊNDICE

5.1 Artigo: Revista - Journal of Medicinal Chemistry**Qualis A1 na área Medicina II****Fator de Impacto 6.054**

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Synthesis, structural characterization, and prospects for new Cobalt (II) complexes with thiocarbamoyl-pyrazoline ligands as promising antifungal agents

Bianca Boni Dias^a, Fabiana Gomes da Silva Dantas^b, Fernanda de Oliveira Galvão^a, Wellington Jhon Cupozak-Pinheiro^c, Heberton Wender^d, Lucas Pizzuti^e, Persiely Pires Rosa^c, Kátia Veronica Tenório^c, Claudia Cristina Gatto^f, Melyssa Negri^g, Gleison Antônio Casagrande^{e*}, Kelly Mari Pires de Oliveira^{a,b*}.

^a Faculdade de Ciências da Saúde, Universidade Federal da Grande Dourados, Dourados, MS 79070-900, Brazil.

^b Faculdade de Ciências Biológicas e Ambientais, Universidade Federal da Grande Dourados, Dourados, MS 79804-970, Brazil.

^c Faculdade de Ciências Exatas e Tecnologia, Universidade Federal da Grande Dourados, MS 79.804-970, Brazil.

^d Instituto de Física, Universidade Federal do Mato Grosso do Sul, Campo Grande, MS 79070-900, Brasil

^e Grupo de Pesquisa em Síntese e Caracterização Molecular de Mato Grosso do Sul, Instituto de Química, Universidade Federal de Mato Grosso do Sul (Laboratório 2), Campo Grande, MS 79074-460, Brazil.

^f Laboratório de Síntese Inorgânica e Cristalografia, Universidade de Brasília, Campus Universitário Darcy Ribeiro, 70.904-970 Brasília, DF, Brazil

^g Departamento de Análises Clínicas e Biomedicina, Universidade Estadual de Maringá, Maringá, PR 87020-900, Brazil.

Corresponding authors

E-mail: gleisoncasag@gmail.com, Tel: +55 67 3345 3595

Email: kellyoliveira@ufgd.edu.br, Tel: +55 67 98159 5606

Abstract¹

Candida spp. cause invasive fungal infections. One species, *Candida glabrata*, may present intrinsic resistance to conventional antifungal agents, thereby increasing mortality rates in hospitalized patients. In this context, metal complexes present an alternative for the development of new antifungal drugs owing to their biological and pharmacological activities demonstrated in studies in the last decades. Accordingly, in this study we have synthesized and characterized two new Co(II) complexes with thiocarbamoyl-pyrazoline ligands to assess their antimicrobial, mutagenic, and cytotoxic potential. For antimicrobial activity, the broth microdilution method was performed against ATCC strains of *Candida* spp. and fluconazole dose-dependent isolates of *C. glabrata* obtained from urine samples. The Ames test was used to assess mutagenic potential. The MTS assay was performed with HeLa, SiHa, and Vero cells to determine cytotoxicity. Both complexes exhibited fungistatic and fungicidal activity for the yeasts used in the study, demonstrating greater potential for *C. glabrata* ATCC 2001 and the *C. glabrata* CG66 isolate with a Minimum Inhibitory Concentration MIC from 3.90 to 7.81 $\mu\text{g mL}^{-1}$ and fungicidal action from 7.81 to 15.62 $\mu\text{g mL}^{-1}$. The complexes inhibited and degraded biofilms by up to 90% and did not present mutagenic and cytotoxic potential at the concentrations evaluated for MIC. Thus, the complexes examined herein suggest promising alternatives for the development of new antifungal drugs.

Keywords: *Candida glabrata*, Microbial resistance, Pyrazole, Antifungal agent

¹ CFU, colony-forming units; FIC, fractional inhibitory concentration; FICI, fractional inhibitory concentration index; IFIs, invasive fungal infections; MFC, minimum fungicidal concentration; MIC, minimum inhibitory concentration; PBS, phosphate-buffered saline; SD: Sabouraud Dextrose; SEM, scanning electron microscopy

1 Introduction

Opportunistic infections caused by yeasts belonging to *Candida* have become an aggravating factor in public health as some species have presented an intrinsic resistance or acquired some resistance mechanism to conventional treatments [1,2]. *Candida* species are part of the normal microbiota of human beings; they colonize the respiratory, gastrointestinal, and reproductive systems as well as the skin and oral cavity. However, an imbalance of this microbiota can favor the multiplication of these opportunistic pathogens and lead to the development of invasive fungal infections [3,4].

Candida glabrata is one of the main *Candida* species which is most frequently isolated in hospitalized patients with urinary tract infections and is normally associated with candidemia and candiduria [5]. This yeast is capable of developing biofilms in cellular tissues or in abiotic environments, such as those in the urinary tubes of hospitalized patients, through adhesion in multilayers [6]. In addition, *C. glabrata* has haploid genome characteristics, unlike *C. albicans* and other species of the genus that have a diploid genome [7]. This genomic characteristic can promote a secondary resistance system which demonstrates a large, rapid ability to develop tolerance and resistance to antifungal drugs, such as the overexpression of several resistance genes [8-10].

Resistant *C. glabrata* can cause invasive fungal infections (IFIs), which are correlated with high rates of morbidity and mortality in hospitalized patients and patients with immunological diseases [11]. Treatment for IFIs is strictly limited to four classes of drugs: triazoles, polyenes, echinocandins, and pyrimidine analogs, with the last class not commercialized in Brazil [12]. Surveillance studies have reported the resistance of *C. glabrata* isolates to triazoles, and more recently, to echinocandins, thus characterizing this species as resistant to multiple drugs [13]. The difficulty in finding new antifungal agents is related to the yeast's cellular structure. As it is a eukaryote, it presents a lower

number of targets for antifungal agents, in addition to hindering the diffusion of compounds through the cytoplasmic membrane [14].

Heterocycles of the pyrazole family have antifungal activity [15]; however, they can also present toxicity and low solubility [16]. The use of metal complexes combined with a bioactive ligand can enhance pharmacological activities, such as improving bioavailability or reinforcing the action of an existing drug [17]. Recently, cobalt(II) complexes containing pyrazole ligands were tested against *C. glabrata* strains and were shown to be more effective than their free ligands [18]. Cobalt complexes containing arylhydrazones also shown to be effective compounds against the fungus *Penicillium chrysogenum* [19].

In the search for new antifungal agents, the coordination of metal complexes to heterocyclic ligands is a promising alternative as it allows the replacement of organic groups in order to improve liposolubility [20]. In order to contribute to the scientific advance and aiming to explore new promising metallodrugs for facing fungal resistance and fungal infection, we describe in this paper the synthesis and structural characterization of two new Co(II) complexes containing thiocarbamoyl-pyrazoline ligands and their biological activity against yeast species of *Candida* and resistant isolates of *C. glabrata*. Furthermore, we also examined cytotoxic and mutagenic activities of such Co(II) compounds..

2 Materials and Methods

2.1 General procedures

Ligands L1 (1-thiocarbamoyl-5-(4-chlorophenyl)-3-phenyl-4,5-dihydro-1H-pyrazole) and L2 (1-thiocarbamoyl-5-(4-bromophenyl)-3-phenyl-4,5-dihydro-1H-pyrazole) were prepared based on the methods reported in the literature [21]. The solvents (grade AR) were obtained commercially and used in the synthesis without further

purification. Elemental analyses (CHN) were conducted using a PerkinElmer 2400 analyzer. The FTIR spectra were acquired on a JASCO-4100 spectrophotometer using KBr pellets. Melting points were determined using an Instrutherm DF-3600 apparatus and were not corrected. The X-ray data were collected using a Bruker APEX II CCD diffractometer with an area detector with graphite-monochromatic Mo-K α radiation. The crystalline structures of the complexes were resolved using the SHELX package [22]. All the non-hydrogen atoms were refined with anisotropic displacement parameters, and the hydrogen atoms were included in their theoretically ideal positions. Table S1 provides more detailed information to structural X-ray diffraction.

2.2 Synthesis of the complexes

Through shaking, we added 0.2 mmol of the ligand (0.0632 g) to a solution of 0.1 mmol (0.0234 g) of cobalt(II) chloride hexahydrate in 4 mL (1:1) of acetone/acetonitrile. After a reaction time of 3 h at room temperature, a light green solid was obtained through filtration. Green single crystals (Figures S1 and S2) suitable for X-ray diffraction were obtained by recrystallizing this green solid in a methanol/acetonitrile/acetone mixture in the proportion 1:1:1. The physical properties of the synthesized complexes are detailed below:

Complex 1: Elemental analysis of theoretical CHN (calculated) for $C_{32}H_{28}CoCl_4N_6S_2$ (761.17 g. mol⁻¹): C = 50.49%, H = 3.67%, and N = 11.03%. Experimental: C = 50.74%, H = 3.71%, and N = 11.20%. Melting point: 215 °C. Yield: 85%. FTIR data: 3364 [v(N-H)]; 1514-1462 [v(C=N)]; 1593-1462 [v(C=C)]; 1344 [v(C=S)]; 579 [v(C-Cl)].

Complex 2: Elemental analysis of theoretical CHN (calculated) for $C_{32}H_{28}CoCl_2Br_2N_6S_2$ (850.07 g. mol⁻¹): C = 45.21%, H = 3.29%, and N = 9.88%. Experimental: C = 44.93%, H = 3.24%, and N = 9.87%. Melting point: 230 °C. Yield:

79%. FTIR data: 3357 [v(N-H)]; 1514-1447 [v(C=N)]; 1600-1447 [v(C=C)]; 1343 [v(C=S)]; 560 [v(C-Br)].

2.3 Microorganisms and culture conditions

To assess the antifungal activity of complexes 1 and 2, six reference strains, *Candida tropicalis* (ATCC 750), *C. parapsilosis* (ATCC 22019), *C. albicans* (ATCC 70231), *C. albicans* (ATCC 90028), *C. glabrata* (ATCC 2001), and *C. krusei* (ATCC 6558) and six clinical isolates of *C. glabrata* (CG10, CG25, CG26, CG37, CG61, and CG66) were used. The isolates were obtained from urine samples at the University Hospital of the Federal University of Grande Dourados and were stored in the Laboratory of Applied Microbiology of the same university.

Before each trial, the reference strains and clinical isolates were subcultured on Sabouraud Dextrose (SD) agar (Difco, Sparks, United States of America (USA)) and incubated at 35 °C for 24 to 48 h. The growing colonies were subcultured in CHROMagarCandida® (Difco, Tlalnepanitla, Mexico) to investigate the purity of the culture and the color of the colony. Yeasts that grew in the selective and differential medium were identified according to conventional methodology.

2.4 Screening for antifungal activity

The minimum inhibitory concentration (MIC) of complexes 1 and 2 was evaluated by the broth microdilution technique in flat-bottom 96-well microplates (Kasvi), in accordance with the standards of the Clinical and Laboratory Standards Institute (CLSI M27-A3) [23].

The complexes diluted in 3% DMSO and 97% sterilized distilled water underwent successive dilutions in microplates containing 100 µL of RPMI-1640 medium (Sigma-

Aldrich, St. Louis, USA), obtaining concentrations ranging from 1.95 to 1000 $\mu\text{g mL}^{-1}$, and were inoculated with 100 μL of suspension containing 2.5×10^3 CFU mL^{-1} of each yeast. The microplates were incubated for 48 h at 35 °C, and the reading was performed visually. MIC was defined as the lowest concentration of the complex capable of inhibiting microbial growth. Fluconazole was used as a reference drug.

To evaluate the minimum fungicidal concentration (MFC), aliquots from wells that did not show microbial growth were removed and inoculated in SD agar. The plates were incubated for 48 h at 35 °C. MFC was defined as the lowest concentration wherein there was no growth of colony-forming units (CFU). The assays were duplicated at two distinct moments.

2.5 Activity of the complexes combined with fluconazole

The checkerboard assay was conducted to assess the antifungal activity of the complexes in combination with fluconazole based on the methodology proposed by De Castro [24]. The standard strain of *C. glabrata* ATCC 2001 and the isolate *C. glabrata* CG66 were used for the assay.

To determine the combinatorial action against *C. glabrata* ATCC, 0.95–125 $\mu\text{g mL}^{-1}$ concentrations of both complexes and 0.25–32 $\mu\text{g mL}^{-1}$ of fluconazole were tested. To determine the combinatorial action against isolate CG66, the concentrations of complex 2 and fluconazole were the same as those used for the ATCC strain and varied from 125–0.475 $\mu\text{g mL}^{-1}$ for complex 1. A matrix of combinations of different concentrations in flat-bottom 96-well microplates (Kasvi, São José dos Pinhais, Brazil) was formed, and the microplates were incubated for 48 h at 35 °C.

Interpretation of the results was based on the fractional inhibitory concentration index (FICI) = (MIC of the complexes in combination/MIC of the complexes alone) +

(MIC of fluconazole in combination/MIC of fluconazole alone). The criteria for interpretation were the following: synergistic effect ($FICI \leq 0.5$), additive effect ($0.5 < FICI < 1$), indifferent effect ($1 \leq FICI < 4$), or antagonistic effect ($FICI \geq 4$) [25].

2.6 Mechanisms of antifungal action of the complexes

Two mechanisms of action of complexes 1 and 2 were tested: the rupture of the pathways of cell wall biosynthesis (sorbitol assay) and the disruption of the permeability of the ionic membrane (ergosterol assay).

2.6.1 Sorbitol assay

The MIC of complexes 1 and 2 was determined by the broth microdilution technique, as described above, and evaluated in the presence of sorbitol (osmotic protector). The microorganisms were adjusted to a concentration of 2.5×10^5 CFU mL⁻¹ in RPMI-1640 medium supplemented with sorbitol (final concentration of 0.8 M; Sigma-Aldrich). The concentrations of the complexes, ranging from 1.9 to 1000 µg mL⁻¹, were added to 96-well microplates along with the standardized suspensions of *C. glabrata* ATCC 2001 and isolate *C. glabrata* CG66. Caspofungin diacetate (Sigma-Aldrich) was used as the positive control of the assay. The microplates were incubated for 48 h and 7 days at 35 °C [26].

2.6.2 Ergosterol activity

The MICs of complexes 1 and 2 were determined by the broth microdilution technique, as detailed above, in the absence or presence of exogenous ergosterol. Serial dilutions of the complexes, 1.9–1000 µg mL⁻¹, along with RPMI-1640 medium were added to 96-well microplates. The suspension of *C. glabrata* (ATCC 2001 and CG66) at

a concentration of 2.5×10^5 CFU mL⁻¹ was supplemented with increasing concentrations of ergosterol (100, 200, and 400 µg mL⁻¹) and added to the microplates. Amphotericin B (Sigma-Aldrich) was used as the positive control of the assay. The plates were incubated at 35 °C for 24 h [26].

2.7 Evaluation of antibiofilm activity

Given the antifungal activity of the complexes, their ability to inhibit biofilm formation and degrade preformed biofilms of *C. glabrata* (ATCC 2001 and CG66) was tested. The concentrations of the complexes used in these tests were based on the results of the antifungal susceptibility test. The assay was conducted in accordance with Silva [27], with some adaptations.

For inoculum preparation, the microorganisms were cultured in SD broth for 24 h at 37 °C and shaken at 80 rpm. Immediately afterward, the cells were washed thrice with phosphate-buffered saline (PBS), centrifuged at $5000 \times g$ for 5 min, resuspended in RPMI-1640 medium, and adjusted to a concentration of 1×10^8 CFU mL⁻¹.

2.7.1 Effect of complexes 1 and 2 on biofilm formation

The concentrations sub-MIC, MIC, 2.5× MIC, 5× MIC, and 10× MIC were used to evaluate the inhibitory effect of complexes 1 and 2 on biofilm formation. We added 100 µL of the standardized suspension of the microorganisms and 100 µL of the concentrations of the complexes diluted in RPMI-1640 medium in 96-well microplates. The microplates were incubated at 37 °C for 48 h at 80 rpm.

After the incubation period, the volume of the wells was removed, and the biofilms were washed thrice with PBS to remove weakly adhered cells. Quantification of biofilms was conducted using the XTT method (2,3-bis(2-methoxy-4-nitro-5-

sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) [28]. RPMI-1640 medium and the inoculum were used as the positive control, and only the medium was used as the negative control. The percentage inhibition of biofilm formation was calculated based on the equation: %Inhibition = (absorbance of the positive control–absorbance of the sample)/absorbance of the positive control × 100.

2.7.2 Effect of complexes 1 and 2 on preformed biofilms

The effect of complexes 1 and 2 on preformed biofilms was evaluated at the same concentrations as described in section 2.7.1. We added 200 µL of the standardized suspension (1×10^7 CFU mL⁻¹) of the microorganisms to 96-well microplates and incubated them at 37 °C at 80 rpm for 48 h. Subsequently, the biofilms were washed once with PBS and treated with 100 µL of the complexes in the concentrations sub-MIC, MIC, 2.5× MIC, 5× MIC, and 10× MIC for 24 h. Biofilms were washed twice with PBS and quantified by the XTT method [28].

2.8 Scanning electron microscopy (SEM)

SEM was employed to observe the structure of biofilms formed by *C. glabrata* ATCC 2001 after cultivation in RPMI-1640 medium and treatment with complexes 1 and 2 in the concentrations sub-MIC and 10× MIC. The formation of biofilms proceeded as described in the previous section [2.7.1] in 24-well polystyrene microplates. The biofilms were fixed in a solution containing 2.5% glutaraldehyde (Sigma-Aldrich) in sodium cacodylate (0.1 M, pH 7.4; Sigma-Aldrich) for 24 h at 4 °C, and coupons (1 × 1 cm) from the bottom of the wells were cut with a scalpel blade. The biofilms were dehydrated with a series of ethanol solutions (30%, 50%, 70%, 80%, 85%, 90%, 95%, and 100%; 15 min

each) and subjected to supercritical drying in CO₂. The coupons were metallized with gold and observed under a SEM JSM-6380LV (Jeol, Peabody, USA).

2.9 Cytotoxicity

The cytotoxicity of complexes 1 and 2 was evaluated according to the Capoci protocol [29], using the reduction method of the MTS reagent (3 [4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium) in HeLa (Henrietta Lacks - human cervical adenocarcinoma) and SiHa (HPV-16-positive cervical carcinoma cells) tumor cell lines and Vero non-tumor cells (line established from kidney cells of the African green monkey *Cercopithecus aethiops*).

The HeLa and SiHa strains were grown in Dulbecco's modified Eagle's medium (Gibco, Gaithersburg, USA), and the Vero strain was grown in RPMI-1640 medium. Both media were supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco). The cultures were incubated at 37 °C at 5% CO₂. After 80% confluence, the cells were adjusted to 2×10^5 cells mL⁻¹ in their respective media without the addition of penicillin/streptomycin, and 200 μL of that suspension was added to a 96-well microplate. After 24 h of incubation, the cells were washed thrice with PBS, and then treated with different concentrations (50, 150, 500, 1500, and 5000 μg mL⁻¹) of the complexes. As controls, the cells were exposed to the respective quantities of the diluent (DMSO and distilled water) and culture medium without the complex. After 24 h of incubation at 37 °C and 5% CO₂, cytotoxicity was measured as the reduction of MTS in Dulbecco's modified Eagle's medium without phenol red. After 3 h of incubation in the dark, the microplate was read by measuring the optical density at 490 nm (Biochrom, Holliston, USA). The assay was performed in triplicate.

The results were expressed as a percentage of cell viability (%CV), calculated by the following equation: %CV = absorbance of the sample/absorbance of the control \times 100. Concentrations that presented cell viability below 50% were considered cytotoxic.

2.10 Ames test

Evaluation of the mutagenic potential of complexes 1 and 2 was performed by the Ames test using the microsuspension method described by Kado et al. [30]. The strains of *Salmonella typhimurium* TA98, TA100, and TA102 were used at a concentration of 1×10^8 cells mL⁻¹ in the absence or presence of metabolic activation S9 (Molecular Toxicology Inc. Boone, USA).

We added 50 μ L of 0.2 M phosphate buffer or S9 fraction, 5 μ L of the complexes (concentrations 50, 150, 500, 1500, and 5000 μ g mL⁻¹), and 50 μ L of the bacterial suspension to the test tubes. The tubes were pre-incubated for 90 min at 37 °C. After the incubation period, 2 mL of top agar (0.6% agar, 0.6% NaCl, 0.05 mM L-histidine, 0.05 mM biotin, pH 7.4, 45 °C) was added to the tubes and the mixture was poured into plates with minimal agar (1.5% agar, Voguel-Boner solution, and 10% glucose solution). The plates were incubated at 37 °C for 48 to 66 h, and the revertant His⁺ colonies were subsequently counted.

The positive controls used in the assays without metabolic activation were the following: 4-nitro-o-phenylenediamine (10 μ g/plate) for the line TA98, sodium azide (2.5 μ g/plate) for the line TA100, and mitomycin C (0.5 μ g/plate) for TA102. In the assays with metabolic activation, the compound 2-antramine (0.63 μ g/plate) was used for all strains. DMSO was used as the negative control. The tests were performed in triplicate.

To evaluate the mutagenic potential, we used the equation $IM = X1/X2$, where IM is the mutagenicity index, X1 is the average number of revertant colonies in the test plate, and X2 is the average number of revertant colonies in the negative control plate. The

sample was considered mutagenic when IM was greater than or equal to 2. When IM was equal to or less than 0.7, the sample was considered a potentially cytotoxic agent.

2.11 Statistical Analysis

The data were analyzed using GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA) with the ANOVA performed for all the assays and the Tukey's post-test for activity in biofilm formation and preformed biofilms. The results of the Ames test were analyzed using the statistical program Salanal version 1.0 (U.S. Environmental Protection Agency, Monitoring Systems Laboratory, Las Vegas, NV, USA from the Research Triangle Institute, RTP, Research Triangle Park, NC, USA).

3 Results

3.1 Synthesis and structural characterization

The Co(II) complexes were obtained through the direct reaction between cobalt(II) chloride hexahydrate and the respective ligands, based on the methodology published in the literature [31], as shown below in Figure 1:

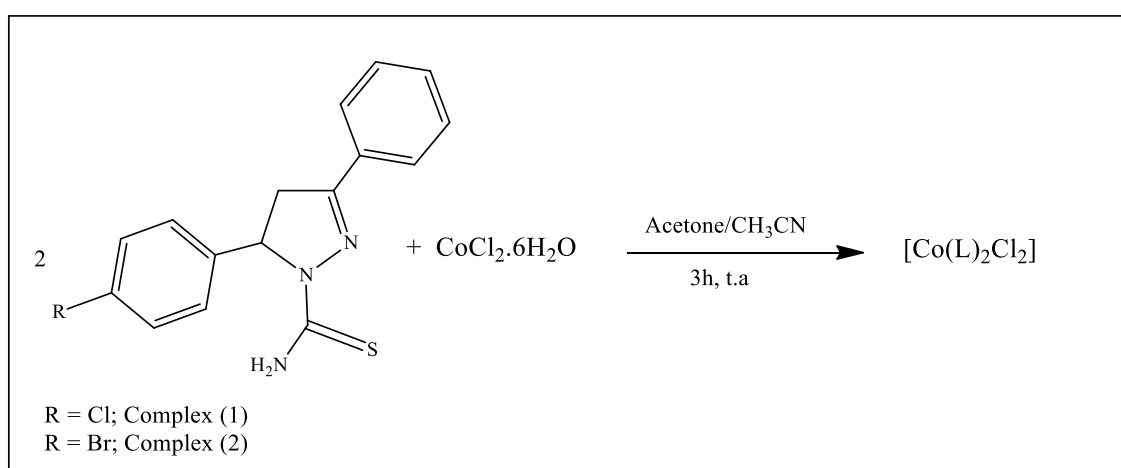


Figure 1. Synthesis for obtaining complexes 1 and 2.

The prepared complexes **1** and **2** have been presented as isostructural neutral complexes of the general formula $[\text{Co}(\text{L})_2\text{Cl}_2]$, where two molecules of the thiocarbamoyl-pyrazoline ligand are coordinated to the $\text{Co}(\text{II})$ atom. Figures 2 and 3 show the crystalline and molecular structures of complexes **1** and **2**. Table 1 gathers the most relevant bond lengths and angles present in the prepared compounds.

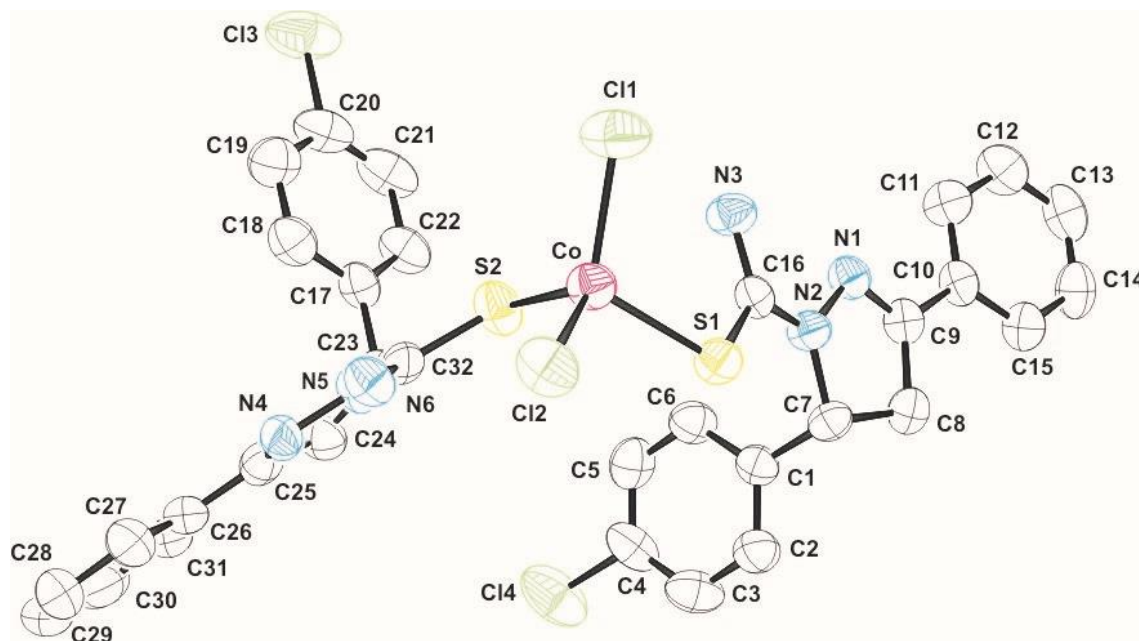


Figure 2. Molecular structure of complex 1. The hydrogen atoms have been omitted for clarity. Thermal ellipsoids drawn at the 50% probability level.

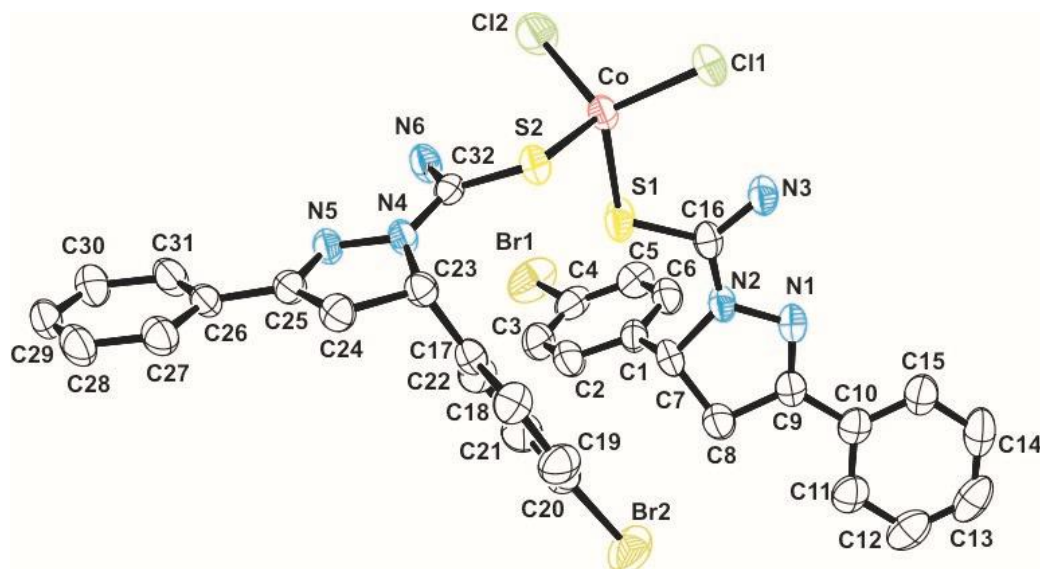


Figure 3. Molecular structure of complex 2. The hydrogen atoms have been omitted for clarity. Thermal ellipsoids drawn at the 50% probability level.

Table 1. Most relevant bond lengths and angles involving the prepared complexes

| Bond Length | Complex 1 | Complex 2 |
|--------------------|------------------|------------------|
| Co(1)–Cl(1) | 2.242 (1) | 2.247 (9) |
| Co(1)–Cl(2) | 2.250(1) | 2.242 (1) |
| Co–S(1) | 2.331(1) | 2.342 (1) |
| Co–S(2) | 2.346(1) | 2.336 (8) |
| N(1)–N(2) | 1.409 (4) | 1.395 (3) |
| N(4)–N(5) | 1.400(5) | 1.401(3) |
| Bond Angles | Complex 1 | Complex 2 |
| S(1)–Co–S(2) | 99.26 (5) | 98.69 (3) |
| Cl(1)–Co–Cl(2) | 115.18(5) | 115.26(4) |
| Cl(1)–Co–S(1) | 112.68 (5) | 114.48 (4) |
| Cl(2)–Co–S(1) | 104.99 (5) | 109.35(4) |
| Cl(1)–Co–S(2) | 109.70 (5) | 104.69 (3) |
| Cl(2)–Co–S(2) | 113.86 (5) | 113.14 (4) |

Bond lengths (Å) and angles (°) of complexes 1 and 2.

According to the bond angles listed at table 1, S(1)–Co–S(2) of $99.26(5)^\circ$, Cl(1)–Co–S(2) of $109.70(5)^\circ$, Cl(1)–Co–Cl(2) of $115.18(5)^\circ$, and Cl(2)–Co–S(2) of $113.86(5)^\circ$ for complex 1, we could determine a distorted tetrahedral coordination sphere for the Co(II) atom (Figure 4).

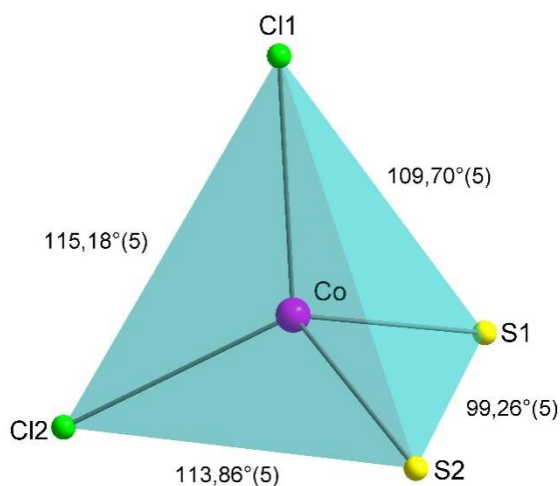


Figure 4. Coordination sphere for the Co(II) atom in complex 1. Complex 2 presented a similar coordination geometry.

The tetrahedral coordination geometry for the Co(II) atom is completed by two chlorine and two sulphur atoms from the thiocarbamoyl moiety of the ligand as previously published in the literature in similar compound [32].

3.2 Infrared spectroscopy

Infrared spectroscopy was used to complement the X-ray diffraction data in order to prove the presence of the main vibrational modes of the free ligands in comparison with the prepared complexes. Table 2 displays the main vibrational modes and frequencies for the prepared compounds. The infrared spectra of ligands L1 and L2 and complexes 1 and 2 are shown in Figures S3-S6.

Table 2. Vibrational modes and frequencies of ligands L1 and L2 and complexes **1** and **2**

| Vibrational modes | Frequencies (cm ⁻¹) | | | |
|--------------------------------|---------------------------------|-----------|-----------|-----------|
| | L1 | L2 | Complex 1 | Complex 2 |
| $\delta_{ax}(\text{C-H})_{Ar}$ | 3144–3055 | 3154 | 3167–3024 | 3167–3055 |
| $\nu(\text{NH}_2)$ | 3514–3265 | 3389–3272 | 3396–3265 | 3410–3258 |
| $\nu(\text{C=C})_{Ar}$ | 1468 | 1469 | 1465 | 1448 |
| $\nu(\text{C=N})$ | 1571 | 1580 | 1514 | 1514 |
| $\nu(\text{C=S})$ | 1365 | 1383 | 1344 | 1343 |
| $\delta_{fp}(\text{C-H})$ | 990–754 | 1010–767 | 1010–754 | 1010–755 |
| $\delta(\text{C-H})_{Ar}$ | 688 | 694 | 688 | 688 |
| $\nu(\text{C-Cl})$ | 579 | --- | 579 | --- |
| $\nu(\text{C-Br})$ | --- | 597 | --- | 560 |

The presence of the main vibrational modes of the ligands can also be observed in the infrared spectrum of the prepared complexes. The data for complexes **1** and **2** obtained by infrared spectroscopy confirm the complexation of the ligands to the metallic center of Co(II) via the sulfur atom of the thiocarbamoyl unit (C=S). This coordination can be proven by decreasing the vibrational frequencies of the C=S moiety in the complexes (Complex 1 = 1344 cm⁻¹, Complex 2 = 1343 cm⁻¹), when compared with the free ligands (L1 = 1365 cm⁻¹, L2 = 1383 cm⁻¹), respectively [33].

3.3 Screening for antifungal activity

Antifungal screening of the complexes was evaluated against planktonic cells of the *Candida* species. For both complexes, the MIC and MFC ranged from 15.62 to 1000 $\mu\text{g mL}^{-1}$ (Table 3).

As the complexes had the lowest MIC against *C. glabrata* (15.62 $\mu\text{g/mL}$), they were tested against resistant clinical isolates of this species. In this case, the MIC of complex 1 varied from 3.90 to 15.62 $\mu\text{g mL}^{-1}$ and that of complex 2 ranged from 7.81 to 31.25 $\mu\text{g mL}^{-1}$ (Table 3).

In equimolar concentrations of the complexes, the salt $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ exhibited antifungal activity against all *Candida* strains. On the other hand, the free ligands, L1 and L2, did not have the potential to inhibit the yeasts tested (Table S-2).

Table 3. Minimum inhibitory concentration ($\mu\text{g mL}^{-1}$) of complexes 1 and 2 against ATCC strains of *Candida* species and tested clinical isolates obtained from urine

| | MIC | MFC | MIC | MFC | MIC |
|-----------------------------------|-----------|-------|-----------|-------|------|
| | Complex 1 | | Complex 2 | | FLC |
| Yeast | | | | | |
| <i>C. albicans</i> ATCC 10231 | 1000 | 1000 | 1000 | 1000 | 2** |
| <i>C. albicans</i> ATCC 90028 | 1000 | 1000 | 1000 | 1000 | 2** |
| <i>C. glabrata</i> ATCC 2001 | 15.62 | 15.62 | 15.62 | 15.62 | 8** |
| <i>C. krusei</i> ATCC 6558 | 500 | 500 | 250 | 250 | 32 |
| <i>C. parapsilosis</i> ATCC 22019 | 500 | 500 | 500 | 500 | 2** |
| <i>C. tropicalis</i> ATCC 750 | 1000 | 1000 | 1000 | 1000 | 1** |
| Isolate | | | | | |
| <i>C. glabrata</i> CG10 | 7.81 | 15.62 | 7.81 | 31.25 | 16** |
| <i>C. glabrata</i> CG25 | 15.62 | 62.5 | 31.25 | 62.5 | 16** |
| <i>C. glabrata</i> CG26 | 15.62 | 62.5 | 15.62 | 31.25 | 2** |
| <i>C. glabrata</i> CG37 | 7.81 | 15.62 | 15.62 | 31.25 | 16** |
| <i>C. glabrata</i> CG61 | 7.81 | 15.62 | 15.62 | 15.62 | 16** |
| <i>C. glabrata</i> CG66 | 3.90 | >1000 | 7.81 | 15.62 | 16** |

MIC: minimum inhibitory concentration ($\mu\text{g mL}^{-1}$); MFC: minimum fungicidal concentration ($\mu\text{g mL}^{-1}$); FLC: fluconazole; **: Dose-dependent.

3.4 Effect of the complexes combined with fluconazole

The combinatorial activity of the complexes with the antifungal agent fluconazole was assessed. Complex **1** presented an additive effect against *C. glabrata* ATCC 2001 and an indifferent effect against the isolate *C. glabrata* CG66 when combined with fluconazole. Complex **2**, in contrast, demonstrated an indifferent effect when combined with fluconazole against *C. glabrata* ATCC 2001 and *C. glabrata* CG66 (Table 4).

Table 4. Antifungal activity of complexes 1 and 2 in combination with fluconazole against *Candida glabrata*

| | MIC ($\mu\text{g mL}^{-1}$) | | FIC | FICI | Result |
|-------------|-------------------------------|----------|------|------|-------------|
| | Alone | Combined | | | |
| ATCC 2001 | | | | | |
| Complex 1 | 15.62 | 1.9 | 0.12 | 0.62 | Additive |
| Fluconazole | 4 | 2 | 0.5 | | |
| Complex 2 | 15.62 | 3.9 | 0.24 | 1.24 | Indifferent |
| Fluconazole | 4 | 4 | 1 | | |
| CG66 | | | | | |
| Complex 1 | 3.9 | 3.9 | 1 | 2 | Indifferent |
| Fluconazole | 16 | 16 | 1 | | |
| Complex 2 | 7.81 | 7.81 | 1.00 | 1.5 | Indifferent |
| Fluconazole | 16 | 8 | 0,5 | | |

MIC: minimum inhibitory concentration, FIC: fractional inhibitory concentration, FICI: fractional inhibitory concentration index.

3.5 Mechanisms of antifungal action of the complexes

Sorbitol and ergosterol assays were performed to evaluate the mechanism of action of the complexes on the yeasts' structure. The results of the sorbitol assay reveal that the complexes do not interfere with the cell wall biosynthesis of *C. glabrata* (Table 5). However, it was observed that MIC in the presence of ergosterol concentrations increased in both complexes, suggesting that they act in disrupting the permeability of the ionic membrane (Table 6).

Table 5. Activity of complexes **1** and **2** on cell wall biosynthesis (sorbitol) of *Candida glabrata* ATCC 2001. Values are expressed in $\mu\text{g mL}^{-1}$.

| Agent | MIC ($\mu\text{g mL}^{-1}$) | |
|-------------|-------------------------------|------------------|
| | With Sorbitol | Without Sorbitol |
| Complex 1 | 62.5 | 62.5 |
| Complex 2 | 31.25 | 31.25 |
| Caspofungin | 15.6 | 7.81 |

Table 6. Effects of different concentrations of exogenous ergosterol on the antifungal activity of complexes **1** and **2** for *Candida glabrata* ATCC 2001

| Agent | MIC in the absence of Ergosterol ($\mu\text{g mL}^{-1}$) | MIC in the presence of different concentrations of ergosterol ($\mu\text{g mL}^{-1}$) | | |
|-----------------------|--|---|-------|-------|
| | | 100 | 200 | 400 |
| Complex 1 | 15.62 | 31.25 | 31.25 | 31.25 |
| Complex 2 | 15.62 | 15.62 | 31.25 | 62.5 |
| Amphotericin B | 31.25 | 62.5 | 62.5 | 62.5 |

MIC: minimum inhibitory concentration

3.6 Antibiofilm activity of the complexes

Considering the promising antifungal activity of the complexes on planktonic cells, their ability to interfere with biofilm formation and preformed biofilms was tested. The complexes significantly reduced the formation of *C. glabrata* biofilms (Figure 5). Complex 2 was more effective than complex 1 in both degrading and inhibiting the formation of *C. glabrata* ATCC 2001 biofilms. Complex 2 presented a concentration of $156.2 \mu\text{g mL}^{-1}$ and inhibited biofilm formation by 90% and degraded 59% of the preformed biofilms, whereas complex 1 at the same concentration inhibited formation by 80% and degraded 54% of the preformed biofilms. Moreover, complex 1 inhibited biofilm formation by 77% and degraded 50% of the preformed biofilms of the isolate *C. glabrata* CG66 at a concentration of $39 \mu\text{g mL}^{-1}$, whereas complex 2 inhibited formation by 94% and degraded 53% at a concentration of $78.2 \mu\text{g mL}^{-1}$ of the isolate (Figure 5).

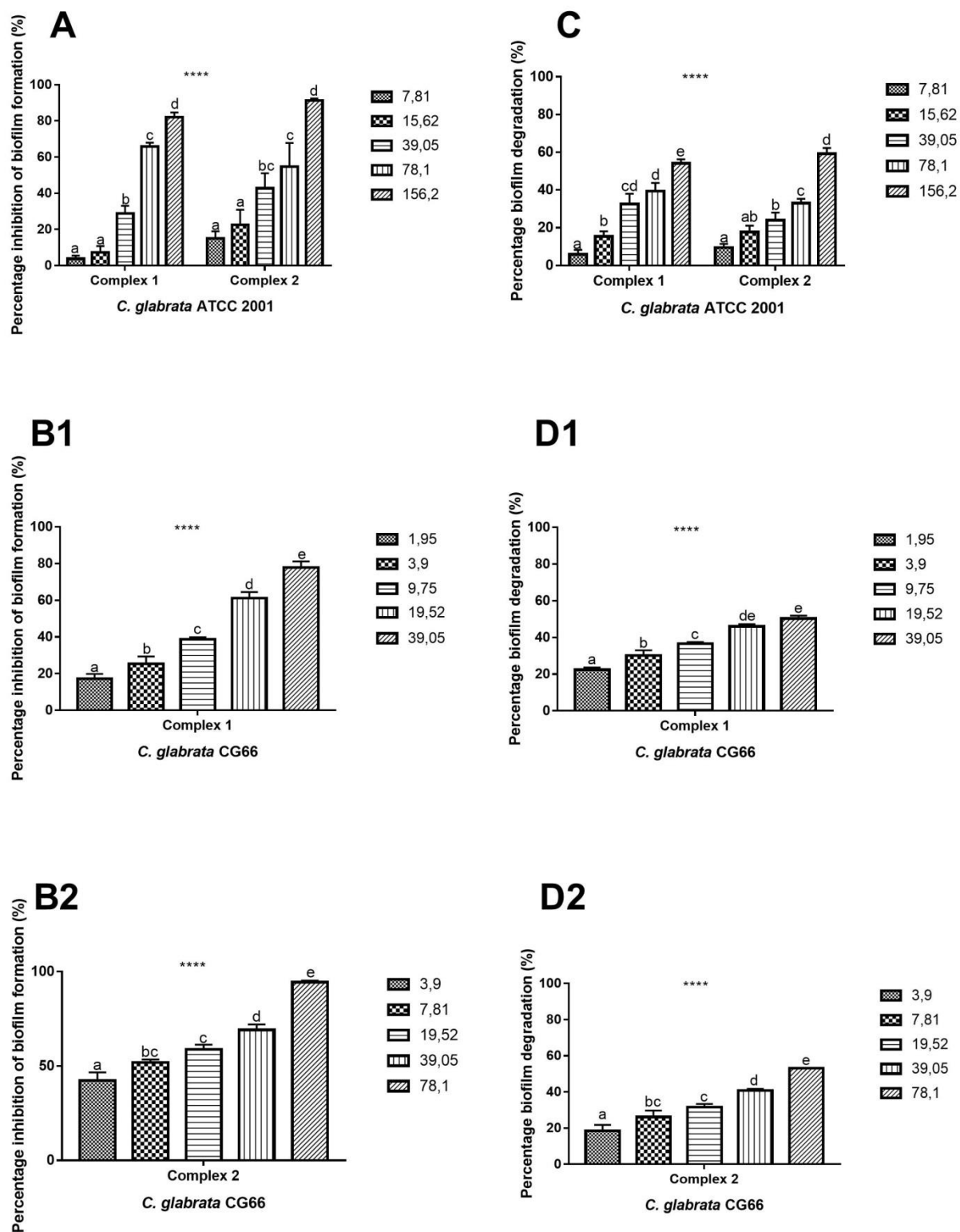


Figure 5. Percentage inhibition and dispersal of biofilms of *Candida glabrata* ATCC 2001 and isolate *C. glabrata* CG66 treated with complexes 1 and 2. A, B1, and B2 represent inhibition of biofilm formation; C, D1, and D2 represent the activity in the dispersal of biofilm, analyzed at the concentrations sub-MIC, MIC, 2.5× MIC, 5× MIC, and 10× MIC. MIC: minimum inhibitory concentration.

3.7 SEM

The images obtained through SEM confirmed the activity of complexes **1** and **2** in inhibiting the biofilm formation of *C. glabrata* ATCC (Figure 6). It is possible to observe a significant reduction in the biofilms treated with the complexes by comparing the images of the positive control (A) with those of the biofilms treated with the sub-MIC (B1 and C1) and 10× MIC (B2 and C2) concentrations.

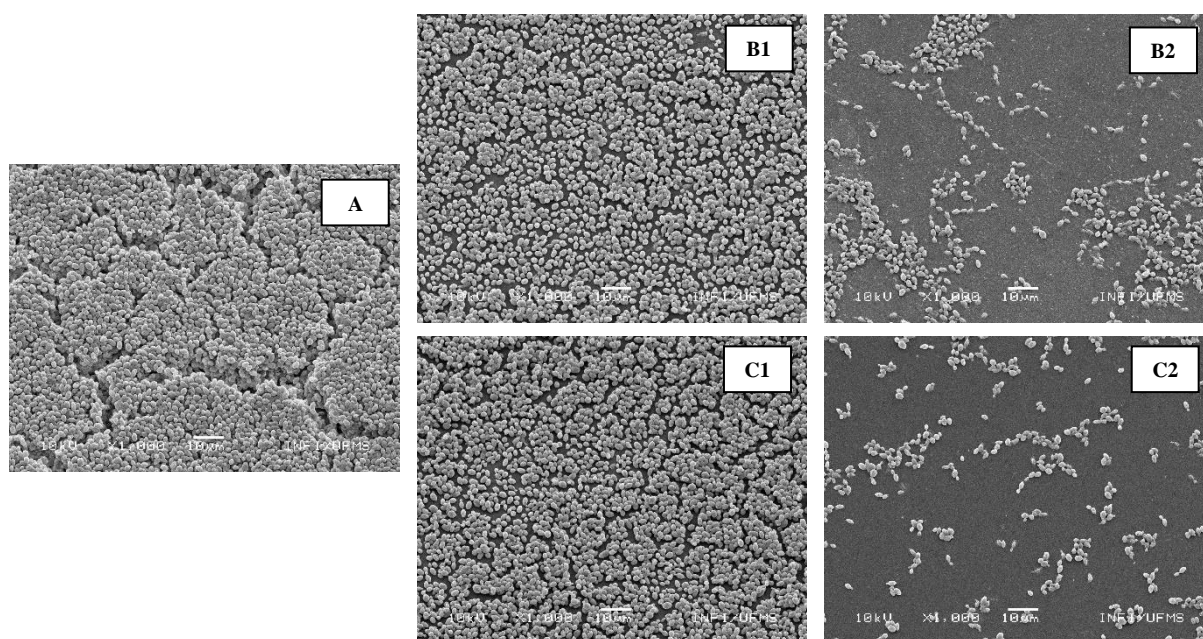


Figure 6. Scanning electron microscope micrographs of *Candida glabrata* ATCC 2001 biofilms treated with complexes **1** and **2**. **A** Untreated Biofilm; **B1 and B2** are biofilms treated with complex 2; **C1 and C2** are treated with complex 1; **B1 and C1** sub-MIC; and **B2 and C2** 10× MIC. MIC: minimum inhibitory concentration.

3.8 Cytotoxicity

Once the complexes demonstrated promising antifungal activity, their cytotoxic potential was tested to examine potential toxic effects. Therefore, tumor cells, HeLa and SiHa, and non-tumor cells, Vero, were treated with different concentrations of the complexes. The results showed that the complexes presented cytotoxic potential at high concentrations, 1500 and 5000 $\mu\text{g mL}^{-1}$, but exhibited a cell viability greater than 50% at lower

concentrations, 50 and 150 $\mu\text{g mL}^{-1}$, which does not indicate cytotoxic potential (Figure 7).

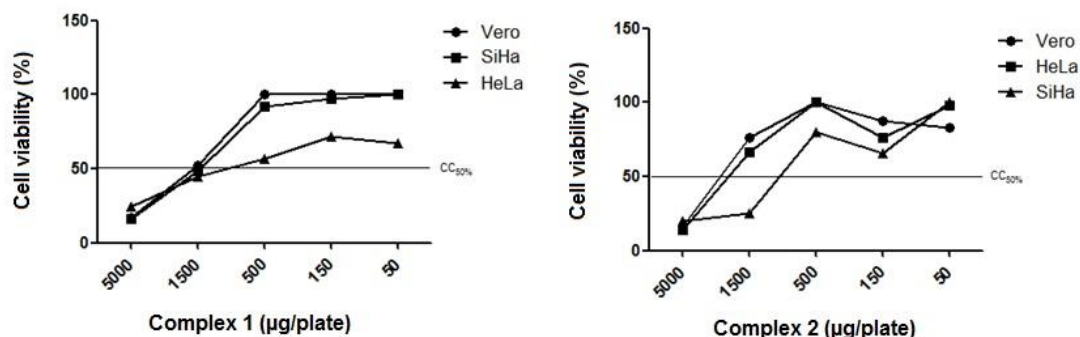


Figure 7. Percentage cell viability of the lines Vero, SiHa, and HeLa treated with complexes 1 and 2

3.9 Ames test

The mutagenic potential of the complexes was also assessed through the Ames test using lines of *Salmonella typhimurium*. Both complexes presented cytotoxic potential at a concentration of 5000 $\mu\text{g mL}^{-1}$ in the presence of the exogenous metabolic activation system (Table 7) as the mutagenicity index was less than 0.7. As there was no significant increase in the number of revertant colonies per plate in the other concentrations, the mutagenicity ratio was less than two, and a positive dose-response relationship was not observed among the concentrations evaluated.

Table 7. Mutagenic activity expressed by the average number of revertant colonies per plate and standard deviation of complexes 1 and 2 against *Salmonella typhimurium* lines (TA98, TA100, and TA102) in the presence or absence of an exogenous metabolic activation system

| $\mu\text{g}/\text{plate}$ | TA98 | | TA100 | | TA102 | |
|----------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| | S9 - | S9 + | S9 - | S9 + | S9 - | S9 + |
| Complex 1 | | | | | | |
| 0^a | 31 \pm 1 | 30 \pm 2 | 162 \pm 8 | 172 \pm 3 | 372 \pm 9 | 394 \pm 7 |
| 50 | 36 \pm 2 | 29 \pm 1 | 147 \pm 2 | 181 \pm 8 | 457 \pm 6 | 462 \pm 2 |
| 150 | 34 \pm 5 | 30 \pm 7 | 150 \pm 8 | 160 \pm 7 | 446 \pm 6 | 444 \pm 5 |
| 500 | 25 \pm 3 | 38 \pm 4 | 138 \pm 8 | 139 \pm 3 | 443 \pm 6 | 419 \pm 6 |
| 1500 | 30 \pm 2 | 35 \pm 8 | 125 \pm 4 | 138 \pm 6 | 349 \pm 1 | 367 \pm 9 |
| 5000 | 31 \pm 3 | 32 \pm 6 | 121 \pm 3 | 112 \pm 8 | 277 \pm 6 | 261 \pm 6 |
| Complex 2 | | | | | | |
| 50 | 30 \pm 3 | 37 \pm 1 | 157 \pm 9 | 187 \pm 2 | 477 \pm 8 | 481 \pm 6 |
| 150 | 30 \pm 1 | 42 \pm 2 | 145 \pm 4 | 173 \pm 7 | 450 \pm 5 | 471 \pm 5 |
| 500 | 30 \pm 2 | 32 \pm 7 | 180 \pm 6 | 153 \pm 9 | 417 \pm 6 | 440 \pm 6 |
| 1500 | 32 \pm 2 | 35 \pm 1 | 150 \pm 5 | 131 \pm 3 | 351 \pm 6 | 369 \pm 7 |
| 5000 | 31 \pm 2 | 33 \pm 1 | 180 \pm 5 | 126 \pm 4 | 276 \pm 6 | 287 \pm 7 |
| C+ | 250 \pm 4 ^b | 305 \pm 4 ^c | 702 \pm 2 ^d | 720 \pm 8 ^c | 998 \pm 6 ^e | 950 \pm 4 ^c |

^aNegative control: DMSO; Positive control (C+): ^b4-nitro-o-phenylenediamine (10 $\mu\text{g}/\text{plate}$); ^c2-aminoanthracene (1.5 $\mu\text{g}/\text{plate}$); ^dsodium azide (2.5 $\mu\text{g}/\text{plate}$); ^eMitomycin C (0.5 $\mu\text{g}/\text{plate}$).

4 General considerations

The fungal and invasive infections caused by *Candida* have increased worldwide, becoming a global health challenge [15]. *Candida* species are opportunistic pathogens that are commonly related to urinary tract infections affecting mainly women and children [5]. However, the treatment of these infections still poses a challenge owing to the limited number of antifungal drugs and the increased resistance of microorganisms to conventional antimicrobial drugs [2]. Therefore, the synthesis and application of two

promising cobalt(II) complexes with thiocarbamoyl-pyrazoline ligands as antifungal agents presented good results in this work.

C. glabrata has been reported as the second most frequent species in infections caused by *Candida* and as the most common pathogen related to cases of resistance in hospital environments [34,35]. It is therefore considered a multidrug-resistant species [15,36]. This species is also known to cause superficial and invasive infections in the elderly and immunocompromised patients [37]. Recent studies have evidenced the emergence of these infections in cases of urinary infections acquired in health institutions and in the community [38,39].

Both complexes showed fungistatic and fungicidal activity against the *Candida* species evaluated herein and were more effective in inhibiting the species *C. glabrata*, presenting a lower MIC value of $3.9 \mu\text{g mL}^{-1}$ against the clinical isolates, a value lower than that presented by fluconazole ($16 \mu\text{g mL}^{-1}$) in the assay. This information is relevant because fluconazole, which is one of the most traditional antifungal drugs used, exhibits only fungistatic action, and can promote yeast resistance and biofilm maturation if used without caution [40-41].

Usually, recurrent infections by *C. glabrata* that have already been treated with the line of azoles used in conventional treatments are treated with echinocandins. Nevertheless, isolates of *C. glabrata* that are resistant to fluconazole may also present co-resistance to echinocandins, thereby demonstrating that the exacerbated use of azoles and echinocandins may lead to the development of multidrug-resistant strains of *C. glabrata* [15]. Thus, the fungicidal action demonstrated by the complexes, with MFC values of $15.26\text{--}62.5 \mu\text{g mL}^{-1}$, is an important characteristic that can contribute to decreasing the proliferation of multidrug-resistant microorganisms in immunocompromised patients [42].

Biofilm adhesion and formation are virulence factors that, in addition to contributing to the pathogenesis of *C. glabrata*, are also related to the species' resistance to azoles and echinocandins [15]. It is common for patients to use urinary catheters in hospital environments, a fact that facilitates the adhesion of microorganisms and a case of urinary infection [43]. The prepared complexes demonstrated promising activity on biofilms of *C. glabrata*. The low concentration of the complexes used to inhibit and degrade biofilms deserves attention, with the biofilm inhibitory action beginning at the sub-MIC concentration level, as evidenced by SEM.

According to Sardi et al. [26] concentrations that are 2 to 1000 times greater than those for planktonic cells are needed to degrade or remove biofilm. The complexes stood out for their potent inhibitory and dispersal activity of the biofilm of the clinical isolate *C. glabrata* CG66 at a concentration of 10× MIC, with inhibition values of 50% to 94%, which confirms the effectiveness of the antibiofilm activity of the complexes.

In some cases, a combination therapy with two antifungal agents is used to control the infection and improve the patient's response and as a strategy to combat microbial development and/or resistance [44,45]. When evaluated in combination with fluconazole, the complexes showed an additive effect for *C. glabrata* ATCC, and this effect is aligned with the perspective of trying to reduce dosages or undesirable effects caused by drugs used in conventional treatments [46].

We currently do not have a large number of antifungal drugs [47], owing to the yeast's own structure [3,48]. As they are eukaryotes, antimicrobial agents act mainly on cell wall biosynthesis and membrane permeability. Accordingly, because of the promising results obtained in the antifungal activity assays, the mechanisms of action of the complexes on *C. glabrata* were examined. The results showed that the complexes present no activity in cell wall biosynthesis, as the MIC value did not change in the

presence of an osmotic protector, such as sorbitol. In contrast, an increase of 4× MIC was observed in the complexes when evaluated in the presence of exogenous ergosterol, indicating that the complexes bind to the ergosterol of the membrane and interfere in the permeability to the ions.

Considering the promising antifungal and antibiofilm activity of the complexes, their pharmacological safety was evaluated. Cytotoxicity was assessed against one non-tumor and two tumor cell lines. The complexes exhibited cytotoxic potential only at the highest concentrations (5000 and 1500 $\mu\text{g mL}^{-1}$) for all the cells. However, these values are 100 times higher than the MIC values of the complexes against yeasts; therefore, they can be considered safe in terms of cytotoxic potential as they showed a viability percentage close to 100% in the MIC concentrations.

The mutagenic potential of the complexes was assessed by the Ames test, as recommended by the international agencies, the Food and Drug Administration (FDA), the European Medicines Agency (EMA), and the Brazilian Health Regulatory Agency (ANVISA) which regulate the development of new drugs [49]. Our results showed that the complexes do not have the potential to induce gene mutations through the replacement of base pairs (TA100 and TA102) or displacement of the reading frame (TA98) in the presence or absence of an exogenous metabolic activation system. In other words, the complexes do not act as direct- or indirect-acting mutagens. Therefore, the results of the Ames test, an essential preclinical assay for assessing the genotoxic potential of candidates for new drugs [50], suggest that Co(II) complexes with thiocarbamoyl-pyrazoline ligands are safe.

5 Conclusion

The two new cobalt(II) complexes **1** and **2** containing thiocarbamoyl-pyrazoline ligands were evaluated for their pharmacological potential. Both showed antifungal and antibiofilm activity and stood out in the control of *Candida glabrata* ATCC 2001 and the resistant clinical isolate *C. glabrata* CG66 obtained from a urine sample. None of the complexes exhibited mutagenic or cytotoxic activity at the concentrations applied in the assays. In this context, the present study indicates cobalt(II) complexes as potential candidates for new drugs in the field of anti-*C. glabrata* therapy.

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Appendix A. Supplementary Data

CCDC 2016353 and 2016354 contains the supplementary crystallographic data for **1** and **2**. These data can be obtained free of charge at <http://www.ccdc.cam.ac.uk/conts/retrieving.html>, or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: (+44) 1223-336-033; or e-mail: deposit@ccdc.cam.ac.uk

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6. CONCLUSÃO

Os novos complexos testados apresentam atividade antifúngica, sendo sua melhor eficácia para *C. glabrata* ATCC 2001 e para isolado clínico resistente de *C. glabrata* CG66 proveniente de amostra de urina;

Os complexos apresentam atividade antibiofilme, para biofilme formados e biofilme pré-formado, sendo o complexo 1 o mais eficaz para biofilmes formados e préformados de *C. glabrata* ATCC 2001 e o complexo 2 mais eficaz para biofilmes formados e pré-formados de isolado resistente de *C. glabrata*;

O mecanismo de ação dos complexos é no ergosterol, onde atuam na perturbação da permeabilidade da membrana iônica;

Apresentam atividade sinérgica aditiva somente para *C. glabrata* ATCC 2001;

Os complexos não apresentam potencial mutagênico e citotóxico nas concentrações utilizadas em nenhum ensaio;

Apesar de ambos complexos apresentarem atividades promissoras, o complexo I foi o mais eficaz para células planctônicas e sesséis para isolado clínico resistente de *C. glabrata* CG66, sendo assim, um possível medicamento para o tratamento anti-*Candida glabrata*.

7. ANEXOS

Tabela S-8. Dados cristalográficos e refinamento estrutural dos complexos **1** e **2**.

| Descrições | Complexo 1 | Complexo 2 |
|---|--|--|
| Fórmula Molecular | $C_{32}H_{28}CoCl_4N_6S_2$ | $C_{32}H_{28}CoCl_2Br_2N_6S_2$ |
| Massa Molecular (g/mol) | 761,17 | 850,07 |
| Radiação utilizada Mo-K α | $\lambda=0,71073$ | $\lambda=0,71073$ |
| T $^\circ$ (K) | 296(2) | 296(2) |
| Sistema Cristalino | Triclínico | Triclínico |
| Grupo Espacial | $P\bar{1}$ | $P\bar{1}$ |
| Parâmetros de Cella | | |
| <i>a</i> (Å) | 10,5115(9) | 10,6337(3) |
| <i>b</i> (Å) | 13,4145(10) | 13,4271(3) |
| <i>c</i> (Å) | 14,3540(12) | 14,3150(4) |
| α ($^\circ$) | 62,712(5) | 62,8300(10) |
| β ($^\circ$) | 71,756(6) | 70,619(2) |
| γ ($^\circ$) | 78,191(6) | 77,777(2) |
| Volume (Å 3) | 1703,9(3) | 1710,90(8) |
| Número de fórmulas elementares | Z = 2 | Z=2 |
| Densidade Calculada g/cm 3 | 1,484 | 1,651 |
| Coefficiente linear de absorção (mm $^{-1}$) | 0,973 | 3,148 |
| F (000) | 778 | 850 |
| Dimensão do cristal (mm) | 0,137 x 0,105 x 0,081 | 0,477 x 0,290 x 0,214 |
| Região de varredura angular | 1,650 a 26,573 $^\circ$ | 1,661 a 28,479 $^\circ$ |
| Índices de varredura | -13 \leq h \leq 13 -16 \leq k \leq 16 -18 \leq l \leq 17 | -14 \leq h \leq 14 -17 \leq k \leq 17 -19 \leq l \leq 19 |
| Número de reflexões coletadas | 28770 | 36291 |
| Número de reflexões independentes | 7074 [R(int) = 0,1010] | 8631 [R(int) = 0,0538] |
| Dados/Restrições/Parâmetro | 7074 / 0 / 431 | 8631 / 0 / 518 |
| Índice R Final [I > 2sigma(I)] | R1 = 0,0545 wR2 = 0,1094 | R1 = 0,0431 wR2 = 0,0902 |
| Índice R (dados totais) | R1 = 0,1452 wR2 = 0,1423 | |
| Densidade eletrônica residual. (e/Å 3) | 0,328 e -0,344 | R1 = 0,0854 wR2 = 0,1048 |
| | | 0,894 e -1,085 |

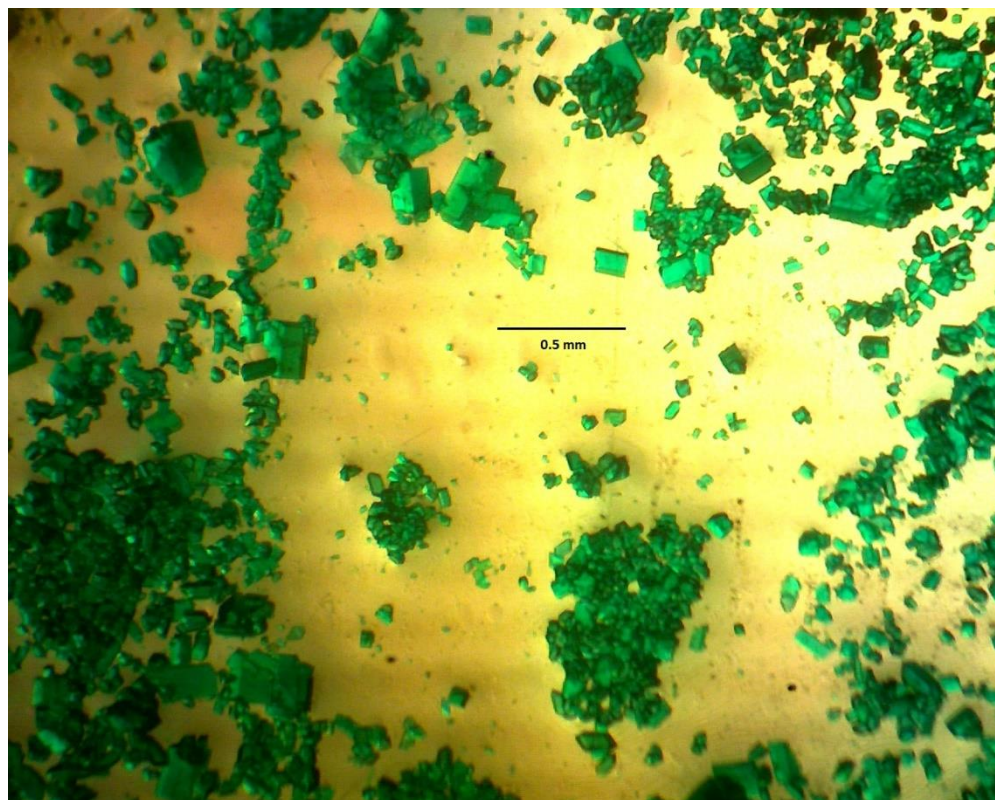


Figura S-8. Cristais únicos do complexo (1) obtidos após recristalização em metanol / acetonitrila / acetona na proporção de volume (1: 1: 1).

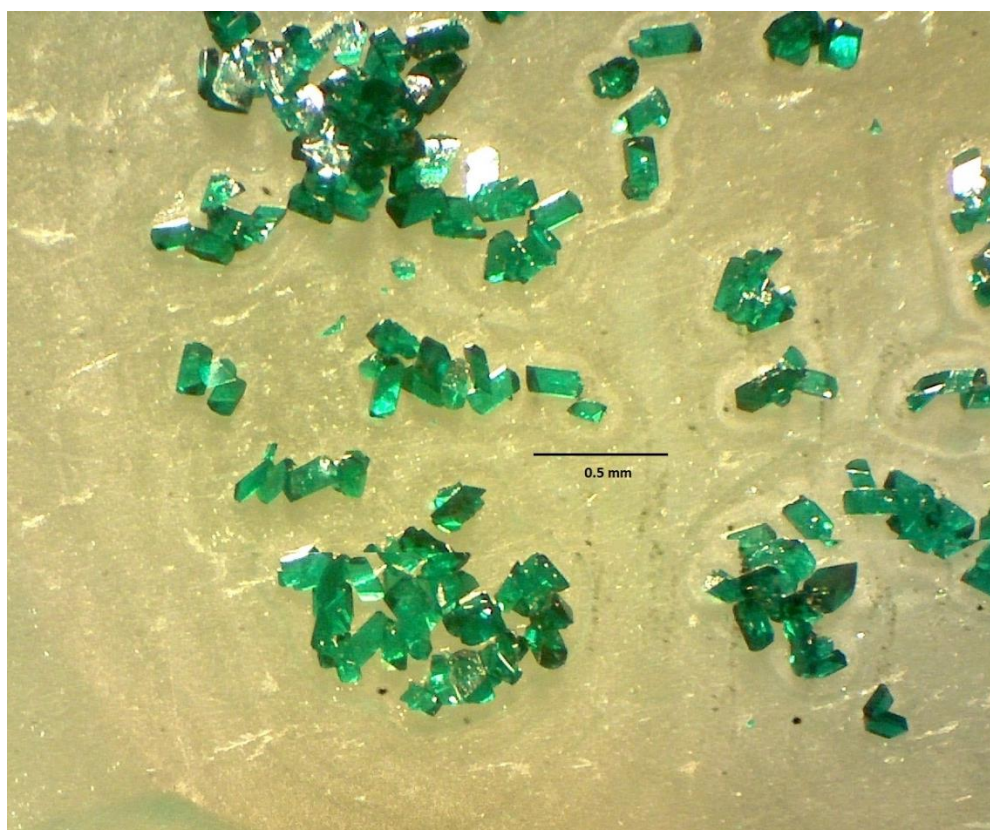


Figura S-9. Cristais únicos do complexo (2) obtidos após recristalização em metanol / acetonitrila / acetona na proporção de volume (1: 1: 1).

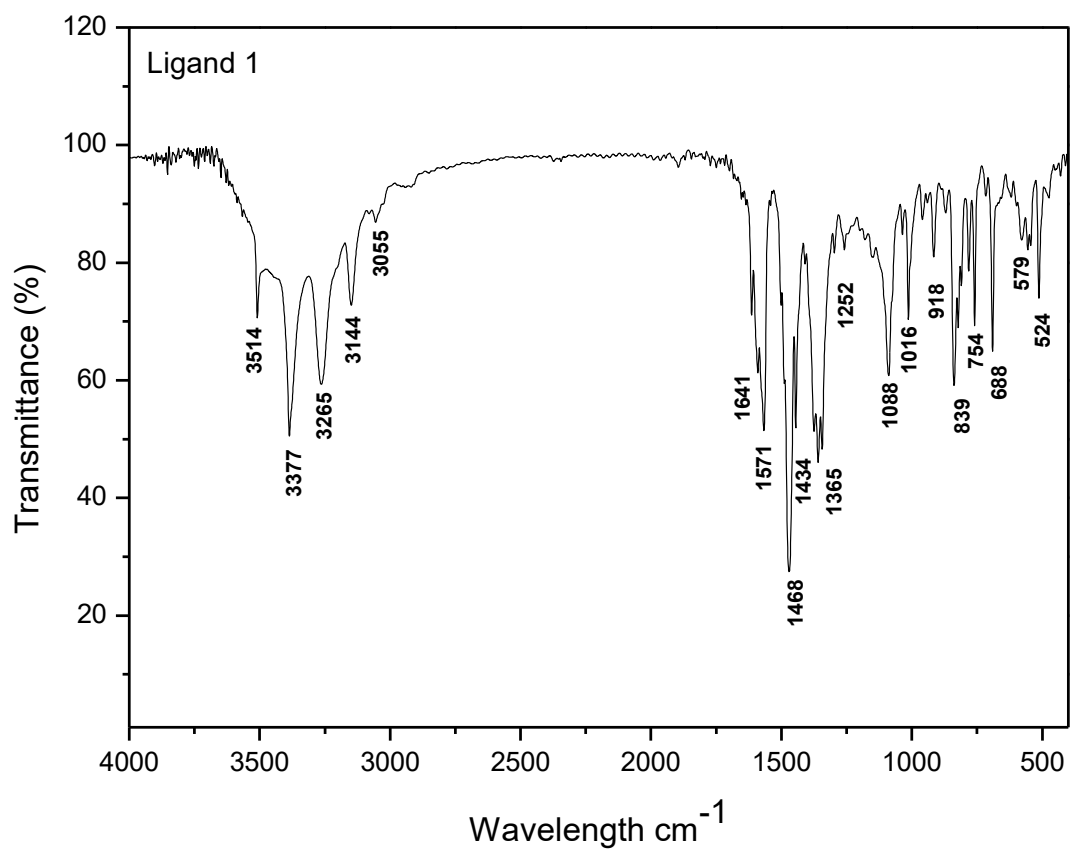


Figura S-10. Espectro de infravermelho do ligante 1.

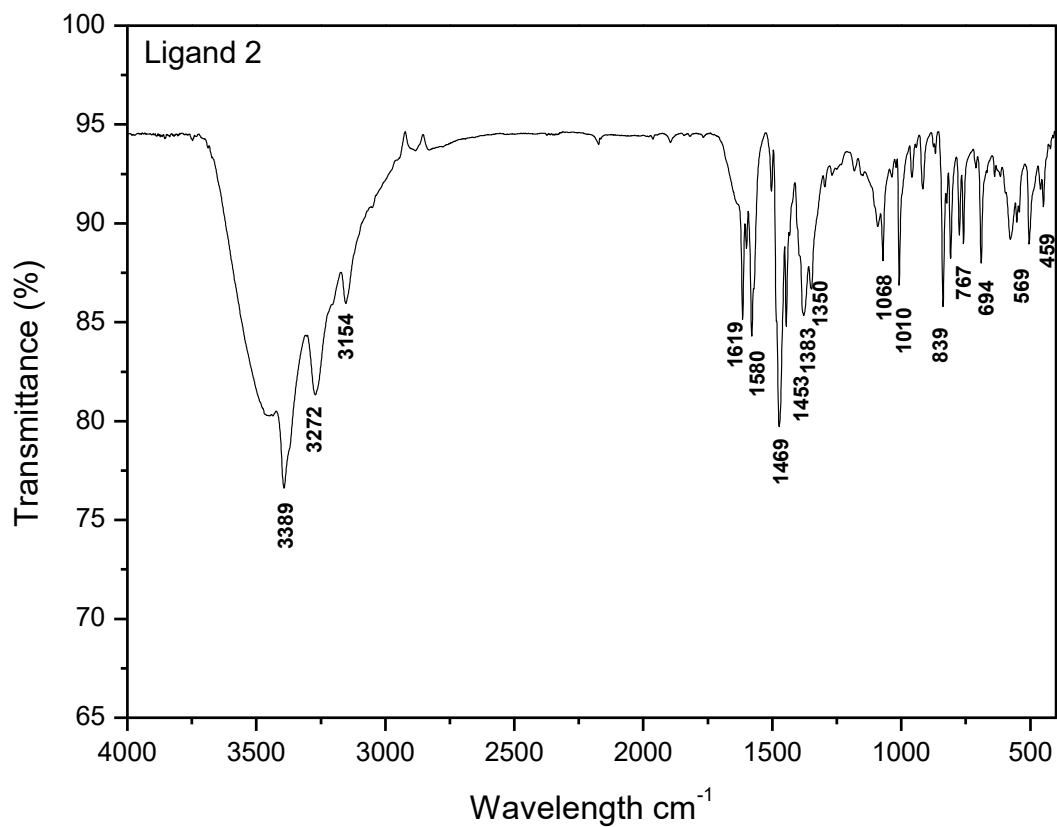


Figura S-11. Espectro de infravermelho do ligante 2.

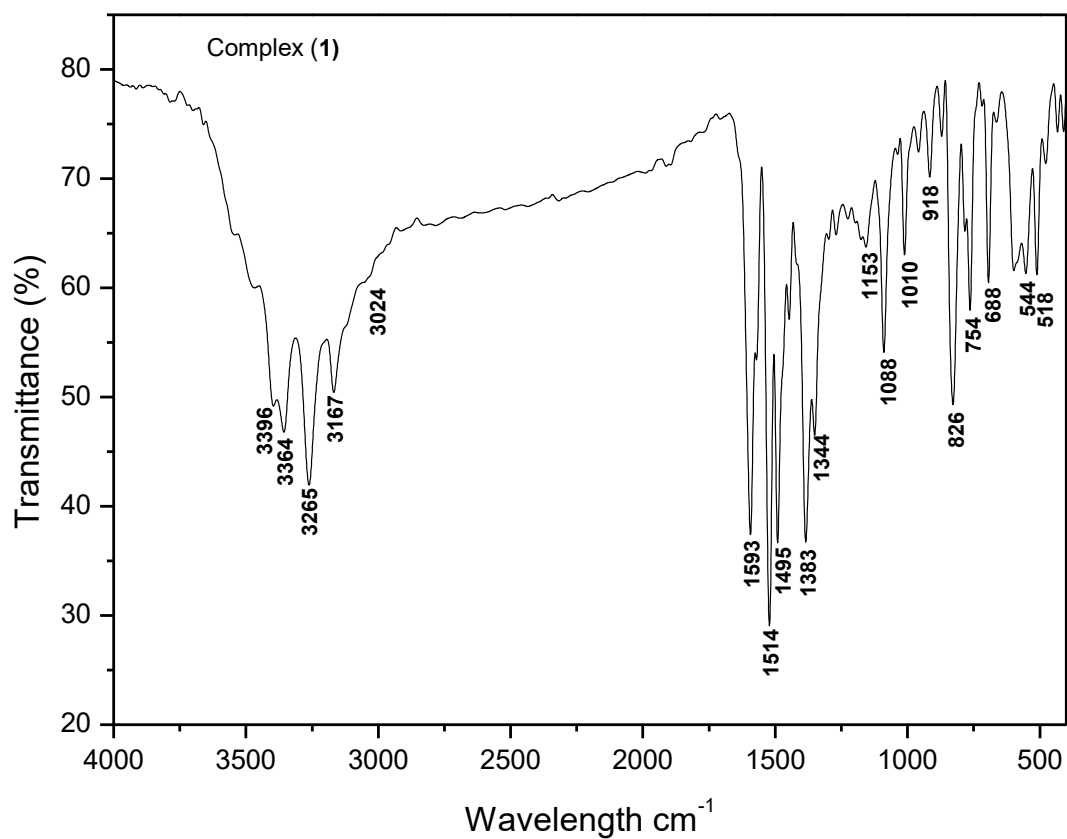


Figura S-12. Espectro infravermelho do complex (1).

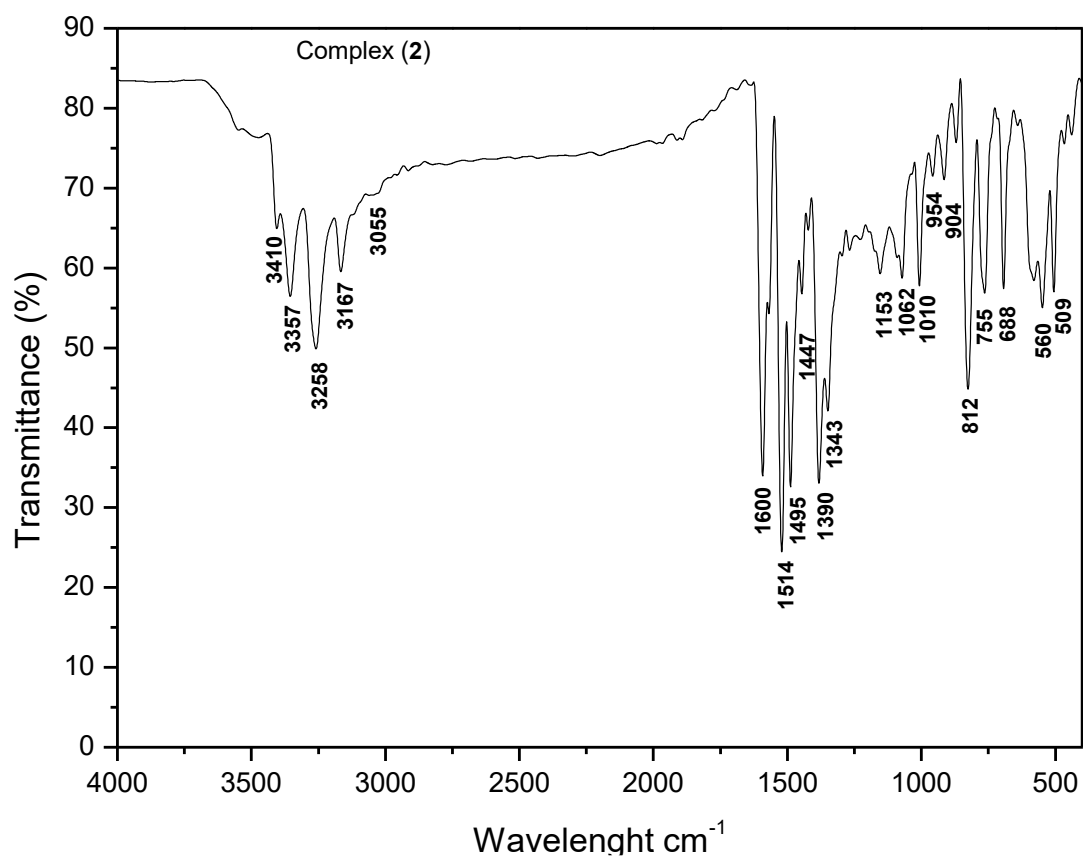


Figura S-13. Espectro infravermelho do complexo (2).

Tabela S-9. Concentrações inibitórias mínimas (mg.L^{-1}) nos ensaios antimicrobianos para leveduras utilizando ligantes livres.

| Levedura | CIM | CFM | CIM | CFM | CIM | CFM |
|---|-------------------|-------|-----|-----|-----|-----|
| | CoCl ₂ | | Br | | Cl | |
| <i>Candida tropicalis</i> ATCC 750 | 125 | 125 | - | - | - | - |
| <i>Candida parapsilosis</i> ATCC 22019 | 31,25 | 31,25 | - | - | - | - |
| <i>Candida albicans</i> ATCC 10231 | 250 | 125 | - | - | - | - |
| <i>Candida albicans</i> ATCC 90028 | 125 | 250 | - | - | - | - |
| <i>Candida glabrata</i> ATCC 2001 | 15,12 | 31,25 | - | - | - | - |
| <i>Candida kruse</i> ATCC 6558 | 31,25 | 125 | - | - | - | - |

CIM: concentração inibitória mínima; CFM: concentração fungicida mínima; -: não houve inibição.