ADRIANA ARAÚJO DE ALMEIDA APOLONIO

EXTRATOS DE *Annona coriacea* MART. (ANNONACEAE), *Cochlospermum regium* (MART & SCHRANK) PILGER (BIXACEAE) E *Myracrodruon urundeuva* ALLEMÃO (ANACARDIACEAE) COM POTENCIAL PARA TRATAMENTO DE INFECÇÕES CAUSADAS POR *Candida albicans* e *Cryptococcus* spp.

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Tese apresentada ao Programa de Pós-Graduação Saúde e Desenvolvimento na Região Centro-Oeste da Universidade Federal de Mato Grosso do Sul, para obtenção do título de doutor.

Orientadora: Profa. Dra. Marilene Rodrigues Chang

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FOLHA DE APROVAÇÃO

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Tese apresentada como requisito para obtenção do título de doutor pelo Programa de Pós-Graduação Saúde e Desenvolvimento na Região Centro-Oeste da Universidade Federal de Mato Grosso do Sul, sob a orientação da Profa. Dra. Marilene Rodrigues Chang.

Nota/Conceito: _____

Campo Grande (MS), _____ de _____ de _____

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"... e você aprende que realmente pode suportar... que realmente é forte e que pode ir muito mais longe depois de pensar que não se pode mais. E que realmente a vida tem valor e que você tem valor diante da vida!"

Veronica Shoffstall

Aos meus pais, Celso e Nilza, que são a minha inspiração e que sempre me incentivaram pela busca do conhecimento.

Ao meu esposo, Gleyson, que com amor e ternura sempre me apoiou e compreendeu pelos momentos de dedicação a este trabalho.

Dedico

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RESUMO

APOLONIO AAA. EXTRATOS DE *Annona coriacea* MART. (ANNONACEAE), *Cochlospermum regium* (MART & SCHRANK) PILGER (BIXACEAE) E *Myracrodruon urundeuva* ALLEMÃO (ANACARDIACEAE) COM POTENCIAL PARA TRATAMENTO DE INFECÇÕES CAUSADAS POR *Candida albicans* e *Cryptococcus* spp.

[Doutorado – Universidade Federal de Mato Grosso do Sul, Brasil].

As infecções causadas por leveduras apresentam altas taxas de morbidade e mortalidade e sua ocorrência tem aumentado globalmente. Entre as doenças causadas por leveduras, a candidíase e criptococose são consideradas oportunísticas e ocorrem com maior frequência em todo o mundo. A resistência aos antifúngicos e a formação de biofilme são agravantes no tratamento de infecções fúngicas. O presente estudo teve como objetivo avaliar atividade antifúngica de extratos de plantas medicinais brasileiras frente a espécies de *Candida* e *Cryptococcus*. As plantas medicinais utilizadas no estudo foram *Myracrodruon urundeuva*, *Annona coriacea* e *Cochlospermum regium*. Para a determinação da concentração inibitória mínima foi utilizada a técnica de microdiluição em caldo. A susceptibilidade antimicrobiana frente a fármacos (fluconazol, nistatina e clorexidina) combinados com os extratos provenientes de *C. regium* e *M. urundeuva* foi realizada pelo método "*checkerboard*". A capacidade *C. regium* de inibir biofilmes de *Cryptococcus gattii* foi avaliada em microplacas de poliestireno de 96 poços. Os extratos etanólicos da casca e folhas de *A. coriacea* apresentaram atividade inibitória de *C. neoformans* e *C. gattii* na concentração de 1500 µg/mL. O extrato etanólico das folhas de *C. regium* apresentaram atividade antifúngica nas concentrações de 62,5 a 250 µg/mL e capacidade de reduzir a formação de biofilmes de *C. gattii*. O extrato aquoso da entrecasca de *M. urundeuva* apresentou atividade anti-*Candida albicans*. Os resultados obtidos auxiliam na descoberta de novos compostos bioativos como alternativas promissoras na terapia de infecções causadas por leveduras.

Palavras-chave: Infecções fúngicas, candidíase, criptococose, plantas medicinais

ABSTRACT

APOLONIO AAA. EXTRACTS FROM *Annona coriacea* MART. (ANNONACEAE), *Cochlospermum regium* (MART & SCHRANK) PILGER (BIXACEAE) AND *Myracrodruon urundeuva* ALLEMÃO (ANACARDIACEAE) WITH POTENTIAL FOR TREATMENT OF INFECTIONS CAUSED BY *Candida albicans* and *Cryptococcus* spp.

[PhD – Universidade Federal de Mato Grosso do Sul, Brazil].

Yeast infections have high rates of morbidity and mortality and their occurrence has increased globally. Among diseases caused by yeast, candidiasis and cryptococcosis are considered opportunistic and the most frequent around the world. Resistance to antifungals and biofilm formation are aggravating in the treatment of fungal infections. The present study aimed to evaluate the antifungal activity of extracts from Brazilian medicinal plants against *Candida* and *Cryptococcus* species. The medicinal plants used in the study were *Myracrodruon urundeuva*, *Annona coriacea* and *Cochlospermum regium*. For the determination of the minimum inhibitory concentration, the broth microdilution technique was used. The antimicrobial susceptibility of drugs (fluconazole, nystatin and chlorhexidine) combined with extracts from *C. regium* and *M. urundeuva* was performed by the checkerboard method. *C. regium* ability to inhibit *Cryptococcus gattii* biofilms was evaluated in 96-well polystyrene microplates. The ethanolic extracts of the bark and leaves of *A. coriacea* presented inhibitory activity of *C. neoformans* and *C. gattii* at the concentration of 1500 µg/mL. The ethanolic extract of the leaves of *C. regium* presented antifungal activity at concentrations of 62.5 to 250 µg/mL and capacity to reduce the formation of *C. gattii* biofilms. The aqueous extract from the *M. urundeuva* bark showed anti-*Candida albicans* activity.The results obtained helps in the discovery of new bioactive compounds as promising alternatives in the therapy of yeast infections.

Keywords: Fungal infections, candidiasis, cryptococcosis, medicinal plants

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1 INTRODUÇÃO

As doenças causadas por fungos leveduriformes, também chamadas de leveduroses, têm ganhado destaque nas últimas décadas por tratar-se de um problema emergente de saúde pública. As leveduroses podem ser causadas por leveduras presentes no ambiente, onde vivem no solo, plantas e animais, mas também por aquelas presentes na microbiota normal do ser humano (LACAZ et al., 2002).

Infecções sistêmicas causadas por leveduras do gênero *Candida* e *Cryptococcus* estão associadas a altas taxas de morbidade e mortalidade e sua ocorrência tem aumentado nos últimos anos (ALMEIDA et al., 2013; CHANG et al., 2008; PARK et al., 2009; TSUJISAKI et al., 2013). Este aumento pode ser devido a vários fatores, como condição imunológica do hospedeiro, mecanismos de virulência ou resistência aos antifúngicos. Candidíase e criptococose são consideradas oportunísticas e ocorrem com frequência em todo o mundo (CHEN et al., 2018; LIMA et al., 2017; RAUTEMAA-RICHARDSON; RICHARDSON, 2017; TSUJISAKI et al., 2013).

Leveduras do gênero *Candida* podem causar desde infecções que variam de superficiais, mucocutâneas e até sistêmicas (COLOMBO; GUIMARÃES, 2003). *Candida* spp. são os principais fungos relacionados a infecções hospitalares, como candidíase oral e/ou esofágica, candidúria e candidíase hematogênica (ALENCAR et al., 2017; ALMEIDA et al., 2013; CHANG et al. 2008; JABRA-RIZK et al., 2016; SULEYMAN; ALANGADEN, 2016).

A criptococose é uma doença de ocorrência mundial causada pelas leveduras encapsuladas dos complexos *Cryptococcus neoformans* e *C. gattii* (KWON-CHUNG et al. 2017). A doença é predominantemente oportunista, pois afeta pacientes imunocomprometidos, porém, o acometimento de imunocompetentes também tem sido descrito (DE AGUIAR et al., 2017; LIZARAZO et al, 2014). Estudos recentes mostraram que apesar de não reconhecida pela Organização Mundial da Saúde, criptococose é uma doença negligenciada e está relacionada a elevada taxa de mortalidade que varia de 20 e 60% em pacientes tratados e, atinge 100% em não tratados (ARMSTRONG-JAMES; MEINTJES; BROWN, 2014; MOLLOY et al., 2017).

Diferente do arsenal de antibióticos para o tratamento de infecções bacterianas, o número de antifúngicos disponíveis no comércio mundial é limitado. Atualmente, os principais agentes antifúngicos utilizados nos tratamentos de infecções causadas por

leveduras são das classes de azólicos, polienos, pirimidina fluorada (5-fluocitosina) e equinocandinas (COLOMBO et al., 2013; MORIO et al., 2017; PERFECT et al., 2010).

No entanto, estudos têm mostrado a emergência de leveduras resistentes a estes antifúngicos (ALENCAR et al., 2017; MATTOS et al., 2017; SILVA et al., 2016). *Candida albicans*, espécie mais frequentemente envolvida em infecções causadas por leveduras do gênero *Candida*, pode apresentar resistência aos azólicos (PERON et al., 2016; WHALEY et al., 2017). *Cryptococcus neoformans* é intrinsecamente resistente as equinocandinas (HUANG et al., 2016) e *C. gattii* pode apresentar heteroresistência ao fluconazol (VARMA; KWON-CHUNG, 2010). Portanto, o tratamento pode ficar limitado à polienos, que apesar de eficiente, são altamente tóxicos (LANIADO-LABORIN; CABRALES-VARGAS, 2009).

Candida spp. e *Cryptococcus* spp. têm a habilidade de formar biofilmes, comunidade microbiana incorporada em uma matriz extracelular que se liga a uma ampla gama de superfícies (DESAI; MITCHELL, 2015; MARTINEZ; CASADEVALL, 2015; FANNING; MITCHELL, 2012). A formação de biofilme é um agravante no tratamento de doenças causadas por essas leveduras, pois pode aumentar a resistência aos antifúngicos até 1000 vezes (RAMAGE et al., 2012).

Diante das dificuldades no tratamento de candidíase e criptococose, é de grande importância a busca por novos compostos com atividade antifúngica para minimizar as limitações das opções terapêuticas disponíveis. Uma alternativa promissora é o uso de plantas medicinais (SOLIMAN et al., 2017; ZHANG et al., 2017). Estudos têm mostrado que alguns compostos vegetais possuem atividade sinérgica *in vitro* com antifúngicos convencionais e apresentam redução na concentração de ambas substâncias (CASTRO et al., 2015; SHARIFZADEH et al., 2017). Também tem sido avaliada a atividade sobre biofilmes fúngicos e compostos provenientes de plantas podem reduzir ou inibir a formação de biofilmes (KUMARI et al., 2017; MANOHARAN; LEE; LEE, 2017).

Apesar da ampla diversidade de plantas na região Centro-Oeste, assim como outras regiões do Brasil, ainda são poucos os estudos que mostram a atividade antifúngica de plantas medicinais frente a *Candida* e *Cryptococcus*.

Assim, este estudo vem de encontro com a necessidade de avaliar novos compostos de origem vegetal com atividade antifúngica para o tratamento de candidíase e criptococose, bem como avaliar sua atividade sobre biofilmes fúngicos. No presente estudo foi avaliada a atividade antifúngica de *Myracrodruon urundeuva* Allemão

(Anacardiaceae), *Annona coriacea* Mart. (Annonaceae) e *Cochlospermum regium* (Mart & Schrank) Pilger (Bixaceae) frente a *C. albicans* e leveduras dos complexos de *C. neoformans* e *C. gattii*.

2 REVISÃO BIBLIOGRÁFICA

2.1 PRINCIPAIS DOENÇAS OPORTUNÍSTICAS CAUSADAS POR LEVEDURAS

A candidíase e a criptococose são doenças relacionadas principalmente com indivíduos com condições de imunodeficiência severa, como HIV, câncer e transplante de órgãos, que possuem maior risco de adquirir uma doença fúngica oportunística (COLOMBO et al., 2017; LINDENBERG et al., 2008; OLADELE et al., 2017).

2.1.1 CANDIDÍASE

Doenças causadas por leveduras do gênero *Candida*, também chamadas de candidíases, incluem infecções nas mucosas, pele, órgãos e corrente sanguínea. As espécies *C. albicans*, *C. tropicalis*, do complexo *C. parapsilosis*, do complexo *C. glabrata* e *C. krusei* representam importantes agentes em infecções em humanos (MILLS, 2017; TELLES; KARKI; MARSHALL, 2017; TURNER; BUTLER, 2014;).

Candida albicans é uma levedura comensal comumente encontrada na microbiota normal do trato gastrointestinal, mucosas e pele do ser humano e outras espécies de *Candida* podem ser residentes transitórias nestes sítios (LACAZ et al., 2002). No entanto, estas leveduras, diante de condições favoráveis como alterações nos mecanismos de defesa do hospedeiro, presença de dispositivos médicos como cateteres e sonda vesical, uso de antibióticos de amplo espectro e outras condições de risco, podem causar infecções oportunistas (CHANG et al., 2008; HAWKSHEAD et al., 2016; MATTOS et al., 2017). Miramón e Lorenz (2017) referem que mesmo em condições desfavoráveis como a deficiência de nutrientes, espécies de *Candida* podem se adaptar às mudanças ambientais dos hospedeiros rapidamente.

Entre todas as espécies de *Candida*, *C. albicans* é o agente infeccioso mais prevalente (CHANG et al., 2008; MATTOS et al., 2017). Estudo de revisão mostra que em casos de infecções sistêmicas causadas por esta espécie, a taxa de mortalidade associada é de aproximadamente 40% em infecções sistêmicas (DADAR et al., 2018). Isso provavelmente se deve ao fato da presença de diferentes fatores de virulência (formação de pseudo-hifas e hifas verdadeiras, enzimas hidrolíticas) (COTTIER; MU¨HLSCHLEGEL, 2009; GIOLO; SVIDZINSKI, 2010; KUMAMOTO; VINCES, 2005; MAYER; WILSON; HUBE, 2013; WHITEWAY; BACHEWICH, 2007).

2.1.2 CRIPTOCOCOSE

A criptococose é uma doença oportunística emergente com distribuição mundial e é causada por fungos do gênero *Cryptococcus*. Uma característica importante do gênero é a presença de cápsula composta por polissacarídeos complexos que são sintetizados dentro da célula fúngica, sendo constituída principalmente por glucuronoxilomanana. Esta cápsula desempenha papel importante na virulência, pois pode atuar como escudo na parede celular e evitar a fagocitose por macrófagos (O'MEARA; ALSPAUGH, 2012).

Os agentes etiológicos da criptococose, inicialmente foram agrupados em duas variedades que incluíam cinco sorotipos: *Cryptococcus neoformans* var. *neoformans* (sorotipos A, D e AD) e *Cryptococcus neoformans* var. *gattii* (sorotipos B e C) (KWON-CHUNG; POLACHECK; BENNETT, 1982). Com o avanço dos métodos moleculares, estas variedades foram reclassificadas como espécies distintas *C. neoformans* e *C. gattii* (KWON-CHUNG et al., 2002; KWON-CHUNG; VARMA, 2006).

Mas recentemente, estudo com genotipagem de *C. neoformans* e *C. gattii,* sugere que cada espécie é composta por pelo menos quatro subgrupos geneticamente diversos (MEYER et al., 2011). Diante dessa diversidade genética, Kwon-Chung et al. (2017) recomendam a utilização dos termos “espécies do complexo *C. neoformans*” e “espécies do complexo *C. gattii*”. Leveduras do complexo *C. neoformans* são responsáveis por doenças graves, relacionadas principalmente com pacientes portadores de HIV ou transplantados. Doenças causadas por espécies pertencentes ao complexo *C. gattii* são mais comuns em indivíduos imunocompetentes e costumam apresentar manifestações graves no sistema nervoso central como meningite, encefalite e meningoencefalite (FRANCO-PAREDES et al., 2015; PERFECT et al., 2010,).

Quanto à ecologia, espécies do complexo *C. neoformans* são globalmente distribuídas e relacionadas com fontes de excrementos de aves, especialmente pombos (*Columba livia*) e solo (KWON-CHUNG et al., 2014). Amostras positivas para estas espécies também podem ser encontradas em áreas frequentadas por galinhas, perus ou ocasionalmente outras espécies de aves, assim como já foi isolado de casca e troncos de árvores e madeira em decomposição (KWON-CHUNG et al., 2014; MITCHELL et al., 2011).

As espécies do complexo *C. gattii* têm sido associadas a espécies arbóreas, material em decomposição e em regiões tropicais e subtropicais (KIDD et al., 2004). Além disso, as espécies dos complexos *C. neoformans* e *C. gattii* podem sobreviver e replicar-se em amebas de vida livre (STEENBERGEN; SHUMAN; CASADEVALL, 2001), sugerindo que esses hospedeiros alternativos possam desempenhar um papel importante para determinar a distribuição e virulência de diferentes linhagens criptocócicas ao redor do mundo (MAY et al., 2016).

Com exceção de casos raros de transmissão iatrogênica (BADDLEY et al., 2011) ou zoonótica (LAGROU et al., 2005), normalmente a criptococose inicia-se com a inalação de células fúngicas (MAY et al., 2016). Uma vez que a levedura se instala nos pulmões, pode produzir criptococose pulmonar ou pneumonia, que pode variar desde quadros assintomáticos e sintomáticos, como febre, tosse, perda de peso e dores no peito (CHAYAKULKEEREE; PERFECT, 2006).

Entre as apresentações clínicas da criptococose, a meningoencefalite é a mais grave e com elevada taxa de mortalidade, principalmente entre indivíduos imunocomprometidos com aproximadamente 180.000 mortes por ano no mundo todo (RAJASINGHAM et al., 2017). A levedura tem tendência a disseminar-se a partir dos pulmões para o sistema nervoso central, onde existem grandes quantidades de catecolaminas, componente utilizado na produção de melanina pela levedura (BIVANCO; MACHADO; MARTINS, 2006). A infecção no sistema nervoso central causa inflamação do espaço subaracnóide e do parênquima cerebral, sendo esta a forma mais aguda da doença (CASADEVALL; PERFECT, 1998; STEENBERGEN; CASADEVALL, 2003).

Outra apresentação clínica da criptococose é a cutânea que pode apresentar lesões como nódulos, edemas, massas na região subcutânea, celulites, abscessos ou úlceras (BIVANCO; MACHADO; MARTINS, 2006; PASA et al., 2012). Além disso, casos de criptococcemia, presença de *Cryptococcus* no sangue, têm sido relatados na literatura (KANDULA et al., 2016; TSUJISAKI et al., 2013; YEUNG et al., 2016).

2.2 BIOFILME FÚNGICO

Uma grande variedade de fungos tem demonstrado a habilidade para colonizar superfícies e formar biofilmes. A formação de biofilmes está associada com infecção persistente, pois podem aumentar a resistência frente aos mecanismos de defesa do

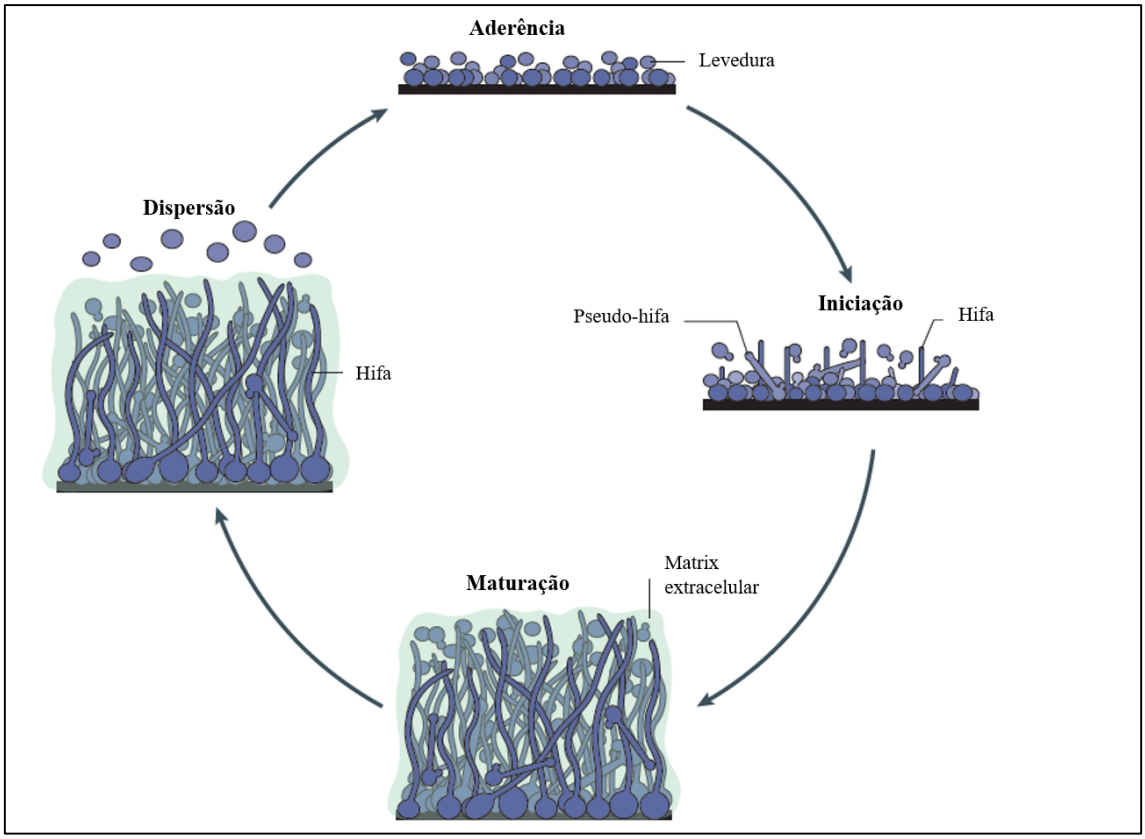
hospedeiro e as drogas antifúngicas (MARTINEZ; CASADEVALL, 2015; SILVA et al., 2017). De acordo com Fanning e Mitchell (2012), um biofilme é definido como uma comunidade microbiana altamente complexa e estruturada incorporada em uma matriz extracelular que se liga a uma ampla gama de superfícies.

Os avanços recentes em técnicas moleculares e microscopia confocal mostraram que a formação de biofilmes é a forma natural e preferida de crescimento microbiano e uma causa importante de infecções humanas persistentes (AKERS et al., 2014; COSTERTON; STEWART; GREENBERG, 1999). Leveduras importantes clinicamente são descritas como micro-organismos formadores de biofilme, entre elas as espécies de *Candida* e *Cryptococcus* (DESAI; MITCHELL, 2015; MARTINEZ; CASADEVALL, 2015).

Vários fatores contribuem para a formação de biofilme como fluxo do meio biológico, fatores imunológicos do hospedeiro e a presença de agentes antimicrobianos (SCORZONI et al., 2017). Resumidamente, a formação de biofilmes fúngicos é dividida em quatro estágios principais (Figuras 1 e 2): Aderência das células fúngicas a uma superfície; Iniciação da proliferação celular, onde formam uma camada basal de células de ancoragem; Maturação em um biofilme complexo e estruturado, no qual as células são encapsuladas pela matriz extracelular; e Dispersão de células leveduriformes do biofilme para semear novos locais (LOHSE et al., 2018; LOPES et al., 2017).

Os biofilmes de *C. albicans* apresentam uma morfologia complexa e são compostos por células leveduriformes, hifas e pseudo-hifas, necessárias para a formação de biofilmes (Figura 1). A matriz extracelular é composta por proteína, carboidrato, lipídeo, ácido nucleico e β-1,3-glucana (ZARNOWSKI et al., 2014). *Candida albicans* pode formar biofilmes em numerosas superfícies bióticas e abióticas (ANDES et al., 2004; DONGARI-BAGTZOGLOU et al., 2009). Também existem biofilmes, como na estomatite dentária, que a formação é resultado de uma combinação biótica (hospedeiro) com superfície abiótica (prótese) (NETT et al., 2010).

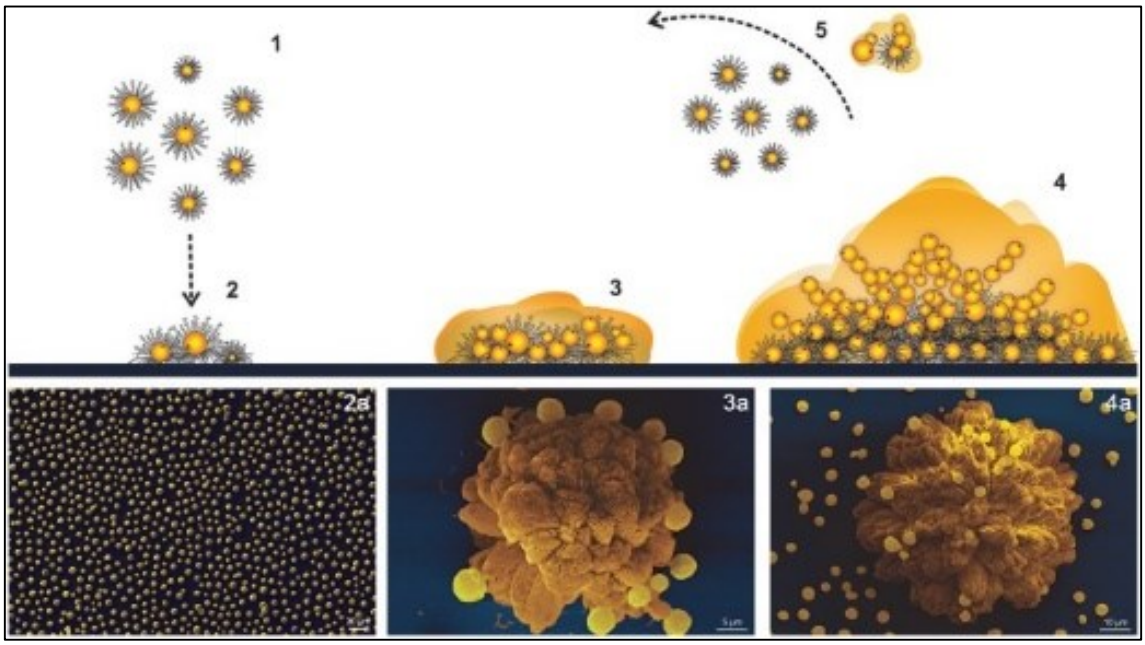
FIGURA 1. ESTÁGIOS DA FORMAÇÃO DE BIOFILME DE *Candida albicans*



Fonte: Adaptado de LOHSE et al. (2018).

Cryptococcus tem a capacidade de formar biofilmes em placas de poliestireno e nas superfícies de dispositivos médicos, como os "*shunts*" utilizados para tratar a hipertensão intracraniana (MARTINEZ; CASADEVALL, 2015). A matriz extracelular é composta por glucuronoxilomanana e açúcares como xilose, manose, glicose e galactoxilomanana (MARTINEZ; CASADEVALL, 2007) e tem a função de proteger as células do biofilme dos estressores circundantes, tanto no contexto da infecção como no ambiente (MARTINEZ; CASADEVALL, 2006; 2007; ALVAREZ; SAYLOR; CASADEVALL, 2008). Recentemente, Lopes et al. (2017) mostraram que os biofilmes de *C. neoformans* apresentam-se em aglomerados com estruturas amorfas e organizados em formato de flor no biofilme maduro (Figura 2).

FIGURA 2. ESTÁGIOS DA FORMAÇÃO DE BIOFILME DE *Cryptococcus neoformans*



Fonte: LOPES et al., 2017.

(1,2) Adesão de células planctônicas; (3) Expansão e modelagem; (4) Biofilme maduro tipo-flor; (5) Dispersão de microcolônias ou células planctônicas. (a) Microscopia eletrônica dos estágios do biofilme

2.3 TRATAMENTO DE INFECÇÕES CAUSADAS POR LEVEDURAS

Atualmente, as drogas antifúngicas disponíveis para o tratamento de candidíase são limitadas as classes: azóis, equinocandinas e polienos (ANTINORI et al., 2016; PAPPAS et al., 2015). O uso de equinocandinas e polienos é recomendado se o paciente apresentar exposição anterior aos azóis e se a infecção for grave para pacientes infectados com *C. glabrata*, uma espécie geralmente resistente a azóis (PAPPAS et al., 2015).

A escolha terapêutica para criptococose é realizada a partir dos antifúngicos anfotericina B (polieno) e fluconazol (azol) combinado ou não a 5-fluocitosina (pirimidina fluorada) (COELHO; CASADEVALL, 2016; MAY et al., 2016; PERFECT et al., 2010).

A anfotericina B e fluconazol são os fármacos de primeira escolha terapêutica. Estas duas classes de antifúngicos agem na membrana celular dos fungos, especificamente no ergosterol, um esterol importante da membrana plasmática fúngica, que é análogo ao colesterol em células de mamíferos. O ergosterol contribui para a

variedade das funções celulares, sendo importante para a fluidez, permeabilidade e a integridade da membrana e para o bom funcionamento de muitas enzimas ligadas à membrana (SCORZONI et al., 2017; TATSUMI et al., 2013). Resumidamente, os alvos das drogas antifúngicas são descritos no Quadro 1.

QUADRO 1. MECANISMO DE AÇÃO DAS CLASSES DE DROGAS ANTIFÚNGICAS

Classes	**Local de ação**	**Mecanismo de ação**
Azóis	Retículo endoplasmático	Inibição da biossíntese do ergosterol
Equinocandinas	Parede celular	Inibe a formação de (1→3)-β-D-glucana
Polienos	Membrana plasmática	Induz a instabilidade da membrana
Flucitosina	Núcleo	Inibe a síntese de RNA/DNA

Fonte: Morio et al. (2017).

Os azóis, que incluem fluconazol, itraconazol, voriconazol e posaconazol, possuem efeito fungistático que bloqueia a biosíntese do ergosterol, visando a enzima lanosterol 14α-desmetilase (relacionada ao gene ERG11) e resulta na inibição do crescimento e replicação da célula fúngica (KATHIRAVAN et al., 2012). Estes antifúngicos causam menos efeitos adversos que a anfotericina B, mas possuem menor potência antifúngica.

As equinocandinas (caspofungina, micafungina e anidulafungina) são antifúngicos fungicidas que atuam na inibição da produção de (1→3)-β-D-glucana, um componente essencial da parede celular fúngica. Contudo, o espectro de ação é menor do que os espectros dos azóis e polienos, pois é limitado a patógenos que dependem destes polímeros (CAMPOY; ADRIO, 2017; ODDS; BROWN; GOW, 2003).

Os polienos (anfotericina B e nistatina) exibem atividade fungicida e atuam na ligação ao ergosterol para formar um complexo capaz de perturbar a membrana criando poros que destroem o gradiente de prótons, o que resulta na saída do citoplasma e outros conteúdos celulares, resultando em morte celular rápida (ODDS; BROWN; GOW, 2003; SCORZONI et al., 2017). Além disso, anfotericina B é um antifúngico de amplo

espectro, mas seu uso é limitado devido ao alto grau de toxicidade. Nistatina, o agente antifúngico mais tóxico dentro da classe, tem sido usado para terapia tópica e localizada por causa dos efeitos adversos (CAMPOY; ADRIO, 2017).

A pirimidinas fluorada, flucitosina (5-fluorocitosina), tem como mecanismo de ação, sua conversão no antimetabólico 5-fluorouracil nas células fúngicas que consequentemente inibe a síntese de DNA (CAMPOY; ADRIO, 2017). No entanto, o início rápido da resistência impede o uso de flucitosina como monoterapia e, consequentemente, só é usado em combinação com outros antifúngicos como a anfotericina B (COELHO; CASADEVALL, 2016). Além disso, 5-FC não está disponível no comércio brasileiro.

2.4 EMERGÊNCIA DE *Candida* sp. E *Cryptococcus* sp. RESISTENTES AOS ANTIFÚNGICOS

Um fator preocupante relacionado à terapêutica da candidíase e criptococose é o surgimento de leveduras resistentes aos antifúngicos. A resistência antifúngica é um processo evolutivo baseado em seleção de micro-organismos que melhoram sua capacidade de sobrevivência para crescerem na presença de drogas (ANDERSON, 2005).

Apesar de não ser comum, *Candida* spp. têm apresentado resistência aos azóis (WHALEY et al., 2017). As infecções causadas por *C. albicans* estão associadas a níveis variáveis de resistência ao fluconazol e depende do tipo de infecção (BERBERI; NOUJEIM; AOUN, 2015; ENWURU et al., 2008; PERON et al., 2016; WHALEY et al., 2017).

A resistência aos azóis de *Cryptococcus* spp. é relatada com frequência (BASSO et al., 2015; BONGOMIN et al., 2018; MPOZA, RHEIN E ABASSI, 2017; SAIJO et al., 2014; SIONOV; CHANG; KWON-CHUNG., 2013) e no Brasil, estudos também têm descrito *Crytococcus* spp. resistentes aos antifúngicos (FAVALESSA et al., 2009; SILVA et al., 2008;), o que demonstra as taxas crescentes de resistência em *Cryptococcus* e as dificuldades clínicas no manejo da criptococose.

2.5 PLANTAS MEDICINAIS COM PROPRIEDADES ANTIFÚNGICAS

Como descrito anteriormente, a emergência de leveduras resistentes e a formação de biofilmes são fatores preocupantes e tem motivado a busca por novos antifúngicos na tentativa de suprir as limitações de drogas antifúngicas disponíveis para o tratamento de infecções causadas por leveduras. Neste sentido, estudos que avaliam o potencial de plantas para o tratamento de infecções fúngicas têm sido estimulados. Sabe-se que plantas medicinais produzem uma variedade de compostos com ação antimicrobiana (NEGRI et al., 2014).

No entanto, ainda são poucos estudos que avaliam a atividade antifúngica de plantas medicinais frente a espécies de *Candida* e *Cryptococcus*. Autores relatam a atividade antifúngica de extratos vegetais semelhante à atividade de anfotericina B (PEREIRA et al., 2014), sinergismo com antifúngicos convencionais (ENDO et al., 2010; SILVA et al., 2011; TOLEDO et al., 2015), frente a leveduras resistentes (SHINOBU-MESQUITA et al., 2015), ação durante a morfogênese (SILVA-ROCHA et al., 2017) e efeitos sobre fatores de virulência, como formação de tubo germinativo de *C. albicans* (SILVA et al., 2011; TAWEECHAISUPAPONG et al., 2005), atividade proteolítica (FREIRES et al., 2014) e formação de biofilmes (SARDI et al., 2017; TOLEDO et al., 2015).

Annona coriacea Mart. (Annonaceae) é uma árvore popularmente conhecida com araticum, araticum liso ou marolo, encontrada no cerrado brasileiro e utilizada popularmente para dermatite e depurativo (SOUZA et al., 2014). Pesquisas recentes demonstraram que esta planta possui atividades antifúngica (DE TOLEDO et al., 2011), antitumoral (FORMAGIO et al., 2015), antiprotozoária (DE TOLEDO et al., 2011; SIQUEIRA et al., 2011), analgésica e anti-inflamatória (SOUSA; DEL-VECHIO-VIEIRA; KAPLAN, 2007).

Cochlospermum regium (Schrank) Pilger (Bixaceae) é conhecida como algodãozinho ou algodãozinho-do-cerrado que pode ser encontrada no cerrado brasileiro, Paraguai e Bolívia. É uma planta medicinal indicada, popularmente, para o tratamento de várias doenças como: artrite, reumatismo, acne, infecções geniturinárias, infecções uterinas, infecções na próstata, colesterol, feridas internas e externas, laxante, depurativo do sangue (NUNES; SILVA; REZENDE, 2003; SOLON; BRANDÃO; SIQUEIRA, 2009). Leme et al. (2017) mostraram que o extrato das folhas de *C. regium*

possui atividade antimicrobiana e antibiofilme frente a *Escherichia coli* e *Candida tropicalis*.

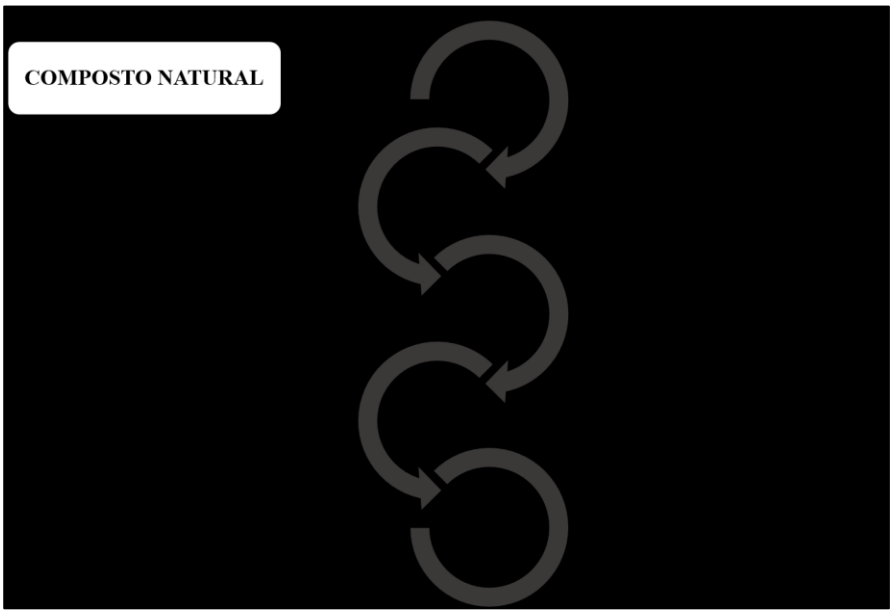
Myracrodruon urundeuva Allemão (Anarcadiaceae) é uma árvore conhecida popularmente como aroeira, aroeira-do-sertão ou urundeúva e pode ser encontrada no Brasil, Bolívia, Paraguai e Argentina (LORENZI; MATOS, 2008). A planta é utilizada pela população para o tratamento de inflamações, dores, infecções, corrimento vaginal, entre outras indicações (DE ALBUQUERQUE et al., 2007; CARTAXO; SOUZA; DE ALBUQUERQUE, 2010). Estudos descrevem diversas atividades biológicas da planta como antioxidante (SÁ et al., 2009), larvicida (SÁ et al., 2009) e antimicrobiana (JANDÚ et al., 2013; SÁ et al., 2009), especialmente sobre bactérias responsáveis por doenças periodontais (BIANCO et al., 2017; GAETTI-JARDIM JÚNIOR et al., 2011). Recentemente, Oliveira et al. (2017) apresentaram que os extratos da entrecasca de *M. urundeuva* possuem potencial para o tratamento de candidíase vulvovaginal.

2.6 ENSAIOS *in vitro* PARA NOVOS ANTIFÚNGICOS

O desenvolvimento de novos medicamentos antifúngicos envolve várias etapas e metodologias *in vitro* e *in vivo* para avaliação da eficácia e segurança de produtos naturais com potencial antifúngico (Scorzoni et al., 2016). Entre os ensaios *in vitro*, a avaliação da atividade antifúngica e a citotoxicidade são os primeiros passos da triagem. A Figura 3 representa todas as etapas *in vitro* que um produto natural deve atingir para ser um candidato para ensaios *in vivo* e posteriormente ser utilizado como um medicamento antifúngico.

Resumidamente, as principais etapas *in vitro* que são necessárias para um candidato promissor a antifúngico, incluem: Atividade antifúngica: Para determinar a concentração inibitória mínima de acordo com os métodos de micro ou macrodiluição em caldo padronizados pelo *Clinical and Laboratory Standards Institute*, com adaptações para produtos naturais. Citotoxicidade: para determinar a viabilidade das células na presença do composto a partir de sistemas celulares *in vitro* e corantes vitais. Índice de seletividade: para determinar se o composto pode ser um protótipo antifúngico de amplo espectro (SCORZONI et al., 2016).

FIGURA 3. ESQUEMA REPRESENTATIVO DAS ETAPAS *in vitro* PARA UM CANDIDATO A DROGA ANTIFÚNGICA



Fonte: Adaptado de Scorzoni et al. (2016).

Além desses ensaios preconizados, a combinação de dois ou mais antifúngicos pode ser uma alternativa para melhorar a terapia e reduzir a toxicidade. O método *in vitro* "*checkerboard*" tem sido amplamente utilizado para avaliar as combinações entre drogas para o tratamento de doenças (BESSA et al., 2018; MARTINEZ-IRUJO et al., 1996; SENGUPTA et al., 2017). Estudos recentes mostraram que a combinação de extratos vegetais com antifúngicos convencionais podem ser opções promissoras para o tratamento de doenças fúngicas (ENDO et al., 2010; SILVA et al., 2011; TOLEDO et al., 2015).

Outro ensaio importante é a avaliação do potencial mutagênico de um novo composto antes dos ensaios clínicos em humanos e sua comercialização. O teste de AMES é um ensaio de triagem que utiliza linhagens de *Salmonella* Typhimurium e detecta substâncias carcinogênicas genotóxicas (MARON; AMES, 1983). Este teste utiliza a fração microsomal S9 que simula um processo de metabolismo em mamíferos, garantindo maior sensibilidade ao teste (ESCOBAR et al., 2013). Além disso, o teste de AMES é um dos ensaios *in vitro* recomendado pela *International Conference On Harmonisation* para avaliar a genotoxicidade de novos fármacos para uso em humanos (ICH, 2012).

3. OBJETIVOS

3.1 OBJETIVO GERAL

Determinar atividade antifúngica de extratos de *Annona coriacea*, *Cochlospermum regium* e *Myracrodruon urundeuva* frente a espécies de *Candida albicans* e *Cryptococcus* spp.

3.2 OBJETIVOS ESPECÍFICOS

- Determinar atividade antifúngica de extratos etanólicos provenientes da casca e folhas de *Annona coriacea* Mart. frente a *Cryptococcus* spp.
- Investigar atividades citotóxica e mutagênica de extratos etanólicos provenientes da casca e folhas de *Annona coriacea* Mart.
- Determinar atividades antifúngica e antibiofilme de extrato etanólico das folhas de *Cochlospermum regium* (Schrank) Pilger frente a *Cryptococcus gattii*.
- Determinar atividade anti-*Candida albicans* de extrato aquoso de *Myracrodruon urundeuva* Allemão.
- Investigar atividade citotóxica de extrato aquoso de *Myracrodruon urundeuva* Allemão.

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5. APÊNDICES

5.1 ARTIGO 1: ATIVIDADE ANTIFÚNGICA DE EXTRATOS ETANÓLICOS DE *Annona coriacea* MART. FRENTE A AGENTES ETIOLÓGICOS DE CRIPTOCOCOSE

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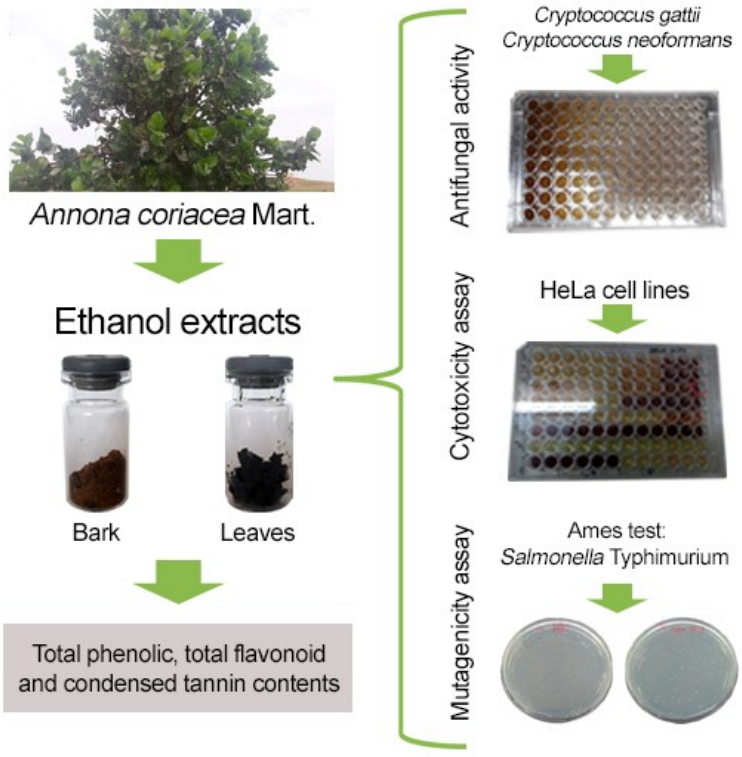
Antifungal activity of *Annona coriacea* Mart. ethanol extracts against the aetiological agents of cryptococcosis



Cryptococcosis is an opportunistic disease with a worldwide distribution. This disease is caused by fungi of the genus *Cryptococcus*, and its treatment is limited to several antifungals. In this study, the antifungal, cytotoxic and mutagenic properties of ethanol extracts from the bark and leaves of *Annona coriacea* were evaluated against the standard *Cryptococcus* species and clinical yeast specimens. Both extracts of *A.*

coriacea showed inhibitory activity of 1.5 mg/mL for all of the yeasts tested. The number of viable cells at the lowest tested concentration was 0.187 mg/mL. The extracts that were tested showed inhibitory activity and reduced the fungal growth of the *C. gattii* species and *C. neoformans* species complexes, suggesting that this plant may be an effective alternative treatment for cryptococcosis.

Keywords: *Cryptococcus neoformans*; *Cryptococcus gattii*; medicinal plant; antifungal activity

1. Introduction

Cryptococcosis is one of the most serious fungal diseases worldwide and afflicts not only immunocompromised individuals but also apparently immunocompetent individuals (Park et al. 2009). Cryptococcal meningitis is a disease considered neglected in HIV carriers (Armstrong-James et al. 2014), which causes over 600,000 deaths per year worldwide (Park et al. 2009). Caused by the *Cryptococcus neoformans* species and the *C. gattii* species complexes (Kwon-Chung et al. 2017), this infection presents substantial therapeutic challenges (Coelho and Casadevall 2016; Kwon-Chung et al. 2017).

The treatment of cryptococcosis is limited to few antifungals. The primary drugs recommended are amphotericin B or fluconazole, isolated or associated with 5-flucytosine (Coelho and Casadevall 2016). The antifungal treatment recommendations for cryptococcal meningoencephalitis indicate that the patients must receive prolonged treatment with fluconazole to eliminate the infection. In addition, the maintenance therapy period may be more than one year in HIV-infected individuals (Perfect et al. 2010).

The nephrotoxicity of amphotericin B (Laniado-Laborin and Cabrales-Vargas 2009) and the increasing number of fluconazole-resistant *Cryptococcus* isolates (Varma and Kwon-Chung 2010) stimulate the search for new drugs of synthetic or vegetal origin that are effective and have minimal toxicity for the control of cryptococcosis. *Annona coriacea* Mart. (Annonaceae) is a tree from the Brazilian Cerrado, popularly known as "*araticum*", "*araticum liso*" or "*marolo*", and recommended by popular medicine for dermatitis and depuration (Souza et al. 2014).

Cryptococcosis remains a challenging management issue with minimal new drug development or recent definitive studies (Perfect et al. 2010). Considering these

limitations, the aim of this study was to evaluate the antifungal properties of ethanol extracts from the bark and leaves of *Annona coriacea* against *Cryptococcus* species.

2. Results and Discussion

The phytochemical analysis of ethanol extracts from the bark and leaves of *Annona coriacea* demonstrated the presence of secondary metabolic compounds, such as total phenols, flavonoids and condensed tannins (Table S2). Chemical studies of the Annonaceae family show that alkaloids are the major chemical constituents detected in this family (Lúcio et al. 2015). The isoquinoline alkaloids can be found in large quantities in species of the genus *Annona* (Rabêlo et al. 2014) and may exhibit biological activities, such as antibacterial (Lall et al. 2017) and antitumour effects (Rinaldi et al. 2017).

Júnior et al. (2016) identified glycoside flavonoids, such as quercetin and luteolin, in hydroethanolic extracts of the leaves of *A. coriacea*. These compounds were also described in the literature as being present in other *Annona* species (Araújo et al. 2017; Formagio et al. 2013). These bioactive compounds are found in large quantities in some plants, and studies with other species of *Annona* show that these compounds may result in important biological activities, such as antimalarial (Pimenta et al. 2014) and anti-*Mycobacterium tuberculosis* (Araujo et al. 2014) activities.

The discovery of anti-cryptococcal compounds is important to assist the pharmaceutical industries in developing new antifungal drugs and increasing the chances of treating cryptococcosis. In this study, yeasts obtained from clinical specimens and from immunocompetent and HIV patients were used (Table S1). The antifungal activity of both *A. coriacea* ethanol extracts obtained by broth microdilution techniques presented minimum inhibitory concentrations (MIC) of 1.5 mg/mL for all of the yeast isolates tested. The ethanol extract from the bark resulted in fungicidal activity (1.5 mg/mL) against the two yeasts isolated from clinical samples - 476 (sputum) and 28 (cerebrospinal fluid) of the species of the complexes of *C. gattii* and *C. neoformans*, respectively (Figure S1). The ethanol extract from the leaves resulted in fungicidal activity (1.5 mg/mL) against two *C. gattii* species complexes from clinical specimens, 476 (sputum) and 32G (cerebrospinal fluid) (Figure S2).

The results obtained from the number of viable cells revealed that the lowest concentration tested (0.187 mg/mL) of both extracts reduced the growth of *Cryptococcus* and that their action was mostly fungistatic, rather than fungicidal. The

ethanol extract of the bark showed a reduction of up to ~ 1.4 $\log_{10}$ at the concentration of 0.187 mg/mL (i.e. reduced to ~ 10X the fungal growth when compared to the positive control) (Figure S1). There was no statistically significant difference ($p > 0.05$) between the lowest tested concentrations of both extracts (0.187, 0.375 and 0.75 mg/mL). However, there was a statistically significant difference ($p < 0.05$) when compared to the concentration of 1.5 mg/mL of the two extracts with the positive control and the other concentrations tested. A study carried out with five plants of the Annonaceae family showed antifungal activity against *Candida* spp. and *Cryptococcus neoformans* (Simo et al., 2017), but the minimum inhibitory concentrations of the plants evaluated against the latter yeast were higher (3.7-15 mg/mL) than those observed in our study.

In vitro cytotoxicity was evaluated in a HeLa cell culture that demonstrated 73.54% and 96.23% cell viability in a concentration of 0.25 mg/mL in the presence of ethanolic extracts of the bark and leaves of *A. coriacea*, respectively. At the concentration of 1 mg/mL, there was a reduction in the cell viability of 45.27% (bark) and 57.72% (leaves). Cell viability assays are performed to evaluate the use of new compounds for pharmaceutical applications. Capoci et al. (2015) have reported good results with the use of MTS tetrazolium because it is bioreduced by human epithelial cells in formazan, which is soluble in tissue culture medium. The ethanol extract from the leaves resulted in higher HeLa cell viability compared to the ethanol extract from the bark at the concentrations used.

The microsuspension method with *S.* Typhimurium strains is an important test recommended by the Organisation for Economic Co-operation and Development (OECD) to detect the mutagenic potential of new drugs (OECD 1997). The mutagenicity assay revealed that the ethanol extracts from the bark and leaves of *A. coriacea* have a low mutagenic potential (Table S3).

3. Conclusions

This study verified the antifungal activity of ethanol extracts from the bark and leaves of *A. coriacea* against *Cryptococcus*. The extracts tested resulted in inhibitory activity and reduced the fungal growth of the *C. gattii* species and the *C. neoformans* species complexes. Cytotoxicity and mutagenicity analyses revealed that the extracts had low cytotoxic and mutagenic potential and could be future alternatives for the treatment of cryptococcosis.

Supplementary material

Experimental details related to this article are available online in Tables S1-S4 and Figures S1 and S2.

Disclosure statement

The authors have no conflicts of interest to declare.

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SUPPLEMENTARY MATERIAL

Antifungal activity of *Annona coriacea* Mart. ethanol extracts against the aetiological agents of cryptococcosis

Experimental

Plant material

The bark and leaves of *Annona coriacea* were collected in the city of Dourados, Mato Grosso do Sul, Brazil, coordinates 22°08'21,53"S 55°08'28,04"O. The collection permit was issued by SISBIO, the Brazilian Biodiversity Authorisation and Information System under number 57730-1. Dr. Zefa Valdivina Pereira of the Herbarium of the Faculty of Biological and Environmental Sciences of the Federal University of Grande Dourados identified the samples. Voucher specimens were deposited in the same herbarium (registration DDMS 5419).

Preparation of ethanol extracts

To prepare the extracts, the plant material was dried in an oven with circulating air at 55 °C and pulverised in a knife mill. The extracts were obtained with 80% ethanol at room temperature. After 72 h, the extracts were filtered, subjected to concentration in a rotary evaporator and subsequently frozen and lyophilised. The extraction yield of the crude extracts was determined from the mass of the material prior to extraction and the mass of extract obtained after the removal of ethanol. The extraction yield was calculated in percentage after weighing.

Total phenolic content

The total phenolic content of the samples was determined using the Folin-Ciocalteau reagent (Djeridane et al. 2006). Briefly, 100 µL of extracts (100 µg/mL) were mixed with 500 µL Folin-Ciocalteau reagent and 1 mL distilled water for 1 min at room temperature. After mixing, 1.5 mL 20% sodium bicarbonate was added and incubated for 2 h in the dark at room temperature. The absorbance was measured at 760 nm using a spectrophotometer. The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram (mg/g) of extract. The methanol solution was used as a blank.

Total flavonoid content

The amount of total flavonoids in the extracts was measured spectrophotometrically as described previously (Lin and Tang 2007). Briefly, 500 µL of each extract (100 µg/mL) was mixed with 1.5 mL 95% ethanol, 0.10 mL 10% aluminium chloride (AlCl3.6H2O), 0.10 mL acetate potassium (CH_3COOK) (1 M), and 2.80 mL distilled water. After incubation for 40 min, the absorbance was measured at 415 nm using a spectrophotometer. To calculate the concentration of flavonoids, we prepared a calibration curve using quercetin as the standard. The flavonoid content was expressed as quercetin equivalents (QE) in milligrams per gram (mg/g) of extract.

Condensed tannin content

Condensed tannin concentrations were determined using the vanillin reaction described by Broadhurst and Jones (1978) and adapted by Agostini-Costa et al. (1999). Briefly, 1 mL of each extract (100 µg/mL) was mixed with 5 mL vanillin–HCl reagent (8% concentrated HCl in methanol and 4% vanillin in methanol) with stirring for 30 s. The absorbance at 490 nm was determined after 15 min. Catechin was used as the reference. The condensed tannin content was expressed as catechin equivalents (CE) in milligrams per gram (mg/g) of extract.

Evaluation of antifungal properties

Microorganisms

Six yeasts of the genus *Cryptococcus* were used in this study: two standard strains from the American Type Culture Collection (ATCC) and four clinical specimens isolated from sputum, cerebrospinal fluid and blood (Table S1). The yeasts were obtained from the collection of the Microbiological Research Laboratory of the Federal University of Mato Grosso do Sul, Campo Grande, Mato Grosso do Sul, Brazil.

Minimum inhibitory concentrations (MIC)

The minimum inhibitory concentration was determined by the broth microdilution technique according to the recommendations of the Clinical and Laboratory Standards Institute (2008) with certain adaptations for the use of natural products. The lyophilised extracts were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA), and successive dilutions (1:2) were subsequently performed in

RPMI-1640 medium (Sigma-Aldrich, USA) in 96-well microplates. The concentrations tested were as follows: 0.187, 0.375, 0.75 and 1.5 mg/mL.

After the yeast were grown in Sabouraud Dextrose Agar (SDA, Difco, USA) for 48 h at 35 °C, a suspension was prepared in sterile saline solution (0.85% NaCl), and the cell density was adjusted with a spectrophotometer at 530 nm with a transmittance of 90% ± 2%. Next, 1:50 and 1:20 dilutions were performed in RPMI-1640 media (Sigma-Aldrich, USA). Aliquots of each extract (100 µL) were dispensed into 96-well plates and further incubated with aliquots (100 µL) of the *Cryptococcus* species tested. The microplates were incubated for 48-72 h at 35 °C and the reading was performed visually. The MIC was defined as the lowest concentration of the extract that was able to inhibit 100% of fungal growth.

The number of viable cells was assessed by the determination of the number of colony forming units (CFUs) through several dilutions after 48 h incubation at 35 °C, and the number of colonies formed were counted. The results were presented as the total of CFUs (log CFUs), and the experiments were repeated in triplicate on three different occasions.

Cytotoxicity assay

The cytotoxicity was evaluated using HeLa cell lines (Henrietta Lacks, cervical adenocarcinoma cell line) (Capoci et al. 2015). HeLa cells were cultured in Dulbecco's Modified Eagle's medium (DMEM, Gibco, Waltham, USA) with 10% foetal bovine serum (FBS, Gibco, Waltham, USA). Cells at a concentration of 2 x 10^5 cells/mL were added to the 96-well microplates (Kasvi, Curitiba, Brazil) and incubated at 37 °C under 5% CO_2 for 24 h. After the incubation period, the adhered cells were washed, treated with different concentrations of the extracts (0.25, 0.5 and 1 mg/mL) and incubated under the same conditions. For growth control (white), the culture medium and the cell suspension were used.

Cell viability was evaluated based on the reduction of MTS (3-[4,5-dimethylthiazol-2-il]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium (Promega, Madison Charter Township, USA) (Malich et al. 1997). After 3 h incubation at 37 °C, the formazan absorbance was measured at a wavelength of 490 nm using an ASYS microplate reader (Biochrom, Holliston, USA). Optical density (OD) values were converted to percent cell viability by dividing the absorbance value of the sample by the

absorbance of the blank and multiplying the result by 100. The assays were performed in triplicate at three different times.

Mutagenicity assay

The mutagenicity was assessed by the microsuspension method developed by Maron and Ames (1983) with modifications described by Kado et al (1983) using extract concentrations of 0.125, 0.25, 0.5, 1 and 2 mg/plate and *Salmonella* Typhimurium strains TA98 and TA100. Mutagenic action was indicated by the reduction in the number of his+ revertants or as the background growth in the minimal glucose agar test plates. The influence of metabolic activation was tested by adding the S9 mix (mixture from several liver enzymes and cofactors).

Briefly, *S.* Typhimurium strains were cultured overnight, concentrated at 10^9 cells/mL per centrifugation (10000 RPM) and resuspended in sodium phosphate buffer [0.2 M, pH 7.4]. In test tubes, 50 µL sodium phosphate buffer or S9 mixture, 5 µL extract or positive and negative control and 50 µL bacterial suspension were added. The mixture was incubated for 90 minutes at 37 °C. After the incubation, 2 mL top agar supplemented with traces of histidine and biotin was added, homogenised and poured into Petri dishes containing minimal glucose agar. After the solidification of the top agar, the plates were incubated at 37 °C for 48 h. After this period, the revertant colonies were counted manually.

The positive controls used were 4-nitro-O-phenylenediamine (0.02 mg/plate) in the absence of S9 and 2-aminoanthracene (0.02 mg/plate) in the presence of S9 for the *S.* Typhimurium TA98 strain, and sodium azide (0.001 mg/plate) in the absence of S9 and 2-aminoanthracene (0.02 mg/plate) in the presence of S9 for the *S.* Typhimurium TA100 strain. DMSO (100 µL/plate) in the absence or presence of S9 was used as the negative control.

The extract concentration was expressed in units of mass/plate. The results were evaluated by the mutagenicity index (MI) according to the equation below:

MI = Number of revertants in the test sample / Number of revertants in the negative control

The sample was considered mutagenic when there was a significant increase in the number of induced revertants and the MI was higher or equal to two in at least one of the tested concentrations. The experiment was performed in triplicate.

Data analysis

The data were analysed using Prism 7.0 software (GraphPad, San Diego, CA, USA). One-way analysis of variance (ANOVA) test was used. All of the tests were performed with a confidence level of 95%. Values of $p \leq 0.05$ were considered to be statistically significant.

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Table S1. Profile of *Cryptococcus* species tested in the study.

Code/Yeasts	Species	Source	Antifungal susceptibility profile (MIC values µg/mL)				Patient with HIV?
			AMB	FLU	ITRA	VOR	
ATCC 56990	*C. gattii*	Cerebrospinal fluid	0.125	4	0.125	0.015	No
476	*C. gattii*	Sputum	0.5	8	0.125	0.125	Yes
32G	*C. gattii*	Cerebrospinal fluid	0.25	2	0.015	0.015	No
ATCC 32045	*C. neoformans*	Fermented fruit juice	0.25	2	0.06	0.125	Not applicable
28	*C. neoformans*	Cerebrospinal fluid	0.25	1	0.06	0.015	No
729	*C. neoformans*	Blood	0.5	2	0.125	0.03	Yes

Table S2. Contents of constituents of ethanol extracts from the bark and leaves of *Annona coriacea.*

Extracts	Total Phenols (mg GAE/g)	Flavonoids (mg QE/g)	Condensed Tannins (mg CE/g)
Bark	178.6	99.6	20.9
Leaves	139.3	79.7	13.4

GAE: Gallic acid equivalent; QE: Quercetin equivalent; CE: Catechin equivalent.

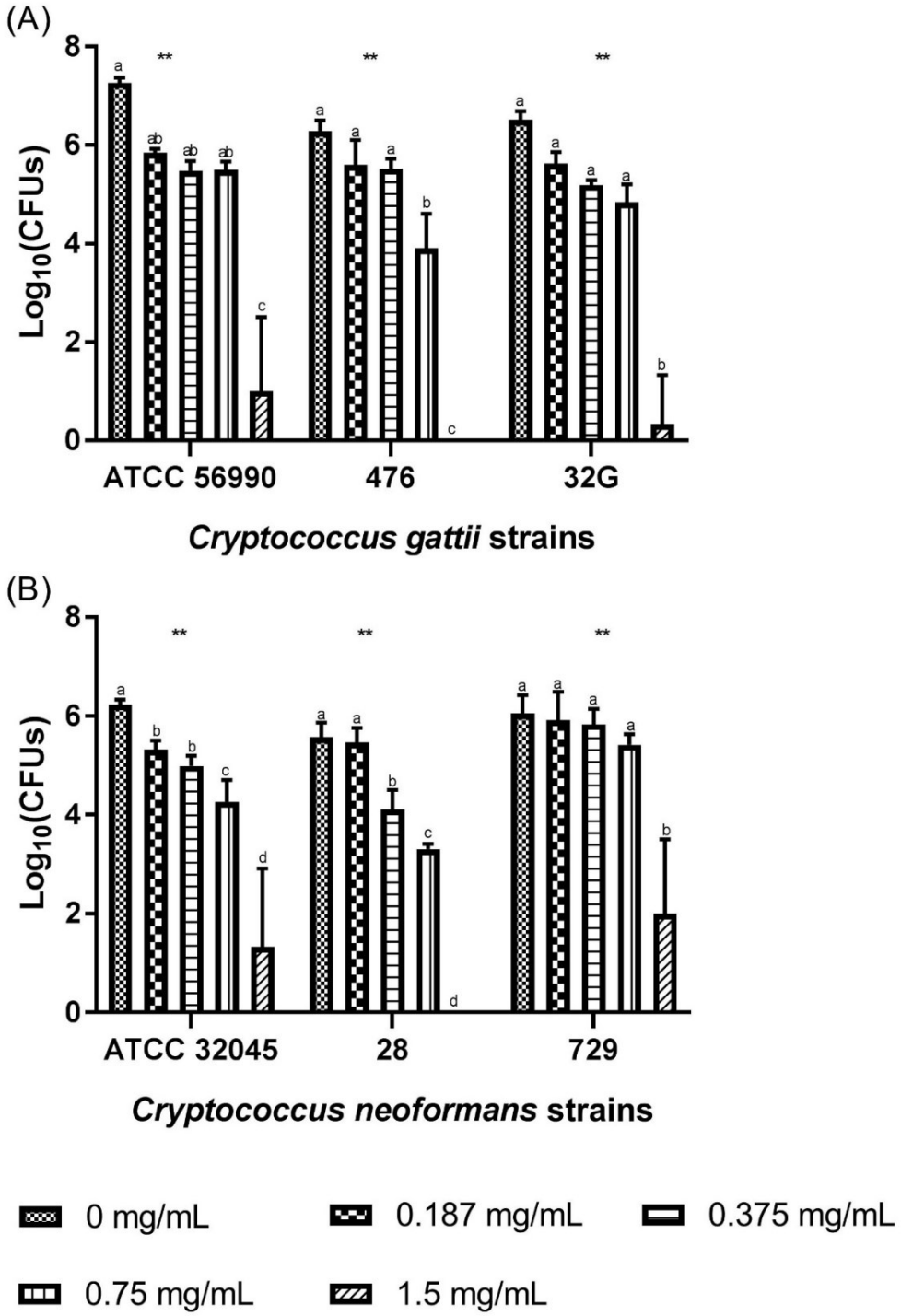

Figure S1. Logarithm of the number of colony forming units (CFUs) of different strains of *Cryptococcus gattii* (A) and *C. neoformans* (B) cultured within different concentrations of the ethanolic extract from the bark of *Annona coriacea*. Error bars represent standard deviations (SD). (**$p < 0.05$), extract concentrations results that are significantly different. In each strain, different letters indicate significant differences ($p < 0.05$).

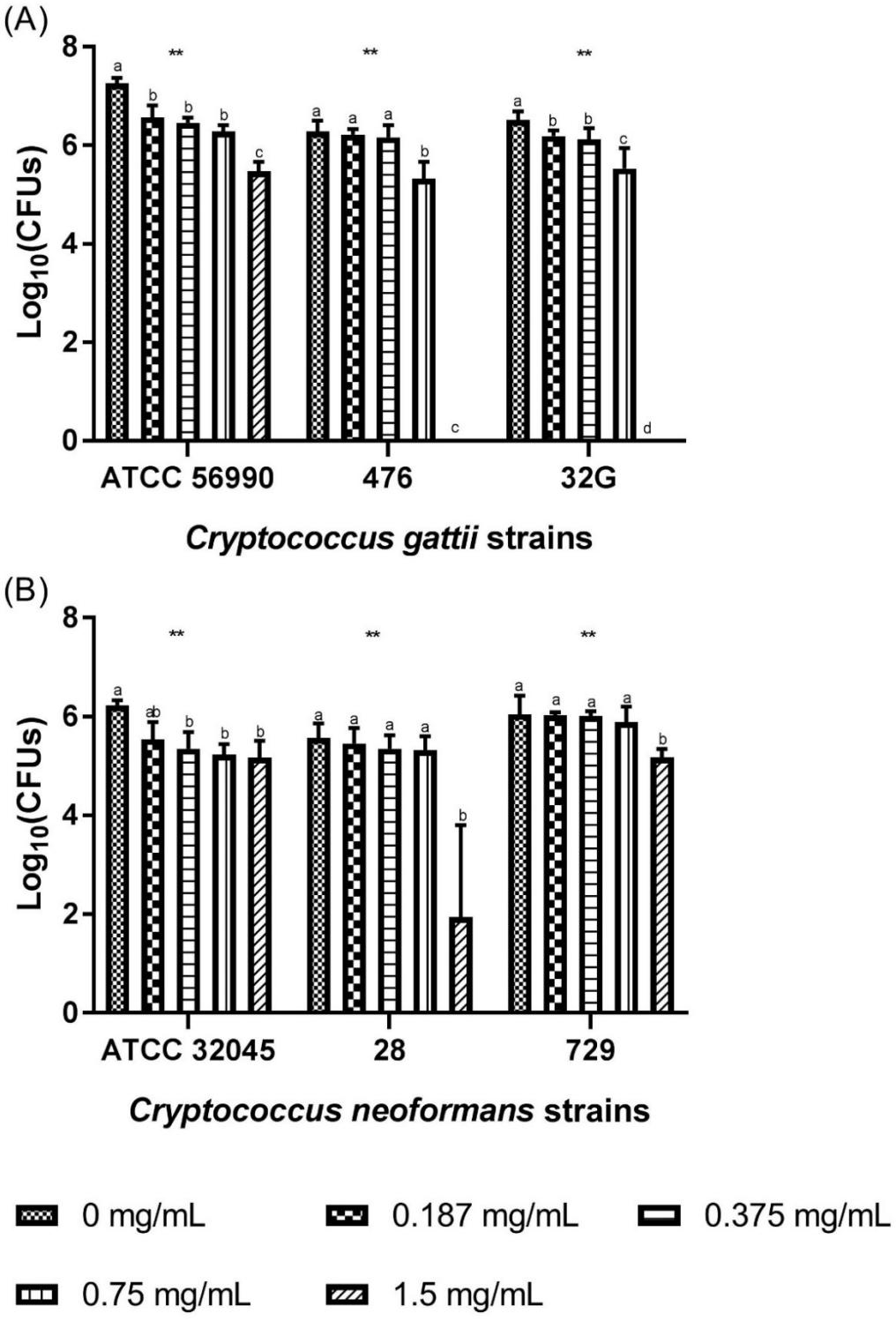

Figure S2. Logarithm of the number of colony forming units (CFUs) of different strains of *Cryptococcus gattii* (A) and *C. neoformans* (B) cultured within different concentrations of the ethanolic extract from the leaves of *Annona coriacea*. Error bars represent standard deviations (SD). (**$p < 0.05$), extract concentrations results that are significantly different. In each strain, different letters indicate significant differences ($p < 0.05$).

Table S3. Mutagenic potential expressed by the mean revertants / plate and mutagenicity index (IM) of the ethanolic extract of the bark of *Anonna coriacea* in the TA 98 and TA 100 strains of *S.* Typhimurium in the presence (+ S9) and absence (S9) of metabolic activation.

Treatment	TA 98		TA 100	
(µg/plate)	S9 -	S9 +	S9 -	S9 +
0 [a]	45,6 ± 4	22,3 ± 6	160 ± 19	126,5 ± 18
125	35 ± 3 (0,76)	33,7 ± 1 (1,5)	142 ± 9 (0,88)	151,5 ± 24 (1,19)
250	38 ± 4 (0,83)	40,3 ± 8 (1,80)	191 ± 53 (1,19)	153,5 ± 5 (1,21)
500	52,7 ± 11 (1,15)	38,3 ± 1 (1,71)*	159 ± 12 (0,99)	131 ± 3 (1,03)
1000	41,3 ± 6 (0,90)	38,3 ± 2 (1,71)*	189,5 ± 4 (1,18)	142,5 ± 3 (1,12)
2000	46 ± 1 (0,98)	45,3 ± 4 (2,0)*	192,5 ± 18 (1,2)	147,5 ± 5 (1,16)
C+	294 ± 2[b]	300 ± 2[c]	500 ± 10[d]	525 ± 10[c]

[a]Negative control: DMSO; Positive control (C +): [b]4-nitro-o-phenylenediamine (10 µg/plate); [c]2-aminoanthracene (1.5 µg/plate); [d]sodium azide (2.5 µg/plate). *$p < 0.05$; **$p < 0.01$ (ANOVA).

Table S4. Mutagenic potential expressed by the mean revertants / plate and mutagenicity index (IM) of the ethanolic extract of the leaves of *Anonna coriacea* in the TA 98 and TA 100 strains of *S. typhimurium* in the presence (+ S9) and absence (S9) of metabolic activation.

Treatment	TA 98		TA 100	
(µg/plate)	S9 -	S9 +	S9 -	S9 +
0.0 [a]	45,6 ± 4	24,6 ± 4	90,3 ± 29	131,6 ± 13
125	24,3 ± 4 (0,53)	33 ± 2 (1,34)	77,3 ± 13 (0,85)	100,3 ± 14 (0,76)
250	30 ± 1 (0,65)	38,3 ± 3 (1,55)*	99,3 ± 4 (1,28)	127 ± 2 (0,96)
500	35,3 ± 5 (0,77)	40 ± 5 (1,62)*	114,3 ± 2 (1,26)	137,6 ± 2 (1,04)
1000	40 ± 6 (0,87)	48,6 ± 6 (1,97)*	70,7 ± 2 (0,78)	113 ± 10 (0,85)
2000	41,6 ± 5 (1,55)	51 ± 3 (2,0)**	136 ± 10 (1,5)	108,6 ± 4 (0,82)
C+	294 ± 2[b]	300 ± 2[c]	500 ± 10[d]	525 ± 10[c]

[a]Negative control: DMSO; Positive control (C +): [b]4-nitro-o-phenylenediamine (10 µg/plate); [c]2-aminoanthracene (1,5 µg/plate); [d]sodium azide (2,5 µg/plate). *$p < 0.05$; **$p < 0.01$ (ANOVA).

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SHORT COMMUNICATION

Antifungal activity of *Annona coriacea* Mart. ethanol extracts against the aetiological agents of cryptococcosis

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ABSTRACT

Cryptococcosis is an opportunistic disease with a worldwide distribution. This disease is caused by fungi of the genus *Cryptococcus*, and its treatment is limited to several antifungals. In this study, the antifungal, cytotoxic and mutagenic properties of ethanol extracts from the bark and leaves of *Annona coriacea* were evaluated against the standard *Cryptococcus* species and clinical yeast specimens. Both extracts of *A. coriacea* showed inhibitory activity of 1.5 mg/mL for all of the yeasts tested. The number of viable cells at the lowest tested concentration was 0.187 mg/mL. The extracts that were tested showed inhibitory activity and reduced the fungal growth of the *Cryptococcus gattii* species and *Cryptococcus neoformans* species complexes, suggesting that this plant may be an effective alternative treatment for cryptococcosis.

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KEYWORDS
Cryptococcus neoformans; *Cryptococcus gattii*; medicinal plant; antifungal activity

5.2 MANUSCRITO 1: EXTRATO ETANÓLICO DAS FOLHAS DE *Cochlospermum regium* (SCHRANK) PILGER É CAPAZ DE CONTROLAR BIOFILME FORMADO POR *Cryptococcus gattii*

Este manuscrito foi aceito para publicação na revista The Scientific World Journal (Qualis CAPES: B1 na área interdisciplinar; Fator de Impacto: 1.55) e está formatado de acordo com as normas exigidas pela revista.

Control of *Cryptococcus gattii* biofilms by an ethanolic extract of *Cochlospermum regium* (Schrank) Pilger leaves

Abstract

Cryptococcus gattii is an etiologic agent of cryptococcosis, a serious disease that affects immunocompromised and immunocompetent patients worldwide. The therapeutic arsenal used to treat cryptococcosis is limited to a few antifungal agents, and the ability of *C. gattii* to form biofilms may hinder treatment and decrease its susceptibility to antifungal agents. The objective of this study was to evaluate the antifungal and antibiofilm activities of an ethanolic extract of *Cochlospermum regium* (Schrank) Pilger leaves against *C. gattii*. The antifungal activity was assessed by measuring the minimum inhibitory concentration using the broth microdilution technique. The antibiofilm activity of the extract was evaluated in 96-well polystyrene microplates, and the biofilms were quantified by counting colony forming units. The extract showed antifungal activity at concentrations of 62.5 to 250 μg/mL. The antibiofilm activity of the extract against *C. gattii* was observed both during biofilm formation and on an already established biofilm. The results showed that the ethanolic extract of the leaves of *C. regium* shows promise for the development of antifungal drugs to treat cryptococcosis and to combat *C. gattii* biofilms.

Keywords: *Cochlospermum*, *Cryptococcus gattii*, herbal therapy, biofilm, plant extracts.

Introduction

Cryptococcosis is a fungal infection that occurs worldwide. Although predominantly the result of opportunistic infections of immunocompromised patients, the incidence of infections among immunocompetent individuals is increasing [1-4]. Recently, studies have shown that cryptococcosis is a neglected tropical disease,

although it is not recognized as such by the World Health Organization [5,6]. Some factors that contributed to this conclusion included high mortality rates in treated patients (ranging from 20 to 60%) that can reach 100% in untreated patients [6].

Despite the importance of this disease, the treatment of cryptococcosis is limited to the antifungals fluconazole and amphotericin B, which are used alone or in combination with 5-flucytosine [7]. The primary etiological agents of cryptococcosis are species of the complexes *Cryptococcus neoformans* and *C. gattii* [8], which respond differently to the treatment established for meningoencephalitis [9]. *C. gattii* is not only more clinically aggressive but also more difficult to control. Species of the *C. gattii* complex can compromise immunocompetent individuals and cause severe diseases of the central nervous system, such as meningitis, encephalitis and meningoencephalitis [9]. In addition, lesions and long-term sequelae more commonly result from infections caused by species of the *C. gattii* complex than those caused by species of the *C. neoformans* complex [10]. Despite this disparity in symptoms, studies on possible new antifungals are primarily aimed at *C. neoformans* [11].

Species of the *C. gattii* complex have some attributes that can hinder antifungal therapy, such as heteroresistance to fluconazole [12]. In these cases, the treatment is limited to amphotericin B, which, although efficient, is highly toxic [13]. Another factor that can hinder antifungal therapies is the polysaccharide capsule, which, particularly for *C. gattii*, helps fungi escape the immune system [14] and decreases their susceptibility to antifungal agents [15]. In addition, the ability to form biofilms on host cells or medical devices, which is also promoted by the capsule, is a major factor associated with the high resistance of *Cryptococcus* spp. to antifungal drugs and host defense mechanisms [16].

In this context, it is of great importance to identify compounds with anti-*Cryptococcus* activity to aid in the treatment of cryptococcosis and alleviate the limitations of the current options. A promising alternative is the use of medicinal plants. Recently, Kumari et al. [17] evaluated the action of six essential oils extracted from medicinal plants on biofilms formed by *Cryptococcus* species, but these authors did not include isolates of *C. gattii*.

Cochlospermum regium (Schrank) Pilger, also known as yellow cotton tree, is a shrub found in the Brazilian cerrado, Paraguay and Bolivia. It is a medicinal plant that is popularly indicated for the treatment of various diseases [18]. Recently, our research group showed antimicrobial and antibiofilm activity of a *C. regium* leaf extract against

Escherichia coli and *Candida tropicalis* [19]. In this context, the aims of the present study were to evaluate the antifungal and antibiofilm activities of an ethanolic extract of *C. regium* leaves (Schrank) Pilger against *C. gattii*.

Materials and Methods

Plant material

The leaves of *C. regium* (Schrank) P. were collected in the cerrado of Mato Grosso do Sul state (22°08'47.2"S; 054°54'54.1"W) under authorization number 57730-1 of the Biodiversity Authorization and Information System (SISBIO). The plant was identified in the herbarium of the Faculty of Biological and Environmental Sciences of the Federal University of Grande Dourados by Prof. Dr. Zefa Valdivina Pereira. A sample of the species was deposited in the herbarium under DDMS registration 5001.

Production of a crude plant extract

The leaves were dried in a circulating air oven at 30 °C and pulverized in a blade mill. The crude extract of the leaves of *C. regium* (ECR) was obtained by mixing 200 g of the powdered plant material with 1000 mL of 95% ethanol and maintaining the mixture at 25 °C for 72 h with shaking every 12 h. The obtained vegetal extract was rotoevaporated (Rotavapor R - 215, Buchi) at 35 °C until complete volatilization of the solvent and then lyophilized using an EC MicroModulyo system coupled to a Savant VLP80 ValuPump vacuum pump.

Cryptococcus gattii isolates and growth conditions

For the antifungal susceptibility and checkerboard assays, three *C. gattii* strains were assayed, including two isolated from sputum specimens (476 and 2164) and one from cerebrospinal fluid (CSF; 32G), obtained from the Microbiological Research Laboratory of the Federal University of Mato Grosso do Sul, Campo Grande, Mato Grosso do Sul, Brazil. A reference strain, *C. gattii* (ATCC 56990, Rockville, MD, USA) was included in all assays.

In each experiment, the yeasts were subcultured in Sabouraud Dextrose Agar (SDA; Sigma-Aldrich) for 48 h at 35 °C. For antifungal susceptibility and checkerboard assays, the density of yeast cells suspended in a saline solution (0.85%) was adjusted to a transmittance of 88% at 530 nm using a spectrophotometer. Next, 1:50 and 1:20 cell dilutions were made in RPMI-1640 medium (Roswell Park Memorial Institute, Sigma-

Aldrich) containing L-glutamine, buffered with 0.165 M MOPS (3-(N-morpholino)propanesulfonic acid, Sigma-Aldrich) and supplemented with 2% glucose.

Antifungal susceptibility assay on planktonic *C. gattii* cells

The antifungal activity of ECR was evaluated using the microdilution technique in broth, according to the standards of the *Clinical and Laboratory Standards Institute* (CLSI, M27-A3) [20], with some modifications for natural products.

The assay was performed in 96-well flat-bottom microplates (Kasvi). The ECR concentrations assayed were 1.95, 3.90, 7.81, 15.62, 31.25, 62.50, 125, 250, 500 and 1000 μg/mL, and the microplates were incubated for 48-72 h at 35 °C.

Minimum inhibitory concentrations (MICs) were determined as the lowest ECR concentration that prevented visible growth of *C. gattii* after incubation. Fluconazole was used as a control, and the assay was performed in duplicate at two different times.

***In vitro* "checkerboard" assay on planktonic *C. gattii* cells**

An assay to evaluate the combined effect of ECR and fluconazole was performed based on the CLSI M27-A3 [20]. The final concentrations assayed were 3.9-500 μg/mL for ECR and 0.5-64 μg/mL for fluconazole. Together, the drugs formed a matrix of combinations of different concentrations in 96-well flat-bottom microplates, which were incubated for 48-72 h at 35 °C.

The interpretation of results was based on the fractional inhibitory concentration index (FICI), defined as follows: FICI=(ECR MIC in combination/ECR ECM alone)+(MIC of fluconazole in combination/MIC of fluconazole alone). The antifungal activity of the extract and the antifungal combination was interpreted as synergistic ($FICI \leq 0.5$), additive effect ($0.5 < FICI < 1$), indifferent ($1 \leq FICI < 4$) or antagonist ($FICI \geq 4$) [21].

Evaluation of the antibiofilm activity of ECR

Growth conditions for C. gattii ATCC 56990. For the ECR activity assays on biofilms, *C. gattii* ATCC 56990 was cultured in Sabouraud dextrose broth (HiMedia) for 24 h at 30 °C with shaking. The cells were collected by centrifugation at 5000 g for five min, washed three times with phosphate-buffered saline (PBS) and resuspended in RMPI-1640 medium. Using a Neubauer chamber, the cell density was adjusted to $1x10^8$ colony forming units (CFUs)/mL [22].

Activity of ECR on biofilm formation by C. gattii ATCC 56990. The effect of ECR on biofilm formation was assessed according to Martinez and Casadevall [22], with minor modifications. The assay was performed in 96-well polystyrene polystyrene microplates, with 2.5, 5 or 10 mg/mL of ECR added simultaneously to wells with the yeast. To form biofilms, the microplates were incubated at 35 °C for 48 h with shaking. A positive control (fungal cells and broth) and a negative control (broth only) were included. *C. gattii* biofilms were washed three times with 0.05% Tween 20 in Tris-phosphate buffer (TBS) to remove the non-adherent cryptococcal cells. The biofilms were characterized by the *C. gattii* viability assay to determine the CFUs, and the experiment was conducted in triplicate.

Activity of ECR on pre-formed biofilms. The effect of ECR on pre-formed biofilms was evaluated according to Martinez and Casadevall [22], with minor modifications. The assay was performed in 96-well bottom polystyrene microplates, with *C. gattii* ATCC 56990 added in RPMI-1640 broth. To form biofilms, the microplates were incubated at 35 °C for 48 h with shaking. *C. gattii* biofilms were carefully washed three times with 0.05% Tween 20 in TBS to remove the non-adherent cryptococcal cells. After washing, the biofilms were treated with 2.5, 5 or 10 mg/mL ECR. The microplates were incubated again at 35 °C for 48 h with shaking. Next, the biofilms were washed three times with 0.05% Tween 20 in TBS to remove the non-adherent cryptococcal cells. A positive control (fungal cells and broth) and a negative control (broth only) were included. The biofilms were characterized by the *C. gattii* viability assay to determine the CFUs, and the experiment was conducted in triplicate.

Viability of C. gattii cells in a biofilm after being treated with ECR. The viability of *C. gattii* strains was determined in each of the biofilm assays described above. After the biofilms were scraped from the wells of the microplates, the resulting cell suspensions were vigorously vortexed for 5 min to disaggregate the cells. Serial dilutions (in PBS) of each cell suspension were prepared and plated by the ASD drop plate technique [23]. Plates were incubated at 35 °C for 24-48 h. After incubation, the CFUs per unit area (Log_{10} CFU/cm^2) of microtiter plate well were enumerated.

Statistical analysis

The results were evaluated by ANOVA, and the means were compared by Tukey's post-test. A value of $p<0.05$ was considered significant for all evaluations.

Statistical and graphical analyses were performed using GraphPad Prism® 7.0 (GraphPad Software, San Diego, CA, USA).

Results

The MIC results for ECR against *C. gattii* ATCC 56990 and three clinical *C. gattii* isolates (two from sputum and one from CSF) are presented in Table 1. All strains were inhibited by ECR, with MICs ranging from 62.5 to 250 µg/mL.

Table 1. Antifungal activity of extract of *C. regium*, alone and in combination with fluconazole, against planktonic *Cryptococcus gattii* cells.

Cryptococcus gattii	**Source**	**MIC ECR (µg/mL)**		**MIC Fluconazole (µg/mL)**		**FICI**	**Interpretation**
		Alone	Combination	Alone	Combination		
32G	CSF	62.5	31.25	2	1	1	Indifferent
476	Sputum	125	31.25	8	8	1.25	Indifferent
2164	Sputum	125	62.5	2	1	1	Indifferent
ATCC 56990	Sputum	250	125	4	2	1	Indifferent

ECR: Ethanolic extract from leaves of *Cochlospermum regium*; MIC: Minimum inhibitory concentration; FICI: Fractional inhibitory concentration index; CSF: Cerebrospinal fluid.

When ECR was evaluated in combination with fluconazole, *C. gattii* was inhibited at sub-MIC levels. However, according to the observed FICI values (1 to 2.5), the combination of the two antifungal drugs was not different from the drugs alone (Table 1).

The activity of ECR on *C. gattii* ATCC 56990 biofilms is presented in Figure 1a (during biofilm formation) and 1b (pre-formed biofilm) by determining the number of viable cells (in Log_{10} CFU/cm^2). When comparing *C. gattii* ATCC 56990 biofilm formation in the presence and absence of ECR, the extract reduced the amount of biofilm formed by 25.33 to 42.58% in a dose-dependent manner, with a maximum observed reduction of 2 log_{10}. For pre-formed biofilms, ECR reduced the amount of biofilm by 7.13 to 15.44% (Table 2).

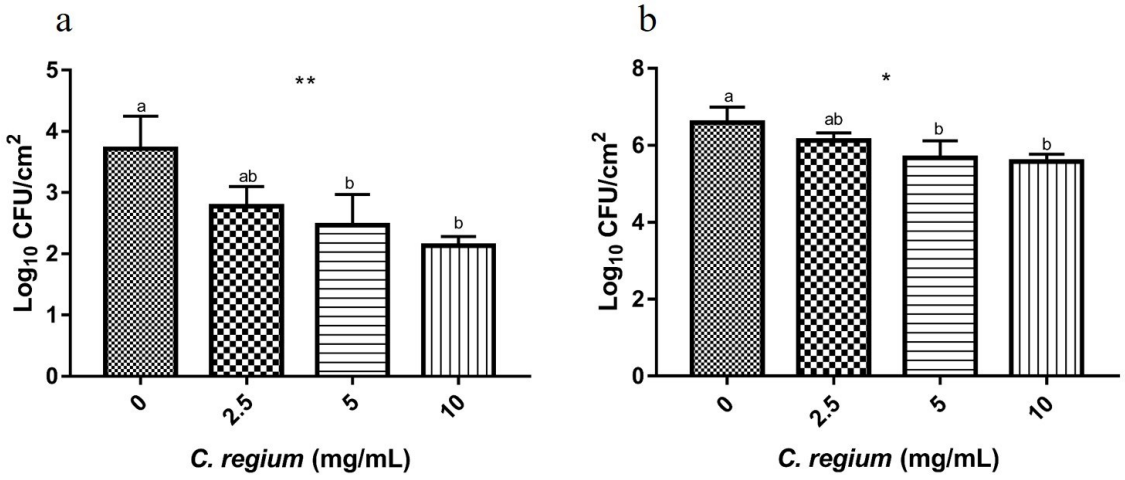

Figure 1. Activity of extract of *C. regium* against *Cryptococcus gattii* (ATCC 56990) biofilms. (a) Evaluation of ECR activity during biofilm formation by ANOVA and Tukey's post-test. ** (p<0.05) indicates a significant difference. Different letters over each column indicate a significant difference between samples (p<0.05). (b) Evaluation of ECR activity on pre-formed biofilms by ANOVA and Tukey's post-test. * (p<0.05) indicates a significant difference. Different letters over each column indicate a significant difference between samples (p<0.05).

Table 2. Effect of extract of the leaves of *C. regium* treatment on *Cryptococcus gattii* (ATCC 56990) biofilms.

ECR concentration (mg/mL)	During biofilm formation		Pre-formed biofilm	
	(Log_{10} CFU/cm^2)	% reduction	(Log_{10} CFU/cm^2)	% reduction
0	3.71	0	6.59	0
2	2.77	25.33	6.12	7.13
5	2.46	33.69	5.67	13.96
10	2.13	42.58	5.57	15.44

ECR: Ethanolic extract from leaves of *Cochlospermum regium.*

Discussion

C. gattii is a primary etiological agent of cryptococcosis and is associated with severe and fatal cases of this disease. This encapsulated yeast can cause serious disease symptoms in humans and cause high levels of mortality. Unlike *C. neoformans*, which

primarily infects immunocompromised patients, *C. gattii* is more often associated with infections of immunocompetent individuals [14].

In Brazil and in other countries where 5-flucytosine is not available, the treatment of *C. gattii*-induced cryptococcosis is limited to two drugs, fluconazole and amphotericin B, which are frequently used in combination. However, this therapeutic choice has been questioned, since these two drugs may have variable interactions, from synergistic to antagonistic [24]. In addition, preventative measures for this disease are non-existent, since it is caused by environmental yeasts that affect humans, primarily by inhalation. Thus, the transmission and virulence potential of this yeast combine to produce high mortality rates, justifying the search for new treatment or control options for this serious infection.

Among the attributes of *Cryptococcus* spp. that contribute to their virulence, the ability to form biofilms on abiotic surfaces is recognized as an important factor [25] and may help these environmental yeasts enter the human body. Thus, new therapeutic options aimed at preventing biofilm formation by cryptococcosis-causing fungi, such as *C. gattii*, would be useful in the prophylaxis of this disease but are currently unavailable, to the best of our knowledge. Unfortunately, neither fluconazole [22] nor amphotericin B [25] perform well on biofilms containing *Cryptococcus* spp.

The search for new compounds with anti-*Cryptococcus* properties is of great importance to maximize treatment options and prevent cryptococcosis. The results obtained in this study show that ECR could be a promising candidate to treat cryptococcosis caused by species of the *C. gattii* complex, since the extract presented MICs of 62 to 250 μg/mL against the *C. gattii* species assayed, exhibiting good to moderate antifungal activity [26].

Considering our previous findings on the antimicrobial activity of ECR against other pathogenic microorganisms [19], we believe this extract is even more promising for the treatment of hospital and domestic surfaces. This is because, considering its proven action against *E. coli*, *Candida tropicalis* and now *Cryptococcus* (specifically the *C. gattii* complex of resistant species), ECR could be used to prevent biofilm formation. It is important to highlight that our previous study showed that ECR is innocuous for animal cells (92% cell viability for VERO lines) and did not show mutagenic potential in an Ames assay [19].

In the present study, we also evaluated the combination of ECR with fluconazole and observed that these antifungal drugs did not show a satisfactory interaction *in vitro*.

Although this could be viewed as an unfavorable result, the concomitant use of both drugs in the same patient without one interfering with the antifungal activity of the other could be allowed. Thus, fluconazole could be used to treat cryptococcosis in parallel with ECR, avoiding the formation of biofilms on cell surfaces and preventing reinfections. Complementary studies could also validate the use of ECR as a coadjuvant in the treatment of cryptococcosis caused by *C. gattii* species.

The formation of biofilms contributes to the permanence of microorganisms in a host and helps protect against antimicrobials [27]. The yeasts of the genus *Cryptococcus* are endowed with a polysaccharide capsule composed primarily of glucuronoxylomannan, which is an important structural component of *Cryptococcus* biofilms [28]. *Cryptococcus* spp. may form biofilms on polystyrene plates and medical devices [28,29] and may be more resistant to amphotericin B and caspofungin [22]. The results of our study showed that ECR could reduce the biomass of a mature biofilm. However, ECR exhibited a superior antibiofilm activity when it was present during *C. gattii* biofilm formation, since an ECR concentration of 10 mg/mL resulted in a reduction of 42.58% of viable *C. gattii* cells compared to the control, indicating its potential use in prophylaxis.

Conclusions

The results of our study showed that an ethanolic extract of *C. regium* leaves showed antifungal activity and especially antibiofilm activity against *C. gattii*. Thus, the results of the present study expand the known antifungal properties of the leaves of this plant. However, additional tests are necessary to characterize the chemical compounds responsible for this antifungal activity. In addition, trials aimed at assessing the applicability of the extract at promoting positive health outcomes should be performed.

Data Availability

Previously reported cytotoxicity, mutagenicity and antimicrobial activity data from *Cochlospermum regium* (Schrank) Pilger leaves ethanolic extract were used to support this study and are available at https://doi.org/10.1155/2017/4687154. This prior study is cited at relevant places within the text as reference [19].

Conflicts of Interest

The authors have no conflicts of interest to declare.

Acknowledgments

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5.3 MANUSCRITO 2: EXTRATO AQUOSO DE *Myracrodruon urundeuva* ALLEMÃO INIBE O CRESCIMENTO DE *Candida albicans* PROVENIENTES DA MUCOSA ORAL DE INDIVÍDUOS PORTADORES DE HIV/AIDS

Este manuscrito está em fase de elaboração e será submetido para publicação na revista Evidence-Based Complementary and Alternative Medicine (Qualis CAPES: A2 na área interdisciplinar; Fator de Impacto: 1.740) e está formatado de acordo com as normas exigidas pela revista.

6. CONCLUSÕES

Extratos provenientes de plantas medicinais podem ser potenciais opções terapêuticas para o tratamento de infecções fúngicas.

Os extratos etanólicos provenientes da casca e folhas de *Annona coriacea* apresentam atividade inibitória ou de redução no crescimento de espécies dos complexos de *Cryptococcus neoformans* e *C. gattii*.

O extrato etanólico das folhas de *Cochlospermum regium* possui atividade anti-*Cryptococcus gattii* e é capaz inibir a formação de biofilmes.

O extrato aquoso da entrecasca de *Myracrodruon urundeuva* apresenta atividade antifúngica frente a isolados de *Candida albicans* provenientes da mucosa oral.

O presente estudo auxilia na descoberta de novos compostos bioativos como alternativas promissoras na terapia de infecções causadas por leveduras. Estudos futuros devem ser realizados para entender os mecanismos de ação e atividade antifúngica *in vivo* dos extratos.

7. ANEXOS

ANEXO A – ARTIGOS PUBLICADOS QUE FORAM REALIZADOS EM PARCERIA DURANTE O DOUTORADO

Mem Inst Oswaldo Cruz, Rio de Janeiro: 1-8, 2016 1

Novel point mutations in the *ERG11* gene in clinical isolates of azole resistant *Candida* species

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The azoles are the class of medications most commonly used to fight infections caused by Candida *sp. Typically, resistance can be attributed to mutations in* ERG11 *gene (CYP51) which encodes the cytochrome P450 14α-demethylase, the primary target for the activity of azoles. The objective of this study was to identify mutations in the coding region of the* ERG11 *gene in clinical isolates of* Candida *species known to be resistant to azoles. We identified three new synonymous mutations in the* ERG11 *gene in the isolates of* Candida glabrata *(C108G, C423T and A1581G) and two new nonsynonymous mutations in the isolates of* Candida krusei *- A497C (Y166S) and G1570A (G524R). The functional consequence of these nonsynonymous mutations was predicted using evolutionary conservation scores. The G524R mutation did not have effect on 14α-demethylase functionality, while the Y166S mutation was found to affect the enzyme. This observation suggests a possible link between the mutation and dose-dependent sensitivity to voriconazole in the clinical isolate of* C. krusei. *Although the presence of the Y166S in phenotype of reduced azole sensitivity observed in isolate* C. krusei *demands investigation, it might contribute to the search of new therapeutic agents against resistant* Candida *isolates.*

Key words: yeasts - *Candida krusei* - voriconazole - 14α-demethylase - Y166S

In Latin American countries, particularly Brazil, *Candida tropicalis* is responsible for 20-24% of all haematogenous infections (Nucci & Colombo 2007, Pfaller & Diekema 2007). It is most commonly seen in patients with neutropenia, diabetes mellitus, and in elderly patients (Sipsas et al. 2009). *Candida glabrata* and *Candida krusei* are the predominant nosocomial fungal pathogens in patients with haematologic malignancies or those undergoing bone marrow transplantation (Goldman et al. 1993, Nucci & Colombo 2007, Pfaller & Diekema 2007).

In the previous decades, there have been many cases of resistance to antifungal agents used in the prophylaxis and treatment of infections caused by *Candida* species (Jiang et al. 2012, Almeida et al. 2013). Mutations and increased expression of genes encoding enzymes responsible for the biosynthesis of ergosterol (Vandeputte et al. 2005, Barker & Rogers 2006) have been identified as the molecular mechanisms responsible for the development of azole resistance in *Candida* species (Barker & Rogers 2006, Berila et al. 2009, Ge et al. 2010, Carvalho et al. 2013).

The azoles, a major class of antifungal compounds, interfere with the ergosterol biosynthesis pathway in fungal membranes by inhibiting the cytochrome P450-dependent enzyme 14α-demethylase (Erg11p or 14DM), synthesised by the *ERG11* gene. Thus, mutations resulting in the increased expression of the *ERG11* gene could confer the yeast species with resistance to azoles by decreasing their drug binding affinity (Barker & Rogers 2006).

Several mutations are clustered into three hot spot regions in *ERG11* gene ranging from amino acids (aa) 105-165, 266-287, and-488 from *Candida albicans*, those regions were associated which *Candida* species resistant to azoles (Marichal et al. 1999, Perea et al. 2001, Chau et al. 2004, Vandeputte et al. 2005, Morio et al. 2010, Flowers et al. 2015, Grossman et al. 2015, Tan et al. 2015).

Vandeputte et al. (2005) found a missense mutation (Y132F) in strains of *C. tropicalis* resistant to fluconazole, which had been previously reported in *C. albicans* by Chau et al. (2004), conferring resistance to this drug. Carvalho et al. (2013), when investigating mutations on the *ERG11* gene in clinical isolates of *C. albicans*, *C. glabrata*, and *C. tropicalis* previously evaluated by fluconazole-susceptibility tests, have identified 14 different missense mutations, five of which had not been previously described, being that one new L321F mutation was identified in *C. albicans* resistant to fluconazole.

Therefore, the search for mutations in the *ERG11* gene in clinically relevant *Candida* species can provide a better understanding of the molecular mechanisms involved in resistance to antifungal agents and aid in epidemiological research. In addition, the genetic and molecular characterisation of resistant *Candida* species could help in the search for new bioactive molecules with antifungal activity. Therefore, the objective was to identify mutations in the coding region of the *ERG11* gene in clinical isolates of *Candida* species known to be resistant to azoles.

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Research Article

In Vitro Control of Uropathogenic Microorganisms with the Ethanolic Extract from the Leaves of *Cochlospermum regium* (Schrank) Pilger

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The roots of *Cochlospermum regium*, popularly known as "algodãozinho-do-cerrado," are used for the treatment of genitourinary infections. However, the removal of their subterranean structures results in the death of the plant, and the use of the leaves becomes a viable alternative. Therefore, the antimicrobial activity of *Cochlospermum regium* leaf's ethanolic extract and its action on the biofilm formation of microorganisms associated with urinary infection were evaluated. The total phenolic compounds, flavoids, and tannins were quantified using the reagents Folin-Ciocalteu, aluminum chloride, and vanillin, respectively. The antimicrobial activity was evaluated by the broth microdilution method and the effect of the extract in the biofilm treatment was measured by the *drop plate* method. Cytotoxicity was evaluated by the method based on the reduction of MTS and the mutagenicity by the Ames test. The ethanolic extract of *C. regium* leaves presented 87.4 mg/EQ of flavonoids, 167.2 mg/EAG of total phenolic compounds, and 21.7 mg/ECA of condensed tannins. It presented reduction of the biofilm formation for *E. coli* and *C. tropicalis* and antimicrobial action of 1 mg/mL and 0.5 mg/mL, respectively. The extract showed no cytotoxicity and mutagenicity at the concentrations tested. This study demonstrated that *C. regium* leaves are a viable option for the treatment of genitourinary infections and for the species preservation.

1. Introduction

Urinary tract infection (UTI) is a public health problem that affects millions of people every year [1]. It is defined as the colonization of pathogenic microorganisms that affect the urinary system tissues causing infection [2]. Urinary infection of bacterial origin is most often caused by enteric Gram-negative bacteria, with *Escherichia coli* being the most predominant microorganism [3, 4]. Opportunistic microorganisms such as yeast are also considered uropathogens, and the genus *Candida* has been reported as one of the most important [5]. Among yeasts of this genus, *Candida tropicalis* is among the most isolated ones in patients diagnosed with urinary infection [6].

A serious nosocomial problem currently faced in relation to these microorganisms is the formation of biofilm, which

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Note

Candidaemia due to *Candida parapsilosis* species complex at a hospital in Brazil: Clinical characteristics and antifungal susceptibility profile

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ABSTRACT

Background: Recent decades have seen a global emergence of candidaemia caused by non-*Candida albicans Candida* species, particularly the *Candida parapsilosis* complex.
Aims: To evaluate the clinical features and antifungal susceptibility profiles of isolates belonging to the *C. parapsilosis* species complex in patients with candidaemia in a midwestern Brazilian tertiary-care teaching hospital.
Methods: Yeast identification was performed using an automated Vitek 2 Compact system. PCR-RFLP was employed for species differentiation.
Results: Five cases of infection by *C. parapsilosis sensu stricto* and two by *Candida orthopsilosis* were found. Of the seven cases, five were adult patients undergoing haemodialysis. The only isolate of *C. parapsilosis sensu stricto* resistant to fluconazole (MIC = 8 μg/ml) was obtained from a patient on a long-term regimen with this drug. This was the only patient who evolved to death.
Conclusions: Resistance to antifungal agents poses a therapeutic challenge, especially for non-*C. albicans Candida* species, and requires continuous monitoring using susceptibility tests because resistance *in vitro* can be predictive of treatment failure. In the present study, *in vitro* antifungal susceptibility proved consistent with clinical outcome.

Candidemia por especies del complejo *Candida parapsilosis* en un hospital de Brasil: características clínicas y perfil de sensibilidad a los antifúngicos

Palabras clave:
Candidemia
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RESUMEN

Antecedentes: En las últimas décadas se ha visto un surgimiento mundial de la candidemia causada por especies de *Candida* no-*C. albicans*, en particular del complejo *Candida parapsilosis*.
Objetivos: Evaluar las características clínicas y los perfiles de sensibilidad antifúngica en aquellos aislamientos del complejo de especies *C. parapsilosis* responsables de candidemia en un hospital universitario de tercer nivel en la región centro-oeste de Brasil.
Métodos: La identificación se realizó en un sistema automatizado Vitek 2 compact. Se utilizó PCR-RFLP para la diferenciación de las especies.
Resultados: Se encontraron cinco casos de candidemia por *C. parapsilosis sensu stricto* y dos por *Candida orthopsilosis*. Cinco eran pacientes adultos sometidos a hemodiálisis. El único aislamiento de *Candida parapsilosis sensu stricto* resistente a fluconazol (CIM, 8 μg/ml) se obtuvo de un paciente en régimen largo de tratamiento con este antifúngico. Este fue el único paciente que murió.

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Short Communication

Variability in the clinical distributions of *Candida* species and the emergence of azole-resistant non-*Candida albicans* species in public hospitals in the Midwest region of Brazil

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Abstract

Introduction: Incidence and antifungal susceptibility of *Candida* spp. from two teaching public hospitals are described. **Methods**: The minimum inhibitory concentrations of fluconazole, voriconazole, itraconazole, and amphotericin B were determined using Clinical Laboratory Standard Institute broth microdilution and genomic differentiation using PCR. **Results**: Of 221 *Candida* isolates, 50.2% were obtained from intensive care unit patients; 71.5% were recovered from urine and 9.1% from bloodstream samples. *Candida parapsilosis sensu stricto* was the most common candidemia agent. **Conclusions:** We observed variations in *Candida* species distribution in hospitals in the same geographic region and documented the emergence of non-*C. albicans* species resistant to azoles.

Keywords: Candidiasis. Candidemia. Epidemiology.

Candida spp. are microorganisms that can cause infections ranging from superficial to systemic infections and are considered the main agents of fungal infections in hospitalized patients. The consequences of invasive candidiasis are severe for both the patient and the institution owing to prolonged hospitalization and increased mortality[1].

Although *Candida albicans* species are the most frequently isolated, the epidemiology of *Candida* infections is changing, with increased incidence of non-*Candida albicans* (NCA) species[1,2,3].

The choice of treatment for candidiasis should be based on the *Candida* species and infection site. In addition, knowledge of the local antifungal susceptibility is of great importance to ensure better patient prognosis.

This study investigated the incidence of *Candida* isolates and their antifungal susceptibility. We performed a prospective study in two public teaching hospitals located in Mato Grosso do Sul State, Brazil, namely University Hospital Maria Aparecida Pedrossian (UH-MAP) and University Hospital of the Federal University of Grande Dourados (UH-FUGD), from March 2013 to March 2014.

This study included *Candida* spp. isolates obtained from different clinical specimens. If patients had more than one isolate of the same species, only the first sample was considered. Data regarding patient age, sex, and hospital units were obtained from the computerized system of each hospital.

The minimum inhibitory concentrations (MICs) of fluconazole, voriconazole, itraconazole, and amphotericin B were determined by using the Clinical Laboratory Standards Institute (CLSI) broth microdilution (BMD) method. For quality control and reproducibility of the tests, American Type Culture Collection (ATCC) strains (*C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019) were included. The MICs were interpreted according to the proposed CLSI breakpoints[4].

Genomic deoxyribonucleic acid (DNA) was extracted and purified using a commercial YeaStar DNA Extraction Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. For the first differentiation between species, multiplex polymerase chain reaction (PCRm) was performed as described by Li *et al.*[5]. The primers used were CL (*Candida lusitaniae*): GTTAGGCGTTGCTCCGAAAT; CP (*Candida parapsilosis* complex): GGCGGAGTATAAAGTAATGGATAG; CT (*Candida tropicalis*): AAGAATTTAACGTGGAAACTTA; CGU

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In vitro antifungal activity of *Myracrodruon urundeuva* Allemão against human vaginal *Candida* species

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ABSTRACT

Myracrodruon urundeuva is a plant native to Brazil, which is used by the indigenous population for the treatment of candidiasis. The aims of this study were to evaluate the antifungal activity of extract against human vaginal *Candida* species and evaluate the possible toxicological activities of *M. urundeuva*. Initially, ethanol extracts, ethyl acetate fractions, and hydroalcoholic fractions of the bark and leaf of *M. urundeuva* were used to determine the minimum inhibitory concentration. The extracts that showed antifungal activity were characterized by liquid chromatography and subjected to toxicity assessment. Toxic, cytotoxic, genotoxic, and mutagenic testing were performed using *Allium cepa* and Ames assays with the ethanol extracts of the bark and leaves. Hemolytic activity was evaluated in erythrocytes and acute toxicity in rats. The ethanol bark extracts showed best activity against *Candida albicans, C. krusei,* and *C. tropicalis* ATCC (4-512 μg/mL). Chemical characterization indicated the presence of flavonoids and tannins in the extracts. Hemolytic activity, genotoxicity, and mutagenicity were not observed. The results of the Ames and *A. cepa* tests were also in agreement, ethanol bark extracts and ethanol leaf extracts of *M. urundeuva* showed absence of mutagenic activity. Similar results were observed in the *A. cepa* assay and acute toxicity test in rats. *M. urundeuva* bark extracts showed potential for the treatment of vaginal infections caused *Candida* species, as a topical.

Key words: Anacardiaceae, candidiasis, *Myracrodruon urundeuva*, traditional medicine.

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Candida albicans isolated from urine: Phenotypic and molecular identification, virulence factors and antifungal susceptibility

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ABSTRACT

Objective: To isolate *Candida albicans* (*C. albicans*) from the urine of hospitalized patients and assess the virulence factors and antifungal susceptibility profiles of the isolates.

Methods: Yeasts were identified using the chromogenic medium CHROMagar™, the VITEK® 2 system, hypertonic Sabouraud broth, tobacco agar, polymerase chain reaction, and DNA sequencing. The evaluated virulence factors were proteinase production, phospholipase production, and biofilm production on polystyrene. The broth microdilution technique was used to determine the minimum inhibitory concentration.

Results: All yeasts isolated from urine were identified as *C. albicans* using both classical and molecular methods. Although 91.3% of the isolates showed no phospholipase activity, 56.5% showed strong proteinase activity and 91.7% produced biofilm. All microorganisms were sensitive to fluconazole, voriconazole and amphotericin B, but 56.5% of the yeasts showed resistance to itraconazole.

Conclusions: *C. albicans* isolates from urine have a high capacity for virulence and can be associated with infectious processes. Furthermore, the high percentage of isolates resistant to itraconazole is important because this antifungal agent is commonly used to treat fungal infections in the hospital environment.

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